SUGGESTED SHORT TITLE

Proteins from Milks of Different Species

ABSTRACT

Comparative Studies on the Proteins of Milks from Different Species

Doctor of Philosophy | Bruce H. Lauer | Agricultural Chemistry

Casein was prepared (acid-precipitation) from the milks of the following mammals: cow, horse, pig, Arctic wolf, barren-ground caribou, dall sheep, fin whale, harp seal, moose, musk-ox, polar bear, and reindeer. The levels of hexose, hexosamine, and sialic acid were determined in each of the casein samples. The caseins and whey proteins were analyzed by polyacrylamide-disc electrophoresis. The conversion of amino acids to their N-trifluoroacetyl n-butyl esters, and gas chromatography of the latter, indicated incomplete recoveries for the amino acids. Thus, a method which made use of standard calibration curves was proposed for the quantitative determination by gas chromatography of amino acids in protein hydrolysates. The amino acid composition of the caseins and other standard proteins was determined. A computer program was written to process the results of amino acid analyses. The levels of strontium-90 and cesium-137 were determined in different tissues of fin whale and harp seal.

COMPARATIVE STUDIES ON THE PROTEINS OF MILKS FROM DIFFERENT SPECIES

by

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CHAPTER I

GENERAL INTRODUCTION

The composition of milk has been the subject of numerous investigations and each worker has had his own reasons for pursuing these studies. Some workers have been interested in the composition of milk insofar as milk is a major factor in the early nutrition of the young. Evans (81) and Ben Shaul (36) have attempted to relate the physiology of an animal to its environment. Jenness and Sloan (139) and other workers (Glass and Jenness (102); Glass, Troolin, and Jenness (103); and Sloan, Jenness, Kenyon, and Regehr (255)) have considered the relation of milk composition to mammalian taxonomy.

Jenness and Sloan (139) recognized that the major criticism which workers in this field face, is that of drawing conclusions from the results of the analysis of a limited number of samples. At first sight, one would be tempted to regard a zoo as a convenient source of animals from which one could obtain milk samples. However, it has been the author's experience that zoo officials are extremely reluctant to make their animals available for milking. The problem

of obtaining milk samples from animals in the wild state is that it is a difficult and costly procedure, especially when each individual worker collects his own samples. The author has been fortunate in that several scientists and other workers, located in remote regions, have been kind enough to provide him with samples of milk. Nevertheless, the author and other workers in this field have reported the results of analysis of single samples of milk from a given species. It is the opinion of those involved in this type of work, that data obtained from one sample are better than no data at all. Jenness and Sloan (139) noted that the variation in milk composition within a species is likely to be less than the variation in milk composition between two or more different species. Thus, the averages based on the data obtained from a small number of samples from a given species, or the results obtained from a single sample from a given species, are adequate for broad comparisons and generalizations.

The author's M.Sc. thesis (159) dealt with the gross composition, fatty acid constitution, and mineral constituents of the milks of the following Arctic and sub-Arctic species: Arctic wolf, husky, fin whale, beluga whale, and mountain goat. The thesis also dealt with the levels of radioactivity (Sr-90 and Cs-137) in these and other milks.

As far as the author is aware, there are only three papers on comparative studies on the carbohydrate content (Johansson and Svennerholm (141)) and electrophoretic properties (Deutsch (68); and Sloan, Jenness, Kenyon, and Regehr (255)) of proteins isolated from the milks of several species. There are no published papers on comparative studies on the amino acid composition of caseins isolated from milks of several species.

The present work is a comparative study on the proteins of the milks of the following Arctic, sub-Arctic, and domestic species: cow (Bos taurus), horse (Equus caballus), pig (Sus scrofa), Arctic wolf (Canis lupus arctos), caribou (Rangifer tarandus groenlandicus), dall sheep (Ovis dalli dalli), fin whale (Balaenoptera physalus), harp seal (Pagophilus groenlandicus), moose (Alces alces), musk-ox (Ovibos moschatus), polar bear (Thalarctos maritimus), and reindeer (Rangifer tarandus).

CHAPTER II

MATERIALS

1. Collection of Milk Samples

1.1. COW (Bos taurus) MILK

Milk (Sample Nos. 1, 2, and 3; 100 cc, each) was collected on three separate occasions from holding tanks located on the Macdonald College Farm, Ste. Anne de Bellevue, Quebec. The samples were preserved by refrigeration (4-6°C).

1.2. HORSE (Equus caballus) MILK

Milk (50 cc) was obtained by hand-milking a horse located on a farm at Stanbridge East, Quebec, about 40 miles S.E. of Montreal. The sample was obtained (September 28, 1969) from an animal which was four months post-partum. The milk was preserved by refrigeration (4-6°C).

1.3. PIG (Sus scrofa) MILK

Milk (50 cc) was obtained from a sow located on the

Macdonald College Farm, Ste. Anne de Bellevue, Quebec. The animal was 23 days post-partum when the sample was obtained (August 15, 1969). The milk was preserved by refrigeration (4-6°C).

1.4. ARCTIC WOLF (Canis lupus arctos) MILK

Milk (60 cc) was obtained from an Arctic wolf (6 years old) held in captivity at Fort Smith, District of Mackenzie, N.W.T. The wolf was caught as a cub on July 17, 1961, on Axel Heiberg Island which is one of the Sverdrup Islands in the Queen Elizabeth group. The wolf gave birth to four cubs on May 24, 1966. The milk, which was obtained 28 days post-partum, was frozen immediately after collection and was maintained in this state until it was analyzed.

1.5. BARREN-GROUND CARIBOU (Rangifer tarandus groenlandicus) MILK

Milk (Sample No. 1; 50 cc) was obtained from a barren-ground caribou (2 years old) which had been captured in the Beverly Lakes area of the Northwest Territories and which subsequently was maintained in captivity at the Wildlife Research Unit of the University of British Columbia. The animal had given birth to a calf on May 18, 1965, and the milk sample was obtained from the mother soon after the

birth of the calf. The sample was frozen immediately after collection and was maintained in this state until it was analyzed.

Milk (Sample No. 2; 50 cc) was obtained from a second animal, but this sample was a composite sample consisting of four individual samples collected on successive days (May 18 to May 21, 1965). The milk was frozen immediately after collection and was maintained in this state until it was analyzed.

Milk (Sample No. 3; 50 cc) was obtained from a third barren-ground caribou captured in the Beverly Lakes area of the Northwest Territories. Specific data pertaining to the collection of this sample are unavailable.

1.6. DALL SHEEP (*Ovis dalli dalli*) MILK

Milk (30 cc) was collected (July 2, 1969) from a dall sheep (6¼ years old; 123 lb) that was shot 16 miles S.W. of Haines Junction, Yukon Territory. The animal was six weeks post-partum. The sample of milk was frozen immediately and was maintained in this state until it was analyzed.

1.7. FIN WHALE (*Balaenoptera physalus*) MILK

Milk (Sample Nos. 1, 2, and 5; 100 cc, each) was

collected from three fin whales (approx. 60 T, each) which were killed (August 15, 1968) near the Emerald Bank, approximately 100 miles east of Blandford, N.S. The milk was frozen immediately after collection and was maintained in this state until it was analyzed.

Milk (Sample Nos. 3 and 4; 100 cc, each) was collected from two fin whales (approx. 60 T, each) which were killed (July 12, 1967) by whalers operating out of Williamsport, Newfoundland. The animals were killed near Grey Island in the North Atlantic (50°49' lat., 55°10' long.). The samples were stored (4-6°C) for five days before they were analyzed.

All samples of whale milk were collected within 10 hours after the animals were killed and were obtained by cutting the mammary glands.

1.8. HARP SEAL (*Pagophilus groenlandicus*) MILK

Milk (150 cc) was obtained from a harp seal which was captured on March 10, 1970 on the ice floes in the Gulf of St. Lawrence. The adult seal and her pup were transported by helicopter to Cap aux Meules (Grindstone Island), Magdalen Islands, where they were maintained in an enclosure. The milk sample was obtained on March 12, 1970 and was preserved with chloroform (0.5% v/v).

1.9. MOOSE (Alces alces) MILK

Milk (Sample No. 1; 50 cc) was obtained from a moose (6 years old) that was killed (August 12, 1969) on the Richardson Highway (3 mile marker) near Anchorage, Alaska. The sample was frozen immediately after collection and was maintained in this state until it was analyzed.

Milk (Sample No. 2; 100 cc) was obtained from a moose (8 years old) that was shot (October 15, 1969) about 16 miles S.W. of Haines Junction, Yukon Territory. The animal was accompanied by a five-year old calf. The sample was preserved with ether (1.5% v/v).

Milk (Sample No. 3; 100 cc) was obtained from the same animal as Sample No. 2, but the sample was preserved by freezing rather than with ether.

1.10. MUSK-OX (Ovibos moschatus) MILK

Milk samples (50 cc, each) were obtained from a musk-ox of an experimental herd maintained at the University of Alaska (Fairbanks, Alaska). Samples were collected on successive days (August 26-30, 1969) immediately after the young had been weaned. The samples were then pooled to make a composite sample (250 cc). This composite sample represented a post-partum interval of 98-102 days. The milk was then

frozen and maintained in this state until it was analyzed.

1.11. POLAR BEAR (Thalarctos maritimus) MILK

Milk (Sample No. 1; 40 cc) was collected from three tagged polar bears near Barrow, Alaska, and was pooled. Data pertaining to each animal is given below:

Sample volume (no. 10 cc vials)	Date of collection	Estimated age of mother (yrs)	No. of cubs	Estimated age of cubs (yrs)
1	April 22, 1968	8	2	1
1	April 29, 1968	11+	2	2
2	April 14, 1968	8	1	2

Milk (Sample No. 2; 15 cc) was obtained (October 22, 1968) from a polar bear (10 years old) near Fort Churchill, Manitoba. The animal (400 lb) was accompanied by two cubs (1 year, 10 months old) weighing approximately 250 lb each. The milk was separated and the skim milk was frozen.

Milk (Sample No. 3; 15 cc) was obtained (October 28, 1968) from a polar bear (15 years old) near Fort Churchill, Manitoba. The animal (480 lb) was accompanied by two cubs (10 months old) weighing approximately 100 lb each. The milk was separated and the skim milk was frozen and

maintained in this state until it was analyzed.

1.12. REINDEER (Rangifer tarandus) MILK

Milk (Sample No. 1; 50 cc) was obtained by hand-milking a reindeer (3½ years old) maintained in captivity in the Helsinki Zoo, Helsinki, Finland. The animal was 70 days post-partum when milked and had delivered its second or third calf on May 5, 1969. The diet of the animal included hay grown locally, prepared commercial pellet feed, and reindeer moss. The sample of milk was frozen immediately after collection and maintained in this state until it was analyzed.

Milk (Sample No. 2; 50 cc) was obtained (July 14, 1969) from a reindeer (7 years old) maintained in captivity at the Loppi (Finland) Experimental Station of Hankkija O.Y. (O.Y. = company)(head office, Helsinki). The animal had delivered its fourth calf on May 20, 1969, and was 55 days post-partum. The animal (70 kg) had been maintained on a commercial feed ration manufactured by Hankkiya O.Y. The milk sample was frozen immediately after collection and maintained in this state until it was analyzed.

Milk (Sample No. 3; 100 cc) was obtained on the day of parturition from an animal (5 years old) maintained on a

Taivalkoski (Finland) commune. The diet of the animal consisted of grass and potatoes. The milk sample was frozen immediately after collection and was maintained in this state until it was analyzed.

The locations of sample collection are shown in Figure 1.

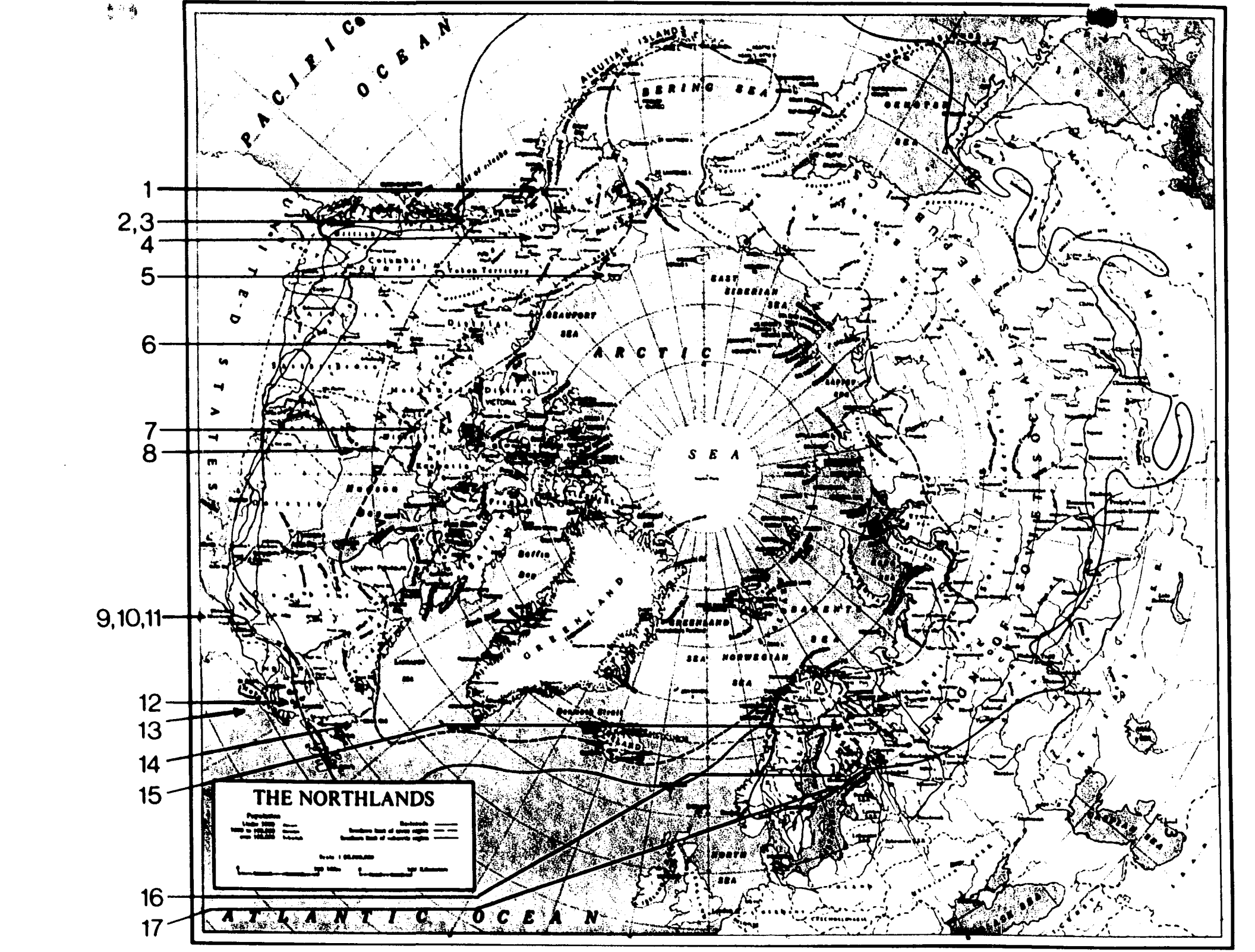
2. Preparation of Caseins and Wheys

The samples of milk that had been frozen were thawed and then centrifuged (4°C; International Model PR-1, head No. 286; 2500 rpm; 1 h). The cream layer was removed and the skim milk was separated from the precipitate that formed with some of the frozen samples. The skim milk was extracted twice with an equal volume of a mixture of petroleum ether and diethyl ether (1:1, v/v). The ether was removed from the skim milk by flash evaporation and hydrochloric acid (0.1 N) was added until pH 4.6 was reached (304). The casein was separated from the whey by centrifugation and was washed several times with distilled water. The casein was suspended in water (volume equal to that of the original skim milk) and sodium hydroxide solution (0.01 N) was added until pH 6.5 was reached. The resultant solution was

Figure 1. Locations of sample collection.

KEY

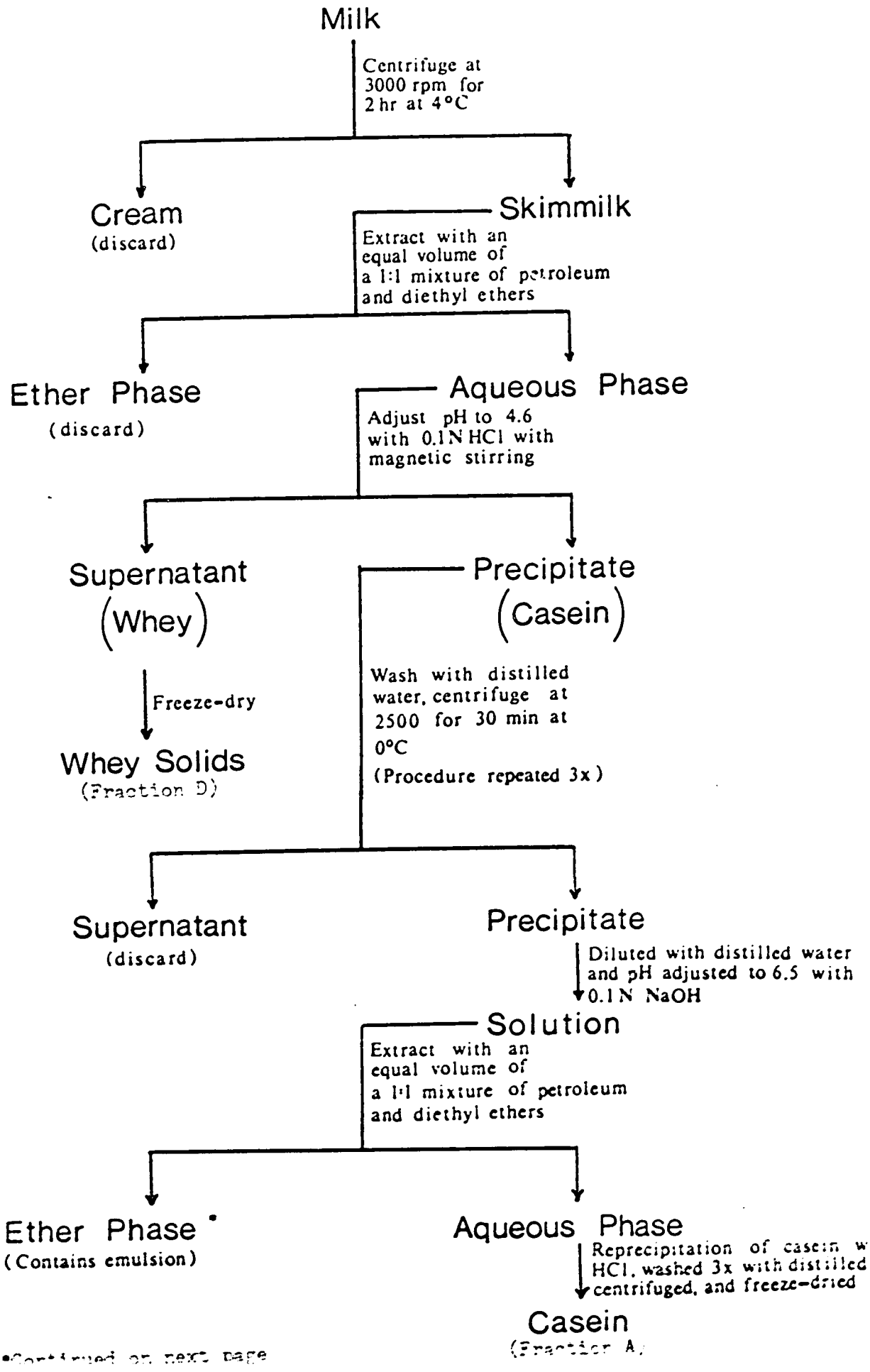
1. Moose	(Sample No. 1)	Anchorage, Alaska
2. Moose	(Sample Nos. 2 & 3	Haines Junction, Yukon, N.W.T.
3. Dall sheep		Haines Junction, Yukon, N.W.T.
4. Musk-ox		Fairbanks, Alaska
5. Polar bear	(Sample No. 1)	Barrow, Alaska
6. Arctic wolf		Fort Smith, District of Mackenzie, N.W.T.
7. Barren-ground caribou	(Sample Nos. 1, 2, & 3)	Beverly Lake, District of Keewatin, N.W.T.
8. Polar bear	(Sample Nos. 2 & 3)	Fort Churchill, Manitoba
9. Cow	(Sample Nos. 1, 2, & 3)	Ste. Anne de Bellevue, Quebec
10. Pig		Ste. Anne de Bellevue, Quebec
11. Horse		Stanbridge East, Quebec
12. Harp seal		Magdalen Islands, Quebec
13. Fin whale	(Sample Nos. 1, 2, & 5)	Blandford, Nova Scotia (Emerald Bank, Atlantic Ocean)
14. Fin whale	(Sample Nos. 3 & 4	Williamsport, Newfoundland (Grey Island, North Atlantic
15. Reindeer	(Sample No. 3)	Taivalkoski, Finland
16. Reindeer	(Sample No. 2)	Loppi, Finland
17. Reindeer	(Sample No. 1)	Helsinki, Finland


PACIFIC OCEAN
ALEUTIAN ISLANDS
BERING SEA
ARCTIC
SEA
ATLANTIC OCEAN
1
2,3
4
5
6
7
8
9,10,11
12
13
14
15
16
17
THE NORTHLANDS

extracted twice with diethyl ether and the casein was precipitated with hydrochloric acid (0.01 N). The casein was washed thoroughly with water and was then lyophilized.

Arctic wolf milk and one sample of fin whale milk (Sample No. 5) yielded a number of other fractions and hence the procedures used for these milks were modifications of that used for the other milks. The two procedures used for Arctic wolf milk and whale milk (Sample No. 5) are summarized in Figures 2 and 3, respectively.

Figure 2. Flow chart showing preparation of casein from Arctic wolf milk.



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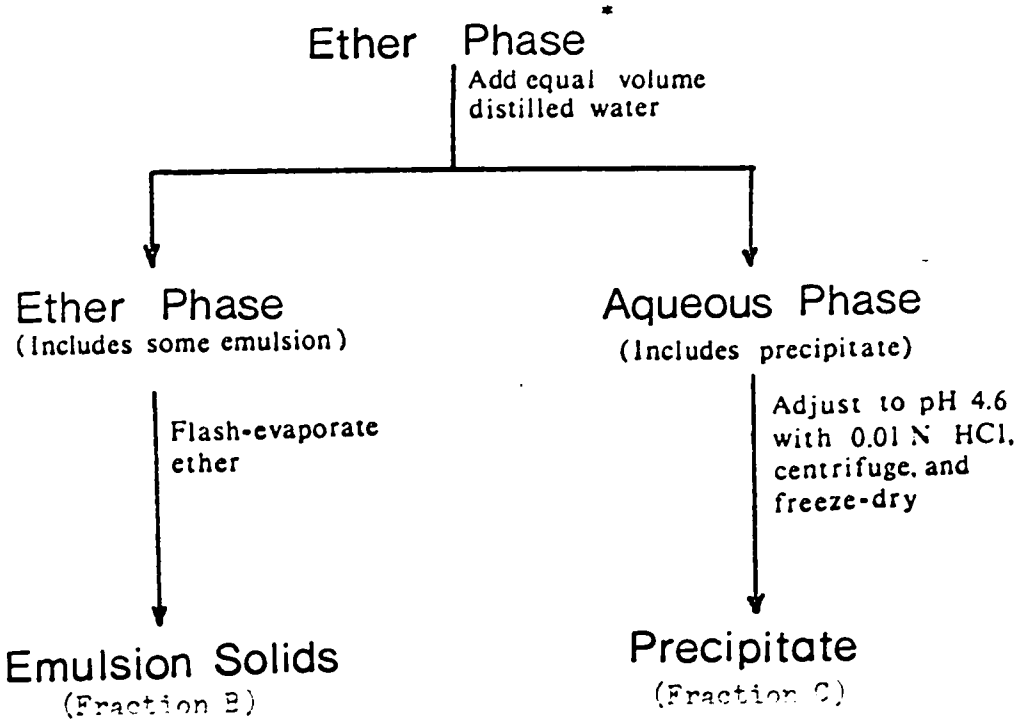
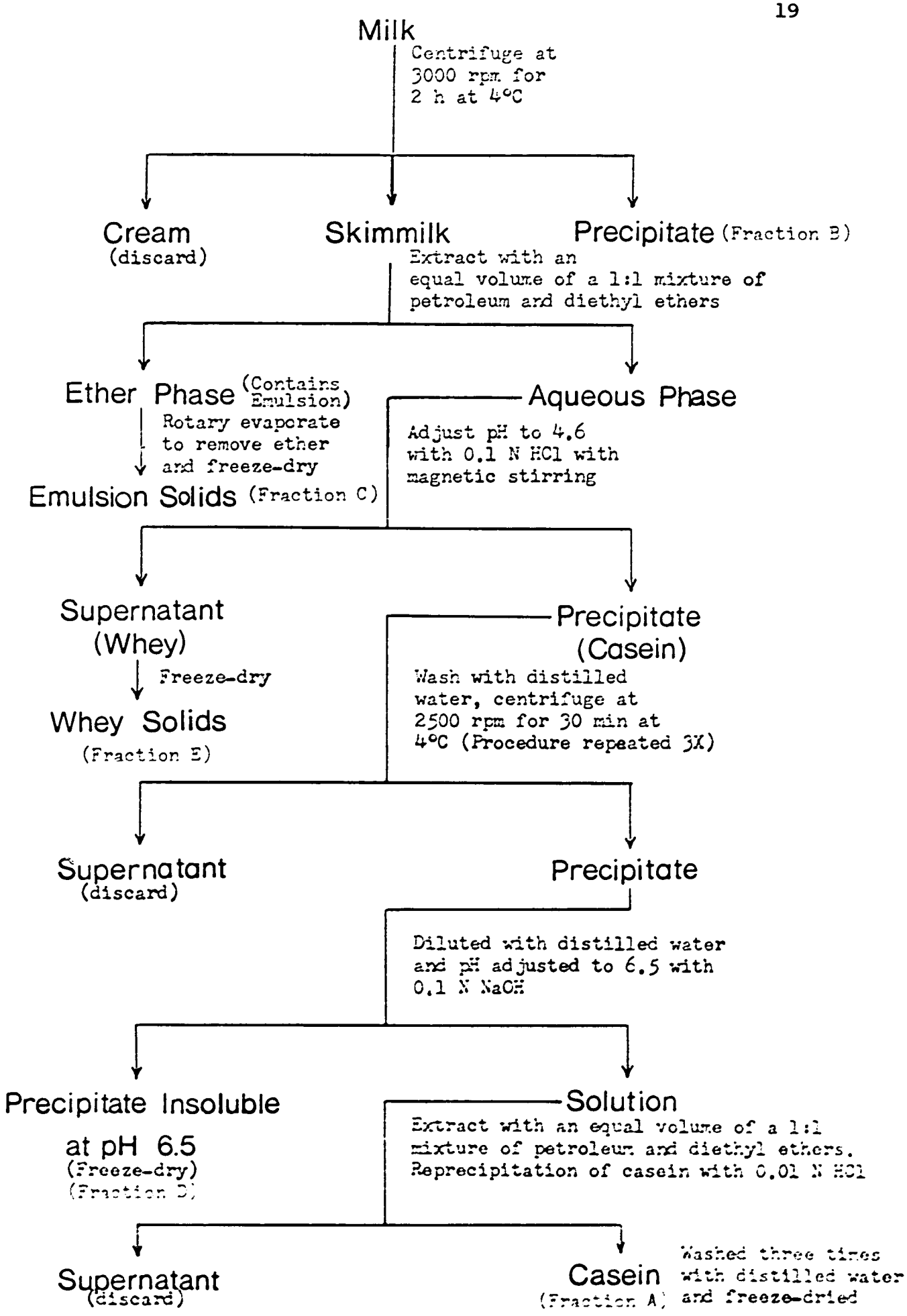


Figure 3. Flow chart showing preparation of casein from fin whale milk (Sample No. 5).


Milk
Centrifuge at 3000 rpm for 2 h at 4°C
Cream (discard)
Skimmilk
Precipitate (Fraction B)
Extract with an equal volume of a 1:1 mixture of petroleum and diethyl ethers
Ether Phase (Contains Emulsion)
Rotary evaporate to remove ether and freeze-dry
Emulsion Solids (Fraction C)
Aqueous Phase
Adjust pH to 4.6 with 0.1 N HCl with magnetic stirring
Supernatant (Whey)
Freeze-dry
Whey Solids (Fraction E)
Precipitate (Casein)
Wash with distilled water, centrifuge at 2500 rpm for 30 min at 4°C (Procedure repeated 3X)
Supernatant (discard)
Precipitate
Diluted with distilled water and pH adjusted to 6.5 with 0.1 N NaOH
Precipitate Insoluble at pH 6.5 (Freeze-dry) (Fraction D)
Solution
Extract with an equal volume of a 1:1 mixture of petroleum and diethyl ethers. Reprecipitation of casein with 0.01 N HCl
Supernatant (discard)
Casein (Fraction A)
Washed three times with distilled water and freeze-dried

CHAPTER III

CARBOHYDRATE CONTENT OF CASEIN

1. Literature Review

1.1. DETERMINATION OF HEXOSES

Several workers have observed that carbohydrates react with certain compounds to give coloured materials.

Dreywood (72) found that the reaction of carbohydrate materials with anthrone in concentrated sulfuric acid gives a permanent green colour. The same worker showed that no colour was produced with the following materials: non-cellulose synthetic resins, organic acids, aldehydes, phenols, fats, terpenes, alkaloids, and proteins.

Morris (214) described a method of applying Dreywood's anthrone reagent to the quantitative analysis of various sugars. He noted that (a) when 100 γ of galactose was subjected to the analytical procedure, the reading on the colorimeter corresponded to that given by 54 γ of glucose; (b) 100 γ of lactose hydrate gave the same reading as 77 γ of glucose; (c) a sample of glycogen that had been hydrolyzed

(100°C; 1 N H_2SO_4; 3 h) gave an identical reading to an unhydrolyzed sample; and (d) proteins produced a red colour with anthrone.

Fairbairn (83) modified the original anthrone reagent so as to afford better temperature control over the reaction (the original colour reagent was 2 g anthrone per liter of concentrated H_2SO_4; the heat for colour development was supplied by the heat of dilution of the sulfuric acid reagent). The reagent suggested by Fairbairn (2 g anthrone per liter of 72% H_2SO_4) also had better-keeping properties than previous reagents (189)(215)(298).

Brieskorn and Berg (48) modified the anthrone method to determine protein-bound hexose in moisture-rich substances (meat, cartilage, casein, etc.).

Dubois, Gilles, Hamilton, Rebers, and Smith (73)(74) discovered that a permanent orange-yellow colour is obtained when carbohydrates carrying a free or potential reducing group are treated with a mixture of phenol and sulfuric acid. Hexoses, hexuronic acids, and their derivatives may be determined by colorimetric measurement at 490 mμ, and pentoses and their derivatives by measurement at 475 mμ. Montgomery (206) has investigated possible interfering substances in the phenol-sulfuric acid procedure. Hexosamines do not form furan derivatives and therefore do not interfere in the

reaction. Lee and Montgomery (165) showed that hexosamines undergo deamination and yield 2,5-anhydro-sugars when treated with nitrous acid. The anhydro-sugars are readily converted to 5-(hydroxymethyl)-2-furfural by acid and can then be determined readily by the phenol-sulfuric acid reagent. Amino acids do not interfere with the method of Dubois et al. unless they have been deaminated (e.g. as in the procedure described above for the determination of hexosamines). Although α- and β-keto acids and aliphatic aldehydes and ketones give a yellow colour with the phenol-H_2SO_4 reagent, no colour was observed with the common acids involved in intermediary metabolism (citric, transaconitic, isocitric, succinic, fumaric, maleic, malonic, malic, and α-hydroxybutyric). The colour produced by lactic acid was very small and hence could be ignored for all practical purposes.

1.2. DETERMINATION OF HEXOSAMINES

The classic method for the determination of hexosamines was developed by Elson and Morgan (80). The procedure is based on the fact that amino sugars are acetylated by acetylacetone in alkaline medium and the acetylated derivatives react with p-dimethylaminobenzaldehyde to produce red pigments.

Anastassiadis and Common (11)(12) obtained water-clear tissue hydrolysates by (a) hydrolysis of tissue samples with Dowex-50 resin suspended in HCl (0.05 N), and (b) elution of the hexosamines from the resin with HCl (2 N). The volume of the eluate was then adjusted to the desired volume and hexosamines were determined on aliquots of the eluate by a modification of the method of Elson and Morgan (10).

Cessi and Piliego (55) noted that non-amino sugars and amino acids interfered with the Elson and Morgan procedure (80). These workers modified the procedure such that the volatile chromogens formed by the reaction of amino sugars with acetylacetone in alkaline solution were separated from the reaction mixture by distillation. Subsequent reaction with p-dimethylaminobenzaldehyde produced a red colour. The method takes advantage of the fact that amino acids in the presence of glucose forms only non-volatile chromogens.

1.3. DETERMINATION OF SIALIC ACIDS

Svennerholm (278)(279) described a method for the quantitative estimation of sialic acids using an orcinol-hydrochloric acid reagent (Bial's reagent). Ketohexoses interfered with the method as they gave a colour indistinguishable from that formed by sialic acids. The author

pointed out, however, that the amount of ketohexoses is low in animal materials and hence does not interfere seriously with the reaction. The author found that sialic acids and fructose gave different colours when they were treated with a resorcinol reagent. In a later publication, Svennerholm (280) reported a method in which interfering materials were eliminated by passing the hydrolysates through ion-exchange (Dowex-2) columns and thereby partially purifying the sialic acids before determination.

Warren (305) determined sialic acids by periodate oxidation in strong acid solution, reaction of the oxidation product (an aldehyde) with 2-thiobarbituric acid, and then extraction of the pigment with cyclohexanone. The sialic acid must be free to be determined by this method. The author applied this method to tissue hydrolysates (80°C; 1 h; 0.1 N H_2SO_4). An important feature of this method is that free, bound, and total sialic acids can be determined by analysis of hydrolyzed and unhydrolyzed samples.

1.4. APPLICATION OF METHODS TO BOVINE CASEIN

Reynolds, Henneberry, and Baker (239) estimated the carbohydrate content of casein by (a) hydrolysis (100°C; 16 h) of the protein with a mixture of dilute hydrochloric

acid (0.05 N) and a cation-exchange resin (Dowex-50), (b) passage of the hydrolysate through a cation-exchange column to remove interfering substances, and (c) measurement of the sugar concentration in the water eluate by the method of Fairbairn (83). The column was rinsed with hydrochloric acid (2 N) and the hexosamines, which were in the resultant acid eluate, were estimated by the method of Anastassiadis and Common (12). Whole bovine casein (15% N) contained 3.85 mg/g total hexose and 2.20 mg/g hexosamine.

Cayen, Henneberry, and Baker (52) investigated the effects of the conditions of hydrolysis and colour reagent (resorcinol, orcinol) on the apparent concentration of sialic acid in whole casein. These workers obtained consistently higher values when the sialic acids were liberated from casein by hydrolysis with a mixture of Dowex 50 x 12 and hydrochloric acid (0.5 h; 95^{o}C) followed by measurement of the colour produced with the resorcinol reagent. The sialic acid content of whole bovine casein was found to be 3.84 mg/g.

Marier, Tessier, and Rose (185) examined the sialic acid content of skimmilk proteins using a modification of Warren's thiobarbituric acid method (305). These workers showed that K-casein and the proteose-peptone fraction were

the only proteinaceous materials of skimmilk which contained marked amounts of sialic acid. The authors also pointed out that proteose-peptone remains in solution at pH 4.5 and therefore the determination of sialic acid can be used as an index of the K-casein concentration of whole acid casein. Since samples of whole acid casein contained 0.26-0.59% sialic acid, and since K-casein contains 2.3% sialic acid, then the proportion of K-casein in whole acid casein was estimated to vary from 11 to 26%.

Huang and Baker (131) investigated the conditions of hydrolysis of casein which would lead to the maximal liberation of sialic acid from the protein and, at the same time, would lead to minimal destruction. The highest concentration of sialic acid in whole casein was found in hydrolysates prepared by hydrolysis of casein for ½ h at 95°C with 0.05 N H_2SO_4. The sialic acid value for casein was found to be 2.90 mg/g.

1.5. CARBOHYDRATE CONTENT OF CASEINS FROM MILKS OF DIFFERENT SPECIES

Johannson and Svennerholm (141) compared the hexose, hexosamine, and sialic acid contents of caseins prepared from the milks of the following mammals: cow, human, domestic sheep, domestic goat, horse, reindeer, and whale.

Baker, Blood, and Chen (28) prepared casein from milks of individual Rocky Mountain bighorn sheep and from Suffolk sheep. The casein from bighorn sheep contained more hexose, hexosamine, and sialic acid than did that from the milk of Suffolk sheep.

Baker, Huang, and Harington (30) and Baker, Hatcher, and Harington (29) have reported values for the carbohydrate content of polar bear milk.

Several workers have investigated the carbohydrate content and in particular, the sialic acid, content of human milk (Baker, Hatcher, and Harington (29); Jordan and Loehr (143); Malpress (183); Alais and Jollès (4)(5); and Maeno and Kiyosawa (180)).

Alais and Jollès (5) have also reported values for the carbohydrate content of the casein of rabbit milk.

Gupta and Ganguli (115) have investigated the sialic acid content of caseins isolated from the milks of Thorparker cows, Red Sindhi cows, non-Indian cows, and buffalo. Casein from Thorparker cows always had the highest sialic acid content and buffalo casein always contained less sialic acid than cow casein.

Other workers (Jenness, Regehr, and Sloan (138); and Roberts, Pettinati, and Bucek (244)) have reported on the

dialyzable carbohydrate content of milks of various species, but these workers have not given values for the carbohydrate content of caseins.

Table 1 is a summary of the results of carbohydrate analyses of various casein preparations as reported by other workers.

2. Methods

2.1. DETERMINATION OF NITROGEN

Total nitrogen (micro-Kjeldahl) was determined by the method described in a publication of the Association of Official Agricultural Chemists (26).

2.2. DETERMINATION OF PHOSPHORUS

Inorganic phosphorus was determined colorimetrically as the phosphate, by a method previously employed by Lauer (159), which is based on the following principles: molybdic acid reacts with phosphate ions in solution to form phosphomolybdic acid which is reduced by ascorbic acid to a coloured compound (molybdenum blue). The intensity of the colour is proportional to the amount of phosphate present in the original solution. The reagents which were required are

Table 1

Carbohydrate content of casein as reported by other workers

Casein	Hexose (%)	Hexosamine (%)	Sialic acid (%)	Reference
Bighorn sheep	0.40	0.16	0.15	(28)
Buffalo	-	-	0.14	(115)
Cow	0.30	0.18	0.30	(30)
	0.39	0.22	-	(294)
	0.24	0.18	0.39	(141)
	3.85	2.20	-	(239)
	-	-	0.38	(52)
	-	-	0.29	(131)
	-	-	0.31	(115)
	0.38	0.35	0.36	(3)
	0.38	0.36	0.36	(5)
	-	-	0.43	(143)
Goat	0.22	0.16	0.30	(141)
	0.39	0.31	0.13	(3)
Horse	0.55	0.44	0.56	(141)
Human	1.98	1.32	0.76	(141)
	2.53	2.48	0.59	(5)
	2.30	2.25	-	(180)
	2.55	2.20	0.37	(4)
	-	-	0.34	(183)
	-	-	0.60	(143)
	2.20	1.72	1.81	(29)
Polar bear	2.80	1.09	1.92	(28)(29) (30)
Reindeer	0.44	0.23	0.46	(141)
Rabbit	1.24	-	-	(5)
	1.57	-	-	(5)
Sheep	0.23	0.15	0.11	(141)
	0.33	0.24	0.09	(5)(3)
Suffolk sheep	0.24	0.10	0.10	(28)
Whale	0.59	0.42	0.37	(141)

as follows:

(1) Molybdic acid reagent

Ammonium molybdate (2.5 g) was added to sulfuric acid (5 N) and the volume of the resultant solution was adjusted to 100 ml with the same acid. Ascorbic acid (10 g) was added to distilled water and the volume of the resultant solution was adjusted to 100 ml with water. The molybdic acid reagent was made by adding distilled water (2 vol) and ascorbic acid solution (1 vol) to the molybdate solution (2 vol).

(2) Phosphorus stock standard solution (250 ppm P)

Potassium phosphate (KH_2PO_4, monobasic, crystal, A.C.S. reagent; 0.109 g) was dissolved in water and the resultant solution was adjusted to a final volume of 100 ml with distilled water. One drop of toluene was added as a preservative.

(3) Diluted phosphorus standard solution (5 ppm P)

A sample (2 ml) of the phosphorus stock standard solution was placed in a volumetric flask (100 ml; Class A, N.B.S.) and the volume of the liquid was adjusted to 100 ml with distilled water.

The procedure for the determination of phosphorus was as follows:

(a) Preparation of a Standard Calibration Curve

Samples (0, 0.5, 1.0, 2.0, 3.0, and 4.0 ml) of the diluted phosphorus standard solution were placed in separate dry test tubes. Distilled water was added to adjust the volume in each tube to 4 ml. Molybdic acid reagent (4 ml) was added to each tube and the contents were mixed. The tubes were placed on a rack in a water bath maintained at 60°C for 10 minutes to develop the colour. The tubes were cooled and the optical densities were recorded at 625 mμ by means of a Bausch and Lomb Spectronic 20 Spectrophotometer. A standard calibration curve was prepared and is illustrated in Figure 4.

(b) Digestion of Casein Samples

Casein digests were prepared by the method of Lindner and Harley (170). Samples of casein (10-15 mg) were placed in an Erlenmeyer flask (25 ml) along with concentrated sulfuric acid (specific gravity, 1.84; 2 ml). The mixture was heated cautiously until the sample was thoroughly charred and then it was cooled to room temperature. Hydrogen peroxide (10 drops, 30%) was added and the mixture was heated for an additional five minutes. The above process of heating, cooling, and addition of peroxide was repeated until a clear and colorless solution was obtained. The reaction mixture was then transferred to a volumetric flask

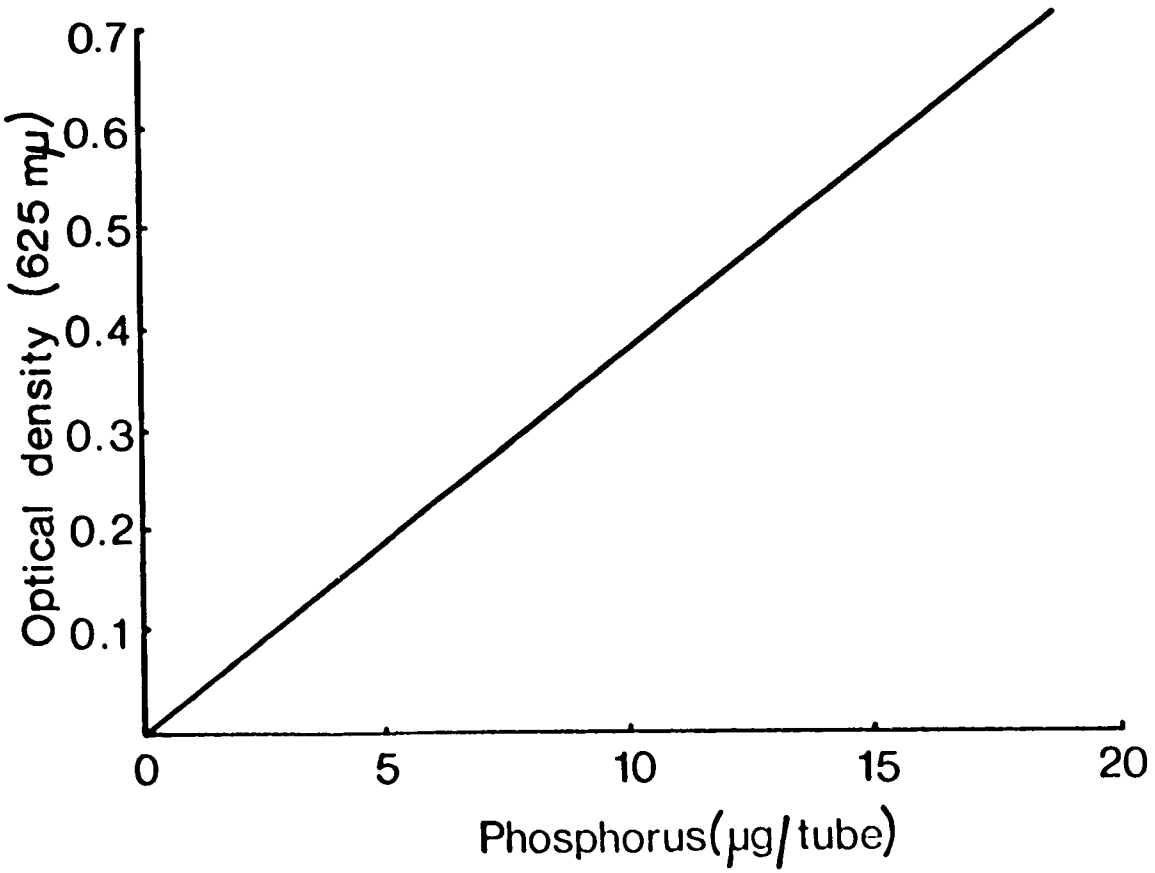


Figure 4. Standard calibration curve for the determination of phosphorus.

and the volume was adjusted to 100 ml.

(c) Determination of Phosphorus in Casein Digests

Samples (0.5-1.0 ml) were placed in separated dry test tubes and the volume of liquid in each tube was adjusted to 4 ml with distilled water. Molybdic acid reagent (4 ml) was added to each tube and the tubes were heated. The optical densities were measured as they were in the preparation of the standard calibration curve. The concentration of phosphorus was ascertained by reference to the standard curve.

2.3. DETERMINATION OF HEXOSE (METHOD I)

Total hexose was determined by the method of Dubois, Gilles, Hamilton, Rebers, and Smith (73)(74)(206). The reagents which were required are as follows:

(1) Sulfuric acid, concentrated (sp. gr. 1.84)

(2) Phenol reagent

Glass-distilled water (20 g) was added to redistilled reagent grade phenol (80 g).

(3) Standard galactose solution (50 μg/ml)

Galactose (50 mg) was placed in a volumetric flask and the volume was adjusted to 1000 ml.

The procedure for the determination of hexose was as follows:

(a) Preparation of a standard calibration curve

Samples (0, 0.1, 0.2, 0.3, 0.4 ..... 2.0 ml) of the standard galactose solution were placed in separate clean dry test tubes along with the phenol reagent (0.05 ml). Distilled water was added to adjust the volume in each tube to 2.0 ml. Concentrated sulfuric acid (5 ml) was added rapidly and the tubes were allowed to stand 10 minutes at room temperature. The tubes were then shaken and placed in a water bath (25°C; 20 min). The absorbance of the solutions was then measured at 490 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer. A standard calibration curve was prepared and is illustrated in Figure 5.

(b) Determination of hexose in casein

Casein samples (1-5 mg) were placed in separate test tubes along with distilled water (2.0 ml), phenol reagent (0.05 ml), and concentrated sulfuric acid (5 ml). The tubes were cooled at room temperature and then in a water bath (25°C; 20 min) and the absorbance of each solution was read at 490 mμ. The concentration of hexose was ascertained by reference to the standard calibration curve.

2.4. DETERMINATION OF HEXOSE (METHOD II) AND HEXOSAMINE

(a) Liberation and purification of hexoses and hexosamines

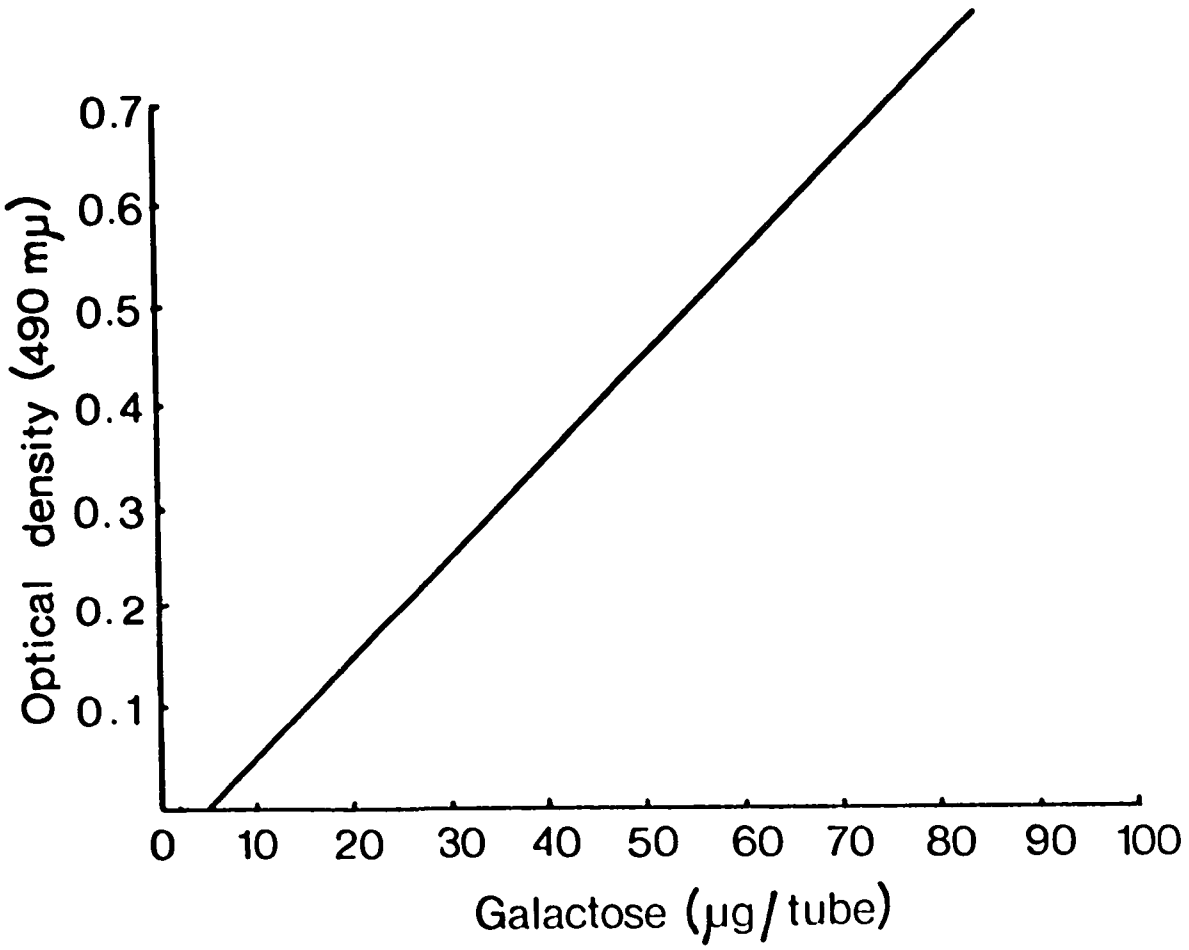


Figure 5. Standard calibration curve for the determination of hexose as galactose.

Sugars were determined by a second method which also enabled determination of hexosamines. The hydrolysis procedure was essentially that of Anastassiadis and Common (11) as modified by Renolds, Henneberry, and Baker (239). Casein (10-50 mg) was hydrolyzed (rotary oven, 100°C; 16 h) in sealed test tubes containing 5 ml of a cation-exchange resin suspension (1 part Dowex 50 x 12; H^+ form; 200-400 mesh + 2 parts hydrochloric acid; 0.05 N). The tubes were then cooled, opened, and their contents were placed on Dowex 50 x 12 (5 cm x 1 cm; H^+ form) columns. The eluates were collected and the columns were washed with distilled water (10 ml). The columns were then washed with hydrochloric acid (10 ml, 2 N) and rinsed with distilled water (2 ml). The water eluates were again passed through the columns to remove any traces of amino compounds and the columns were washed with water (8 ml). The eluates were combined and the final volumes were adjusted to 25 ml. The water eluates contained the sugars that were to be determined. The columns were washed with HCl (8 ml, 2 N) and the eluates were combined with the previous acid eluates. The volumes were adjusted to 25 ml in each case. The acid eluates contained the amino sugars that were to be determined.

(b) Procedure for determination of hexoses in water eluates

Neutral sugars were determined on aliquots (2 ml) of the aqueous eluates by the phenol-sulfuric acid method of Dubois, Gilles, Hamilton, Rebers, and Smith (73)(74)(206). The standard calibration curve is illustrated in Figure 5.

(c) Procedure for determination of hexosamines in acid eluates

Aliquots (10 ml) of the acid eluates were dried in beakers placed under vacuum in a desiccator containing sodium hydroxide flakes. Hexosamines were determined by the method of Cessi and Piliego (55). The reagents which were required for the analysis are as follows:

(1) Acetylacetone reagent

Acetylacetone (redistilled; b.p. 138-140°C; 1 ml) was dissolved in 100 ml of sodium carbonate-sodium bicarbonate buffer (pH 9.8; 23.02 g sodium carbonate + 2.76 g sodium bicarbonate in 1000 ml of solution) which was 0.1 M with respect to sodium chloride.

(2) p-Dimethylaminobenzaldehyde reagent

p-Dimethylaminobenzaldehyde (80 mg) was dissolved in absolute ethanol (100 ml) containing concentrated hydrochloric acid (3.5 ml).

(3) Standard glucosamine solution (50 μg/ml)

Glucosamine hydrochloride (0.05 g) was dissolved

in water and the volume of the resultant solution was adjusted to 1000 ml.

The standard calibration curve was prepared as follows: aliquots (0, 0.5, 1.0, 1.5, and 2.0 ml) of the standard glucosamine solution were placed in separate clean, dry, test tubes. The volume of the liquid in each tube was then adjusted to 2.0 ml. Acetylacetone reagent (5.5 ml) was added to each tube and the tubes were placed in a boiling water bath for 20 minutes. The contents of each tube were transferred with the aid of three portions (2 ml, each) of distilled water, into a distillation apparatus such as that described by Gottschalk (107). Portions (2 ml) of each distillate were collected in volumetric flasks (10 ml) containing p-dimethylaminobenzaldehyde reagent (8 ml). The absorbances of these solutions were then measured at 545 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer. A standard calibration curve was prepared and is illustrated in Figure 6.

Hexosamines were determined in the acid eluates as follows: the residues obtained by drying aliquots of the acid eluates in the desiccator were dissolved in distilled water (2 ml). The procedure described above for the preparation of the standard curve was then followed and the

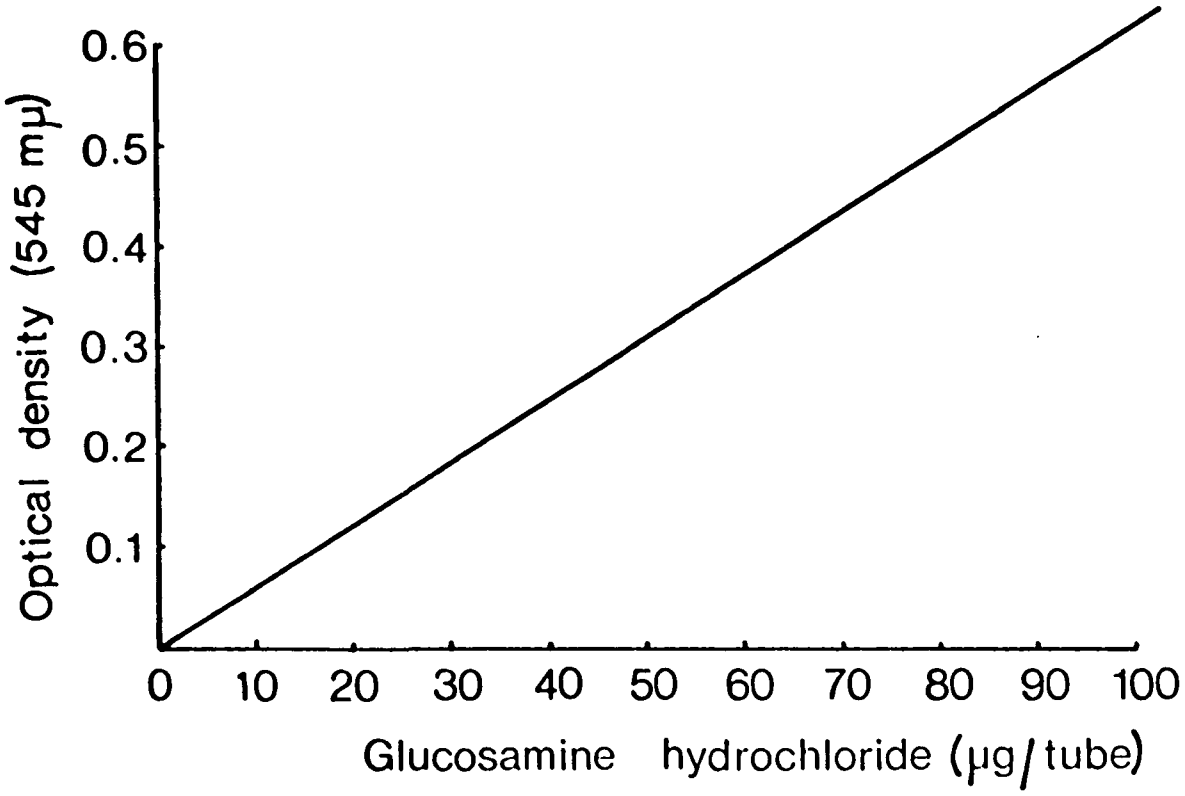


Figure 6. Standard calibration curve for the determination of hexosamine as glucosamine.

absorbances of the final solutions were determined at 545 mμ. The concentration of glucosamine in each sample was ascertained by reference to the standard curve.

2.5. DETERMINATION OF SIALIC ACID

(a) Liberation of sialic acid

Sialic acids were liberated from casein by the method of Huang and Baker (131). Casein samples (10-50 mg) were placed in test tubes (16 x 150 mm) containing dilute sulfuric acid (0.05 N; 6 ml). The tubes were sealed and heated in a rotary oven (95°C; ½ h). The tubes were then opened and the hydrolysates were transferred to volumetric flasks and the volumes were adjusted to 10 ml.

(b) Procedure for determination of sialic acids in hydrolysates

Sialic acids were determined by the method of Warren (305). The reagents which were required are as follows:

(1) Sodium periodate solution

Sodium meta periodate (0.5694 g) was dissolved in 9 M phosphoric acid and the volume of the resultant solution was adjusted to 25 ml with the same acid.

(2) Sodium arsenite solution

Sodium arsenite (10 g) and sodium sulphate (7.10

g) were dissolved in sulfuric acid (0.1 N) and the volume of the resultant solution was adjusted to 100 ml with the same acid.

(3) Thiobarbituric acid solution

Thiobarbituric acid (0.6 g) and sodium sulphate (7.10 g) were dissolved in water and the volume of resultant solution was adjusted to 100 ml.

(4) Standard sialic acid solution (100 ug/ml)

N-acetylneuraminic acid (10 mg) was dissolved in water and the volume of the resultant solution was adjusted to 100 ml.

The standard calibration curve was prepared as follows: aliquots (0, 0.01, 0.02, 0.04, 0.08, 0.12, 0.16, 0.18, and 0.20 ml) of the standard solution were placed in dry centrifuge tubes (screw top; 15 ml, calibrated) and the volume of the solution in each tube was adjusted to 0.2 ml with distilled water. Periodate solution (0.1 ml) was added to each tube and the tubes were shaken and allowed to stand at room temperature for 20 minutes. Arsenite solution (1.0 ml) was added and the tubes were shaken until the yellow-brown colour disappeared. Thiobarbituric acid reagent (3.0 ml) was added and the tubes were shaken, stoppered, and placed in a boiling water bath for 15 minutes. The

tubes were then cooled under cold running water for five minutes. A volume of cyclohexanone equal to the total volume of solution (4.3 ml) was added to each tube. The tubes were shaken twice and centrifuged for three minutes in a clinical centrifuge. The absorbance of the upper layer in each tube was measured at 549 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer. A standard calibration curve was prepared and is illustrated in Figure 7.

Sialic acid was determined in the hydrolysates obtained in (a) as follows:

Samples (0.2 ml) of the diluted hydrolysates were placed in centrifuge tubes (screw top; 15 ml, calibrated) and the procedure for the preparation of the calibration curve described above was followed. The absorbances of the final solutions were measured at 549 mμ and the concentration of sialic acid (as N-acetylneuraminic acid) in each sample was ascertained by reference to the standard curve.

3. Results and Discussion

The caseins which were prepared from the various samples of milk, along with the precipitates obtained on centrifugation of some of the milk samples that had been frozen, were analyzed for their nitrogen, phosphorus, total

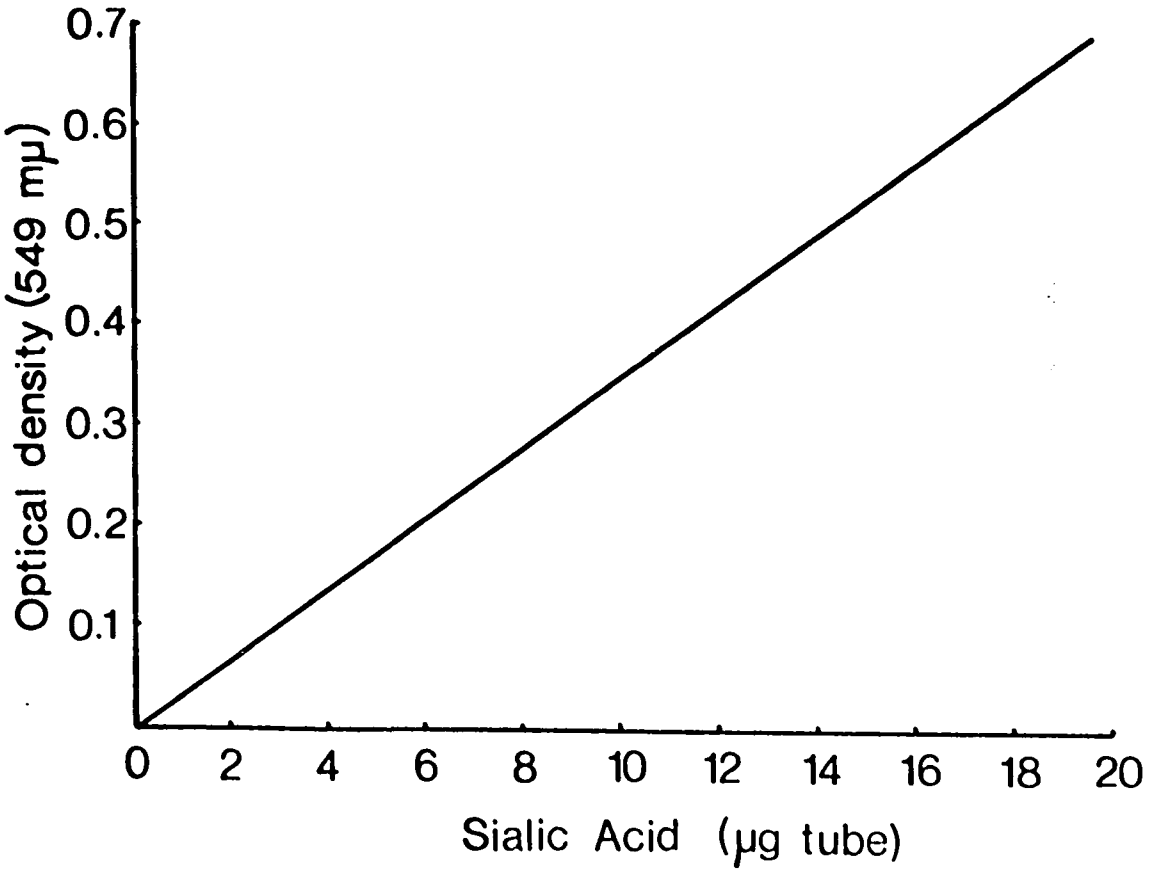


Figure 7. Standard calibration curve for the determination of sialic acid as N-acetylneuraminic acid.

hexose, hexosamine, and sialic acid content. The results of these analyses are presented in Table 2. Each result represents the average of two separate determinations. It will be noted that the carbohydrate content of samples of casein obtained from different individuals of the same species displayed, in some instances, wide differences. The author has made similar observations in connection with the constitution of milks of Arctic and sub-Arctic species (159)(160)(161)(162)(163).

In most instances where the hexose level determined by Method I was greater than 1%, the corresponding level of hexose determined by Method II was lower than that determined by Method I. Conversely, in most instances where the hexose level determined by Method I was less than 1%, the corresponding level of hexose determined by Method II was higher than that determined by Method I. Possible explanations for Method I yielding higher results than Method II could be the following: (a) Method I involves more severe conditions of hydrolysis than Method II. This might lead to release of a greater amount of bound hexose; (b) greater risks of losses are possible in Method II than in Method I due to the resin purification step; and (c) interfering substances might contribute to the higher values obtained in Method I.

Table 2

Carbohydrate content of casein from milks of different species*

Sample	Nitrogen (%)	Phosphorus (%)	Hexose (%) Method I	Hexose (%) Method II	Hexosamine (%)	Sialic Acid (%)
A. Caseins						
Caribou, Sample No. 1	11.2	-	2.86	-	-	0.70
2	12.7	0.82	2.79	2.23	0.28	0.41
3	11.5	0.82	6.15	5.58	0.88	0.67
Dall sheep	11.0	-	1.90	1.76	0.33	0.01
Fin whale, Sample No. 1	9.6	1.21	3.22	1.14	0.18	0.21
2	4.6	-	4.22	-	-	0.17
3	9.3	-	6.73	-	-	0.24
4	11.4	-	3.88	-	-	ND**
5	16.0	1.24	3.17	3.66	1.12	0.10
Harp seal	16.0	0.97	0.95	1.19	0.30	0.43
Moose, Sample No. 1	14.6	0.79	1.45	1.00	0.11	0.25
2	15.3	0.78	0.87	0.79	0.18	0.34
3	15.6	0.82	0.58	0.99	0.17	0.33
Musk-ox	13.7	0.80	0.61	1.00	0.09	ND**
Polar bear, Sample No. 1	11.6	0.79	1.50	1.42	0.60	1.48
2	12.6	-	1.29	-	-	-
3	12.5	-	1.58	1.32	0.58	0.86

Table 2 (cont'd)

Sample	Nitrogen (%)	Phosphorus (%)	Hexose (%) Method I	Hexose (%) Method II	Hexosamine (%)	Sialic Acid (%)
A. Caseins						
Reindeer, Sample No.1	15.3	0.75	0.32	0.61	0.18	0.50
2	16.2	0.75	0.22	0.68	0.15	0.59
3	15.2	0.77	0.91	1.06	0.25	0.51
Cow, Sample No.1	13.2	0.76	0.26	0.59	0.12	0.26
2	15.1	0.87	0.24	0.57	0.09	0.39
3	15.3	-	0.30	-	0.18	0.30
Horse	15.9	0.86	0.43	0.72	0.17	0.18
Pig	15.4	1.00	1.11	1.19	0.27	2.26
B. Precipitates						
Dall sheep	10.1	1.75	1.30	1.27	0.12	ND**
Polar bear, Sample No.2	12.7	-	0.71	0.83	0.30	0.44
3	13.1	-	0.53	0.61	0.16	0.21
Fin whale, Sample No.5	14.9	-	0.78	0.87	0.15	0.08
Polar bear "D"***	13.3	-	0.36	-	0.17	0.60

*Values represent averages of duplicate determinations

**ND = Not detected

***Precipitate obtained by Baker, Hatcher, and Harington (29)

Caribou and fin whale caseins contained the highest concentrations of hexose (3.93%; 4.24%); caribou and polar bear caseins contained the highest concentrations of hexosamines (0.58%; 0.59%); pig and polar bear caseins contained the highest concentrations of sialic acid (2.26%; 1.17%). The average values (milk from three different animals) for the carbohydrate content of polar bear casein (1.45% hexose, 0.59% hexosamine, and 1.22% sialic acid) were somewhat lower than those (2.80% hexose, 1.09% hexosamine, and 1.92% sialic acid) obtained previously (30) with the casein obtained from a single sample of polar bear milk. The casein from the sample of polar bear milk that was frozen before the cream layer was removed, contained about 70% more sialic acid than did that from the sample which was centrifuged before freezing (1.48% and 0.86% sialic acid, respectively). The dall sheep casein contained several times more hexose and hexosamine (1.90%; 0.33%) than did the samples of bighorn sheep casein (0.40%; 0.16%, averages) or domestic sheep casein (see Table 1) which were analyzed previously (3)(5)(28)(141). As only one sample of dall sheep milk was available, it was impossible to check whether or not dall sheep casein does in general contain more carbohydrate than other sheep caseins.

Previous workers have isolated protein fractions from seal (Callorhinus ursinus) milk (Ashworth, Ramaiah, and

Keyes (25)), reindeer (Rangifer tarandus) milk (Aschaffenburg, Gregory, Kon, Rowland, and Thompson (22); Luhtala, Rautiainen, and Antila (174)), and fin whale (Balaenoptera physalus) milk (Ohta, Watarae, Oishi, Ueshiba, Hirose, Yoshizawa, Aikikusa, Sato, and Okano (223)), but as far as the author is aware, there is little or no available information on the carbohydrate constitution of casein obtained from these milks, or of casein obtained from musk-ox (Ovibos moschatus) milk.

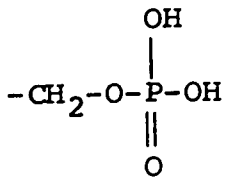
The compositions of the protein precipitates obtained on initial centrifugation of some of the milks are also tabulated in Table 2. Precipitates were present in milks which had been frozen. Previous workers have also encountered similar precipitates in their studies (28)(29). The levels of hexose, hexosamine, and sialic acid in the precipitates were lower than those of the corresponding caseins. This suggests that the precipitates might contain albumin fractions or casein fractions other than α-casein which are known to contain little or no bound carbohydrate.

Marier, Tessier, and Rose (185) suggested that the concentration of sialic acid in whole bovine casein could be used as a measure of the quantity of K-casein in the casein complex, provided that (a) only sialic acid in casein is

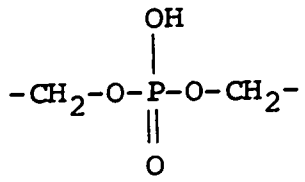
measured, (b) all other casein fractions contain little or no sialic acid, and (c) the sialic acid content of K-casein is known and is constant. These authors found that whole bovine casein contained between 0.26 and 0.58% sialic acid. On the assumption that K-casein contains 2.3% sialic acid, they concluded that bovine casein contains between 11 and 26% K-casein. If one assumes that the sialic acid content of the K-casein fractions of the whole casein prepared from the milks of the various species is about the same as that of bovine K-casein, then the concentrations of K-casein in the various casein preparations would range between 0.0 and 50%, with the exception of pig casein. The high sialic acid content of pig casein might indicate that the distribution of sialic acid in the pig casein complex is different from that in bovine casein or that the sialic acid content of the glycoprotein fraction of pig casein is much higher than that of bovine K-casein.

Gordon and Whittier (105) have pointed out that phosphorus analyses are useful for the characterization of casein because this element is an uncommon constituent of other milk proteins. Jenness and Patton (137) have suggested that the phosphorus is incorporated in α- and β-caseins as esters of phosphoric acid with the hydroxyamino acids serine and threonine. Perlmann (227) has suggested that in α-casein, 40% of

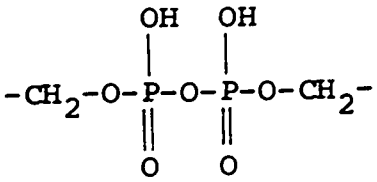
the phosphate is bound as monoesters, 40% as phosphamide diester, and 20% as pyrophosphate. In β-casein, a large amount of phosphate is present as the diester.



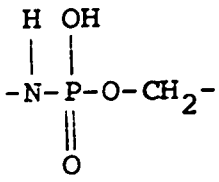
(monoester)



(diester)



(pyrophosphate diester)



(phosphamide diester)

Sloan, Jenness, Kenyon, and Regehr (255) have indicated that the electrophoretic mobilities of casein fractions are partly a function of the content of ester-bound ionizable phosphate groups. These workers concluded that in species having more than one electrophoretic component, the one of greater mobility has the higher phosphorus content. The same workers also provided data on the phosphorus-nitrogen ratios of caseins of various species. These results and

the results of other workers are included in Table 3. The phosphorus-nitrogen ratios calculated from the data supplied by the author are presented in Table 4. The P/N ratios for cow and horse (0.058; 0.054) are in close agreement with results (Table 3) of previous workers (0.056; 0.054). The two precipitates showed exceptionally high values, although values for their corresponding caseins are not available. This fact suggests that the precipitates might contain a relatively large proportion of α-casein. Values for P/N ratios of whole casein seem to be characteristic of each species. However, a discrepancy exists between the P/N ratios for the casein of caribou (0.068) and the casein of reindeer (0.049), especially when one considers that they are both of the species Rangifer tarandus.

The composition of the various protein fractions of Arctic wolf milk and fin whale milk are shown in Tables 5 and 6. The letters assigned to each fraction in these tables correspond to the letters assigned to the various fractions in Figures 2 and 3. It is of interest to note the low nitrogen value obtained for Arctic wolf "casein" and the high value obtained for the "emulsion precipitate from the ether phase." It may well be that this precipitate is, in fact, casein, but further evidence, and in particular that supplied by electrophoretic analyses would have

to be obtained to support this point.

Table 3

Phosphorus-nitrogen ratios of caseins as reported by other workers

Species	P (%)	N (%)	P/N*	Reference
California sea lion (Zalophus californianus)	-	-	0.027	Pilson & Kelly (230)
Cow (Bos taurus)	0.85	15.25	0.056	Alais & Jolles (5)
	0.86	15.63	0.055	Gordon et al. (104)
Goat (Capra hircus)	0.76	15.48	0.049	Davies (66)
Horse (Equus caballus)	0.88	16.44	0.054	Davies (66)
Human (Homo sapiens)	0.48	14.84	0.032	Alais & Jolles (5)
	0.33	14.09	0.023	Maeno & Kiyosawa (180)
	0.68	14.97	0.046	Davies (66)
	0.46	15.14	0.030	Mellander (203)
Mouse (Mus musculus)	1.2	15.34	0.078	Ross & Moore (246)
Rabbit (Oryctolagus cuniculus)	0.54	15.1	0.036	Alais & Jolles (5)
	0.49	14.9	0.033	" "
Rat (Rattus norvegicus)	-	-	0.062	Sloan et al. (255)
Sheep (Ovis aries)	0.86	15.35	0.056	Alais & Jolles (5)
	0.81	15.71	0.052	Davies (66)
Wild ass (Equus asinus)	1.06	16.28	0.065	Davies (66)

*Values have been calculated by the present author based on original authors' results, except for Sloan et al., in which case the value is that cited by those workers.

Table 4

Phosphorus-nitrogen ratio of caseins from milks of different species

Sample	Ratio, P/N
A. Caseins	
Caribou (Rangifer tarandus), Sample No. 2	0.065
3	0.071
Fin whale (Balaenoptera physalus), Sample No. 1	0.126
5	0.078
Harp seal (Phoca groenlandicus)	0.061
Moose (Alces alces), Sample No. 1	0.054
2	0.051
3	0.053
Musk-ox (Ovibos moschatus)	0.058
Polar bear (Thalarctos maritimus), Sample No. 1	0.068
Reindeer (Rangifer tarandus), Sample No. 1	0.049
2	0.046
3	0.051
Cow (Bos taurus), Sample No. 1	0.058
2	0.058
Horse (Equus caballus)	0.054
Pig (Sus scrofa)	0.065
B. Precipitates	
Dall sheep (Ovis dalli dalli)	0.173
Polar bear "D"*	0.117

*See footnote marked (***) in Table 2.

Table 5

Composition of Arctic wolf milk fractions*

	Fraction	Nitrogen (%)	Hexose** (%)
A	Casein	1.10	***
B	Emulsion solids	5.06	57.50
C	Precipitate (from ether phase)	12.96	3.05
D	Whey solids	6.47	42.63

*Refer to Figure 2 for explanation of various fractions. The letters A, B, C, D correspond to the letters A, B, C, D in Figure 2. Results of duplicate analyses.

**Hexose determined by Method I.

***Insufficient sample available for analysis.

Table 6

Composition of fin whale (Sample No. 5) milk fractions*

	Fraction	Nitrogen (%)	Hexose** (%)	Hexosamine (%)	Sialic Acid (%)
A	Casein	15.95	3.17	1.12	0.10
B	Precipitate***	14.87	0.78	0.15	0.08
C	Emulsion solids	10.76	3.33	0.74	0.28
D	Precipitate (protein insoluble at pH 6.5)	9.29	3.50	0.63	0.16
E	Whey solids	13.00	15.93	-	-

*Refer to Figure 3 for explanation of various fractions. The letters A, B, C, D, and E correspond to the letters A, B, C, D, and E in Figure 3. Results of duplicate analyses.

**Hexose determined by Method I.

***Precipitate obtained on initial centrifugation of milk.

CHAPTER IV

ELECTROPHORESIS OF MILK PROTEINS

1. Literature Review

1.1. CASEIN

Many workers have used the electrophoretic technique to study the nature of the various components of the casein complex. The techniques that have been employed are, in the order of their chronological use in casein studies, the following: (a) free moving-boundary electrophoresis, (b) filter paper electrophoresis, (c) starch-gel (vertical and horizontal) electrophoresis, (d) polyacrylamide (plate) gel electrophoresis, and (e) polyacrylamide-gel disc electrophoresis.

(a) Early Work on Casein and Paper Electrophoresis

The first indication that bovine casein comprised three electrophoretic fractions (α-, β-, and γ-caseins, in the order of decreasing mobility) was made by Mellander (202) using free electrophoresis. Further work by Warner (304) did not offer conclusive evidence as to the presence

of γ-casein. Mellander (203) showed in a later paper, that the electrophoretic pattern of human milk casein is similar to that of bovine casein in that the three fractions (α-, β-, and γ-caseins) are present. The α-casein which migrated the farthest, contained more phosphorus (0.95%) than the original whole casein (0.46-0.47%). The presence of γ-casein was later confirmed by investigations of Hipp, Groves, Custer, and McMeekin (125)(126). Excellent reviews of early work on milk proteins have been written by McMeekin and Polis (196) and by Lindqvist (171).

Libbey and Ashworth (168) were among the early workers to employ paper electrophoresis as a means to separate casein. These workers obtained three fractions when casein was subjected to paper electrophoresis at pH 12 employing a KOH-K_2HPO_4 buffer. Based on the areas below the scanning curves, the authors obtained the following relative distribution of casein fractions: α-K, 50%; β, 45%; and γ, 5%.

MacRae and Baker (178) developed a method, based on filter paper electrophoresis, to separate α-, β-, and γ-casein. These authors estimated the dyed proteins on the paper by direct photometry.

Payens (226) and Sode-Mogensen and Lahav (263) recognized that the limitation afforded by paper electrophoresis

was the strong interaction between the paper and the various components of casein. The former author repeated work previously done by Vlodavets and Zhdanova and cited by d'Yachenko (75) in which urea was included in the electrophoresis buffer to reduce interaction between casein components during paper electrophoresis. Payens noted that in the absence of urea, no separation of the casein components was observed. When urea was added to the buffer solution, two well-defined bands appeared; the first band was probably the α-K casein complex, and the second, β-casein. The reasons cited for MacRae and Baker's observations of three bands (α-, β-, and γ-casein) were: (a) the low temperature employed (4°C), and (b) the relatively high pH value of the buffer (pH 8.6). Both these parameters were known to reduce the interaction between casein components (277)(300). Payens stated that MacRae and Baker's α-fraction was probably the undisturbed α-K complex. Payens demonstrated by ultracentrifugation and free electrophoresis that his slowest moving component consisted of two sub-fractions, the first of which was calcium-sensitive (e.g. flocculated by calcium ions)(α_s-casein) and the second of which was insensitive to calcium ions (K-casein).

(b) Starch-Gel Electrophoresis

Wake and Baldwin (303) observed several new components of casein upon starch-gel electrophoresis in concentrated solutions of urea. Most of the new components were found in the α-fraction of the casein; the β-fraction was relatively homogeneous.

McMeekin, Hipp, and Groves (195) described an improved method of preparing α-casein. In addition, these workers were able to separate and characterize by moving-boundary electrophoresis its major component which they designated α_1-casein. Waugh and Von Hippel (311) had previously separated α-casein into calcium-sensitive (α_s-casein (306)) and calcium-insensitive (crude K-casein) fractions. The calcium-sensitive fraction (α_s-casein) was insoluble in calcium chloride whereas the calcium-insensitive fraction (K-casein) was soluble in calcium chloride. Casein fractions other than α_s-, crude K-, or β-casein were assigned the designation "m-fraction" (308). Long, Van Winkle, and Gould (172) referred to their calcium-sensitive fraction of α-casein as α_R-casein. The first revision of the Report of the Committee on Milk Protein Nomenclature, Classification, and Methodology of the Manufacturing Section of the A.D.S.A. for 1958-59 (50) failed to resolve the confusion arising

from names assigned to various α-casein fractions by different workers.

Waugh, Ludwig, Gillespie, Melton, Foley, and Kleiner (309) employed a DEAE-cellulose column and a 4.5 M urea buffer to separate α_s-casein and later showed by starch electrophoresis, that each of the two fractions were heterogeneous. The first fraction which was eluted was termed $\alpha_{s1,2}$-casein as it consisted of α_{s_1}- and α_{s_2}-casein, the former having the greater mobility. The second fraction was called "α_s-casein fractions" (which had unfortunately been termed α_{s_1}-casein in the earlier paper (307)). Lindqvist (171) postulated that the α_{s_1}-caseins were probably two of the genetic variants of α_s-casein reported by Thompson, Kiddy, Pepper, and Zittle (290) and Kiddy, Johnston, and Thompson (149). Both groups of workers used starch-gel electrophoresis.

Wake and Baldwin (303) observed about 20 components in a single starch-gel electropherogram of casein. They eliminated the possibility that some of the bands were artifacts rather than actual protein components in the milk. They prepared casein by the following methods: (a) acid precipitation at 30°C, (b) Na_2SO_4 (20 g/100 ml skim-milk) precipitation (with subsequent removal of globulins

and peptone) at 40°C, and (c) by the method of Waugh and Von Hippel (311) which yielded "first cycle soluble casein." The authors then showed by starch-gel electrophoresis that the caseins prepared by the three methods yielded the same patterns. They demonstrated further that all the bands produced by whole casein could be found in the patterns of α-, β-, and γ-fractions. They concluded that there is always a unique correspondence between a band and a chemical component. Furthermore, most of the preparations of α-, β-, and γ-caseins were heterogeneous. This work, and subsequent work of Neelin, Rose, and Tessier (219), which involved exposure of casein to concentrated solutions of urea, led Manson (184) to believe that the free amino groups of casein might be susceptible to attack by cyanate (urea forms an equilibrium mixture with ammonium cyanate in aqueous solutions) to form carbamoyl derivatives. This author found that treatment of α_s-casein with an aqueous solution of urea reduced the number of free amino groups normally found in α_s-casein by 29%. The amount of reduction indicated that both the ε-amino group of the lysyl residues and the N-terminal arginyl amino group had reacted, since complete substitution of the latter would have caused a reduction of only 6%. The author showed that considerable reaction

occurred between the -amino group of the lysyl residues and this resulted in the production of homocitrulline derivatives. Earlier work by Stark, Stein, and Moore (271) showed that urea rendered a protein (ribonuclease A) less basic. They also discovered that cyanate reacts even more rapidly with -SH groups than with amino groups and that treatment of cysteine with an equimolar amount of cyanate resulted in the formation of S-carbamylcysteine. Stark, Stein, and Moore (271) pointed out that at equilibrium, an 8 M solution of urea (starch gels and polyacrylamide gels are usually 5-7 M) yields at equilibrium a 0.02 M solution of cyanate.

Groves, McMeekin, Hipp, and Gordon (114) developed a method of preparing β- and -casein consisting of fractionation of that portion of casein which is soluble at pH 4 at 2°C, followed by chromatography on ion-exchange cellulose. Starch-gel electrophoresis of β- and -casein fractions revealed that they possessed a higher degree of homogeneity than β- and -caseins prepared by previous methods (125)(126)(304).

Gehrke, Freeark, Oh, and Chun (92) prepared β-casein by chemical fractionation and subsequent purification by column chromatography on DEAE-cellulose. Their procedure involved the fractionation of isoelectric casein by the use

of urea to yield the (α_s-K)-, β-, and γ-fractions. The β-casein fraction was isolated by ammonium sulphate precipitation. The procedure effected a partial separation of γ-casein and β-casein. Analysis of the fractions obtained by chromatography (DEAE-cellulose) revealed two electrophoretically distinct β-caseins which the authors named β_{slow} and β_{fast}. The β_{slow}-casein was the major component. The authors suggested the β_{fast}-casein might be a sub-component of λ-casein.

Waugh and Von Hippel (311) were the first workers to demonstrate that K-casein is responsible for micelle stabilization and is the protein which acts as a substrate for rennin action. Previous work by Waugh and Noble (310) and Noble and Waugh (222) had established that casein micelles consisted, in simplest form, of cores of calcium α_s-caseinate covered by a uniform coat of (low weight ratio) calcium (α_s-K)-caseinate. This work led to new methods of preparation of K-casein.

McKenzie and Wake (193) developed an improved method for the isolation of K-casein using acid casein as a starting material. Starch-gel electrophoresis revealed that K-casein obtained by this procedure had a mobility which was identical to that of the rennin-sensitive fraction of whole

casein.

Neelin, Rose, and Tessier (219) separated acid casein into at least 17 zones using starch-gel electrophoresis. The authors also prepared α-, β-, and Υ-casein by the method of Hipp, Groves, Custer, and McMeekin (126), Υ-casein by the method of Swaisgood and Brunner (281), and K-casein by the methods of Morr (212) and Swaisgood and Brunner (281). The authors analyzed the different fractions by starch-gel electrophoresis and observed that all fractions contained numerous components. However, they did tentatively identify the different zones with certain specific casein fractions. The method of Swaisgood and Brunner (281) yielded the K-casein having the highest stabilizing power for casein micelles. However, the fraction was contaminated with many minor electrophoretic components. The method of Morr (212) gave a similar product. The method of McKenzie and Wake (193) yielded a K-casein which was less contaminated with fast-moving components, but the product had low stabilizing power for casein micelles. Although this K-casein preparation was least contaminated with other casein components, there were some indications that it was partially denatured.

Further work by Neelin (217) established a method for the identification of K-casein by starch-gel electrophoresis. The method was based on the fact that calcium

ions cause α_s-casein (the calcium-sensitive fraction of the α-casein complex) and β-casein to precipitate. The method involved two-dimensional starch-gel electrophoresis in which Ca^{++} was added to the gel when electrophoresis was run in the second dimension.

K-casein has a molecular weight similar to that of α-casein and β-casein when completely disaggregated. The molecular weight increases by a factor of 10 in the absence of urea (226). Neelin (217) subjected K-casein to starch-gel electrophoresis in concentrated urea and 12 zones were produced with mobilities similar to those of the various components of acid casein. When the electrophoretically separated components of K-casein fractions were further subjected to starch-gel electrophoresis in a second dimension in the absence of urea, the fastest zone behaved normally, but the slow-moving K-zone formed an intense band near the origin.

Peterson (228) and Thompson, Kiddy, Pepper, and Zittle (289)(290) had some success in distinguishing variant forms of α_s-casein and β-casein using gel electrophoresis in concentrated urea solutions. Neelin (218) postulated that small amounts of sulphur-containing amino acids in K-casein (140) might give rise to intermolecular disulphide bridges and thus cause unsatisfactory resolution of

variant forms of K-casein. Furthermore, Swaisgood, Brunner, and Lillevik (284) suggested that the basic unit of K-casein was composed of two sub-units (MW=28,000) joined by disulphide bonds, or that the two sub-units were joined by secondary bonds which depended on the disulphide bonds to maintain a particular tertiary structure for the interaction. The addition of mercaptoethanol to the starch gel led to the resolution of K-casein into two zones which occurred singly or in combination.

Schmidt (250) investigated the influence of 2-mercaptoethanol on the starch-gel pattern of K-casein. The author found that the addition of 2-mercaptoethanol led to a satisfactory resolution of the diffuse K-casein zone into a large number of well-defined bands. A fraction which flocculated readily with rennin in the absence of Ca^{++}, was isolated by chromatographic purification in the presence of mercaptoethanol of K-casein obtained by the method of McKenzie and Wake (193).

Schmidt, Both, and de Koning (251) fractionated K-caseins A and B by column chromatography (DEAE-cellulose) using buffer solutions which contained 2-mercaptoethanol. The author used starch-gel electrophoresis in the presence of urea and mercaptoethanol to check the purity of each fraction.

El-Negoumy (77) developed a procedure in which casein was precipitated from milk (acetate buffer; pH 4.6), dispersed in a solution of urea (7 M), and impregnated into strips of filter paper. The filter paper strips were dried and stored for periods up to two years. Insertion of the paper strips into the slots of a starch gel and subsequent separation of the casein indicated that no alteration of the electrophoretic properties of the casein occurred, and sharp bands corresponding to α_s-, β-, and K-caseins were obtained.

Yaguchi and Tarassuk (324) prepared acid casein from the milk of an individual Holstein cow and subjected it to starch-gel electrophoresis. These authors found that the casein contained α_{s_1}-casein B, β-casein A, and K-casein A. The sodium caseinate was fractionated on a column of Sephadex G-200 both in the absence and presence of 6 M urea in the elution buffer. They noted that relatively pure K-casein emerged from the column in the void volume, when urea was used. This fact led Yaguchi, Davies, and Kim (323) to the development of a method for the preparation of K-casein by gel filtration.

The action of rennin on whole casein and K-casein was further studied by El-Negoumy (79) who used high-resolution starch-gel electrophoresis. The author found that K-casein

was the only component of acid casein that was attacked after 50 minutes of enzyme action and that the α_s- and β-caseins remained unchanged. Electrophoretic analysis of the rennin-treated acid casein showed the presence of one major and two minor components with faster mobilities than α_s-casein and two minor components with slower mobilities than K-casein. Electrophoretic analysis of K-casein that had been treated with rennin for 60 minutes revealed the presence of at least 11 breakdown components of para-K-casein.

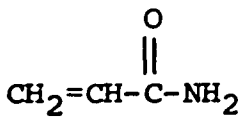
Ganguli and Majumder (90) developed a simple apparatus and procedure for electrophoresis of milk proteins on starch gel in the presence of urea and mercaptoethanol. The apparatus consisted essentially of a petri dish as the gel support and two beakers as the buffer reservoirs. The procedure eliminated the necessity of slicing the gel before staining. The authors obtained two major bands corresponding to α_s- and β-caseins and several minor bands including K-casein, when whole casein was analyzed by their method.

The review on starch-gel electrophoresis that appeared as a National Research Council of Canada publication (6), the bibliography produced by the Connaught Medical Research Laboratories (58), the review by Smithies (261), and the

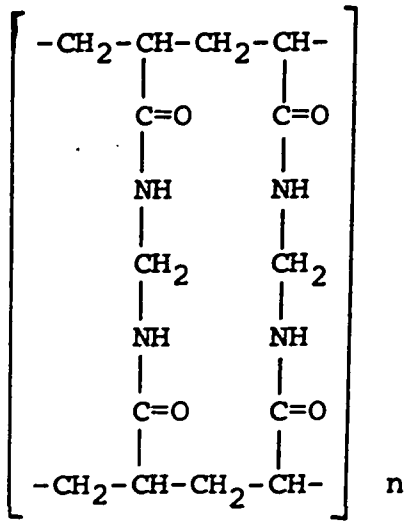
excellent book by Smith (257), are valuable sources of information on starch-gel electrophoresis.

(c) Polyacrylamide (Plate) Electrophoresis

Raymond and Wang (237) described the preparation and properties of acrylamide gels and indicates that these gels might replace starch gels in the electrophoretic analysis of proteins. They showed that the gel is a linear polymer of acrylamide, cross-linked by means of methylene bridges:



acrylamide



The three-dimensional structure, which consists of long hydrocarbon chains is made hydrophilic by the presence of amide groups. There are no hydroxyl or carboxyl groups in the molecule as is the case in starch.

Peterson (228) was one of the earlier workers to recognize the high resolving power of polyacrylamide electrophoresis. He cited the following advantages of

electrophoresis in acrylamide gels over electrophoresis in starch gels: (a) acrylamide gels are easier to prepare than starch gels since they can be used 20 minutes after mixing and do not require heating during preparation and slicing prior to staining; (b) there are no free carboxyl groups such as are found in starch gels which might interfere with the migration of some proteins; and (c) higher voltages can be employed with the cell used for acrylamide electrophoresis (235)(236) than with the flat cell described by Smithies (259)(260) for starch-gel electrophoresis. Peterson obtained bands for all the six phenotypes of β-casein listed by Aschaffenburg (13) (e.g. A/A, A/B, A/C, B/B, B/C, and C/C) who previously used paper electrophoresis. He also obtained bands for all the six phenotypes of α_s-casein described previously by Thompson, Kiddy, Pepper, and Zittle (290) who used starch-gel electrophoresis.

Aschaffenburg (16) employed electrophoresis in polyacrylamide gels (veronal buffer) to phenotype whole milk. For β-lactoglobulin typing, the milk samples were applied without dilution (Conditions: current, 12.5 mA/gel; initial voltage, 130 V; time, 6 h). For casein typing, the samples were applied after dilution (1 volume of milk + 5 volumes of urea-containing buffer (5 M urea)(Conditions: current, 8 mA/gel; initial voltage, 90 V; time, 16 h). In the absence of

urea, the β-lactoglobulins were the fastest-moving components when whole milk was subjected to electrophoresis. The clear resolution of the β-lactoglobulins made phenotyping easy. Unless urea was present, the resolution of the remaining milk proteins was extremely poor. The presence of urea caused the α_s- and β-casein components to form well-defined bands and the β-lactoglobulins to form weak bands in front of the β-caseins.

Polyacrylamide-gel electrophoresis, like starch-gel electrophoresis, has been used extensively in studies on K-casein. Early workers showed that K-casein migrated as a broad unresolved zone in starch-gel electrophoresis (303). Woychik (318) observed similar electrophoretic behavior of K-casein in polyacrylamide gels containing 5% cyanogum. However, in polyacrylamide gels containing 7% cyanogum, the protein was almost totally retained in the sample slot. The high molecular weight of the K-casein complex caused it to remain at the origin due to the sieving effect exerted by the polyacrylamide gel of high cyanogum concentration. Swaisgood and Brunner (282) showed that the molecular weight of K-casein decreases markedly when K-casein is subjected to alkaline conditions. The same phenomenon was observed when K-casein was reduced with 2-mercaptoethanol. This indicated

that disulphide bonding plays an important role in the formation of high molecular weight K-casein. Woychik (320) confirmed the existence of two major K-casein components after having reported previously the presence of three components (319)(321) in reduced whole caseins.

Purkayastha and Rose (233) subjected K-casein to polyacrylamide-gel electrophoresis in the presence of urea and mercaptoethanol and they obtained three prominent bands. The gels were stained first with periodic acid-Schiff's reagent to locate the carbohydrate-containing proteins and then with Amido Black 10B to stain all proteins. The greater mobility of the K-casein was ascribed to the increased charge on it due to the presence of sialic acid. Enzymic removal of the sialic acid from the glycoprotein by neuraminidase decreased the mobility of the glycoprotein. The carbohydrate-containing components were very labile and prolonged storage of freeze-dried K-casein gave rise to a material resembling glycomacropeptide which is a non-dialyzable substance soluble in 12% TCA formed by the action of rennin on K-casein (2)(221).

Kalan and Woychik (144) studied further the action of rennin on K-casein. Electrophoretic analysis (polyacrylamide; pH 9.2) of reduced para-K-casein showed the presence of two

major components.

Kim, Yaguchi, and Rose (151) treated K-caseins A and B with rennin and the corresponding para-K-caseins were separated chromatographically (carboxymethylcellulose) in the presence of mercaptoethanol and urea. They obtained one major and two minor para-K-casein components. The minor components were not observed when para-K-casein was fractionated in the absence of urea. Further observations showed that homocitrullin was present only in the minor components and the major component was richer in lysine. These findings indicated that the heterogeneity in para-K-casein was not due to a "two-point cleavage" by rennin as was thought previously (177)(322), but resulted from the carbamylation of lysine by cyanate which occurred in the urea solution. Polyacrylamide-gel electrophoresis revealed that homocitrullin-free para-K-caseins A and B were identical. This finding was contrary to the results of other workers (79)(144)(176)(322)(323). Kim, Yaguchi, and Rose also showed that the amino acid compositions of the two para-K-caseins were identical. Cole and Mecham (57) substantiated the earlier work of Manson (184) and showed that carbamylation also takes place in polyacrylamide gels containing urea. The end result of carbamylation is to render proteins less basic, since carbamylated residues are neutral.

Yaguchi, Davies, and Kim (323) prepared K-casein from unreduced casein by Sephadex gel filtration using a 0.005 M tris-citrate buffer (pH 8.6) containing 6 M urea. Two fractions (I and II) were eluted from the column. Polyacrylamide-gel electrophoresis indicated that Fraction I was a K-casein of high purity and Fraction II contained very little K-casein. Hill (121) suggested that urea splits K-casein, despite the fact that the above workers reported the absence of para-K-casein components in acid casein fractionated in the presence of urea.

Melachouris and Tuckey (199) used polyacrylamide-gel electrophoresis to study changes that occurred in milk proteins during the ripening of cheddar cheese made from milk heated at different temperatures. At the end of the ripening period (190 days), the electropherograms revealed that the α_s-fraction had been degraded to form seven components, and β-casein had degraded to form six components. No qualitative differences were observed in any of the electrophoretic patterns of proteins from cheeses made from milk which had undergone the different heat treatments (61.7°C for 30 min; 93.3°C, 110.0°C, and 126.7°C for 2.08 sec). The authors concluded that the changes in the α_s- and β-caseins were the result of ripening process.

Melachouris (198) subjected whole acid casein to discontinuous polyacrylamide-gel electrophoresis in the presence of urea and in the absence of mercaptoethanol, and observed twenty well-defined zones. Components which had lower mobilities than β-casein or faster than α_{s_1}-casein appeared as sharp zones. The author also compared electrophoretic patterns obtained by the use of Amido Black and Coomassie Blue for staining. The zones were sharper and the casein components of low concentration appeared more distinct when Coomassie Blue was used. There was less contrast, however, between the components of high and low concentration.

(d) Polyacrylamide-gel disc electrophoresis

Ornstein (224) developed the theory of disc electrophoresis and Davis (67) illustrated its application to the separation of human serum proteins. The main features of this technique may be summarized as follows: (a) individual samples are subjected to electrophoresis in gels supported by glass tubing; (b) the gel has a small cross-sectional area and hence, heat production and thermal gradients, such as those which occur in thick slabs, are reduced; (c) relatively high voltage gradients can be used because of the low heat production and small temperature gradients, and hence,

resolution of a mixture can be effected in a shorter time; and (d) high resolution is achieved because of the possibility of concentration of the starting zones. The process of "steady-state stacking" involves the following: the proteins concentrate themselves into very thin discs behind the chloride ion, one stacked on top of the other in the order of their decreasing mobility. The last disc is followed immediately by glycine. The stacking process occurs in the "spacer gel" (located between the "sample gel" and the smaller pore "running gel". Conditions are arranged such that when the chloride boundary (followed by a stack of discs) passes into a region of smaller pore size (the "running gel"), the mobility of the fastest protein drops below that of glycine, and hence, the glycine overruns the protein discs and runs directly behind the chloride ion. The result is the formation of a sharp boundary at the chloride ion interface. The proteins are then in a uniform, linear-voltage gradient, each in its own very thin starting zone. The proteins are then allowed to migrate as in ordinary zone electrophoresis. Because of the viscous properties of the gel, proteins of equal free mobility, but of different molecular weights (different diffusion constants), will migrate with different mobilities and thus a separation will be effected

(67)(224)(257). The original running gel buffer of tris-HCl (pH 8.6) is changed to a tris-glycine buffer (pH 9.5). The migration of the proteins thus takes place at a higher pH. At the same time, the mobility of the glycinate ion increases as it enters the higher pH range (257).

As far as the author is aware, Hartman and Swanson (117) were the first workers to subject milk proteins to disc electrophoresis. These workers were interested in the changes which took place in mixtures of whey proteins and K-casein when they were heated. McGugan, Zehren, Zehren, and Swanson (191) had shown earlier that when a mixture of casein and β-lactoglobulin was heated (85°C; 30 min; pH 6.9), the β-lactoglobulin was converted to a form which moved with α-casein during moving-boundary electrophoresis (pH 6.9 and 2.45). Hartman and Swanson (117) heated (74.5°C; 30 min) acid whey and β-lactoglobulin in the presence and absence of K-casein. They found that K-casein and β-lactoglobulin interacted and the size of the molecular aggregate prevented the complex from migrating into the small pore region of the polyacrylamide gel. A complex did not form with α-lactalbumin or bovine serum albumin when they were heated with K-casein. The authors noted that the acrylamide disc electrophoretic technique did not separate bovine serum albumin and α-lactalbumin under the conditions described by Davis (67).

The acrylamide concentration was increased to 9% in the small pore gel, and the ionic strength of the tris-HCl buffer (pH 8.9) in the small pore gel was increased from 0.082 to 0.11. The mobility of bovine serum albumin was thus reduced due to its larger molecular size, and the two proteins were separated satisfactorily.

Grindrod and Nick (109) used polyacrylamide-gel electrophoresis to study the effect of hydrogen peroxide on the proteins of skim milk. It should be mentioned that hydrogen peroxide is used (56) as a bactericide in milk used in cheese manufacture and is suggested for use in improving milk quality in developing countries (8)(296). Hydrogen peroxide treatment caused the migration rate of α_s-casein to increase and the migration rate of β-casein to decrease. The migration rate of K-casein was unchanged by the peroxide treatment.

Groves and Kiddy (113) modified the system of polyacrylamide-gel disc electrophoresis recommended by Davis (67) to make possible a study of polymorphism in Υ-casein. Their modification involved essentially the incorporation of urea into the gels. They noted two forms of Υ-casein which they designated as A and B, and reported three phenotypes of Υ-casein (A, AB, and B). The authors used the same convention of nomenclature as was used previously for α_s-

and β-casein; namely, the protein of highest mobility was designated as the A component. In samples where the A form occurs (A and AB phenotypes), another band designated R was found which had a mobility between that of the A and B bands of Y-casein. The authors demonstrated that this was not a Y-casein. The Y-casein AB samples showed a prominent slow-moving band which they designated as S in the unfractionated casein. Further work by Groves (111) and Groves, Gordon, and Kiddy (112) has shed further light on the polymorphic forms of Y- and β-caseins.

Akroyd (1) has written a general article on the application of polyacrylamide-gel disc electrophoresis to the study of milk proteins.

(e) Genetic Polymorphism in Casein

Aschaffenburg (13)(14) used paper electrophoresis to provide the first evidence for variation in the caseins isolated from milks of individual cows. He noted that β-casein existed in three forms, which he termed A, B, and C. Thompson, Kiddy, Johnston, and Weinberg (288) employed starch-gel and polyacrylamide-gel electrophoresis to confirm Aschaffenburg's findings. These workers showed that the A and B alleles occur in Jersey and Holstein cattle, the A, B, and

C alleles occur in Guernsey and Brown Swiss cattle, and only the A allele occurs in Ayrshire cattle.

One year after Aschaffenburg's discovery, Thompson, Kiddy, Pepper, and Zittle (290) observed two polymorphic forms of α_{s_1}-casein, namely the A and B variants. Kiddy, Johnston, and Thompson (149) confirmed the existence of three forms of α_{s_1}-casein which they designated as A, B, and C in the order of decreasing mobility. Grosclaude, Pujolle, Garnier, and Ribadeau-Dumas (110) and Aschaffenburg, Sen, and Thompson (23) offered evidence for the existence of a fourth variant designated as D.

Neelin (218), Woychik (319), and Schmidt (250) reported simultaneously genetic variation in K-casein. Neelin and Schmidt observed only the A and B variants, but Woychik reported the existence of A, B, and C variants. It is now acknowledged that there are only two variants of K-casein, namely, A and B.

El-Negoumy (78) and Groves (111) were the first workers to report genetic variation of Y-caseins. Their work has already been discussed in a previous section of this chapter.

Aschaffenburg (16) and Aschaffenburg and Thymann (24) developed procedures for phenotyping by direct electrophoresis of the whole milk. The first method (16) involved the

use of polyacrylamide gels and required one run to type the β-lactoglobulins, and another run, using urea, to type the α_s- and β-caseins. The second method (24), which employs starch gels, permitted the simultaneous separation of α_s-, β-, and K-caseins, and the β-lactoglobulins. Neither of the methods is suitable for typing α-lactalbumins, as they are masked by the more prominent α_{s_1}-caseins. This is not a serious drawback, however, since genetic variation of α-lactalbumins is restricted to African Zebu cattle.

Thompson (286) indicated that α_{s_1}- and β-caseins can be detected readily by zone electrophoresis methods and that polyacrylamide-gel electrophoresis is superior to starch-gel electrophoresis in resolving the β-casein variants.

Excellent review articles on methods for typing milk protein variants have been written by Aschaffenburg (17)(19), Thompson (287), and Groves (111).

1.2. WHEY PROTEINS

Vandegaer and Miettinen (297) studied the effect of heat on whey proteins. Whey proteins were isolated from untreated milk, milk that had been pasteurized (172°C; 15 sec), and milk that had been sterilized (119°C; 25 min).

Paper electrophoresis resolved the whey proteins from the unheated and the pasteurized milk into five components and from the sterilized milk into one diffuse zone. Brown, Aurand, and Roberts (49) and Melachouris and Tuckey (200) studied the sensitivity to heat of β-lactoglobulin.

Larson and Jenness (158) identified α-lactalbumin in the moving-boundary electrophoretic patterns of whey proteins. This protein was identified by addition of pure α-lactalbumin to the whey protein mixture and observation of the peak area that was enhanced by this addition.

Schulte and Müller (252) prepared whey proteins from cow's milk and subjected the protein to paper electrophoresis (sodium veronal-oxalic acid buffer; pH 7.9). Ninhydrin was used to detect the bands. The β-lactoglobulin zone was located between the α- and β-casein and was masked considerably by them. Two slow-moving components were identified as immunoglobulins. A component not previously identified which moved slower than β-lactoglobulin but faster than the immunoglobulins was identified as α_3-lactoglobulin.

Aschaffenburg and Drewry (20) subjected whey proteins to paper electrophoresis (barbitone buffer; pH 8.6) and noted that individual cows produce either a mixture of two β-lactoglobulins which are electrophoretically distinct, or

the individual β-lactoglobulin components. They named these components β_1- and β_2-lactoglobulins and noted that the type of β-lactoglobulins produced was characteristic for a given cow, which indicated that the synthesis of these β-lactoglobulins was genetically controlled.

Aschaffenburg and Drewry (21) proposed that the nomenclature of the lactoglobulins be changed to reflect current genetical usage. The faster-moving component, β_1, was named β-lactoglobulin A; the slower, β_2, β-lactoglobulin B; and the mixture, $\beta_{1,2}$, β-lactoglobulin AB. The authors suggested that there was no simple or obvious relationship between blood groups and β-lactoglobulin type and that the gene which controlled the formation of milk proteins was probably unrelated to that governing haemoglobin synthesis. They also suggested that the concentration of each of the β-lactoglobulins varied with the casein content of the milk which indicated some type of quantitative relationship between β-lactoglobulin and casein synthesis. Further work on this problem has been done by Kiddy, Townend, Thatcher, and Timasheff (150).

Blumberg and Tombs (44) suggested possible genetic polymorphism of the α-lactalbumins in the milks of Nigerian cattle (White Fulani and Lyre-horned Zebu). They assigned

the names α_A- and α_B-lactalbumins to the fastest- and slowest-moving lactalbumins in paper electrophoresis. The authors noted that the faster-moving α_A-lactalbumin was not found in British or Icelandic breeds of cattle.

Weigt (312) subjected the whey produced by acid precipitation of casein, to paper electrophoresis (veronal buffer; pH 8.6) and identified the following whey proteins: albumin, β-lactoglobulin, α-lactalbumin, pseudoglobulin, and euglobulin.

Bell (34) separated the whey proteins of untreated skim milk by starch-gel electrophoresis. The colloidal casein of milk did not enter the gel within the time that the inserts remained in the gel. He obtained satisfactory separation of serum albumin, α-lactalbumin, and β-lactoglobulin A and B. This author also discovered a new β-lactoglobulin variant, C, which was later shown by Larson and Hageman (157) to be immunologically similar to β-lactoglobulins A and B.

Morr and Kenkare (213) provided additional evidence for the heterogeneity of β-lactoglobulin by the use of polyacrylamide-gel disc electrophoresis and Sephadex gel filtration. Four major bands were obtained by disc electrophoresis and three major bands were obtained by gel filtration of

crystalline β-lactoglobulin.

Review articles on the genetic variants of whey proteins have been published by Bell and McKenzie (35), Kiddy (148), Thompson (286), and Aschaffenburg (17)(19) and the techniques of electrophoresis of whey proteins have been reviewed by El-Negoumy (77), Peterson (228), Melachouris (198), McKenzie and Sawyer (192), and Akroyd (1).

1.3. NOMENCLATURE OF MILK PROTEINS

Nomenclature of milk proteins has been the subject of four reports of the Committee on Milk Protein Nomenclature, Classification, and Methodology of the Manufacturing Section of the American Dairy Science Association (50)(136)(245)(291).

The first Committee on Nomenclature which published its report in 1956 (136), recognized that the classical nomenclature of the milk proteins in terms of casein, lactalbumin, and lactoglobulin, was no longer adequate. The report clarified milk protein nomenclature by presenting a summary of "preferred" usage and it showed the relationship between individual proteins that had been isolated and classical fractions. Table 7 is an excerpt from this first report.

Table 7

Relationship of "preferred" nomenclature to "classical" nomenclature of caseins and whey proteins

Preferred nomenclature	Classical fraction from which isolated
α-Casein	Casein
β-Casein	Casein
γ-Casein	Casein
β-Lactoglobulin	Lactalbumin
α-Lactalbumin	Lactalbumin
Blood serum albumin	Lactalbumin
Immune globulins	
Euglobulins	Lactoglobulin
Pseudoglobulin	Lactoglobulin

The second report (First Revision) of the Committee on Nomenclature was published in 1960 and dealt mainly with terminology that had been introduced by various investigators to designate the components of the α-casein fraction and the β-lactoglobulin fraction. The report recommended that the α-casein component of the casein system of milk be referred to as the α-casein fraction because of its heterogeneity. The authors of this report did not adopt a system of nomenclature of the components of the α-casein complex

because of the complexity of this fraction and because of the conflicting evidence as to the possible sub-fractions that were present in the α-casein complex. The authors deemed it desirable to postpone recommendations for the nomenclature of the α-casein complex until the nature of the complex was more completely understood. Table 8 is a list of sub-fractions of the α-casein fraction based on the data of Brunner, Ernstrom, Hollis, Larson, Whitney, and Zittle (50).

Table 8

Components of α-casein

Name of components	Reference
Ca-sensitive component	
α_s-Casein	(311)*
α_1-Casein	(194)*
α_R-Casein	(172)*
Ca-insensitive components	
K-Casein	(87)(311)**
α_2- or α_z-Casein	(194)**
α_3-Casein	(127)**
λ-Casein	(172)***
m-Casein	(306)(308)***

*Similar characteristics suggest that proteins are similar
**Similar characteristics suggest that proteins are similar
***Similar characteristics suggest that proteins are similar

The committee recommended that "para-K-casein" should be used to designate the primary product of rennin action on K-casein and "α_s-para-K-casein should be used to designate the clots which are formed by the action of rennin on the α_s-casein fraction. The major change in the nomenclature of whey protein recommended in the Second Report involved the nomenclature of the β-lactoglobulins. It was recognized that β-lactoglobulin existed in two forms which were genetically controlled and discernible by paper electrophoresis at pH 8.6. The committee accepted the nomenclature of Aschaffenburg and Drewry (21) for β-lactoglobulin A (the faster-moving component) and β-lactoglobulin B (the slower-moving component).

The third report (Second Revision) of the Committee on Nomenclature was published in 1965 (291), and dealt primarily with genetic polymorphism in the proteins of bovine milk. It recommended that the β-lactoglobulins be referred to as β-lactoglobulins A, B, and C because of the discovery of the C variant by Bell (34). The three genetic forms of β-casein, which were reported to exist either singly or in pairs (14) (288), were designated β-caseins A, B, and C. The committee recommended that the genetic variants (A, B, and C) (149) (289)(290) of the principal α_s-casein fraction (α_{s_1}-casein)

be referred to as α_{s_1}-A, α_{s_1}-B, and α_{s_1}-C. The term α_{s_1}-casein was to refer to those components of the α-casein complex that were precipitated by calcium and were stabilized by K-casein. Two genetic variants of α-lactalbumin, A and B, were reported in the milks of Zebu cattle (15)(38)(44), but only the B form was found in the milk of Western breeds. The report lists definitions of all the major fractions of casein.

The fourth report (Third Revision) of the Committee on Nomenclature was published in 1970 (245) and is the last to be published to date. It incorporates the D variant along with the previously reported three variants of β-lactoglobulin. Grosclaude, Pujolle, Garnier, and Ribadeau-Dumas (110) reported an α_{s_1}-D variant in casein isolated from the milk of French Flamande cattle. The report incorporated the recent findings of Groves (111)(112)(113) and his co-workers concerning the variants of the Y- and β-caseins. The authors stated in their report that "the nomenclature of all milk proteins remains fluid and there seems little likelihood that the situation will stabilize, i.e., that discovery of additional components and variants will cease."

1.4. ELECTROPHORESIS OF PROTEINS FROM MILKS OF DIFFERENT SPECIES

Several workers have subjected casein and whey proteins from the milks of different species to electrophoretic analysis. Very little work on a comparative basis by individual workers, however, has been published. The little work that has been done did not employ the more sophisticated techniques which are available today.

Deutsch (68) subjected the whey proteins isolated from milks of the following mammals to moving-boundary electrophoresis (barbiturate-citrate buffer; pH 8.6): cow, goat, pig (Chester white), pig (Poland-China), human, sheep, and horse. The author showed that the whey proteins of the different animals gave markedly different electrophoretic patterns and, as is found with plasma proteins, each pattern was characteristic of a given species.

Foster, Friedell, Catron, and Dieckmann (86) studied, by moving-boundary electrophoresis, changes that took place in the whey proteins of sow's milk during the early stages of lactation. They observed a marked decrease in the relative concentration of the slowest-moving component during the first 24 hours of lactation.

Dovey and Campbell (71) subjected the caseins of cow,

goat, and rabbit to moving-boundary electrophoresis (veronal buffer; pH 8.6). Cow casein contained α-, β-, and Y-components but goat casein exhibited a complete absence of the Y-component and a different distribution of the α- and β-components from that of cow casein. The α-component of goat casein exhibited some heterogeneity. Rabbit casein contained much less β-casein than did the cow or the goat casein.

Schulte and Müller (253) subjected the whey proteins of goat milk and ewe's milk to paper electrophoresis. They observed that the mobility of the β-lactoglobulins of ewe's and goat's milk was lower than that of the corresponding β-caseins. The mobility of the β-lactoglobulins of cow's milk was between that of the corresponding α- and β-caseins.

Lunsford and Deutsch (175) analyzed the proteins of human milk whey by moving-boundary electrophoresis. They concluded that serum albumin is present in human milk whey to the extent of 2.5%.

Hofman (130) subjected acid casein and α-casein isolated from goat's milk to paper electrophoresis (veronal buffer; pH 7.6; no urea). The acid casein and the α-casein gave two bands in the α-casein region and were identified as α_1- and α_2-casein, according to the nomenclature of McMeekin,

Groves, and Hipp (194). Acid casein (goat) that had been previously dried with alcohol and ether did not give two bands, but only a single α-casein band.

Sowls, Smith, Jenness, Sloan, and Regehr (264) analyzed the casein and the whey of the collared peccary (Pecari tajacu) and domestic sow (Sus scrofa) by paper and by moving-boundary electrophoresis (veronal buffer; pH 8.6; no urea). The caseins of the peccary were similar electrophoretically to those of the sow. They both exhibited two major components in paper electrophoresis, the component of lower mobility being present in much higher concentration. The faster component was split into two sub-fractions by moving-boundary electrophoresis. The whey proteins of the peccary contained similar electrophoretic components to those of the domestic sow, but in different proportions. The colostrum of the collared peccary had a large proportion of slow-moving components.

Sloan, Jenness, Kenyon, and Regehr (255) analyzed by paper electrophoresis, the caseins and the whey proteins isolated from the milks of 40 species representing eight orders. They reached the following conclusions: (a) each species gives a distinct electrophoretic pattern and related species give similar patterns; (b) the caseins from

the different species may be one of three types, i.e. casein giving a single fast-moving component, casein giving a prominent slow-moving component and a faster component, and casein giving two sharply differentiated components; (c) the wheys from the different species contain components with mobility which are similar to those of blood serum albumin and the gamma globulins; and (d) wheys from the milks of primitive orders contain fewer unique protein components than do milks of more advanced orders.

Pilson and Kelly (230) prepared casein from the milk of the California sea lion (Zalophus californianus) and analyzed it by paper electrophoresis (veronal buffer; pH 8.6; no urea). They noted that the mobility of the α-casein band was exactly the same as that of bovine α-casein and that the mobility of the β-casein band was greater than that of bovine β-casein.

Aschaffenburg, Gregory, Kon, Rowland, and Thompson (22) examined the proteins of reindeer (Rangifer tarandus) milk by paper electrophoresis. These workers showed that the casein contained two major components, one with a greater mobility than that of bovine β-casein, and the other with a mobility less than that of bovine α-casein. The whey proteins were complex and comprised at least six components,

the most prominent of which had a mobility which was similar to that of bovine β-lactoglobulin B.

Rozhanskii, Sergeeva, and Kudryashov (247) subjected the whey proteins of mare milk and colostrum to paper electrophoresis. They obtained the following fractions: blood serum albumin, β-lactoglobulin, α-lactalbumin, and the immune globulins.

Prodanski and Petrov (232) isolated and analyzed (paper electrophoresis) the casein and whey proteins of the milks of the following species: cow, ewe, buffalo, and goat. They ascertained the levels of α-, β-, and Y-caseins and the levels of the major whey proteins in these milks.

Hilpert and Enkelmann (123) carried out paper electrophoretic studies on the whey proteins isolated from the milks of the following animals: cow, goat, rabbit, and guinea pig. The authors found that the electropherograms obtained with the different whey samples differed from one species to another. The most remarkable observation was the relatively large amount of β-lactoglobulin in each of the different milks and the authors showed that the amount of this protein differed widely in all species.

Baker, Bertok, and Symes (27) isolated casein and whey proteins from guinea pig milk and analyzed these protein preparations by paper electrophoresis. The casein had

two electrophoretic components, neither of which had the same mobility as the α-, β-, or γ-component of bovine casein. The "α-casein" of guinea pig milk had a mobility which was slightly greater than that of bovine α-casein and the "β-casein" of guinea pig milk had a mobility which was greater than that of bovine β-casein. The whey protein fraction contained a major component which had a mobility similar to that of bovine β-lactoglobulin.

Ganguli and Bhalerao (89) analyzed the caseins of buffalo and cow milk by paper-disc electrophoresis. The mobilities of the α-, β-, and γ-caseins of buffalo milk were lower than the corresponding fractions of bovine milk.

Rao (234) examined the non-micellular proteins of cow and buffalo milks by paper electrophoresis (veronal buffer; pH 8.6). The β-lactoglobulin component of buffalo and cow milks had the same mobility. The α-lactalbumin component of buffalo milk had a higher mobility than that of cow milk.

Ashworth, Ramaiah, and Keyes (25) analyzed by paper electrophoresis (5 M urea), the casein isolated from the milk of the Northern fur seal (Callorhinus ursinus). They observed a major band which had the same mobility as that of bovine β-casein.

Baker, Blood, and Chem (28) isolated the caseins and whey proteins from the milks of individual Rocky Mountain bighorn sheep (Ovis canadensis canadensis) and from Suffolk sheep. The caseins were analyzed by starch-gel electrophoresis (tris-citrate buffer; pH 8.6; urea added) and by polyacrylamide-gel electrophoresis (tris-borate buffer; pH 9.1-9.3; urea added). The whey proteins were analyzed by polyacrylamide electrophoresis (tris buffer; pH 9.1-9.4). Electrophoresis in starch gels showed that the caseins from the milks of bighorn sheep each contained two major components with mobilities similar to those of bovine α_s- and β-caseins. These caseins also contained components which appeared in the same region as the K-casein component of bovine milk. Electrophoresis in polyacrylamide gels revealed that the caseins from the milks of bighorn sheep contained α- and β-components which had slightly lower mobilities than did the corresponding components of bovine casein. Polyacrylamide-gel electrophoresis of the whey proteins revealed that the sheep wheys each contained a component which had the same mobility as that of bovine β-lactoglobulin. The sheep whey did not give a band in the bovine α-lactalbumin region.

Brew and Campbell (47) analyzed the whey proteins of

guinea pig milk by electrophoresis on paper, cellulose acetate, starch gel, and polyacrylamide gel and compared the electropherograms with those obtained for other milks. The authors made the following observations: the major protein component in the wheys from the ruminants buffalo, sheep, and goat corresponds to bovine β-lactoglobulin. The major component of guinea pig whey corresponds to bovine α-lactalbumin. There was no evidence for genetic variation of α-lactalbumin in guinea pig milk.

Baker, Hatcher, and Harington (29) prepared casein and the whey proteins from the milks of the polar bear (Thalarctos maritimus), human, and cow. Initial centrifugation of the polar bear milk resulted in a precipitate which deposited at the bottom of the centrifuge tube. The caseins were subjected to (1) polyacrylamide-gel electrophoresis (no urea added), (2) polyacrylamide electrophoresis (urea added), and (3) starch-gel electrophoresis (2-mercaptoethanol and urea added). The wheys were subjected to polyacrylamide-gel electrophoresis (no urea added). The polar bear casein, the polar bear precipitate, and bovine casein gave similar electropherograms. The α-caseins of the polar bear milk and precipitate had mobilities which were similar to that of bovine α-casein, but the β-caseins

of these two fractions had mobilities which were slightly less than that of bovine β-casein. The electropherograms which were obtained with the whey proteins of polar bear and cow were dissimilar.

Ambrosino, Liberatori, LaVecchia, Sarra, and Ubertalle (7) concluded that the serum albumins, β-lactoglobulins, α-lactalbumins, and immunoglobulins (separated by electrophoresis) of cow milk whey are structurally analogous to the corresponding fractions in sheep, buffalo, and goat milk wheys, since these fractions give cross-reactions with the immune sera for the various milk wheys.

Lemon and Poole (166) analyzed the whey proteins of grey kangaroo (Macropus giganteus) milk by starch-gel electrophoresis. Three components were noted, namely, the albumins, and two fractions which the authors refer to as "pre-albumins", p1 and p2. These two fractions are, in fact, "post-albumins", and probably refer to the α-lactalbumins and β-lactoglobulins.

Jenness, Erickson, and Craighead (134) prepared the caseins and whey proteins from 25 individual bears (black bears (Ursus americanus), brown bears (Ursus arctos), and polar bears (Thalarctos maritimus)). The caseins and wheys were analyzed by electrophoretic analysis on polyacrylamide

gels (Caseins: tris-buffer; pH 9.2; gels contained 4.5 M urea and sample solutions contained mercaptoethanol. Wheys: veronal buffer; pH 8.6). The authors were unable to resolve bear caseins into well-defined bands as was possible with bovine casein and, hence, were unable to observe any precise differences in the casein components of the different milks or to determine whether or not polymorphism existed within a given species. Although the wheys contained a component which corresponded to blood serum albumin, they did not contain many of the slower-moving serum proteins. The electrophoretic patterns obtained with the whey samples indicated outstanding differences in the whey protein constitution, despite the fact that the blood proteins of the different animals were remarkably similar.

Kraeling and Gerrits (153) analyzed the whey proteins of sow's milk by polyacrylamide-gel electrophoresis (veronal buffer; pH 8.6). They noted two components in the fastest-moving region and designated them as A and B. Individual sows produced one or both of these components.

2. Methods

2.1. ELECTROPHORESIS OF CASEIN

The method employed for polyacrylamide-gel disc

electrophoresis of casein was essentially that of Ornstein (224) and Davis (67) as modified for casein by Groves and Kiddy (113).

(a) Reagents

The stock solutions that were required for the electrophoretic analysis of casein are summarized in Table 9. The solutions were prepared using glass-distilled water and, when not in use, were stored in stoppered brown glass bottles in the coldroom. The working solutions, which were prepared fresh on the day that they were needed, were made from the stock solutions listed in Table 9. The proportions of the stock solutions required to make the working solutions are tabulated in Table 10. Other solutions which were required are as follows:

(1) Fixative-stain solution

Amido Schwartz or Buffalo Black (1.0 g) was dissolved in acetic acid (7% v/v) and the resultant solution was adjusted to a final volume of 100 ml with the same acid.

(2) Destaining solution (also used for storage of the gels)

Glacial acetic acid (70 ml) was diluted to a final volume of 1000 ml with distilled water.

(3) Bromphenol blue solution (0.001% w/v)

Table 9

Stock solutions required for polyacrylamide-gel disc electrophoresis of casein

(A)		(B)	
1 N HCl	24 ml	1 N HCl	48 ml
TRIS[a]	18.3 g	TRIS[a]	5.98 g
TEMED[b]	0.115 ml	TEMED[b]	0.46 ml
Urea	24.0 g	Urea	24.0 g
Water to (pH 8.9)	100 ml	Water to (pH 6.7)	100 ml

(C)		(D)	
Acrylamide[c]	28.0 g	Acrylamide[c]	10.0 g
BIS[d]	0.735 g	BIS[d]	2.5 g
Urea	24.0 g	Urea	24.0 g
Water to	100 ml	Water to	100 ml

(E)		(F)	
Riboflavin	4.0 mg	Urea	24.0 g
Urea	24.0 g	Water to	100 ml
Water to	100 ml		

[a]TRIS: 2-amino-2-(hydroxymethyl)-1,3-propanediol; or tris (hydroxymethyl) aminomethane.

[b]TEMED: N,N,N',N'-tetramethylehtylenediamine (Eastman 8178).

[c]Acrylamide: Eastman 5521.

[d]BIS: N,N'-methylenebisacrylamide (Eastman 8383).

All of the above reagents are available from Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N.Y.

Table 10

Working solutions required for polyacrylamide-gel disc electrophoresis of casein

Small-pore solution No.1*	Small-pore solution No.2*	Large-pore solution	Stock buffer solution for reservoirs
1 parts A	Ammonium persulphate 0.14 g	1 part B	TRIS
1 parts C	Urea 24.0 g	2 parts D	Glycine
	Water to 100 ml	1 part E	Water to 2 liters
		4 parts F	(pH 8.3)
(pH 8.8-9.0)		(pH 6.6-6.8)	

*Small-pore gel solution is made immediately prior to pouring gels, by mixing equal parts of small-pore solution No. 1 and small-pore solution No. 2.

Bromphenol blue (0.010 g) was dissolved in distilled water and the volume of the resultant solution was adjusted to a final volume of 1000 ml with distilled water.

(b) Procedure

The stock solutions were removed from the cold room and were allowed to warm to room temperature. Meanwhile, eight glass tubes (length=83 mm; o.d.=7 mm; i.d.=5 mm) which had been previously cleaned with chromic acid and rinsed with distilled water were placed vertically in a rack. The

bottoms of the tubes were inserted into holes formed by removing 8 circular discs (diameter=7 mm) of rubber from a flat piece of surgical rubber (thickness=¼ in). The holes extended through ½ the thickness of the rubber. The rubber thus formed the base of the tube rack and the bottom opening of each tube was thus sealed.

The working solutions were then prepared as shown in Table 10.

The glass tubes were filled with small-pore solution (made by mixing equal volumes of small-pore solution No. 1 and small-pore solution No. 2) to a level 13 mm from the top of each tube. This was done by means of a small disposable pipette fitted with a rubber bulb. A water layer (3 mm) was carefully placed on top of the small-pore gel solution and care was taken not to cause mixing at the interface. This step ensured a flat meniscus on top of the small-pore gel. Polymerization was allowed to take place for 45 min, during which time the gels were protected from strong light.

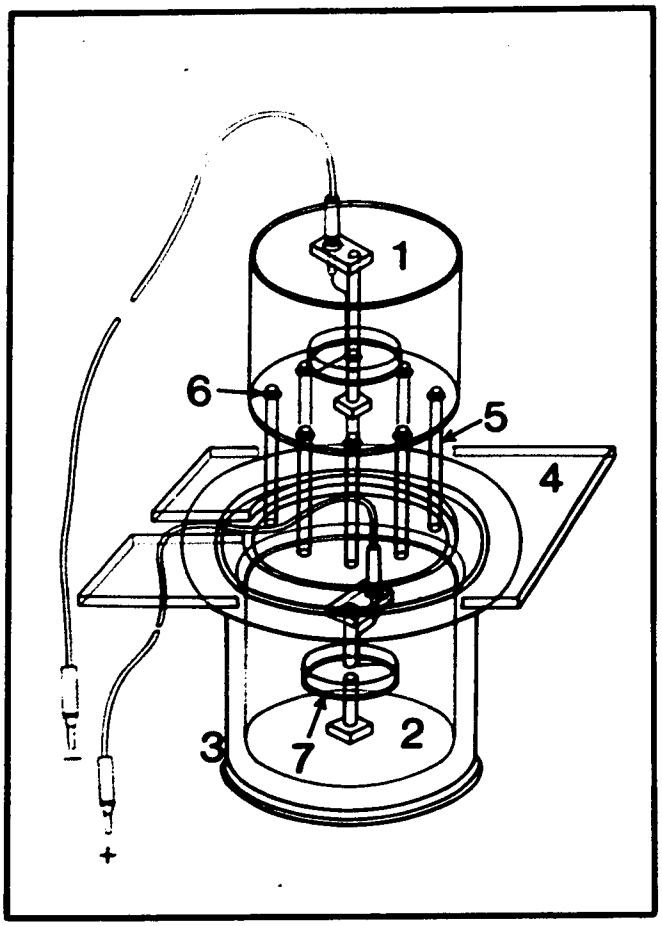
The water layer was drawn off by means of small filter paper wicks and the tubes were then filled with large-pore gel solution to a level 5 mm from the top of each tube (the volume of large-pore solution was about 0.15 ml). A water layer (3 mm) was carefully placed on top of the large-pore gel solution as described above. The rack containing the

gel tubes was then placed between two fluorescent lights and the spacer gel was allowed to photo-polymerize for 20 min.

The tube rack was removed from the intense light, the water layer was drawn off in the same manner as described above, and the remainder of the tube was filled with large-pore solution (about 0.1 ml) containing about 200 µg casein. The tube rack was again placed between the fluorescent lights and the sample gel was allowed to photo-polymerize for 20 min.

The tubes were removed from the tube rack and inserted into the rubber grommets of the upper reservoir of the electrophoresis apparatus shown in Figure 8. Buffer was added to the upper and lower reservoir so that the level of the liquid just covered each electrode. Bromphenol blue (0.001%; 1 ml) was added to the buffer in the upper reservoir. The upper reservoir was then placed and supported over the lower reservoir such that the tubes extended to a depth of approximately ¼ inch below the surface of the buffer in the lower reservoir. The power supply was connected (anode, lower reservoir; cathode, upper reservoir) so as to provide a constant current of 2 mA per tube (16 mA, total). The electrophoresis was conducted in the

Figure 8. Disc electrophoresis apparatus.



1 Upper reservoir

2 Lower reservoir

3 Desiccator support

4 Seat for [1]

5 Gel tube

6 Rubber grommet

7 Electrode

cold room and the run took about 1 h 45 min. The run was over when the marker dye (Bromphenol Blue) reached the bottom of the tubes.

The gels were removed from the tubes with the aid of cold, running tap water and a long needle, which was passed between the gel and the inner wall of each tube. The gels were placed in individual labelled petri dishes and were stained for 1 h with the fixative-stain solution (Amido Schwartz; 1% w/v in 7% v/v acetic acid). At the end of the staining procedure, excess stain solution was removed by rinsing each gel with destaining solution (7% v/v acetic acid) and final destaining was accomplished electrolytically. The gels were placed on a rack, which in turn was placed in a TLC tank which had two thin rectangular pieces of carbon which served as electrodes at each side of the tank. The tank was filled with destaining solution and a high potential (300 V) was maintained across the electrodes. The solution in the tank was replaced as often as necessary (about 5 or 6 times). The gels were then removed from the rack and were photographed immediately.

2.2. ELECTROPHORESIS OF WHEY PROTEINS

The method employed for polyacrylamide-gel disc

electrophoresis of whey proteins was essentially that of Ornstein (224) and Davis (67).

(a) Reagents

The gels did not contain urea as did those for casein since high concentrations of urea cause denaturation of the β-lactoglobulins (1). The stock solutions that were required for the electrophoretic analysis of wheys are summarized in Table 11. The working solutions, which were prepared fresh on the day that they were needed, were made from the stock solutions shown in Table 11. The proportions of the stock solutions required to make the working solutions are tabulated in Table 12. The other solutions which were required are the same as those described in Section 2.1.(a).

(b) Procedure

The procedure followed for electrophoresis of whey proteins is exactly the same as that described in Section 2.1.(b). Since the wheys contained considerable and varying amounts of total hexose (Table 13), a weight of whey was taken which contained 200 μg of protein material.

Table 11

Stock solutions required for polyacrylamide-gel disc electrophoresis of whey proteins

(A)			(B)		
1 N HCl		48 ml	1 N HCl		48 ml
TRIS*		36.6 g	TRIS*		5.98 g
TEMED*		0.23 ml	TEMED*		0.46 ml
Water	to	100 ml	Water	to	100 ml
(C)			**(D)**		
Acrylamide*		28.0 g	Acrylamide*		10.0 g
BIS*		0.735 g	BIS*		2.5 g
Water	to	100 ml	Water	to	100 ml
(E)			**(F)**		
Riboflavin		4.0 mg	Sucrose		40.0 g
Water	to	100 ml	Water	to	100 ml

*See footnotes at bottom of Table 9.

Table 12

Working solutions required for polyacrylamide-gel disc electrophoresis of whey proteins

Small-pore solution No.1*	Small-pore solution No.2*	Large-pore solution	Stock buffer solution for reservoirs
1 part A	Ammonium persulphate 0.14 g	1 part B	TRIS 6.0 g
2 parts C		2 parts D	Glycine 28.8 g
1 part water	Water to 100 ml	1 part E	Water to 1 liter
(pH 8.8-9.0)		4 parts F	(pH 8.3)
		(pH 6.6-6.8)	

*Small-pore gel solution is made immediately prior to pouring gels by mixing equal parts of small-pore solution No. 1 and small-pore solution No. 2.

Table 13

Carbohydrate content of whey solids from milks of different species*

	Nitrogen (%)	Carbohydrate** (%)
Cow, Sample No.2	1.29	75.9
Horse	2.93	42.7
Pig	4.90	30.8
Arctic wolf	6.47	42.6
Caribou, Sample No.1	6.46	61.5
2	6.56	59.4
Dall sheep	4.01	64.0
Fin whale, Sample No.5	13.0	15.9
Harp seal	11.9	12.2
Moose, Sample No.1	5.95	8.26
2	13.9	4.10
3	12.8	4.51
Musk-ox	7.00	13.9
Polar bear, Sample No.1	9.18	3.69
2	9.66	6.00
3	8.37	6.64
Reindeer, Sample No.1	4.54	25.0
2	4.35	45.2
3	7.73	15.9

*Values represent averages of duplicate determinations.

**Determined by Method I.

3. Results and Discussion

3.1. ELECTROPHORESIS OF CASEIN

The electropherograms for the caseins of cow No. 1 and reindeer Nos. 1-3 are shown in Figure 9; of cow No. 1 and moose Nos. 1-3 in Figure 10; of cow No. 1 and caribou Nos. 1-3 in Figure 11; of cow No. 1 and horse, pig, and musk-ox in Figure 12; of cow Nos. 1 & 2 and fin whale No. 1 in Figure 13.

It should be recalled (Figure 3) that Sample No. 5 of fin whale milk yielded a precipitate (Fraction B; 14.9% N) upon initial centrifugation of the milk. Extraction of the skimmilk with diethyl ether caused an emulsion to form in the ethereal layer. This protein fraction (Fraction C; 10.8% N, 1.30% P) was recovered from the emulsion by evaporation of the ether layer by means of a rotary evaporator. When the acid-precipitated casein was suspended in water and the reaction adjusted to pH 6.5, some of the casein did not go into solution. The protein which was insoluble at pH 6.5 (Fraction D; 9.3% N, 0.42% P) was recovered by filtration. Cow No. 1 casein, fin whale No. 5 casein (Fraction A), the emulsion protein fraction (Fraction C), the protein insoluble at pH 6.5 (Fraction D), and the initial precipitate

(Fraction B) are shown in Figure 14.

Figure 15 shows the electropherograms of the caseins of cow Nos. 1 & 2, polar bear Nos. 1-3, and the precipitates obtained by initial centrifugation of polar bear milks Nos. 2 & 3 and a precipitate (Pattern No. 31) obtained by previous workers (Baker, Hatcher, and Harington (29)) from polar bear milk.

Figure 16 shows the electropherograms of the caseins of the milks of cow No. 1, dall sheep, and harp seal, and the precipitate obtained by initial centrifugation of dall sheep milk.

Examination of Figures 9 and 11 reveals that the patterns obtained for the caseins of reindeer (Rangifer tarandus) milk are remarkably similar to those obtained for the caseins of caribou (Rangifer tarandus groenlandicus) milk. Both these caseins gave patterns in which the β-casein bands migrated a distance slightly greater than that of the β-casein band of bovine casein. The α_{s_1}-casein bands of both reindeer and caribou casein migrated a shorter distance than the α_{s_1}-component of bovine casein. These observations are in agreement with the observations of Aschaffenburg, Gregory, Kon, Rowland, and Thompson (22). These workers noted that reindeer casein contained two electrophoretic (paper electrophoresis) components, one with a greater

mobility than that of bovine β-casein, and the other with a mobility less than that of bovine α-casein milk. The similarity between the electrophoretic patterns obtained for reindeer and caribou caseins is a further indication that related species produce caseins which give similar electrophoretic patterns (see Sloan, Jenness, Kenyon, and Regehr (255)).

Previous workers have observed three genetic types of bovine β-casein (A, B, and C) using urea-gel electrophoresis at high pH (Aschaffenburg (13)(14), Peterson (228), and Thompson, Kiddy, Johnston, and Weinberg (288)). Close examination of Pattern Nos. 2, 3, and 4 (Figure 9) suggests that there is evidence of genetic polymorphism in the β- and α_{s_1}-caseins of reindeer milk. With respect to β-caseins, reindeer No. 1 could be typed β-casein B (β-Cn B) while reindeer Nos. 2 & 3 could represent β-Cn AC. It is likely that if additional samples had been available for analysis, caseins typed either β-Cn A and β-Cn C would be found. The separations shown in Pattern Nos. 3 & 4 were not sufficiently clear to offer conclusive evidence for the existence of β-Cn A and C variants. The author is also aware of the fact that the β-Cn A components can be further resolved by electrophoresis (pH 2.8) and that the three forms β-Cn A^1, β-Cn A^2, and β-Cn A^3 can be separated (Peterson and Kopfler

(229), Aschaffenburg (18), Aschaffenburg, Sen, and Thompson (23), and Thompson (287). Further research might be directed in this area when a larger number of samples becomes available. Reindeer No. 1 might have the B variant of α_{s_1}-casein and reindeer Nos. 2 & 3 might have the AB variant.

Reference to Pattern Nos. 10, 11, & 12 (Figure 11) suggests genetic polymorphism in the caseins of caribou milk. With respect to β-casein, caribou No. 1 could have the β-Cn B variant; caribou No. 2, the β-Cn AC variant; and caribou No. 3, the β-Cn BC variant. Although polymorphism seems to be evident in the α_{s_1}-caseins, the variants cannot be stated with certainty.

Pattern Nos. 7 & 8 (Figure 10) indicate complex patterns for two of the moose casein samples. These two samples seem to contain very little β-casein. The α_{s_1}-caseins of all samples of moose casein have approximately the same mobility as that of bovine α_{s_1}-casein. The mobility of the β-casein in moose milk, like that of the β-casein in the milks of the reindeer and caribou, was higher than that observed for bovine β-casein. It is interesting to note that the two samples which had small amounts of β-casein contained very prominent bands in the region corresponding to bovine "T.S. (temperature-sensitive)-casein."

Temperature-sensitive casein occurs evidently in all milks (245). Groves and Kiddy (113) have described a method for the preparation of Y-casein by extraction of acid casein at pH 4 (2°C) followed by chromatography on DEAE-cellulose columns (0.005 M phosphate buffer, pH 8.3). The protein fraction which is eluted with the front is designated "temperature-sensitive" since it is soluble at 2°C but precipitates at 25°C. (The Y-casein fraction is eluted at 0.02 M phosphate concentration.) The variants A and B of the T.S.-caseins have the same mobility at alkaline pH values and hence give a single band (111).

Variation in the β-casein of the moose samples seems to be evident. Pattern No. 6 could represent β-Cn AB and Pattern Nos. 7 & 8, β-Cn B.

Examination of Figure 12 reveals that horse, pig, and musk-ox caseins also exhibit complex electrophoretic patterns. Musk-ox casein did not show any predominant band in the β- or α_{s_1}-casein regions. Both horse and pig caseins had intermediate bands in the region between the β- and α_{s_1}-caseins. The α_{s_1}-casein of horse milk migrated a shorter distance than did that of bovine milk; the α_{s_1}-casein of pig milk migrated a greater distance than did that of bovine milk. The β-casein component of pig casein is present in

much greater concentration than is the α_{s_1}-component. Previous workers (Sowls, Smith, Jenness, Sloan, and Regehr (264) made a similar observation with the casein from the milk of the collared peccary (Pecari tajacu) and the domestic sow (Sus scrofa).

The fin whale casein (Sample No. 1)(Figure 13) did not have any predominant band in the β-casein region, but did exhibit a band which corresponded to bovine α_{s_1}-casein. The P/N ration for this casein (see Chapter III) was 0.126 (the highest value the present author has observed for casein) compared with 0.058 for bovine casein. It is not surprising, therefore, to observe a relatively large proportion of α_{s_1}-casein in this sample since a high content of phosphorus in casein generally reflects a relatively high concentration of the component having the highest electrophoretic mobility.

Figure 14 deals with the various fractions obtained in the preparation of the fifth sample of fin whale casein. Comparison of Pattern No. 24 (Figure 14) with Pattern No. 19 (Figure 13) reveals that the two samples of fin whale casein are similar in that neither appears to contain a major β-casein component. In both samples, the α_s-casein complex seems to be the predominant band. However, the

precipitate (Pattern No. 21) obtained on initial centrifugation of the milk contained a band which could be a slow-moving β-casein. The arrow in Pattern No. 23 (Fraction D) indicates a faint band in the α_s-casein region which did not show up in the photographic process. The electropherogram (Pattern No. 22) of the fraction recovered from the ethereal emulsion (Fraction C) did not reveal a band in the region between β-casein and α_{s_1}-casein which was observed with the electropherograms of the precipitate (Fraction B) and the casein (Fraction A).

The polar bear caseins (Figure 15) and precipitates were similar to each other and differed from bovine casein in that they contained slower-moving α_{s_1}- and β-caseins; they also contained a band in the region between the α_{s_1}-casein and the β-casein components. Baker, Hatcher, and Harington (29) also observed relatively slow-moving β-caseins in polar bear milk.

The dall sheep precipitate (Pattern No. 35, Figure 16), like the other precipitates, gave a band in the β-casein region which was more prominent than the corresponding band in the casein (Pattern No. 34). The α_{s_1}-casein was slower-moving than was that of bovine casein. Dall sheep milk, like that of the fin whale, had relatively

little β-casein and a large concentration of α_{s_1}-casein compared to bovine milk.

The harp seal casein (Pattern No. 36) gave two major bands in the β-casein region and one band in the region of the α_{s_1}-casein of bovine milk. One of the bands in the β-casein region of harp seal milk was much slower-moving than that of bovine milk.

3.2. ELECTROPHORESIS OF WHEY PROTEINS

Electropherograms obtained with the whey proteins from the milk of cow No. 2, reindeer Nos. 1, 2, & 3, moose Nos. 2 & 3, musk-ox, and harp seal are shown in Figure 17; of cow No. 2, horse, pig, polar bear Nos. 1, 2, & 3, and caribou Nos. 1 & 2 in Figure 18; and of cow No. 2, Arctic wolf, dall sheep, moose Nos. 1 & 4*, and fin whale No. 5 in Figure 19. In addition, Figure 19 shows the electrophoretic pattern obtained from bovine serum albumin.

The three main regions of the electropherograms of bovine whey have been labelled A, B, and C. Region A comprises the immune globulins which is a fraction pre-formed in the blood and which contains antibodies (1). This fraction remains at the origin because of the high molecular

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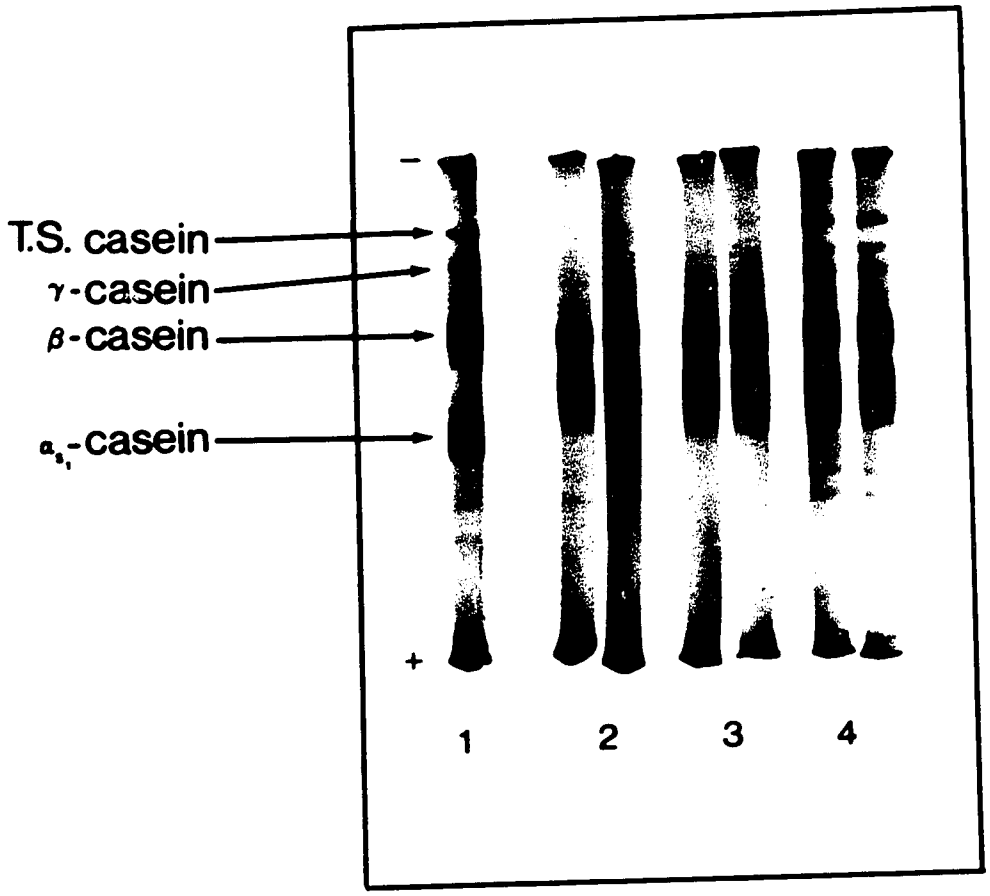
*This whey was isolated from a sample of moose milk for which data pertaining to sample collection are unavailable.

weight of its protein constitutents (pseudo-globulin, MW= 180,000; euglobulin, MW = 800,000). The usual sequence of bands, in the order of increasing mobility, is: immune globulins, serum albumin, α-lactalbumin, and β-lactoglobulins B and A (1). The B region probably contains bovine serum albumin and α-lactalbumin. Close examination of the patterns for bovine whey did not reveal any separation of the bovine serum albumin (BSA) and α-lactalbumin components. Hartman and Swanson (117) noted that disc electrophoresis by the method of Ornstein (224) and Davis (67) did not resolve BSA and α-lactalbumin. Thus, it is likely that the most prominent band in the B region of bovine whey (Pattern Nos. 1, 9, & 17) represents a mixture of BSA and α-lactalbumin. It will be noted by examination of Pattern No. 23 (Figure 19) that the mobility of the fastest-moving component of the commercial preparation of BSA corresponds to the mobility of the prominent band in the bovine whey B region. Since α-lactalbumin is present in bovine whey in a larger concentration than is BSA (137), the author has concluded that the prominent B band is in all probability an unresolved mixture. The two bands in the C region could be the A and B variants of β-lactoglobulin which are likely to be present in a pooled sample of milk.

The patterns which were obtained with the different wheys were extremely complex and hence are difficult to interpret. Other workers (22)(29)(68)(123)(255) have also obtained complex patterns with whey proteins of different species. In general, the wheys from different animals of the same species gave similar patterns. Examination of Figures 17 and 18 shows that the samples of whey from the same species (reindeer (Pattern Nos. 2, 3, & 4), moose (Pattern Nos. 5 & 6), polar bear (Pattern Nos. 12 & 13), and caribou (Pattern Nos. 15 & 16)) gave similar electrophoretic patterns. The wheys of reindeer, musk-ox, moose, harp seal, and fin whale contain a high concentration of a protein with a mobility similar to that of bovine β-lactoglobulin. The wheys of horse, pig, polar bear, Arctic wolf, and dall sheep contain a high concentration of protein with a mobility similar to that of the bovine albumins (bovine serum albumin + α-lactalbumin). Several whey samples contained high concentrations of proteins that could be immune globulins.

Figure 9. Electrophoretic patterns of cow and reindeer caseins.



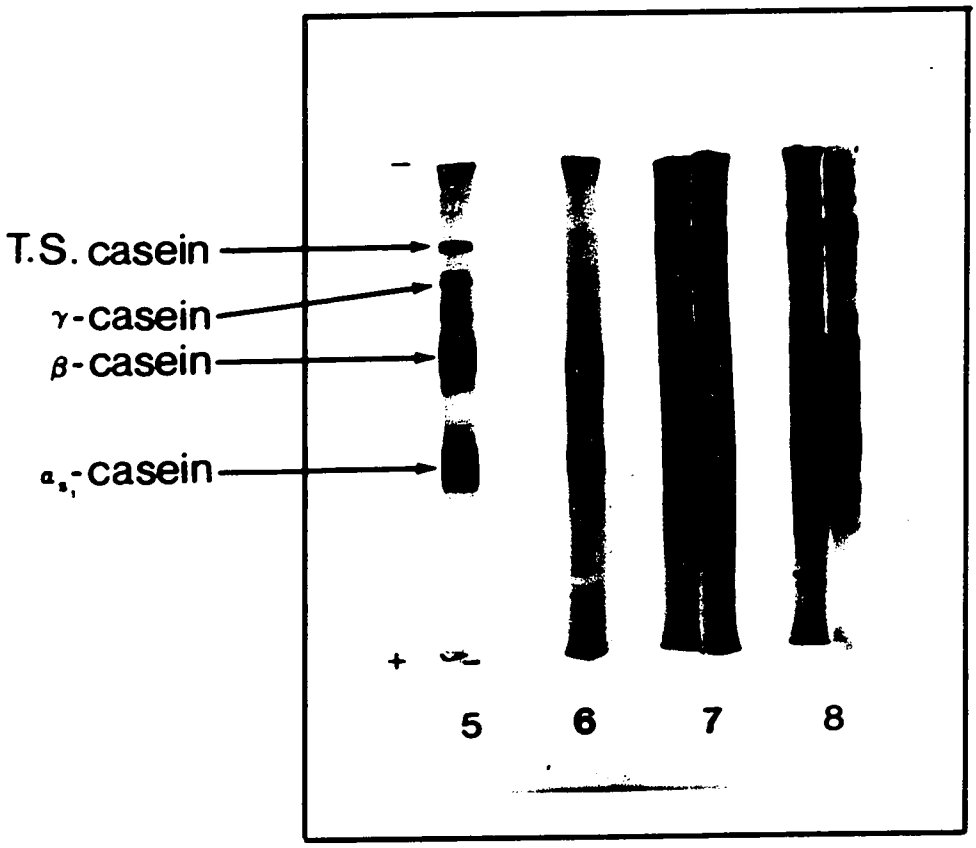
1 Cow 1

2 Reindeer 1

3 Reindeer 2

4 Reindeer 3

Figure 10. Electrophoretic patterns of cow and moose caseins.



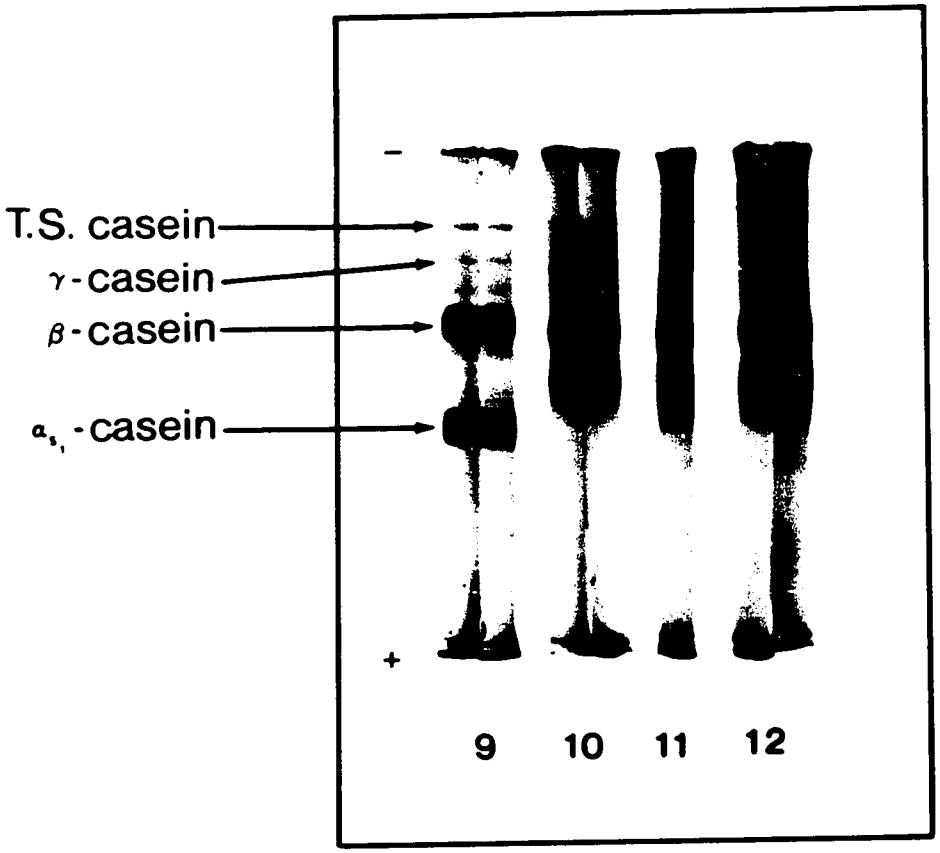
5 Cow 1

6 Moose 1

7 Moose 2

8 Moose 3

Figure 11. Electrophoretic patterns of cow and caribou caseins.



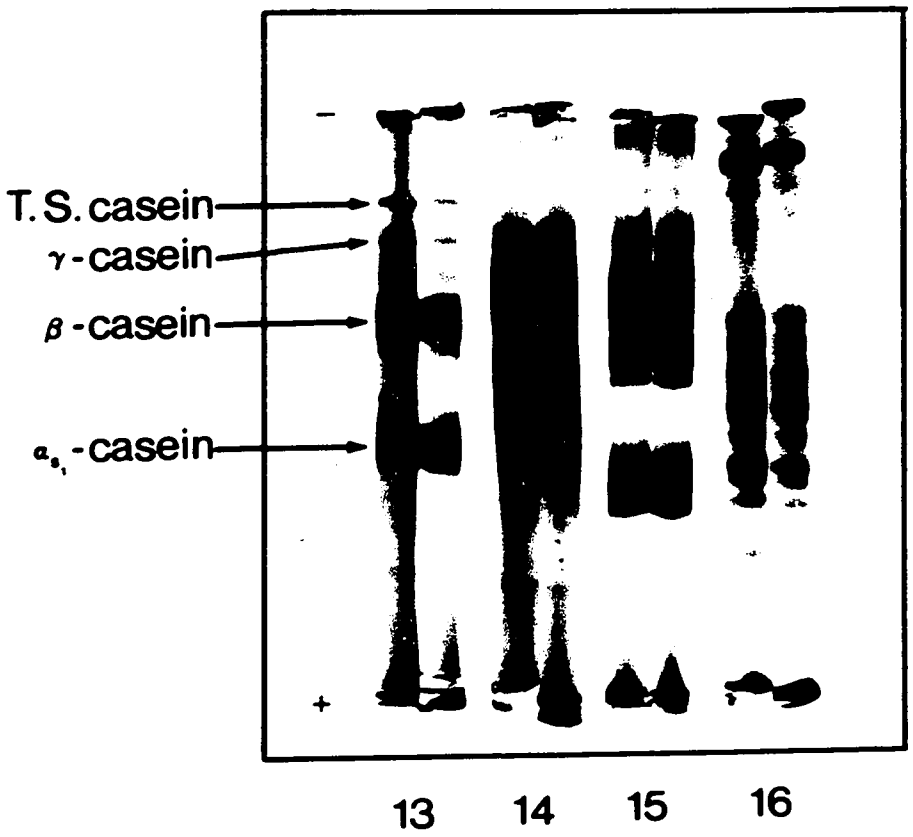
9 Cow 1

10 Caribou 1

11 Caribou 2

12 Caribou 3

Figure 12. Electrophoretic patterns of cow, horse, pig, and musk-ox caseins



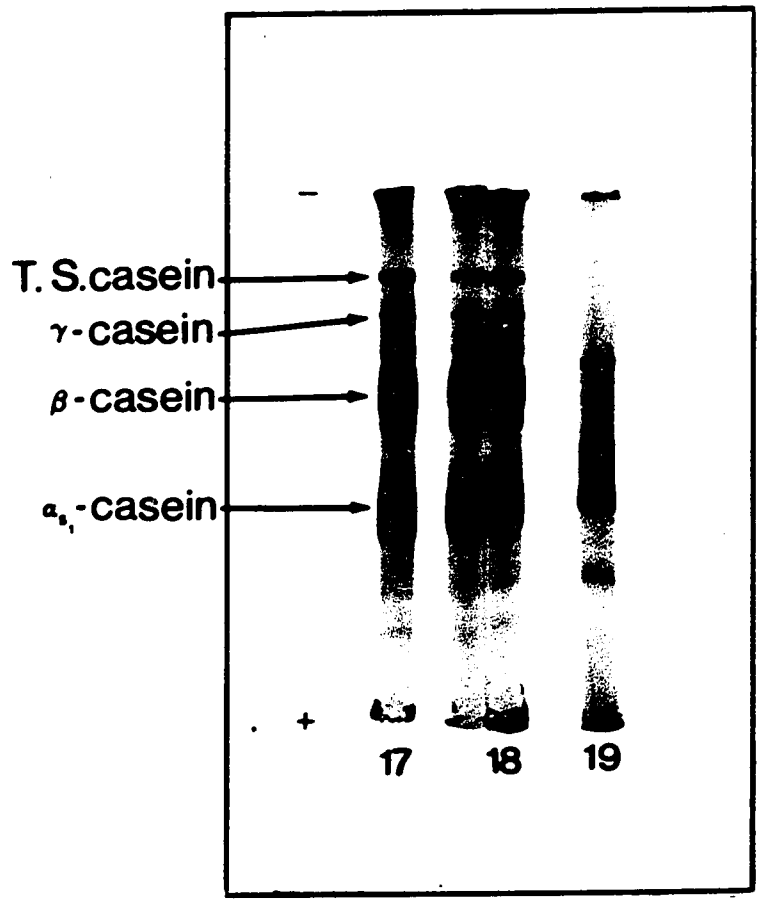
13 Cow 1

14 Horse

15 Pig

16 Musk-Ox

Figure 13. Electrophoretic patterns of cow and fin whale caseins.



17 Cow 1

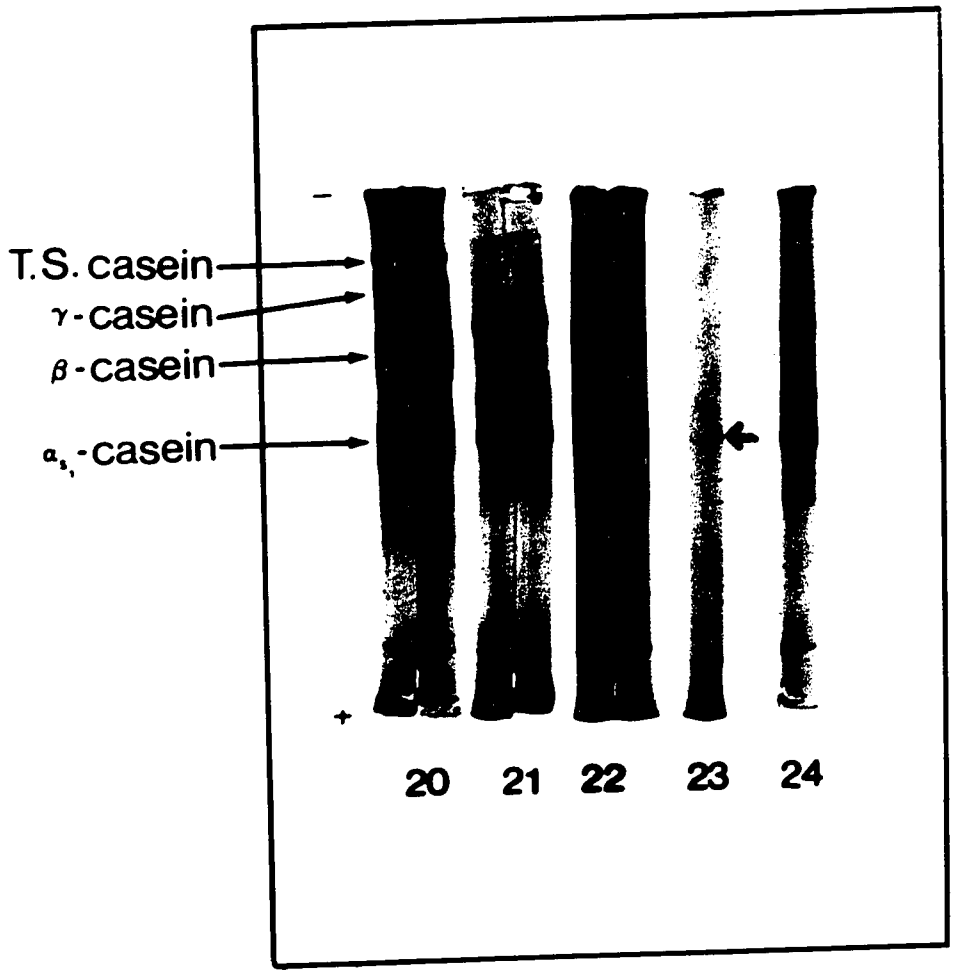
18 Cow 2

19 Fin Whale 1

Figure 14. Electrophoretic patterns of cow and fin whale caseins, and other fractions of fin whale milk.

Pattern No.	Fraction*
21	B
22	C
23	D
24	A

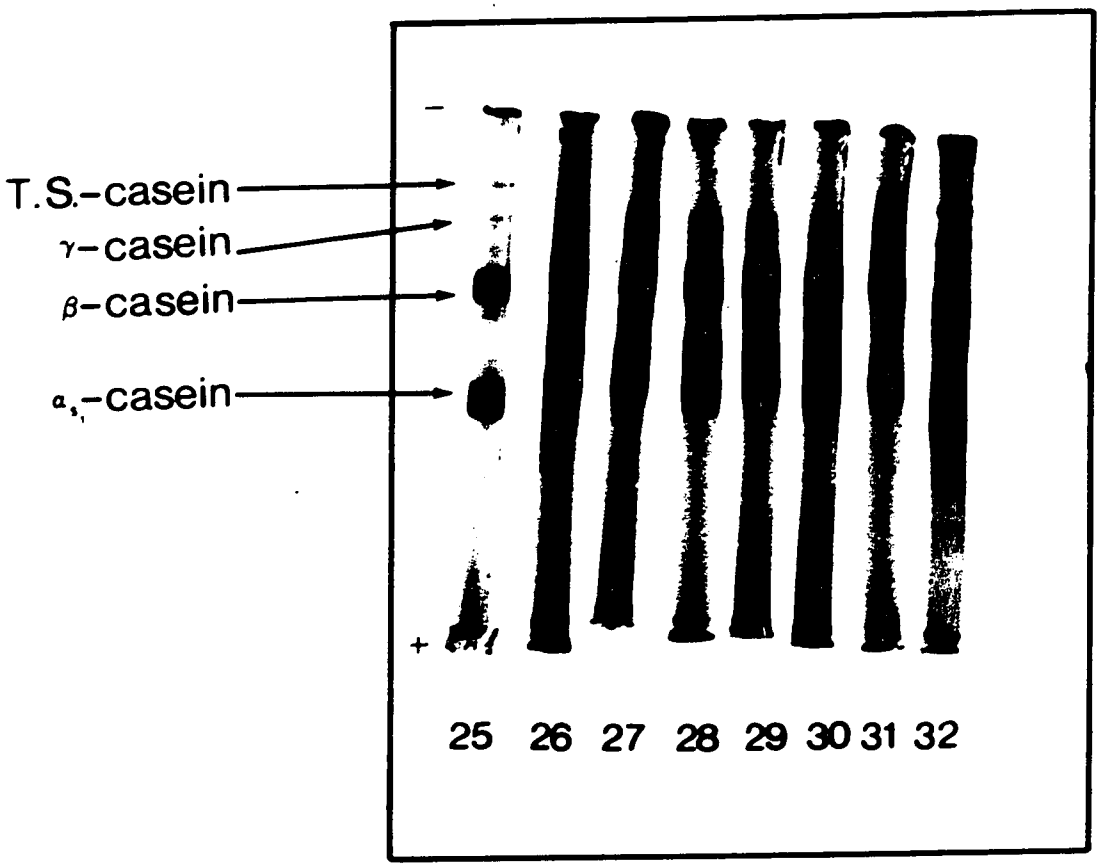
*See Figure 3.



20	Cow 1	
21	Fin Whale 5	Precipitate
22		Emulsion protein
23		Protein insoluble at pH 6.5
24		Casein

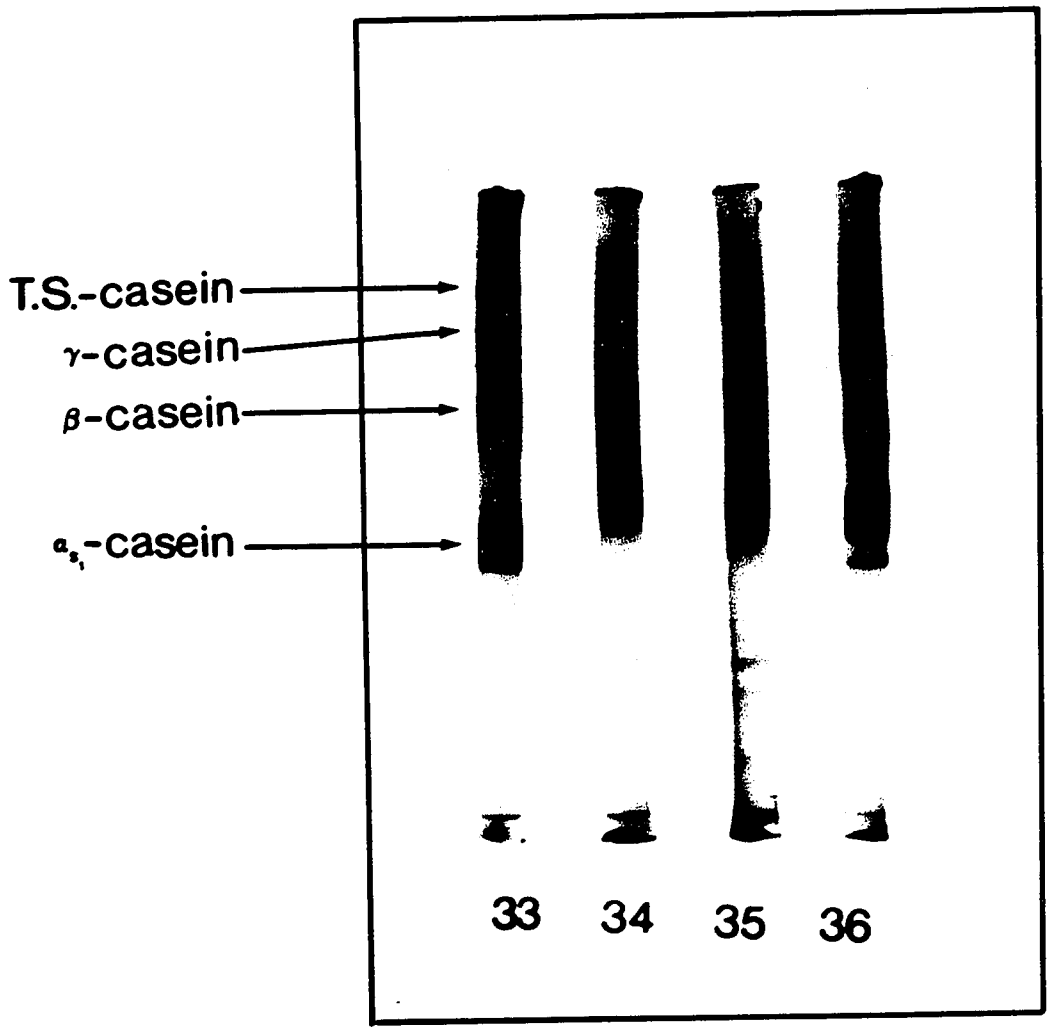
Figure 15. Electrophoretic patterns of cow and polar bear caseins, and the precipitates obtained from polar bear milk.

*Precipitate "D" of Baker, Hatcher, and Harington (29)



25	Cow 1	
26	Polar Bear	1
27		2
28		3
29	Polar Bear Precipitate	2
30		3
31		•
32	Cow 2	

Figure 16. Electrophoretic patterns of cow, dall sheep, and harp seal caseins, and the precipitate obtained from dall sheep milk.



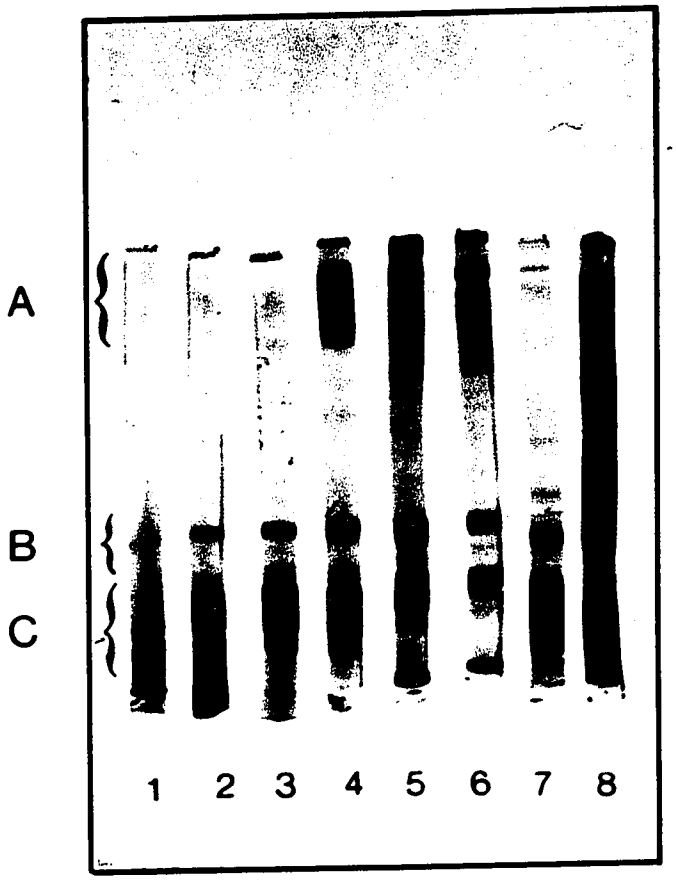
33 Cow 1

34 Dall Sheep

35 Dall Sheep Precipitate

36 Harp Seal

Figure 17. Electrophoretic patterns of cow, reindeer, moose, musk-ox, and harp seal whey proteins.



1 Cow 2

2 Reindeer 1

3 Reindeer 2

4 Reindeer 3

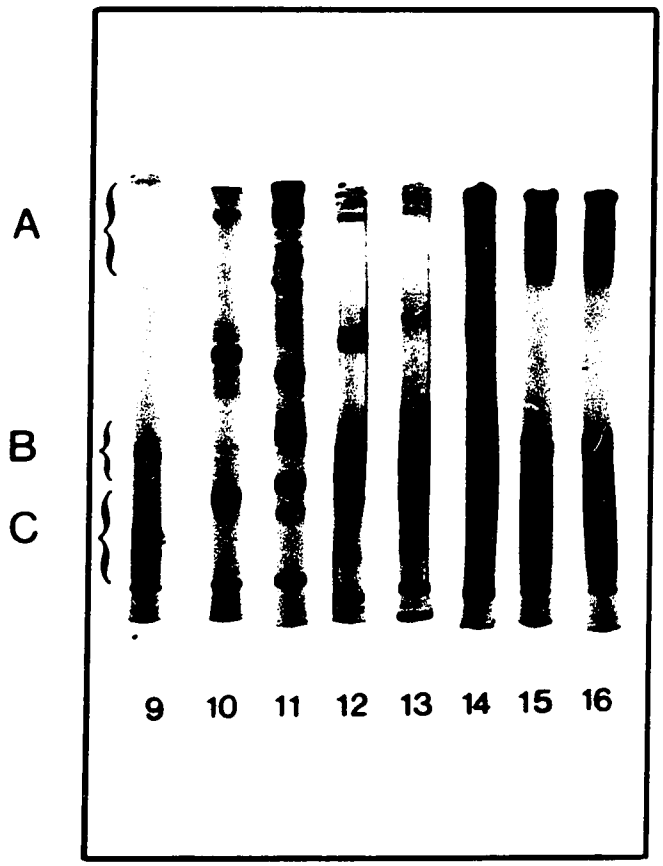
5 Moose 2

6 Moose 3

7 Musk Ox

8 Harp Seal

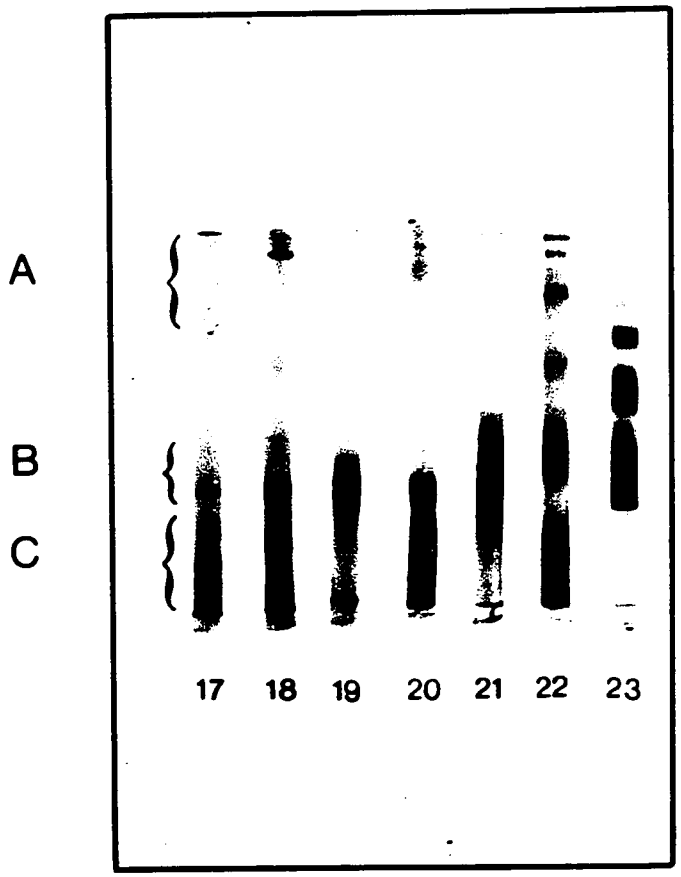
Figure 18. Electrophoretic patterns of cow, horse, pig, polar bear, and caribou whey proteins.



9	Cow	2
10	Horse	
11	Pig	
12	Polar Bear	2
13	Polar Bear	3
14	Polar Bear	1
15	Caribou	1
16	Caribou	2

Figure 19. Electrophoretic patterns of cow, Arctic wolf, dall sheep, moose, and fin whale whey proteins, and bovine serum albumin.

*Whey obtained from a fourth sample of moose milk. Data pertaining to sample collection is unavailable.



17 Cow 2

18 Arctic Wolf

19 Dall Sheep

20 Moose 1

21 Moose

22 Fin Whale 5

23 Bovine Serum Albumin

CHAPTER V

AMINO ACID COMPOSITION OF CASEIN

1. Literature Review

1.1. AMINO ACID ANALYSIS BY EARLY AND CURRENT METHODS

Until about 1950, amino acids were determined in proteins by: (a) methods involving distillation of amino acid esters under reduced pressure, (b) colorimetric methods, and (c) miscellaneous methods, such as selective precipitation of individual amino acids from hydrolysates, and microbiological assay.

Esterification of amino acids decreases the polarity of the molecules and hence, they become more volatile. Emil Fischer pioneered the development of a technique to separate amino acid ethyl esters by fractional distillation under reduced pressure. Since decomposition occurred readily, this technique required skill and experience on the part of the operator and recoveries of roughly 66% were considered satisfactory (88).

Some amino acids, particularly tryptophan, tyrosine,

and cysteine, may be analyzed by colorimetric techniques. This involves the absorption of light of a specific wavelength by coloured derivatives of the amino acid (120)(254).

Microbiological assay is based on the fact that certain mutant strains of microorganisms require specific amino acids for growth. The procedure involves preparation of a culture medium containing all essential substances for growth except the amino acid to be determined. A standard curve is prepared by the addition of known amounts of the missing amino acid and measurement of the growth of the microorganism, as revealed by turbidity or by titration of lactic acid which is produced by lactic acid bacteria (88) (314).

The calcium or barium salts of the dicarboxylic amino acids can be separated from an aqueous solution by precipitation with alcohol. Arginine can be separated as the flavianate, employing 2,4-dinitro-1-napthol-7-sulfonic acid (88). Bergmann (88) and his co-workers discovered a large number of inorganic complex salts which could be used to precipitate selectively certain amino acids (70)(88).

Procedures developed in the 1950's for the analysis of amino acids included paper chromatography and chromatography on ion-exchange resins which eventually led to the development of the amino acid analyzer. The 1960's saw the

development of thin-layer chromatographic and gas chromatographic amino acid analysis. Paper chromatography involves spotting the amino acid standard or hydrolysate at one end of a rectangular piece of filter paper and developing the chromatogram by use of various solvent mixtures. The paper is dipped in ninhydrin solution and then scanned with a densitometer. Standard calibration curves are drawn for each amino acid and the concentration of the amino acid in the hydrolysate is ascertained by a calculation based on the densitometer reading obtained from the chromatogram of the hydrolysate.

The principle of thin-layer chromatography is the same as that of paper chromatography except that the solid support is a thin layer of powdered adsorbent such as silica gel, coated on a glass plate. The development time for thin-layer chromatography is much shorter (about 1 h) than that necessary for a paper chromatogram (10-20 h).

There are many published papers on the application of paper and thin-layer chromatography to the quantitative determination of amino acids (Baker and Khan (31); Smith (258); Roberts, Kolor, and Bucek (243); Khan, Baker, and Van Horn (147); Barbiroli (32); and Pelszeghy and Abraham (85)).

The method involving the use of ion-exchange resins

is the most widely used method for amino acid analysis today. Martin and Synge (186) were the first workers to use a chromatographic method for the separation and estimation of amino acids. Moore and Stein at first used starch columns (208) (274), but later changed to polystyrene ion-exchange resins (209)(210)(211)(265). The method developed by Moore and Stein for amino acid analysis using ion-exchange resins may be summarized as follows: the protein is hydrolyzed (20-70 h, 110°C, 6 N HCl) and the resultant hydrolysate is freed from HCl by evaporation. The reaction of the hydrolysate is adjusted to pH 2 and the hydrolysate is placed on a column of sulfonated cation-exchange resin. Two columns are used, one for basic amino acids and the other for neutral and acidic amino acids. Buffers are pumped through the columns at constant rates and as the pH rises, amino acids are eluted from the column. The column effluent is mixed with ninhydrin reagent and this mixture is continuously monitored by a recording spectrophotometer (570 mμ for all amino acids except proline and hydroxyproline; 440 mμ for the latter two amino acids). The area under each curve is proportional to the amount of amino acid present. Suitable standard solutions are subjected to the same procedure in order to calibrate the instrument. Cysteine and cystine cannot be distinguished.

Tryptophan is destroyed by acid hydrolysis and hence cannot be determined by this method.

1.2. GAS-LIQUID PHASE CHROMATOGRAPHY OF AMINO ACIDS

(a) Introduction

Gas chromatography of fatty acids and carbohydrates has become a powerful tool for the separation of these substances; gas chromatography of amino acids has not been developed to the same degree. Before the advent of gas chromatography, there were no rapid, convenient, or sensitive methods for the determination of fatty acids or sugars, and therefore much attention was given to the development of gas chromatographic methods for these materials (37)(285). When gas chromatographic equipment came into common usage in the 1960's, the analysis of amino acids by the Moore and Stein (265) technique was already a standard method and sophisticated apparatus was being developed. Blau (40) thus concluded that much of the urgency had been removed from the need for a gas chromatographic method of amino acid analysis.

The twenty amino acids commonly found in proteins have dissimilar structures. This makes it difficult to select a reaction that will produce a satisfactory volatile derivative for each amino acid (40). Table 14 shows the frequency

of occurrence of some of the groupings in the common amino acids.

Table 14

Structure and occurrence of various functional groups in amino acids

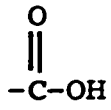
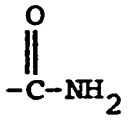
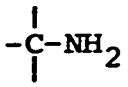
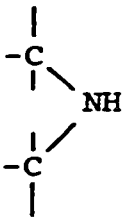
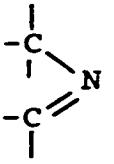
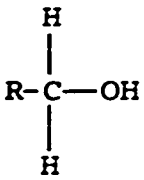
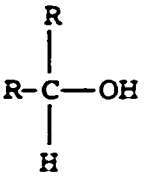
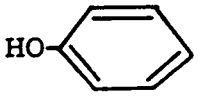
Group	Structure	Found In
Carboxyl (α)		All
(Side chain)	"	Aspartic acid, glutamic acid
Carboxamide		Asparagine, glutamine
Primary amino (α)		All, except proline, hydroxyproline
(Side chain)	"	Lysine, ornithine
Secondary Amino (Aliphatic)		Proline, hydroxyproline
(Heterocyclic)	"	Tryptophan, histidine
Tertiary amino (Heterocyclic)		Histidine
Primary alcohol		Serine
Secondary alcohol		Threonine, hydroxy-proline

Table 14 (cont'd)

Group	Structure	Found in
Phenol		Tyrosine
Thiol	-SH	Cysteine
Thioether	$-S-CH_3$	Methionine
Guanidino	$-N(H)-C(=NH)-NH_2$	Arginine

The amino acids commonly found in proteins are white crystalline solids which are stable at room temperature. Amino acids do not exhibit sharp melting points at high temperatures, but undergo decomposition over temperature ranges of several degrees (197). This is due to the high polarity of amino acids and results in their similarity to inorganic crystalline salts rather than to organic crystalline solids (88)(262).

Amino acids exhibit wide differences in their solubilities in water. Cystine and tyrosine are the least soluble (0.01 and 0.05 g/100 cc at 25°C, respectively) and proline and hydroxyproline (162 and 36 g/100 cc, respectively) are the most soluble of all the naturally-occurring amino acids. Cystine and tyrosine may be dissolved by

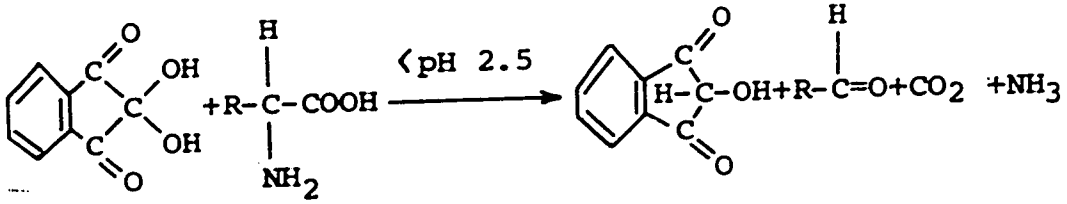
addition of acid or alkali and as a result are soluble at pH values lower than 2.5 or higher than 9 (197). In general, amino acids are only slightly soluble in absolute ethanol (118)(197). Proline, however, is soluble in ethanol (1.6 g/100 cc at 20°C). The hydrochlorides of the neutral amino acids and the dihydrochlorides of the basic amino acids are more soluble in water than are the corresponding free amino acids. Amino acid hydrochlorides are very soluble in ethanol (197).

Table 15 is a summary of some of the properties of the more common naturally-occurring amino acids.

(b) Derivatives

i) Degradation Products

Hunter, Dimick, and Corse (132) subjected leucine and isoleucine to ninhydrin oxidation (Equation 1). The



(1)

aldehydes thus obtained were subjected to gas chromatography. Two peaks were obtained and were identified, although they were not completely resolved.

Table 15

Physical properties of the common amino acids[a]

Amino acid	Molecular weight	Decomposition temperature (°C)	Solubility in water (g/100 cc)					
			0°C	25°C	50°C	65°C	75°C	100°C
Alanine	89.09	297	12.73	16.65	21.79	-	28.51	37.30
Cystine	240.30	260-261	-	0.011	0.024	-	0.052	0.114
Glutamic Acid	147.13	247-249	-	0.864	2.19	-	5.53	14.00
Glycine	75.07	233-290	-	25.0	39.1	-	54.4	67.2
Histidine	155.16	277-287	-	4.19	-	-	-	-
Hydroxyproline	131.13	274[b]	28.86	36.11	45.18	51.67	-	-
Isoleucine	131.17	168-170[c]	3.79	4.12	4.82	-	6.08	8.26
Leucine	131.17	284 145-148[c]	2.27	2.43	2.89	-	3.82	5.64
Phenylalanine	165.19	293-295 283	1.98	2.96	4.43	-	6.62	9.90
Proline	115.13	220-222	127.0	162.0	206.7	239.0	-	-
Tryptophan	204.22	289	0.023	1.14	1.71	-	2.80	4.99
Tyrosine	181.19	342-344	0.020	0.045	0.105	-	0.244	0.565
Valine	117.15	315[b]	8.34	8.85	9.62	10.24	-	-
Methionine	149.21	280-281[b]	1.82[e]	3.38[e]	6.07[e]	-	10.52[e]	17.60[e]
Serine	105.09	228	2.20[e]	5.02[e]	10.3[e]	-	19.2[e]	32.2[e]
Threonine	119.12	255-257	-	20.5[e]	-	-	-	-
Arginine	174.20	230-244	Sat'd soln ctns 15% (w/w) arginine at 21°C					
Aspartic Acid	133.10	270-271	0.267 (.2°C), 0.404 (9.5°C), 0.518 (16.4°C), 0.751 (31.5°C), 0.926 (40°C), 5.37 (97°C)					
Cysteine	121.16	175-178[d]	Freely soluble in water					
Lysine	146.14	210-224.5	Freely soluble in water					

[a]Data compiled from Merck Index. Solubilities are given for L-form unless otherwise indicated.
[b]Melting point.
[c]Sublimation occurs in this temperature range.
[d]Hydrochloride.
[e]DL-form.

Bayer (33) subjected the aldehydes formed by oxidation (hypochlorite) of various amino acids to gas chromatography (Equation 2). Alkaline hypochlorite caused deamination and

$$R\text{-}CH\cdot NH_2\text{-}COOH + NaOCl + H_2O \longrightarrow RCHO + NH_3 + NaCl + CO_2 \quad (2)$$

decarboxylation of the amino acid to give the corresponding aldehyde containing one less carbon atom. Aliphatic amino acids underwent the reaction quantitatively and no side products were formed. The author pointed out, however, that the reaction has limited application in the analysis of protein hydrolysates, since acidic and sulfur-containing amino acids give complex mixtures of volatile substances, and in some instances, two different amino acids give the same aldehyde (e.g. methionine and aminobutyric acid both yield propionaldehyde). Bayer separated six aldehydes, obtained from the following amino acids: alanine, α-aminobutyric acid, norvaline, valine, norleucine, and leucine.

Bier and Teitelbaum (39) carried out ninhydrin oxidation of some amino acids in aqueous solution, extraction of the aldehydes with solvent (carbon tetrachloride or ethylene dichloride), and injection of aliquots of the solvent layer onto a silicone column. These workers separated acetaldehyde (alanine), propionaldehyde (2-aminobutyric acid), isobutraldehyde (valine), and 3-methylbutraldehyde (leucine).

Zlatkis, Oró, and Kimball (326) oxidized amino acids to yield aldehydes with one less carbon atom by injecting them into a heated microreactor (140°C) containing ninhydrin coated on firebrick (30%). The amino acid mixtures which they used contained alanine, α-amino-n-butyric acid, valine, norvaline, leucine, isoleucine, and norleucine. Glycine reacts under these conditions to form formaldehyde which then polymerizes. The aldehydes obtained from phenylalanine and methionine had a low volatility and required higher operating temperatures and special stationary phases. As the aldehydes emerged from the column, they were cracked to methane over a nickel catalyst (Equation 3).

$$C_nH_{2n}CHO+(n+2)H_2 \xrightarrow[425^oC]{\text{catalyst}} (n+1)CH_4+H_2O \qquad (3)$$

The water was then removed by means of a drying column and the analysis was made by a thermal conductivity cell. This eliminated calibrations which normally are necessary for individual aldehydes because of differences in their thermal conductivities. One microgram of an amino acid could be detected by this technique.

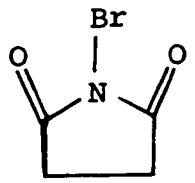
Ülehla (295) used a combination of pyrolysis and gas chromatography in the determination of amino acids. The breakdown products which resulted when a red-hot wire was dipped into amino acid solutions (5% in 2 N KOH) were carried

by means of a stream of argon onto a gas chromatographic column. The following amino acids gave specific pyrolytic spectra: alanine, α-aminobutyric acid, valine, leucine, isoleucine, arginine, lysine, phenylalanine, serine, threonine, methionine, proline, and hydroxyproline. However, Fales and Pisano (84) point out that conditions of pyrolysis are difficult to reproduce and therefore suggested that this approach to quantitative analysis of the amino acids is not feasible.

Stack (266) subjected amino acids and proteins to pyrolysis (280-320°C) in the injection port of a gas chromatograph. Collagen was characterized by two main peaks, which accounted for 18 and 20% of the total peak area. Peaks corresponding to those given by collagen were obtained when a mixture of glycine, proline, and hydroxyproline (3:2:1) was pyrolyzed under the same conditions.

Kanomata and Mashiko (145) subjected amino acids and proteins to a combination of pyrolysis and gas chromatography. They analyzed casein and egg albumin by this method and the results were compared to those obtained with an amino acid analyzer. The results obtained by the two methods were almost identical. Histidine, glycine, and serine could not be detected by pyrolysis gas chromatography.

Stevenson and Luck (276) studied the reaction of N-bromosuccinimide (NBS) with aqueous solutions of some α-amino



(N-bromosuccinimide)

acids to form the nitrile corresponding to the decarboxylated parent compound (Equations 4 & 5). The authors pointed out that small amounts of aldehydes containing one carbon atom less than the original amino acid may form (Equation 5).

$$RCH(NH_2)COOH \xrightarrow{NBS} \underset{I}{[RCH(NH_2)Br]} + CO_2 \qquad (4)$$

$$\underset{I}{[RCH(NH_2)Br]} \xrightarrow[-2HBr]{NBS} RCN$$

$$\downarrow H_3O^+ \qquad (5)$$

$$RCHO + NH_4Br \xrightarrow{1.5\ NBS} \tfrac{1}{2}N_2 + \tfrac{5}{2}HBr$$

The nitriles were then determined by gas chromatography. The amino acids that were studied included: alanine, α-aminobutyric acid, norvaline, valine, leucine, and phenylalanine.

McGregor and Carpenter (190) prepared the nitriles of some amino acids by hypobromite oxidation in strong alkali (Equation 6).

$$H_2NCH(R)COO^- + 20Br^- + OH^- \rightarrow RC{\equiv}N + CO_3^{=} + 2Br^- + 2H_2O \qquad (6)$$

The nitriles from alanine, valine, leucine, glutamic acid, and lysine were then subjected to gas chromatography.

Melamed and Renard (201) heated α-monoamino-monocarboxylic acids with a mixture of concentrated HCl and HNO_3 and obtained (approx. 100% yield) the corresponding α-chloro-acids (Equation 7). After methylation of the α-chloro-acids by means of diazomethane (Equation 8), the chloro-acid esters were subjected to gas chromatography.

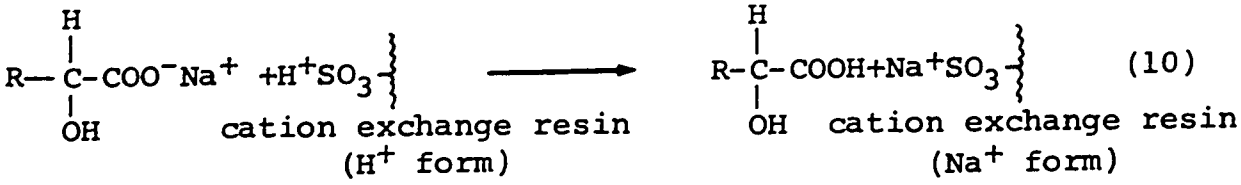
$$R\text{-}CHNH_2COOH + HCl + HNO_3 \longrightarrow R\text{-}CHCl\text{-}COOH + N_2O + H_2O \qquad (7)$$

$$R\text{-}CHCl\text{-}COOH + CH_2N_2 \longrightarrow R\text{-}CHCl\text{-}\overset{\overset{O}{\|}}{C}OCH_3 + N_2 \qquad (8)$$

The chloro-acid methyl ester corresponding to glycine, alanine, α-aminobutyric acid, valine, norvaline, leucine, isoleucine, and norleucine were separated.

Liberti (169) determined glycine, valine, alanine, leucine, serine, aspartic acid, and glutamic acid by gas chromatography. The amino acids were converted to hydroxy acids by treatment with sodium nitrite (Equations 9 & 10), which were then converted to methyl esters by means of diazomethane (Equation 11). The author pointed out that the method works well only for amino acids which react with sodium nitrite (α-amino acids).

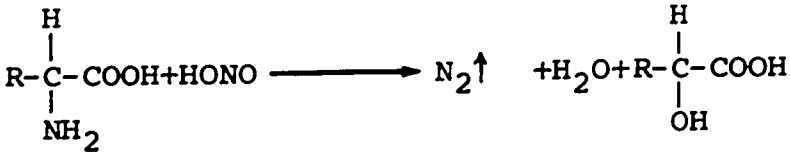
$$R\text{-}\underset{\underset{NH_2}{|}}{\overset{\overset{H}{|}}{C}}\text{-}COOH + NaNO_2 \longrightarrow N_2\uparrow + H_2O + R\text{-}\underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}}\text{-}COO^-Na^+ \qquad (9)$$



(10)

(11)

Wagner and Rausch (301) treated amino acids with HNO_2 (Equation 12) and the resultant hydroxy acids were converted to their methyl esters by means of diazomethane prior to gas chromatography (Equation 13). Although the yields of the



(12)

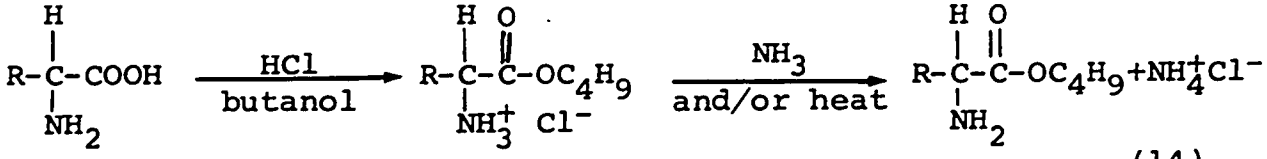
(13)

hydroxy acids were low (20-72%), they were reproducible. The authors were able to separate mixtures containing valine, leucine, isoleucine, threonine, methionine, phenylalanine, histidine, and arginine. The separation of leucine and isoleucine was not complete.

ii) Amino Acid Esters

Saroff, Karmen, and Healy (249) injected the

hydrochlorides of amino acid butyl esters (dissolved in a butanolic-HCl solution) directly onto a gas chromatographic column. The addition of ammonia to the carrier gas (nitrogen) liberated the esters from their hydrochlorides (Equation 14). The authors show a chromatogram in which the following amino acids were separated: alanine, valine, glycine,



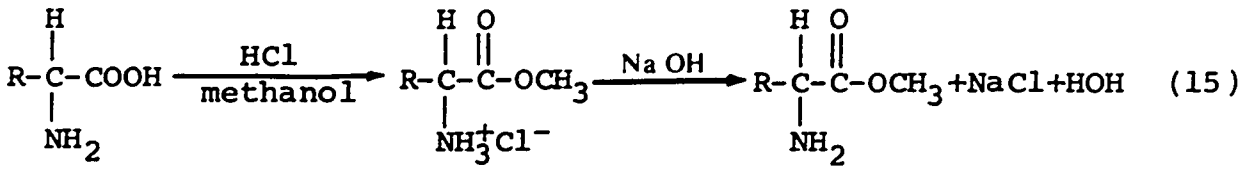
(14)

leucine, isoleucine, and proline. In addition to these amino acids, the authors claim satisfactory separation of aspartic acid, threonine, methionine, serine, glutamic acid, phenylalanine, lysine, and hydroxyproline.

Nicholls, Makisumi, and Saroff (220) demonstrated that acid salts of 15 of the 20 common amino acids were amenable to gas chromatographic analysis by virtue of their ease of dissociation under the influence of heat. The hydrochloride and hydroacetate salts of the methyl esters of amino acids were studied and it was found that the hydroacetate salts dissociated more readily than the hydrochloride salts at 120-200°C. The salts of lysine and arginine did not dissociate and they separated as their free bases. Cysteine, histidine, tyrosine, and tryptophan could not be separated

satisfactorily as the methyl esters of their free bases. The pairs glycine-alanine, leucine-isoleucine, and phenylalanine-hydroxyproline, were unresolved.

Bayer (33) also prepared methyl esters of amino acids contained in neutralized dried protein hydrolysates by treatment of the hydrolysates with saturated methanolic-HCl. The hydrochlorides of the amino acid methyl esters produced were converted to the free esters by the addition of 2 N NaOH followed by extraction with ether (Equation 15). The ether



(15)

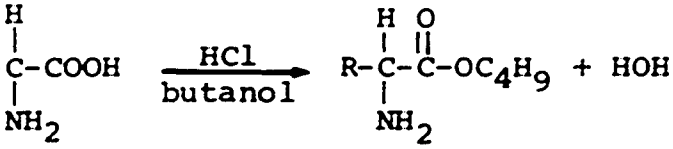
solutions were either injected directly, or the ether was evaporated and the resultant mixture of methyl esters was injected into the gas chromatograph. Bayer applied this technique to the analysis of albumin, casein, and transferrin, although the separation was limited to 7 amino acids.

iii) N-Acyl Amino Acid Esters

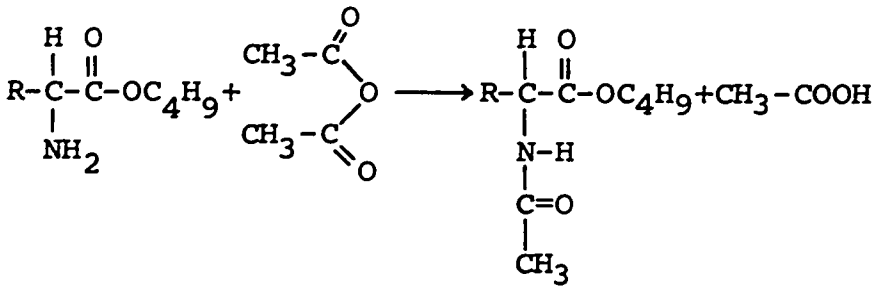
Bayer (33) was one of the early workers to observe that the methyl ester of an amino acid was not a satisfactory derivative because of the affinity of the amino group for the stationary phase. The author made some attempts to separate the N-trifluoracetyl esters of the amino acids and he was

able to separate glycine, valine, leucine, and proline.

Youngs (325) converted the amino acids to their N-acetyl butyl esters prior to gas chromatography (Equations 16 & 17). Earlier experiments by this author, in which he used the N-acetylated ethyl esters, proved unsuccessful since the ester of glycine tended to crystallize from the mixture and consequently a homogeneous sample could not be taken for injection. The basic steps in the procedure used by the author were as follows: a mixture of amino acids was dissolved in butyl alcohol and the solution was saturated with anhydrous HCl gas. One-half of the butyl alcohol was removed slowly by distillation at atmospheric pressure and the remainder was removed by vacuum distillation leaving a viscous liquid consisting of the ester hydrochlorides. Acetic anhydride was then allowed to react with the esters for one hour, after which the unreacted acetic anhydride was removed by vacuum distillation. The viscous oil was then injected directly into the gas chromatograph. Glycine, alanine, valine, leucine, isoleucine, and proline were determined in synthetic mixtures of pure amino acids and in protein (gelatin) hydrolysates.

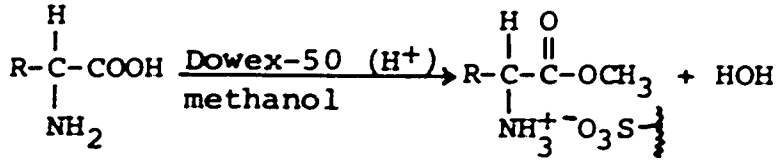


(16)

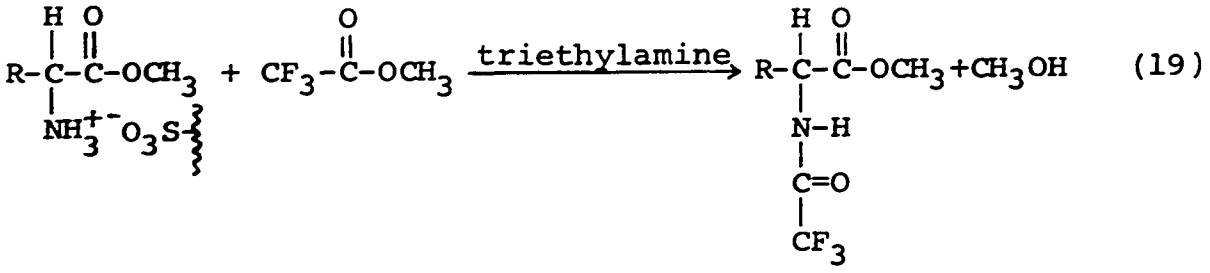


(17)

Saroff and Karmen (248) prepared the N-trifluoroacetyl methyl esters of several amino acids by esterification followed by acylation of the amino groups. They used a sulfonated polystyrene resin (Dowex-50; H^+ form) as a catalyst for esterification instead of dry HCl which they used previously (249), and which caused an undesirable component to appear as a shoulder on the emergence curve of glycine. The amino acids were added to anhydrous methanol containing Dowex-50 (Equation 18). The mixture was refluxed, cooled, and the methyl alcohol decanted. After rinsing the resin with anhydrous methanol, the methyl ester of trifluoroacetic acid in methanol was added to the resin, the solution was made alkaline by the addition of triethylamine, and the resultant alkaline solution was refluxed (Equation 19). The solution was cooled, decanted from the resin, and a sample was injected into the gas chromatograph without further treatment.



(18)



(19)

It was assumed that histidine formed the di-trifluoroacetyl methyl ester in solution, but analysis showed that histidine was mono-acetylated and that the derivative was in the form of the imidazolium salt. The N,S-di-trifluoroacetyl methyl ester of cysteine underwent a change in methanol and, hence, it was assumed that the mono-acyl derivative was formed. Analysis showed that the derivative of arginine contained three trifluoroacetyl groups. However, the analysis was not sufficiently precise to make possible a distinction between the di-trifluoroacetylated derivative (in the form of the trifluoroacetate salt) and the tri-trifluoroacetylated derivative. The following amino acids were analyzed as their N-trifluoroacetyl methyl esters: alanine, valine, isoleucine, leucine, glycine, proline, aspartic acid, threonine, methionine, serine, glutamic acid, phenylalanine, hydroxyproline, and lysine. The authors chose ionization detectors for their work because of their high sensitivity to organic substances. It was difficult to determine whether the failure to obtain identical peak areas for equal amounts of different amino

acids was the result of variation in detector response with different derivatives, or was the result of unequal yields in the synthesis of the derivatives.

Makisumi and Saroff (182) investigated the preparation, properties, and gas chromatography of the N-trifluoroacetyl methyl esters of the amino acids. This study illustrated the similarity in the properties of the mono-and the di-acetylated derivatives of cysteine, histidine, hydroxyproline, serine, threonine, tryptophan and tyrosine. The di-trifluoro-acetylated derivatives of hydroxyproline, serine, threonine, and tyrosine decomposed to give the mono-trifluoroacetylated derivatives on exposure to moisture or when dissolved in methanol. The di-trifluoroacetylated derivative of trypto-phan was formed readily from the methyl ester hydrochloride when treated with pure trifluoroacetic anhydride. This der-ivative is not converted to the mono-trifluoroacetylated derivative with the same ease as are the same derivatives of hydroxyproline, serine, threonine, and tyrosine. In fact, the di-acetylated derivative of tryptophan is stable when dissolved in methyl alcohol.

Weygand, Kolb, Prox, Tilak, and Tomida (315) subjected trifluoroacetyl derivatives of amino acids and dipeptide methyl esters to gas chromatography. The methyl esters were

prepared by refluxing the amino acid hydrochlorides in methanolic-HCl for two hours. The solvent was removed and the methyl ester hydrochlorides were added to a mixture of methanol, triethylamine, and methyltrifluoroacetate. The solvent was evaporated and the derivatives were isolated by extraction with ethyl acetate. Derivatives of the following amino acids were successfully chromatographed: glycine, valine, alanine, leucine, isoleucine, aspartic acid, proline, methionine, phenylalanine, and lysine.

Wagner and Winkler (302) prepared the N-trifluoroacetyl methyl esters of amino acids by a method which involved acetylation followed by esterification. Trifluoroacetic anhydride was added to a solution of the amino acid in trilfuoroacetic acid and the reaction mixture was allowed to stand (-10 to 0°C) for at least four hours. The solution was evaporated under vacuum and the residue was dissolved in methyl acetate. Esterification was effected by use of diazomethane in ether. These authors found that the ε-amino group of lysine did not react with trifluoroacetic anhydride under these conditions. Since basic amino acids are somewhat insoluble in methyl acetate, difficulty was encountered with esterification involving the use of ethereal diazomethane. The threonine derivative gave two peaks which the authors

suggested was due, in part, to an N→O acyl shift. The authors used this method for the separation of valine, leucine, threonine, methionine, and phenylalanine.

Johnson, Scott, and Meister (142) subjected N-acetylamino acid n-amyl esters to gas chromatography. They developed a procedure for the preparation of the derivatives and suggested conditions for the separation of 35 amino acids, including 18 of the common amino acids. Studies were first carried out with 7 amino acids to determine which of the following N-acetyl derivatives could be separated most successfully: n-butyl, isobutyl, n-amyl, and isoamyl. The superior separation of the n-amyl esters of acetylated alanine and valine led these workers to carry out subsequent studies with N-acetyl n-amyl amino acid esters. Recovery experiments indicated that amino acids could be converted to their amyl esters in 90% yield. These workers also showed that there was no difference in the chromatographic behavior of D- and DL-derivatives. The esterification procedure was as follows: the amino acid (1-10 mg) was suspended in the alcohol (20 ml) which was then saturated with anhydrous HBr. The mixture was heated at 165°C (30 min), and part of the alcohol was removed by distillation at atmospheric pressure. The remainder was evaporated in a vacuum rotary evaporator

(60°C). The resultant mixture of ester hydrobromides was then mixed with acetic anhydride (8 ml) and the mixture was allowed to stand for five minutes (26°C). The solution was evaporated in a rotary evaporator under vacuum (60°C) and the residue was dissolved in benzene or n-amyl alcohol prior to gas chromatography. Analysis indicated that the acetylation of the hydroxyl groups of serine, threonine, tyrosine, and hydroxyproline occurred and that both carboxyl groups of glutamic acid and aspartic acid were esterified. Cystine did not form a stable derivative and histidine and arginine were esterified in poor yield under the conditions of the experiment. No peak was observed for tryptophan.

Zomzely, Marco, and Emery (327) were able to separate derivatives (n-butyl N-trifluoroacetyl) of 22 naturally occurring amino acids. The N-trifluoroacetyl derivative is more volatile than the corresponding N-acetyl derivative (325), and this resulted in faster column elution. They noted that there was no difference in the chromatographic behavior of L- and DL-derivatives (142) and that the response of the detector (hydrogen flame) was not the same for equal amounts of the different amino acids. The authors cited the explanation of Dewar (69) who suggested that the hydrogen flame detector responds primarily to the C-H bond and that

functional groups such as carbonyl, carboxyl, ether, and amino, tend to cause a decrease in response. Thus, the lysine derivative which has two trifluoroacetylated amino groups would produce a smaller response than the derivative of alanine, for example, which contains no interfering groups. The procedure used by the authors was as follows: the amino acid(s) (10 mg) was dissolved in a mixture of 1-butanol (20 ml) containing anhydrous HCl (5%), dimethylformamide (10% of the volume of butanol; this compound is necessary to esterify the basic amino acids), and dibutoxypropane (used as a "water scavenger"; 3x the amount required to react with the water of esterification). The reaction mixture was placed in an Erlenmeyer flask which was fitted with a $CaCl_2$ drying tube, and heated (55-60°C) for three hours with constant stirring. The alcohol was evaporated in a rotary evaporator (60°C) and the resultant mixture of butyl ester hydrochlorides was neutralized with Na_2CO_3 (1 N) and extracted three times with methylene chloride (10 ml portions). The combined extract was evaporated (30°C) in a rotary evaporator and water was removed from the residue by azeotropic distillation using methylene chloride (10 ml). The residue was dissolved in methylene chloride (2-5 ml) containing dimethylformamide (2%). Trifluoroacetic anhydride (0.2-0.5 ml)

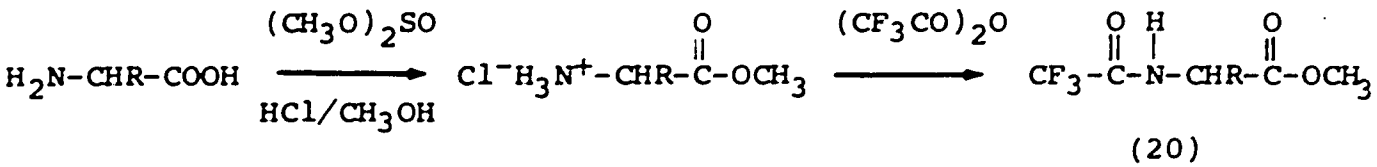
was added and acetylation was carried out at 28°C (30 min). Excess reagents were removed *in vacuo* and the residue was dissolved in acetone prior to gas chromatography. The use of mild esterification conditions preserved amino acids such as tryptophan, cystine, cysteine, arginine, and histidine. Preliminary studies indicated that the amino acids were converted to their n-butyl esters in 90% yield.

Graff, Wein, and Winitz (108) converted 30 amino acids to their N-acetyl n-propyl esters which were used as standards in gas-liquid chromatography. The method involved: (a) azeotropic removal of water from the reaction mixture (n-propanol-benzene containing HCl) during esterification, (b) acylation of the n-propyl ester with acetic anhydride, (c) liquid-liquid extraction of the amino acid derivatives from the reaction mixture, (d) removal of the solvent by evaporation, and (e) dilution of the residual syrup with fresh solvent prior to gas chromatography.

Losse, Losse, and Stoeck (173) prepared the N-formyl methyl esters of some amino acids by reaction of the amino acids with formic acid in acetic anhydride followed by esterification with diazomethane in methanol-ether. They claimed that the derivatives were formed in at least 95% yields. The derivatives of glycine, alanine, valine, leucine, norvaline,

and proline were separated on one column and derivatives of aspartic acid, glutamic acid, methionine, and phenylalanine were separated on another column.

Cruickshank and Sheehan (60) developed a method for the preparation of N-trifluoroacetyl methyl esters of 21 naturally occurring amino acids. The sample of amino acids was suspended in methanol (5 ml) which was then saturated with anhydrous HCl. Dimethyl sulfite (1 ml) was added and the mixture was heated (steam bath) under reflux for ½ h, precautions being taken to exclude moisture. The excess methanol and dimethyl sulfite were then removed under reduced pressure and the residue of methylester hydrochlorides was dried under high vacuum. Trifluoroacetic anhydride (1 ml) was added to the flask containing the methyl ester hydrochlorides and the flask was heated (10 min) under reflux. The excess of trifluoroacetic anhydride and trifluoroacetic acid was evaporated under nitrogen and the residue (20-120 μM) was dissolved in trifluoroacetic anhydride (0.1 ml)(Equation 20).



(20)

Experiments were also performed in which the amino acids were first acetylated and then esterified by means of diazomethane.

When the amino acid derivatives were dissolved in solvents other than trifluoroacetic anhydride and then injected into the gas chromatograph, the normal derivative for arginine would be replaced by a substance with a lower retention time. The coefficients of variation for relative (to the derivative of alanine) peak areas, obtained by six repetitions of the procedure using a standard mixture of amino acids, varied from ± 2.3 to ± 9.7%, with the exception of cysteine (± 30%) and histidine (± 15.7%).

Hagen and Black (116) devised a method for the separation and estimation of the N-trifluoroacetyl methyl esters of 19 amino acids in a protein hydrolysate. Methanol (20 ml) was placed in a round bottom flask (100 ml), the amino acid mixture was added, and the flask was shaken at room temperature. The flask was then cooled (dry ice) and thionyl chloride (2-4 ml) was added dropwise to the mixture with constant agitation. The flask was allowed to stand (40°C, 2 h), precautions being taken to exclude moisture. The reaction mixture was then concentrated (rotary evaporator, room temperature), the reaction mixture was cooled again, and trifluoroacetic anhydride (1-2 ml) was added. The reaction mixture was allowed to stand (room temperature, 2 h), excess reagents were removed by means of a rotary evaporator (room temperature), and the residue was dissolved in dry methanol and the

volume was adjusted to 5 ml. Up to 20 µl of this solution was injected onto the gas chromatographic column. The authors did not report the recoveries of individual amino acids. The concentration of amino acid in a hydrolysate was ascertained by reference to a standard calibration curve drawn for each amino acid.

Darbre and Blau (62), who worked with the N-trifluoroacetyl n-amyl esters of the amino acids, observed that the derivatives of cysteine, hydroxyproline, serine, threonine, and tyrosine underwent degradation on standing at room temperature. Weygand and Rinno (316) had previously observed that the O-TFA* groups of serine and threonine were sensitive to moisture. In addition, Bourne, Tatlow, and Tatlow (46) had shown that esters of trifluoroacetic acid could easily be "hydrolyzed"**, even by alcohols. Darbre and Blau noted that acyl derivatives were stable for a considerable period of time if they were dissolved in methyl ethyl ketone or nitromethane that had been carefully dried, but were unstable in benzene, ethylene dichloride, and n-amyl

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*TFA = trifluoroacetyl.

**The author realizes that "hydrolysis" is a process of decomposition which involves the addition of water. In this case, the "hydrolysis" by alcohol of amino acid acyl esters is actually a process of interesterification; hence, the quotation marks.

trifluoroacetate. An experiment was performed to study the rate of hydrolysis in a mixture of water and methyl ethyl ketone of the O-TFA and S-TFA groups of the N-trifluoroacetyl n-amyl esters of cysteine, hydroxyproline, serine, threonine, and tyrosine. The results indicated that the hydrolysis followed first order kinetics. The phenolic O-TFA group of tyrosine was most easily hydrolyzed, followed by the S-TFA group of cysteine, the O-TFA group of serine, the O-TFA group of hydroxyproline, and the O-TFA group of threonine (Figure 20). The hydrolysis led to the formation of less volatile N-trifluoroacetyl n-amyl derivatives which had free -OH or -SH groups. The hydrolysis did not go to completion. The authors suggested that quantitative yields of the N-mono-TFA derivative are not realized because an intra-molecular N→O acyl shift with rapid hydrolysis of the resultant O-mono-TFA compound. These workers showed that the derivatives of tyrosine and hydroxyproline were stable (for at least 1 h at 200°C) and suggested that about 10% of the derivatives of cysteine, serine, and threonine decomposed at 145°C during the first hour of heating, possibly due to the presence of traces of oxygen in the carrier gas (nitrogen).

Makisumi, Nicholls, and Saroff (181) prepared the N-acetyl and the N-trifluoroacetyl derivatives of the methyl,

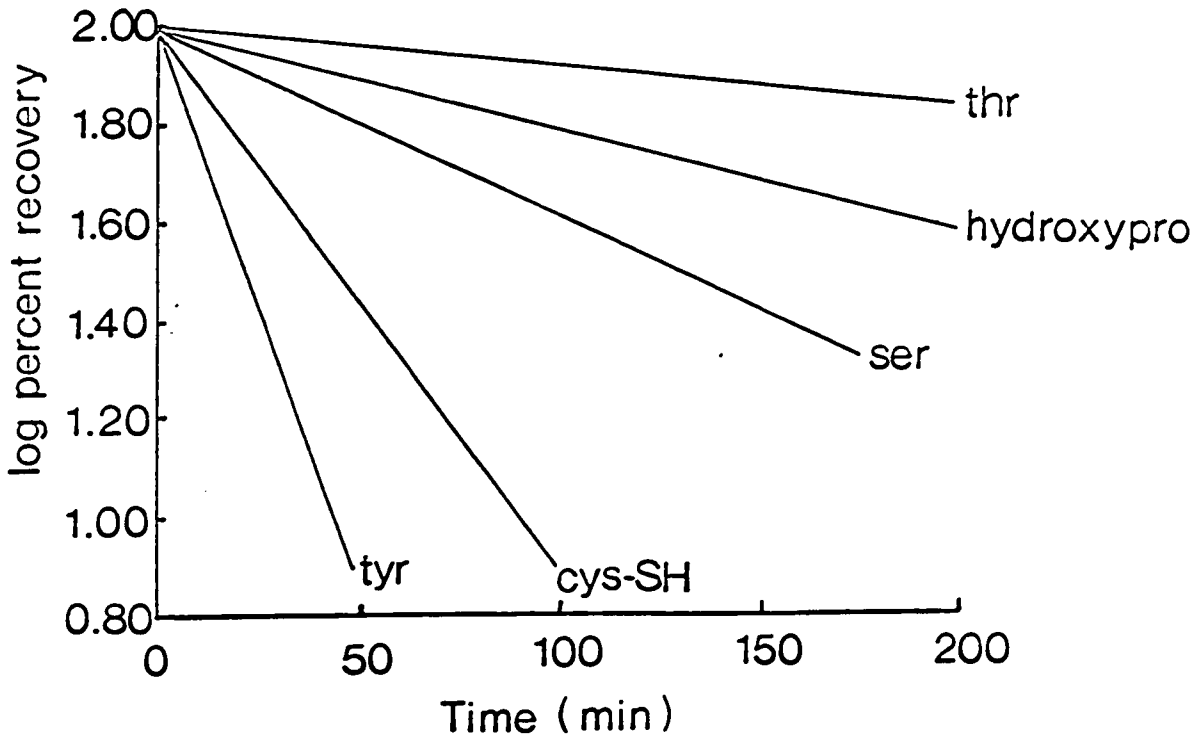


Figure 20. Hydrolysis of S-TFA and O-TFA groups of certain trifluoroacetylated n-amyl amino acid esters.[a,b]

[a]From: Darbre and Blau (62).

[b]Hydrolysis carried out in methyl ethyl ketone containing water (5% v/v).

ethyl, butyl, and amyl esters of several amino acids and investigated the suitability of the different derivatives for gas chromatography. They concluded that, all other factors being equal, the derivative of choice is the one with the lowest retention time; thus, the trifluoroacetyl methyl esters should be the most satisfactory for gas chromatography.

Darbre and Blau (61) prepared the N-trifluoroacetyl n-amyl esters of alanine, glycine, valine, leucine, isoleucine, serine, and threonine. The amino acid (2 mg) was placed in a test tube with amyl alcohol (1-pentanol; 2 ml). The tube was maintained at 108°C and a continuous stream of HCl was passed (25 min) through the alcohol by means of a Pasteur pipette. The excess alcohol was removed under vacuum by means of a rotary evaporator. Trifluoroacetic anhydride (0.1 ml) was added to the residue and the tube was allowed to stand for one hour at room temperature, after which the excess trifluoroacetic anhydride was removed by means of a rotary evaporator (room temperature). The N-trifluoroacetyl n-amyl esters were dissolved in methyl ethyl ketone or nitromethane (100-200 µl). The authors were unable to separate all seven derivatives in a single run and found it necessary to use two different columns.

Unfortunately, temperature programming could not be

used to improve the separation because isothermal conditions were a requirement of the type of detector (gas density balance) used in this study. In subsequent publications, Blau and Darbre applied their method to the separation of leucine, cysteine, proline, hydroxyproline, methionine, phenylalanine, aspartic acid, and glutamic acid (41) and to the separation of aspartic acid, lysine, ornithine, and tryptophan (42).

Trifluoroacetylation of the n-pentyl esters of arginine, tryptophan, and cystine produced derivatives having very long retention times. In addition, histidine did not form a derivative that could be detected using a gas chromatograph. Thus, Islam and Darbre (133) preferred to use the N-trifluoroacetyl methyl ester derivatives. The amino acids (2 mg, total) were placed in a stoppered test tube with dry methanol (2 ml). The tube was then placed in an oil bath ($70 \pm 2^{o}C$) and dry HCl was bubbled through the reaction mixture (30 min). The mixture was then evaporated to dryness by means of a rotary evaporator, first using a water pump and finally using an oil pump (65). The mixture of amino acid methyl ester hydrochlorides was treated with trifluoroacetic anhydride in methylene chloride (4:1, 0.2 ml) and the reaction mixture was allowed to stand for 20 min at room temperature. To avoid losses of the esters, the solvent was removed at $0^{o}C$ by means of a rotary evaporator. The authors

used a silicone column for the separation of the derivatives, since Darbre and Blau (63) had reported earlier that non-silicone polar stationary phases might catalyze the break-down of O-TFA and S-TFA groups. Darbre and Blau (63) were unable to obtain consistent results in their studies on cysteine, hydroxyproline, serine, threonine, and tyrosine. Table 16 lists the stationary phases with which Darbre and Blau (63) got complete or partial loss of the peaks for these five amino acid derivatives. The authors point out that most of these phases contained polar groupings and that these groupings were probably the ones which acted as catalysts in the decomposition of trifluoroacetyl derivatives of amino acids containing hydroxyl and sulfhydryl groups.

Islam and Darbre (133) showed that the size of the ester group affects greatly the order of emergence of some amino acids from the column. For example the methyl ester of phenylalanine has a longer retention time than that of glutamic acid, but the reverse is true for the n-pentyl (133) and the n-butyl (97) esters. The methyl ester derivatives of hydroxyproline and methionine appear after aspartic acid and the reverse is true with the n-pentyl (133) and n-butyl (97) esters. Islam and Darbre separated the derivatives made from amino acids in hydrolysates of insulin and gelatin.

Table 16

Stationary phases which cause breakdown of trifluoroacetylated esters of cysteine and hydroxyl amino acids[a]

Phase	Synonym	Polarity[b]
EGS	Ethylene glycol succinate	0.59
GE-XF-1105		0.59
TTP	Tri-o-tolyl phosphate	0.61
GMS	Glyceryl monostearate	0.64
GMHS	Glyceryl monohydroxystearate	0.70
Admul S57	Polyglycerol ester of hardened tallow fatty acids (Advita Ltd)	0.73
Admul 19	Polyglycerol ester of mixed fatty acids	0.76
SPAN's	Sorbitan monolaurate,-monooleate,-dioleate, and -trioleate	0.7-0.9
GMR	Glyceryl monoricinoleate	0.84
PPS	Polypropylene sebacate	0.89
NPGSb	Neopentyl glycol-sebacic acid polyester	0.90
GE XE-60	Cyanoethyl silicone elastomer	0.92
Organometallic liquids		0.6-1.5
Versamid 900	Polyamide from dimerized linoleic acid and ethylene diamine	1.08
NPGG	Neopentyl glycol-glutaric acid polyester	1.11
TWEEN's	Polyoxyethylene Sorbitan monostearate, -tristearate, -monooleate, -trioleate	1.0-1.6
Hi-Eff 8B	Cyclohexane dimethanol-succinic acid polyester	1.17
DEGA	Diethylene glycol-adipic acid polyester	1.19
GE XF-1150		1.19
GE SF-1066		1.22
GE SF-1034		1.37
BDS	Butane-1,4-diol-succinic acid polyester	1.42

Table 16 (cont'd)

Phase	Synonym	Polarity
Ucon 50-HB-2000		1.50
ECNSS-S		1.53
EGCNSS-S	Ethylene glycol succinate-cyanoethyl copolymer	1.54
PEG-L	Polyethylene glycol lauryl ether	1.64
PEG-S		1.69
DEGS	Diethylene glycol-succinic acid polyester	1.70
Carbowaxes		1.8 (approx)
EAA		2.09
Antarox-CO-990		2.15

[a]Data of Darbre and Blau (63).

[b]"Polarity" is expressed as the retention time of the glycine derivative relative to that of the leucine derivative. The larger the value, the greater the "polarity".

The authors pointed out the following advantages of using methyl esters: (a) they are easily prepared in a single step operation; (b) the methyl esters emerge from the column sooner than do higher esters because of their higher volatility; and (c) the lower operating temperatures made possible by the use of methyl esters reduce column bleeding and associated "noise", base-line drift, and the risk of derivative decomposition at higher temperatures (65).

In the first two (Lamkin and Gehrke (156) and Gehrke, Lamkin, Stalling, and Shahrokhi (93)) of several papers of which Gehrke was either an author or co-author, the authors reported the separation of amino acids as their n-butyl N-trifluoroacetyl esters. The amino acid or mixture (<60 mg) of amino acids was placed in a glass-stoppered flat-bottom flask (125 ml) and HCl in methanol (1.2 N) was added to the mixture (10 ml). The mixture was stirred (25°C, 30 min) by means of a magnetic stirrer, and excess methanol was removed by vacuum distillation (60°C) when the esterification was complete. Butanolic-HCl (1.2 N) was added to the mixture (10 ml) which was then heated (90°C) for three hours with constant agitation. The butanolic-HCl was then removed from the reaction mixture by vacuum distillation. The n-butyl ester hydrochlorides were dissolved in methylene chloride (5 ml) to which was

added trifluoroacetic anhydride (0.50 ml) and the flask containing the reaction mixture was placed on a magnetic stirrer. The flask was stirred (room temperature) for two hours and excess reagents were then removed by vacuum distillation. The resultant mixture of derivatives was dissolved in anhydrous chloroform prior to chromatography. The authors claimed that glutamic acid gave the dibutyl ester and tyrosine and lysine gave the di-trifluoroacetyl derivatives. Methionine was not converted to the sulfoxide. All carboxyl groups were esterified and all amino, imino, phenolic, hydroxy, sulfhydryl, and imidazole groups were trifluoroacetylated. They also claimed that tryptophan gave the mono- and the di-acyl derivatives. Arginine was converted to the α-trifluoroacetyl n-butyl ester ω-trifluoroacetate salt and hence could not be chromatographed successfully.

Lamkin and Gehrke (156) showed that the trifluoroacetyl esters are more volatile than the corresponding acetyl esters and that the methyl esters are more volatile than the n-butyl esters. They pointed out that if volatility is too great, then losses will occur during the concentration step which follows trifluoroacetylation. They chose the n-butyl N-trifluoroacetyl esters as they represented a satisfactory compromise between volatility and ease of chromatographic

separation. Table 17 (156) shows the retention temperatures for different derivatives of four amino acids. The authors noted that excessively high temperatures in the flash heater (injection port) caused decomposition of certain derivatives with the production of extraneous peaks. They lowered the temperature of the flash heater from 223 to 142°C and noted considerable reduction in the size of the major extraneous peak of threonine. They finally solved the problem of decomposition by elimination of the flash heater and by using direct "on-column" injection. Recoveries, as determined by peak areas, were essentially quantitative for derivatives which had been allowed to stand for 90 hours or more, and no instability of the derivatives was indicated. Extraneous peaks due to hydrolysis of the O-trifluoroacetyl ester upon standing prior to chromatography were noted for hydroxy amino acids, but hydrolysis was prevented by storing the derivative in a solvent containing a small amount of trifluoroacetic anhydride. Table 18 lists some of the physical constants of amino acid n-butyl ester hydrochlorides and n-butyl N-trifluoroacetyl esters.

The method developed by Gehrke, Lamkin, Stalling, and Shahrohki (93) gave the trifluoroacetate salt of arginine which was unsuitable for gas chromatography. Although the

Table 17

Retention temperatures of amino acid derivatives[a]

Amino Acid	Retention temperature[b] (°C)				
	N-trifluoroacetyl methyl ester A[c]	N-acetyl methyl ester A[c]	N-trifluoroacetyl n-butyl ester A[c]	N-trifluoroacetyl n-butyl ester B[d]	N-acetyl n-butyl ester B[d]
Valine	56	65	109	71	110
Phenylalanine	133	141	167	130	156
Glutamic Acid	123	137	180	144	170
Lysine	200	e	209	173	216 (tailed)

[a]Data of Lamkin and Gehrke (156).

[b]Five μl of solution (10 mg of amino acid converted to derivative and dissolved in 2.00 ml $CHCl_3$) were injected directly onto the chromatographic column without the use of a flash heater.

[c,d]Indicates different conditions of chromatography. Refer to paper for details.

[e]Not eluted under the chromatographic conditions employed.

Table 18

Physical constants of amino acid n-butyl ester hydrochlorides and n-butyl N-trifluoroacetyl esters*

Amino acid	n-Butyl ester HCl, m.p. (°C)	n-Butyl N-trifluoroacetyl ester b.p. (°C/mm Hg)	m.p. (°C)
Valine	59.2-60.0	98.0-99.0 /43	
Isoleucine	liquid	94.8-95.3 / 1.7	
Methionine	57.6-59.0	126.0-129.0 /18	
Glutamic acid	62.7-63.2	159.2-161.0 /13	
Tyrosine	161.8-164.0	70-83 $/10^{-3}$	96.9-98.0
Lysine	liquid	85-90 $/10^{-3}$	86.0-87.3

*Data of Lamkin and Gehrke (156) or cited by Lamkin and Gehrke (tyrosine and lysine).

hot metal flash heater of the injection port caused formation of the desired tri-acyl arginine derivative from the salt upon injection, this was accompanied by partial decomposition to the ornithine derivative (Figure 21). This led Stalling and Gehrke (267) to the development of a method for the formation of the tri-acyl derivative of arginine which involved a high-temperature acylation reaction (150°C, 5 min) in a centrifuge tube (12 ml) sealed with a teflon-lined cap. High-temperature acylation ensured the conversion of arginine

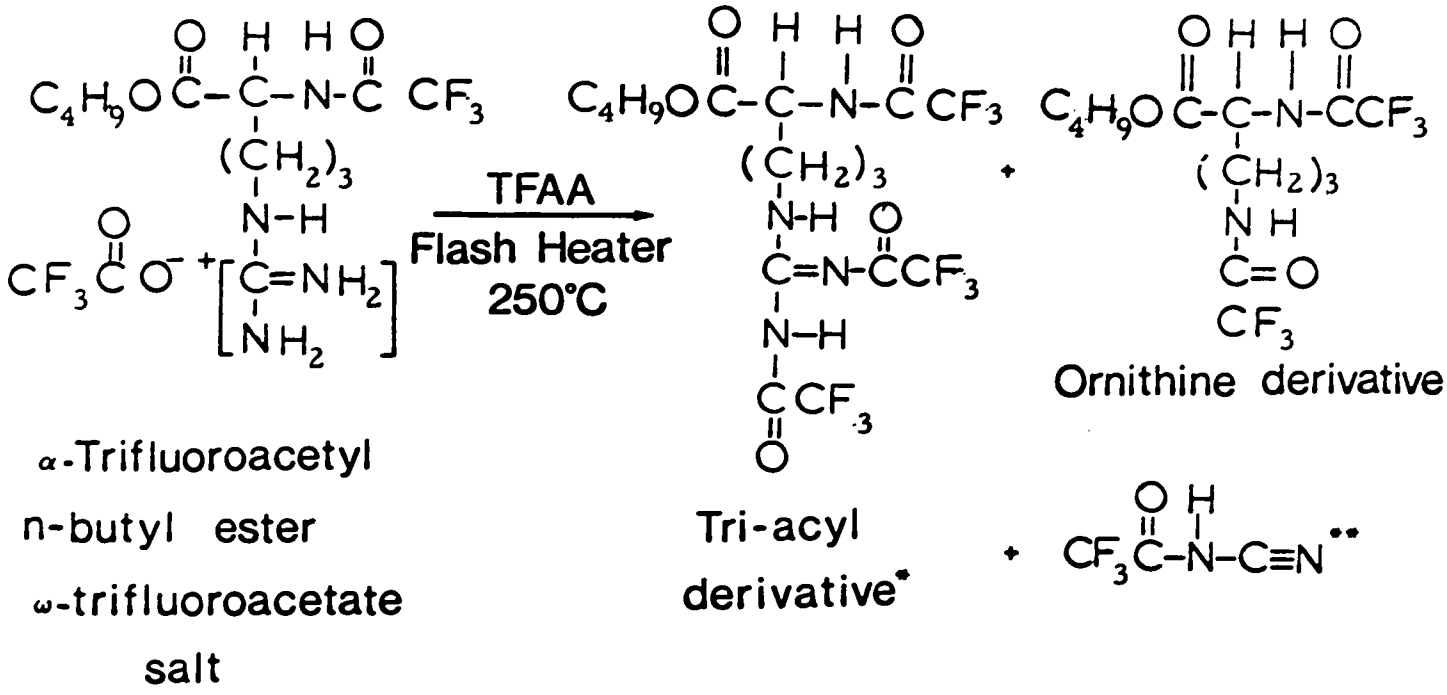


Figure 21. Reactions of arginine in the flash heater*.

*From: Stalling and Gehrke (267)

to the tri-acyl derivative, and direct "on-column" injection avoided adverse decomposition (<1%) to ornithine. High-temperature acylation gave relative peak areas for the other 18 protein amino acids which were identical to the peak areas obtained by acylation at room temperature for two hours. Tryptophan was also converted quantitatively to the di-acyl derivative. Figure 22 illustrates the acylation reactions of arginine.

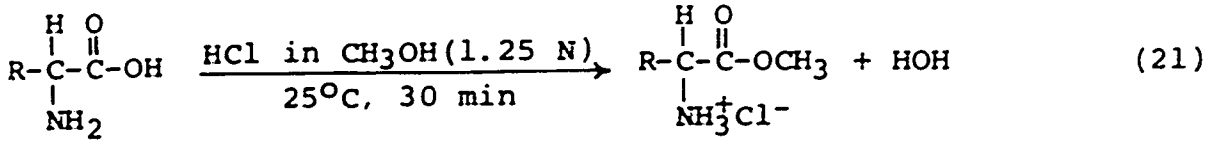
Gehrke and Shahrokhi (96) obtained complete resolution of 20 n-butyl N-trifluoroacetylamino acid esters by use of a mixed stationary phase column of 0.75/0.25 w/w % of DEGS/EGSS-X. The authors applied the method to the analysis of bovine serum albumin and K-casein hydrolysates.

Stalling, Gille, and Gehrke (269) tried to increase the yields of the n-butyl esters of threonine and cystine, which were less than 95% (93). Cystine and threonine (20 mg samples) were converted to their methyl esters. These were subjected to the interesterification reaction (10 ml butanol) under different conditions (90°C for 180 min and 100°C for 150 min with HCl concentrations of 1.25 and 3.25 meq/ml) and the yields of the n-butyl ester (hydrochlorides) were determined. They found that there was less methyl ester remaining after interesterification at 100°C than at

90°C and that the HCl concentration did not affect the conversion as markedly as did the esterification temperature. They claimed that the yields were increased to 100% for threonine and 98.2% for cystine. The yields of the other amino acids were not affected.

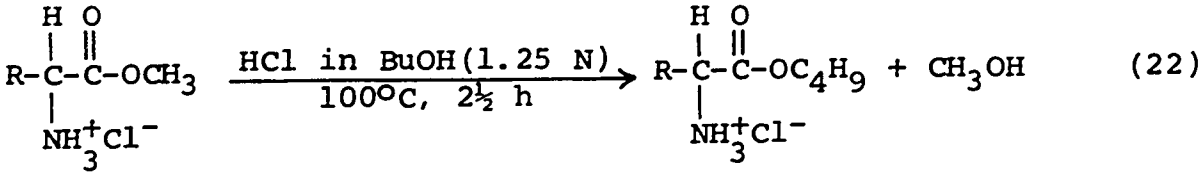
Gehrke and Stalling (97) published a paper in 1967 in which they recommended a procedure for routine determination of amino acids in protein hydrolysates. They claimed an average yield of 98 ± 3% for 20 amino acids and reported excellent agreement between the results obtained by their method and those obtained by ion-exchange methods. For quantitative analysis, the amount of amino acid injected was 0.05-35 μg. Results obtained for the analysis of bovine serum albumin, K-casein, and soybean protein by gas chromatography and ion-exchange methods were given. A more detailed examination of the method and practical application of the method are given in a publication of the Analytical BioChemistry Laboratories, Inc. (9). The basic steps involved in the method are outlined below (Equation Nos. 21, 22, and 23).

(a) Esterification of amino acids to form methyl ester hydrochlorides



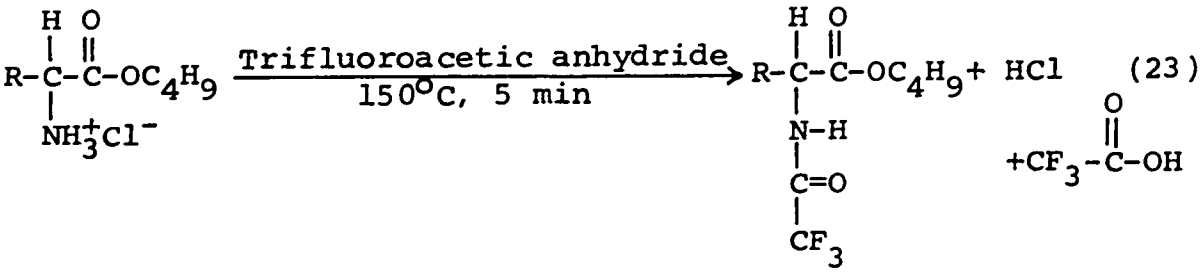
(21)

(b) Interesterification of amino acid methyl ester hydrochlorides to form n-butyl ester hydrochlorides



(22)

(c) High-temperature acylation of n-butyl ester hydrochlorides to form N-trifluoroacetyl n-butyl esters



(23)

Gehrke, Zumwalt, and Wall (100) investigated several factors which influenced the performance of the chromatographic system, including the separation characteristics of various polyesters of neopentyl glycol (succinate, adipate, sebacate, and brassylate) and of support materials which had undergone different heat treatments. Eight amino acid derivatives were chromatographed under identical conditions and the retention volumes (relative to alanine=1) were plotted against the carbon atom number of the dicarboxylic acid. The graph (Figure 23) indicated that maximum separation occurred at carbon number 10, that is, with neopentyl glycol sebacate. Inspection of the structure of the amino acid n-butyl N-trifluoroacetyl esters indicates that there are 10

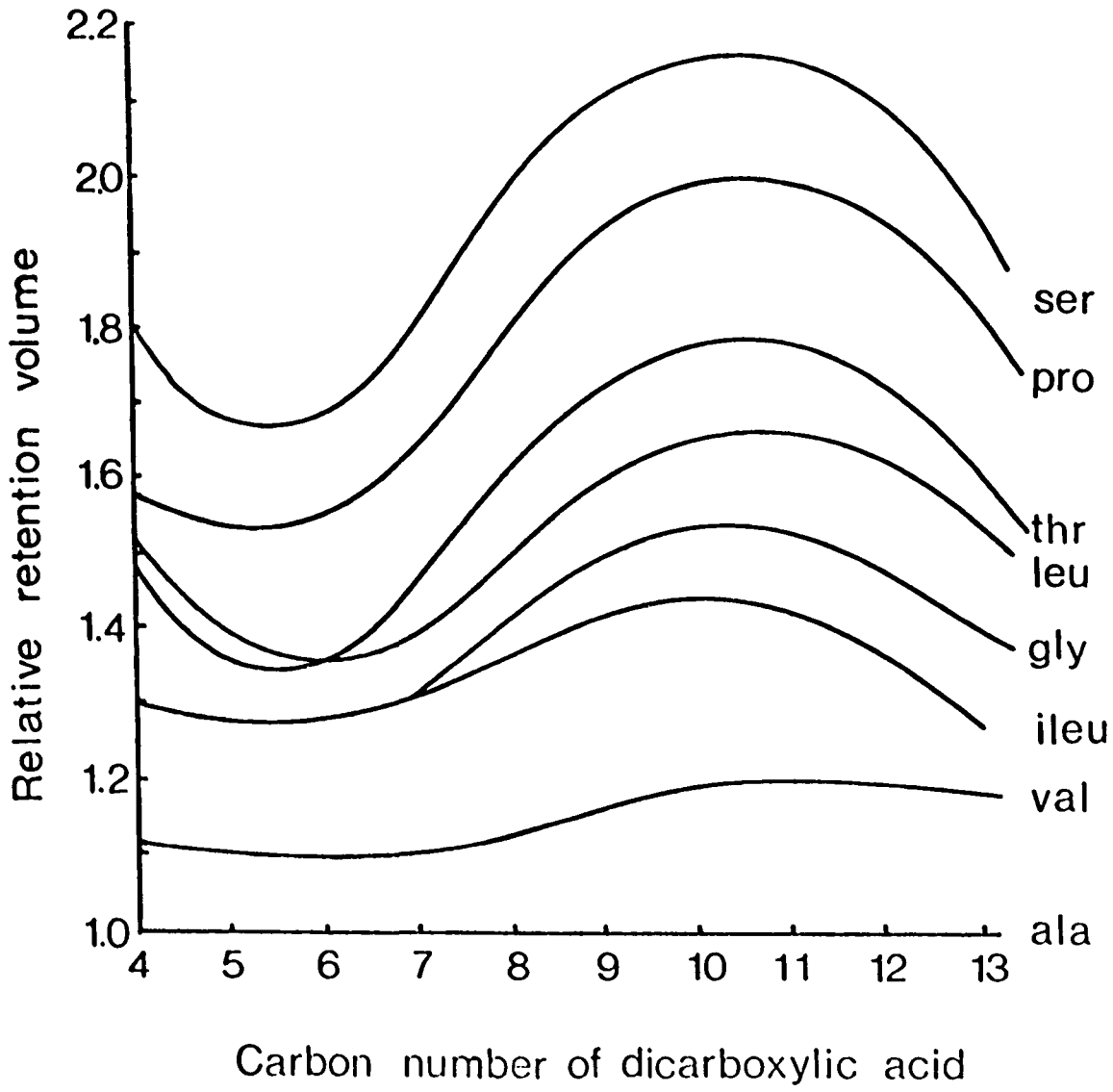
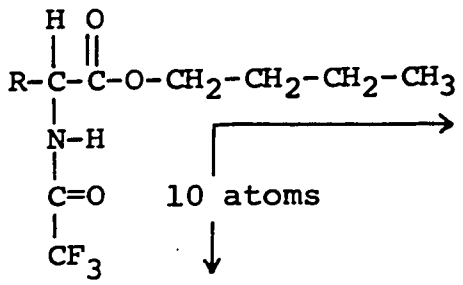


Figure 23. Relative* retention volumes of N-TFA n-butyl esters of amino acids chromatographed on polyesters of neopentyl glycol**.

*Retention volume of alanine = 1

**From Gehrke, Zumwalt, and Wall (100)

atoms from the trifluoromethyl group to the terminal methyl group of the butyl ester.



This fact led these workers to suggest that the maximum separation obtained with neopentyl glycol sebacate might be the result of preferential orientation of the aliphatic portion of the amino acid with the $-CH_2-$ groups of the polyester, and of the polar trifluoroacetyl groups with the ester groups in the polyester. The separation ability of ethylene glycol adipate (0.325% on acid-washed, heat-treated (450-600°C, 15 hr) Chromosorb G (80/100 mesh)) was found to be superior, however, to neopentyl glycol sebacate. Arginine, histidine, and cystine were not eluted from either columns because of interaction with the liquid phase. The best separation of these amino acid derivatives was obtained with an OV-17 (siloxane) liquid phase (1.5% w/w) coated on high-performance Chromosorb G (80/100 mesh).

Stefanovic and Walker (273) studied the effect of liquid phase (ethylene glycol adipate) loading (0.5, 0.7, 0.8, 1.0, and 2.0% w/w EGA on acid-washed Chromosorb W (80/100

mesh)) on the separation of n-butyl N-trifluoroacetyl amino acid derivatives. They found that 0.65% ethylene glycol adipate accomplished the separation of 17 of the common amino acids. Low responses were obtained for arginine, histidine, and cystine, and the authors suggested that this indicated incorrect preparation of the derivatives, or decomposition on the column, or chemical reaction with the polar stationary phase.

Roach and Gehrke (241) studied the various factors which affected the separation of the n-butyl N-trifluoroacetyl esters; namely, the effect of liquid phase loading (0.325-0.65% w/w EGA), the effect of heat-treatment of acid-washed Chromosorb W, and the effect of mesh size of the dried support (60/80, 80/100, and 100/200 mesh). The latter parameter did not markedly affect separations and the best separations were obtained with 0.65 w/w % EGA coated on 80/100 mesh acid-washed Chromosorb W (dried at 140°C for 12 hr).

Roach, Gehrke, and Zumwalt (242) developed a method by which histidine could be analyzed as its di-acyl n-butyl N-trifluoroacetyl ester. Evaporation of excess acetylating reagent and solvent in the final concentration step in the derivatization procedure caused the di-acyl derivative of histidine to be converted to the monoacyl derivative. Lamkin

and Gehrke (156) had shown previously that the storage of derivatives in a solvent containing a small amount of trifluoroacetic anhydride prevented hydrolysis of the di-acyl derivatives of tyrosine, serine, threonine, and hydroxyproline to their mono-acyl derivatives. Roach and his coworkers showed that the injection of 4 μl of trifluoroacetic anhydride immediately after the appearance of the methionine peak resulted in quantitative reproducible conversion of the mono-acyl derivative of histidine to the di-acyl derivative, and elution of the histidine derivative precisely between the derivatives of tyrosine and glutamic acid.

Roach and Gehrke (240) shortened their earlier procedure (97) by elimination of the step involved in the formation of the methyl ester. The amino acids (~2 mg) were dissolved in butanolic-HCl (3 N, 150 μl per 100 μl total amino acids) with the aid of ultrasonic mixing (15 seconds). The authors showed that 19 of the common amino acids were esterified quantitatively with butanolic-HCl (3 N) in 15 min at 100°C. Isoleucine required an esterification time of 35 min. Tryptophan, however, underwent some decomposition (about 15%) under these conditions. After removal of the solvent under nitrogen (100°C), the water of esterification was removed by azeotropic distillation using methylene

chloride (150 μl). A mixture of methylene chloride and trifluoroacetic anhydride (3:1, 80 μl for each 100 μg amino acids) was added to the screw-topped (teflon) culture tube which was then heated at 150°C for 5 min. The sample was then injected directly into the gas chromatograph. Gehrke and Zumwalt (98) applied this method to the determination of 20 protein amino acids and 50 non-protein amino acids. This work dealt in particular with the ion-exchange clean-up procedures required for biological samples and the application of the method to the analysis of blood, urine, and geochemical samples, including those of Apollos' 11 and 12 (99).

Zumwalt, Roach, and Gehrke (329) adapted their earlier methods to the quantitative analysis of free amino acids in complex biological substances, such as blood plasma and urine. The authors showed that the optimal ratio of trifluoroacetic acid to total amino acids is 50:1, with respect to reproducibility of acylation, stability of the derivative, and maintenance of a minimal sample volume. They confirmed the earlier observation of Lamkin and Gehrke (156) that solutions of N-trifluoroacetyl n-butyl esters may be concentrated with less evaporative loss than solutions of N-trifluoroacetyl methyl esters.

Gehrke and Leimer (94) studied the effect of salts on the derivatization and chromatography of the N-trifluoroacetyl n-butyl esters of amino acids. The ions which were classified as interfering were: Mn^{++}, Co^{++}, Ni^{++}, Zn^{++}, Sn^{++}, Pb^{++}, Cr^{+++}, Fe^{+++}, and oxalate. It was suggested that this interference could be due to (a) reduced volatility of the internal standard, (b) reduced volatility of the amino acids, and (c) reduced response due to chelation of the amino acid. The following ions did not interfere: Na^{+}, K^{+}, Cu^{+}, Ag^{+}, Mg^{++}, Ca^{++}, Ba^{++}, Hg^{++}, Al^{+++}, Cl^{-}, Br^{-}, CH_3COO^{-}, $SO_4^{=}$, and $PO_4^{\equiv}$.

Zumwalt, Kuo, and Gehrke (328) developed a venting system which permitted the injection of a total derivatized sample (50-100 μl) on a standard packed analytical column. This device makes possible the analysis of extremely small (nanogram) quantities of biological samples or samples containing very low concentrations of amino acids. The device prevents the relatively large volume of solvent and acylating reagent from passing through the column to the detector. The authors reported data on the amino acid composition of corn grain and soybean meal hydrolysates, and data on the free amino acid composition of bovine blood plasma and human urine.

Coulter and Hann (59) prepared the N-acetyl n-propyl

esters of the common amino acids. The amino acid solution or protein hydrolysate (10^{-6} to 10^{-8} moles of amino acid in 0.1 ml) was evaporated to dryness by means of a vigorous stream of nitrogen passing through a steam-jacketed tube (100°C). Propanolic-HCl (8 M; 0.4 ml) was added and the tube was heated (100°C; 10 min). The excess solvent was removed by nitrogen evaporation. The propylation step was then repeated. Acetylation was effected by addition of a freshly-prepared mixture of pyridine and acetic anhydride (4:1 v/v; 0.4 ml) and the mixture was allowed to stand (25°C; 5 min). Excess reagent was removed by nitrogen evaporation and the esters were dissolved in ethyl acetate (5 μM/ml) prior to injection into the gas chromatograph.

Lachovitzki and Björklund (155) converted amino acids to their n-butyl N-trifluoroacetyl esters prior to chromatography. A mixture of dried amino acids (~47.5 μM) was placed in a Pyrex ampoule and methanol (2 ml) was added to the mixture. The ampoule was placed in an oil bath (70°C, 30 min) during which time HCl gas was bubbled through the mixture by means of a Pasteur pipette. The solvent was removed by means of a rotary evaporator, after which filtered dry air was permitted into the ampoule. The methyl esters were either acylated and chromatographed or interesterified

with an n-butanol-HCl mixture prior to acylation and chromatography. Acylation was accomplished by addition of trifluoroacetic anhydride (0.2 ml) to either the dry methyl esters or the dry butyl esters. The stoppered tubes (teflon-lined caps) were either allowed to stand at room temperature (60 min) or were sealed and heated to 150°C (5 min). Excess trifluoroacetic anhydride was removed from the flask by rotary evaporation (0°C) under vacuum. Methylene chloride (100-200 µl) was added to the derivatives before an aliquot (1.0-2.5 µl) was injected into the chromatograph. Interesterification of the methyl esters of serine, aspartic acid, phenylalanine, glutamic acid, and lysine with n-butanol (70°C; 30 min) was incomplete and each of these amino acids gave two peaks. Interesterification according to the method of Stalling, Gille, and Gehrke (269), however, gave more satisfactory results. Incomplete resolution was noted for the following: valine-threonine, isoleucine-glycine, and aspartic acid-phenylalanine-methionine. Arginine, histidine, and cystine did not give satisfactory peaks. Acylation with trifluoroacetic anhydride (22°C for 60 min or 150°C for 5 min) did not give significantly different results. The authors obtained 11 peaks when a sample of amino acids from a bovine serum albumin hydrolysate was derivatized and

chromatographed.

McBride and Klingman (188) tried to find a stable liquid phase or mixture of phases which could be used with single column operation to separate nanomolar quantities of the n-butyl N-trifluoroacetyl esters of amino acids isolated from biological systems. These workers employed the procedures developed by Gehrke and co-workers (93)(96)(97)(156)(267)(269) for the preparation of derivatives. The separation (18 amino acid esters) was most successfully completed using a column of phenyldiethanolamine succinate (PDEAS, 1.2%) on Gas Chrom A (60/80 mesh). The authors presented chromatograms of the n-butyl N-trifluoroacetyl esters of free amino acids of a rat superior cervical ganglion and of amino acids obtained from a beef insulin hydrolysate.

Carlström (51) chromatographed the N-trifluoroacetyl n-butyl esters of the free amino acids of serum and the amino acids obtained from serum protein hydrolysates. Butanolic-HCl (1.2 N; 1 ml) was added to test tubes containing evaporated hydrolysates and standards (5 mg). The stoppered tubes were heated (90°C*) and shaken for 3 h. The samples

*Roach and Gehrke (240) showed later that the amino acids were sufficiently soluble in 1.5 N butanolic-HCl, but the esterification time was less rapid than with 3.0 N butanolic-HCl. They also showed that the yield of threonine butyl ester from the methyl ester was increased from 90.4 to 100.0 % when the temperature of interesterification was increased from 90 to 100°C, and the yield of cystine butyl ester increased from 88.0 to 98.2% with the same increase in temperature.

were then evaporated to dryness (60°C; rotary evaporator). Trifluoroacetic anhydride (0.1 ml) was then added to the cooled tubes and the tubes were allowed to stand overnight. The esters were then extracted with chloroform, the extract was evaporated to dryness, and the esters were dissolved in ethyl methyl ketone (0.1 ml) prior to gas chromatography. The author was able to identify only 11 out of the 20 common amino acids. The author did not use an internal standard and he expressed his final results as the percentage of each amino acid in relation to the total weight of amino acids identified in the sample.

Table 19 is a summary of the operating conditions used by different workers to separate amino acid derivatives.

1.3. AMINO ACID ANALYSIS OF CASEIN

Several workers have reported values for the amino acid composition of casein isolated from bovine and other milks.

Gordon, Semmett, Cable, and Morris (104) determined the amino acid composition of bovine casein and the α- and β-fractions. They showed that α-casein and β-casein differ considerably in the content of several amino acids. These differences are reflected in some physical properties of

Table 19

Gas chromatography of amino acids: operating conditions employed by different workers

Source and Reference	Derivative Used	No.of Amino Acids Separated	Type of of Column and Length	Type of Carrier Gas and Flow Rate (ml/min)	Type of Detector	Stationary Phase and Solid Support	Column Temperature (°C)
. Degradation Products							
Bayer (33)	Aldehydes	6	Cu, 2m	H_2, 45	K	Dinonyl phthalate on Sterchamol	92
Bier and Teitelbaum (39)	Aldehydes	4	-	(20 psi)	K	Silicone Dow-Corning 200	78
Hunter et al. (132)	Aldehydes	2	10 ft	He, 23	-	Silicone-Celite mixture	69
Kanomata & Mashiko (145)	Pyrolytic products	6	-	He, -	-	Hexanedione + tetra-ethylene glycol dimethyl ether + dioctyl phthalate	-
		3	-	He, -	-	Silicone DC-550	-
Liberti (169)	α-Hydroxy-acid methyl esters	7	1 m	- , 60	-	Silicone DC-550 (30%)	80-140
McGregor & Carpenter (190)	Nitriles	2	5 ft	He, 60		Carbowax	73,75

McGregor & Carpenter (190)	Nitriles	3	5 ft	He, 60		LAC-446	27,32,40
		2	5 ft	He, 60		Silicone (GE SF-96)	28
Melamed & Renard (201)	α-Chloro-acid methyl esters	8	4 m	H_2, 33-41	-	Polyethylene glycol (PEG)(2 m) followed by silicone oil-stearic acid mixture (2 m)	130
Stack (266)	Pyrolytic products	-	-	N_2, -	H	Carbowax-1500 (15%), butane-1,4-diol succinate(8%), or neopentyl glycol succinate (NPGS)(1%) on Chromosorb	-
Stevenson & Luck (276)	Nitriles	6	180x0.5 cm	He, 30	-	Tetrahydroxyethyl ethylenediamine on firebrick	110-230
	Aldehydes	6	180x0.5 cm	He, 30	-	Dow-Corning Silicone Oil No. 710	-
Úlehla (295)	Pyrolytic products	13	-	Ar, -	-	Reoplex 400 (30%) or Squalene (20%) on celite	-
Wagner & Rausch (301)	α-Hydroxy-acid methyl esters	8	-	-	-	Silicone oil	160
Zlatkis et al. (326)	Aldehydes	7	Cu 10 ft x ¼in	H_2, 100	TC	Equal mixture of ethylene and propylene carbonates (10%) on aqua regia-treated C-22 Firebrick (30/60 mesh)	25

. Amino Acid Esters

Bayer (33)	Methyl esters	5	Cu, 2m	H_2, 45	K	Silicone high-vacuum grease (30%) on Sterchamol	140
	"	2	"	H_2, 43	"	"	187
	"	4	"	H_2, 100	"	"	138
	"	3	"	H_2, 45	"	"	191
Nicholls et al. (220)	Methyl esters	16	G 6 ft x 3/16 in	N_2, 60	H	Neopentyl glycol succinate (NPGS)(2%) on Fluoropak 80	120-180
Saroff et al. (249)	Butyl esters (hydrochlorides)	6	6 ft x ¼ in	N_2, 50 + NH_3, 8	H	Polyethylene glycol adipate (PGA)(22%) on Chromosorb W (50/100 mesh)	131

. N-acyl Amino Acid Esters

Bayer (33)	Methyl N-trifluoroacetyl esters	4	Cu, 2 m	H_2, 45	K	Silicone high-vacuum grease and sodium caproate on Sterchamol	190
Blau & Darbre (41)	n-Amyl N-trifluoroacetyl esters	8	SS 182x0.5 cm	N_2, 38	GD	Silicone (MS-710)(15%) or cyanoethyl silicone elastomer (GE XE-60) on Silocel C-22 (Firebrick)	185°C (MS-710) 190°C (XE-60)

Blau & Darbre (42)	n-Amyl N-trifluoroacetyl esters	4	SS 182x0.5 cm	N_2, 38	GD	XF-1105 or XF-1150 (5%) on Silocel C-22 (Firebrick)	150
Carlström (51)	n-Butyl N-trifluoroacetyl esters	11	SS 2m x 1/8 in	He, 30	H	Carbowax 1500 (1%) on AW-DMCS-Chromosorb W (80/100 mesh)	100-235 (8°C/min)
Coulter & Hann (59)	n-Propyl N-acetyl esters	20	G 106x0.3 cm	N_2, 30	H	Mixture (1:1) of Carbowax 6000 (0.7%), and Carbowax 6000 (0.7%) + tetracyanoethyl-pentaerythritol (0.05%) on HP-Chromosorb G (80/100 mesh)	100-240 (6°C/min)
Cruickshank & Sheehan (60)	Methyl N-trifluoroacetyl esters	21	SS 2 ftx0.15 cm	Ar, 18	A	Neopentyl glycol succinate (NPGS)(5%) on Gas Chrom P (80/100 mesh)	65-210*
Darbre & Blau (61)	n-Amyl N-trifluoroacetyl esters	7	SS 182x0.5 cm	N_2, 30	GD	Sorbitan dioleate (SPAN-80)(5%) on AW-DDS-Silocel C-22 Firebrick	152
	"		"	N_2, 0.83	"	XE-60 (10%) on AW-DDS-Silocel C-22 (Firebrick)	140
Darbre & Blau (64)	n-Amyl N-trifluoroacetyl esters	9	G 5mx0.25 cm	N_2, 24	GD	Mixture (5%) of XE-60 (60%) and MS-550 (40%) on Anakrom ABS (90/100 mesh)	135

Darbre & Blau (64)	"	"	"	"	H	"	
	"	11	G 3.2mx0.4cm	N_2, 38	GD	Same as Darbre & Blau (61)	170
Gehrke et al. (93)	n-Butyl N-trifluoro-acetyl esters	19	G 1m x 0.4cm	N_2, 38	H	DEGS/EGSS-X (0.75/0.25 w/w %) on AW-Chromosorb W (60/80 mesh)	67-218 (3.3°C/min)
Gehrke et al. (100)	n-Butyl N-trifluoro-acetyl esters	8	G 1.5mx0.4cm	N_2, 64	H	NPGS, NPGA, NPGSb, NPGB (0.5%) and EGA (0.325%)(5 separate columns) on AW-Chromosorb G (80/100 mesh)	65-220 (2°C/min)
Gehrke & Leimer (94)	n-Butyl N-trifluoro-acetyl esters	20	(See Roach and Gehrke (240)(241)(242) for operating conditions)				
Gehrke & Shahrokhi (96)	n-Butyl N-trifluoro-acetyl esters	20	(See Gehrke and Stalling (97) for operating conditions)				
Gehrke & Stalling (97)	n-Butyl N-trifluoro-acetyl esters	20	G 1.0mx0.3cm	N_2, 38	H	DEGS/EGSS-X (0.75/0.25 w/w %) on AW-Chromosorb W (60/80 mesh)	67-218 (3.3°C/min)
	"	15	G 1.5mx0.4cm	N_2, 70	H	DC-550 (5%) on AW-Chromosorb W (60/80 mesh)	-

Graff et al. (108)	n-Propyl N-acetyl esters	30	-	-	-	Polyethylene glycol (PEG) on Chromosorb W	-
Hagen & Black (116)	Methyl N-trifluoro-acetyl esters	19	SS or Cu 20 ftx¼ in	N_2, 80		Carbowax 20M (1%) on Diatoport S (80/100 mesh)	80-185*
			2 ftx¼ in	N_2, 80		Carbowax 20M (1%) on Diatoport S (80/100 mesh)	20-270 (in 2-3 min)
			10 ftx¼ in	N_2, 50		Carbowax 1540 on Diatoport S	120-170*
Islam & Darbre (133)	Methyl N-trifluoro-acetyl esters	16	G 3.25mx0.25cm	N_2, 15	H	Mixture (2.5% w/w) of XE-30 (46%), QF-1 (27%), and MS-200, 1000 S (27%) on Diatoport S (80/100 mesh)	110
Johnson et al. (142)	n-Amyl N-acetyl esters	14	G 8 ftx0.5cm	Ar, 60	S	Polyethylene glycol (PEG)(Carbowax 1540) (1%) on AW-Chromosorb W (80/100 mesh)	125-148
	"	7	G 2 ftx0.5cm	Ar, 240	S	Polyethylene glycol (PEG)(Carbowax 1540) (0.5%) on AW-Chromosorb W(80/100 mesh)	148
Lachovitzki & Björklund (155)	n-Butyl N-trifluoro-acetyl esters	16	G 3.75mx0.2cm	N_2, 23	H	XE-30(cyanoethyl silicone gum)(5% w/w) coated on diatomaceous earth (80/100 mesh)	90-230*

Lamkin & Gehrke (156)	n-Butyl N-trifluoro-acetyl esters	5	SS 2.5mx0.47cm	Ar, 46	S	Carbowax 1500 (0.25%) coated on AW-Chromosorb W(30/60 mesh)	150
	"	4	G 1mx0.3cm	N_2, 38	H	NPGS (2%) on AW-Chromosorb W (80/100 mesh)	41-218*
	"	19	"	"	H	NPGS (1%) on Gas Chrom A (60/80 mesh)	67-218 (3.3°C/min)
Losse et al. (173)	Methyl N-formyl esters	10	-	-	-	High vacuum grease (25%) on Silica gel	-
Makisumi & Saroff (182)	Methyl N-trifluoro-acetyl esters	21	SS 19inx3/32in	N_2, 13.3	H	Neopentyl glycol succinate(2%) on Chromosorb W	204
	"	-	SS 15ftx3/32in	N_2, 29.8	H	"	161
	"	-	SS 15ftx3/32in	N_2, 11.4	H	"	137
McBride & Klingman (188)	n-Butyl N-trifluoro-acetyl esters	18	G 1.1mx0.4cm	N_2, 43	H	Phenyldiethanolamine succinate (PDEAS)(1.2%) on Gas Chrom A (60/80 mesh)	70-200*
Regis Chemical Co.(238)	n-Butyl N-trifluoro-acetyl esters	20	G 1.5mx0.37cm	N_2, -	H	NPGS(0.5 w/w %) on AW-Chromosorb G (80/100 mesh)	75-160 (2°C/min)

Roach et al. (242)	n-Butyl N-trifluoro-acetyl esters	2	G 1.5mx0.4cm	-	H	OV-17(1.5% w/w) on HP-Chromosorb G (80/100 mesh)	140-240 (6°C/min)
Roach & Gehrke (240)(241)	"	17	"	-	H	Stabilized EGA(0.65%) on AW-Chromosorb (dried at 140°C for 12 h)(80/100 mesh)	80-220 (6°C/min)
Saroff & Karman (248)	Methyl N-trifluoro-acetyl esters	6	6 ftx¼in	Ar, 80	A	Polyethylene glycol adipate (PEGA)(22%) on Chromosorb W	162
	"	6	"	N_2, 50	H	"	181
Stalling et al. (269)	n-Butyl N-trifluoro-acetyl esters	18	G 1mx0.3cm	N_2, 38	H	DEGS, EGSS-X(0.75/0.25 w/w %) on AW-Chromosorb W(60/80 mesh)	60-220 (3.3°C/min)
	"	2	"	"	H	GE XE-30(cyanomethyl silicone)(1% w/w) on AW-Chromosorb W (60/80 mesh)	100-250 (7.9°C/min)
Stefanovic & Walker (273)	n-Butyl N-trifluoro-acetyl esters	20	SS 1mx0.4cm	N_2, 60	H	EGA(0.65%) on AW-Chromosorb W (80/100 mesh)	80-230 (4°C/min)
Wagner & Winkler (302)	n-Methyl N-trifluoro-acetyl	6		0.4 atm (inlet) 0.05 atm (outlet)	-	Apiezon-L(17.5%) and sodium caproate(2.5%) on Sterchamol	167

Weygand et al. (315)	n-Methyl N-trifluoro-acetyl	10	1-2m	He	-	Silicone grease or Reoplex on Celite	160, 190, 204 (3 separate runs)
Youngs (325)	n-Butyl N-acetyl	6	Cu 6ftx¼in	He, 1.3	-	Hydrogenated vegetable (Safflower) oil (20%) on Firebrick (20/40 mesh)	220
Zomzely et al. (327)	n-Butyl N-trifluoro-acetyl	22	SS 2.0mx0.63cm	N_2, 128	-	Neopentyl glycol succinate (NPGS)(1%) on Gas Chrom A(60/80 mesh)	75*
Zumwalt et al. (329)	n-Butyl N-trifluoro-acetyl	20	(See Roach and Gehrke (240)(241)(242) for operating conditions)				

[a]Abbreviations for types of columns: Cu = copper; G = glass; SS = stainless steel.

[b]The first symbol indicates the type of carrier gas and the second symbol indicates the flow rate (ml/min).

[c]Abbreviations for types of detectors: TC = thermal conductivity; K = katharometer; H = hydrogen flame ionization; GD = gas density balance; A = argon ionization; S = strontium-90 ionization.

*Multiple programming rates employed.

the casein such as solubility and electrophoretic mobility. These workers concluded that although whole casein is heterogeneous, it is well-characterized with respect to its amino acid composition.

Baker and Khan (31) determined the amino acid composition of undesalted casein hydrolysates by quantitative chromatography on buffered filter paper. The coefficients of variation for the determination of individual amino acids was highest for glycine (7.4%) and lowest for arginine (2.3%).

Kugenev and Medvedeva (154) isolated casein from the milks of cow, buffalo, and goat. The acid hydrolysates of these caseins were then subjected to paper chromatography and the content of 11 amino acids was determined. Casein from goat milk contained higher levels of histidine, lysine, aspartic acid, serine, and tyrosine than did the other caseins.

Lee, Mehta, and Lucia (164) determined the amino acid composition of goat milk casein and compared it with the caseins of cow and human milks. Goat milk casein contained higher levels of glycine and lower levels of arginine and methionine than those reported previously for bovine casein. The authors suggested that the lower biological value of goat milk casein might be due to its relatively low methionine content.

Hoeller (129) compared the amino acid composition of goat milk casein with that of cow casein. He reported that goat milk casein contained more histidine, methionine, proline, and threonine, and less arginine, glutamic acid, isoleucine, serine, tyrosine, and valine than did bovine casein.

Ganguli, Prabhakaran, and Iya (91) isolated casein from buffalo milk and cow milk. The two caseins were hydrolyzed and the resultant hydrolysates were subjected to paper chromatography. They found that the composition of buffalo casein was almost identical to that of bovine casein.

Ashworth, Ramaiah, and Keyes (25) analyzed the casein isolated from the milk of the Northern fur seal (Callorhinus ursinus) and found it to be similar in composition to bovine casein, except for somewhat lower amounts of dicarboxylic acids (glutamic and aspartic). The authors suggested that the lower content of these amino acids might account for the lower electrophoretic mobility (veronal buffer; pH 8.6; urea) of the major protein fraction as compared with that of bovine casein.

Luhtala, Rautiainen, and Antila (174) analyzed the "protein" of reindeer milk. The authors did not indicate whether the amino acid analysis represented the composition of "casein" or "total protein" (i.e. casein + whey proteins).

Heathcote and Haworth (119) determined the amino acid

composition of casein by subjecting hydrolysates to thin-layer chromatography. The values which they obtained were in close agreement with those obtained by ion-exchange procedures.

Williamson (317) was one of the first workers to report on the amino acid composition of human casein. He found that human casein contained over three times as much cystine as did cow casein and less methionine than cow casein. The author found that there was no significant difference in the levels (millimoles) of sulfur-containing amino acids between the two caseins.

Alais and Jollès (4) analyzed human casein and reported important differences in the levels of glycine, methionine, aspartic acid, and lysine as compared with the values reported by Williamson (317). The authors found that human casein contained more cystine and less basic amino acids than did bovine casein.

Nagasawa, Kiyosawa, and Kuwahara (216) provided comparative data on the amino acid composition of human colostral, transitional, and mature milk caseins. The contents of aspartic acid, glycine, alanine, cystine, arginine, and tryptophan in colostral casein were higher than those in mature milk casein, but the contents of glutamic acid, leucine, proline, leucine, and lysine in colostral casein were lower than those

in mature milk caseins.

Values for the amino acid composition of bovine casein as reported by different workers are tabulated in Table 20. Comparative data on the amino acid composition of caseins isolated from milks of different species are tabulated in Table 21.

2. Experimental

2.1. INSTRUMENTAL

(a) Gas Chromatograph

The instrument employed for amino acid analysis was a dual-column F & M Model 810 Research Gas Chromatograph equipped with dual hydrogen flame ionization detectors. The instrument contained a Minneapolis-Honeywell (Brown) recorder (-0.2 to 1.0 mv). Compressed gases (nitrogen, air, and hydrogen) were obtained from Ohio Medical Products Canada Ltd. Drying tubes containing "tell-tale" silica gel G and Linde molecular sieve 5A (Hewlett-Packard, Avondale Division, Avondale, Pa.; Cat. No. 8501-5208) were inserted in each of the gas lines between the tank and the inlet port of the gas chromatograph. Single column operation was used for amino acid analysis. The glass column (8 ft x ¼ in) was packed

Table 20

Amino acid composition of bovine whole casein as reported by different workers[a]

Amino Acid	Reference No.									
	(179)	(104)	(317)	(91)[b]	(154)[b]	(31)[b]	(272)[c]	(119)[d]	(119)[e]	(104)[f]
Glycine	0.4	2.7	0.4	8.90	1.6	2.05	1.97	2.4	2.2	1.9
Serine	5.45	6.3	5.0		4.0	4.64	5.67	6.4	6.3	5.9
Alanine	2.3	3.0	2.3	2.98	2.3	3.84	3.31	3.0	3.3	3.5
Valine	6.64	7.2	5.3	6.58	-	6.84	6.79	6.5	6.3	7.2
Leucine	10.8	9.2	14.4	13.55	-	10.6	9.39	8.8	8.2	10.3
Isoleucine	6.0	6.1	5.2		-	5.77	5.91	5.6	5.0	7.6
Proline	9.8	11.3	8.1	-	-	9.84	10.4	10.1	10.5	11.6
Phenylalanine	5.54	5.0	5.5	4.99	-	5.19	4.82	4.8	5.0	5.5
Cystine	0.34	0.34	0.4	-	-	-	-	0.4	1.4	0.34
Cysteine	-	-	-	-	-	-	-	-	-	-
Methionine	2.88	2.8	3.1	2.48	-	3.32	2.94	2.9	3.1	3.1
Tryptophan	1.22	1.2	1.3	1.30	-	1.35	1.24	-	-	1.2
Arginine	3.76	4.1	3.9	4.71	1.9	4.48	3.72	3.8	3.9	4.0
Histidine	2.25	3.1	2.0	1.38	2.9	3.67	3.00	3.1	3.2	3.2
Lysine	6.85	8.2	6.0	8.47	5.8	7.35	7.68	7.8	8.1	8.3
Aspartic Acid	5.8	7.1	4.2	28.63	3.5	7.63	6.95	7.6	7.3	7.2
Glutamic Acid	21.7	22.4	21.9		11.8	20.8	20.8	22.0	20.8	22.0
Threonine	4.35	4.9	4.6	4.31	2.7	4.43	4.26	5.5	6.9	4.6
Tyrosine	5.99	6.3	5.5	4.80	3.1	6.04	5.62	5.8	4.9	6.2

[a]Values expressed as g amino acid per 100 g casein.
[b]Values obtained by paper chromatography.
[c]Values obtained by microbiological assay.
[d]Values obtained by thin-layer chromatography.
[e]Values obtained by amino acid analyzer.
[f]Values cited by authors (104) from literature.

Table 21

Amino acid composition of caseins isolated from milks of different species as reported by different workers[a]

Amino Acid	Casein										
	Goat (154)[b]	Goat (164)[c]	Goat (179)	Buffalo (91)[b]	Buffalo (154)[b]	Human (216)[c]	Human (4)[c]	Human (179) (317)	Northern fur seal (25)[c]	Rat (101)	Reindeer[d] (174)[c]
Glycine	1.2	1.41	-	8.85	1.3	1.37	1.8	0	1.3	1.65	2.12
Serine	4.4	4.10	0.7		3.9	4.25	4.3	5.4	2.4	-	5.18
Alanine	1.9	2.65	1.8	2.37	2.0	3.00	2.8	2.0	3.9	-	2.92
Valine	-	6.47	6.0	5.58	-	7.48	4.85	5.0	5.9	5.02	5.89
Leucine	-	9.43	11.4	13.26	-	10.39	10.8	12.2	8.9	5.54	8.95
Isoleucine	-	5.24	-		-	5.90	4.4	6.3	4.2	4.67	4.34
Proline	-	8.29	6.9	-	-	13.86	10.9	8.9	8.1	7.84	8.79
Phenylalanine	-	3.77	2.0	4.46	-	3.56	3.8	5.8	3.7	3.75	4.38
Cystine	-	Tr[e]	Tr[e]	-	-	0.48	0.95	0.64	1.78	3.99	0.74
Cysteine	-	-	-	-	-	-	-	-	-	-	-
Methionine	-	1.35	-	2.01	-	2.05	0.7	2.4	2.08	1.87	2.27
Tryptophan	1.5	-	1.37	1.45	-	1.06	2.4	1.4	-	4.57	-
Arginine	1.9	1.34	2.66	2.78	1.2	2.79	2.05	3.43	4.2	3.42	3.58
Histidine	3.5	3.65	1.85	1.62	2.9	2.47	2.15	1.77	2.4	2.50	3.39
Lysine	6.8	5.24	3.7	7.56	5.5	5.79	3.85	5.4	5.0	5.28	10.27
Aspartic Acid	5.2	7.62	1.7	27.28	3.6	6.82	7.5	4.6	6.6	5.78	5.99
Glutamic Acid	11.3	18.27	12.1		9.3	21.87	18.3	20.9	16.9	20.20	21.67
Threonine	2.4	4.38	-	3.74	2.1	4.05	4.35	4.5	3.5	4.49	4.24
Tyrosine	5.0	3.22	4.87	4.21	-	5.28	5.1	5.7	3.9	3.85	5.28

[a]Values expressed as g amino acid per 100 g casein.
[b]Values obtained by paper chromatography.
[c]Values obtained by amino acid analyses.
[d]Reindeer "protein".
[e]Trace.

with neopentylglycol sebacate (NPGS; 0.5%) on acid-washed Chromosorb G (80/100 mesh). The column was not packed in the region which was inserted into the injection port. The brass Swagelok fittings were coated with a Teflon coating ("Fluoro-glide", Chemplast, Inc., Wayne, N.J. 97470, U.S.A.).

The chromatographic conditions were as follows:

Column temperature:	Initial 67°C, final 210°C
Program rate:	4°C/min
Detector temperature:	250°C
Injection port temperature:	230°C
Range:	10
Attenuation:	2
Chart speed:	0.25 in/min
Carrier gas (N_2) flow rate:	60 ml/min (Tank, 60 psig; Rotameter 2)
Scavenger (air) flow rate:	250 ml/min (Tank, 18 psig)
Hydrogen flow rate:	120 ml/min (Tank, 16 psig)

(b) Preparation of Columns

Neopentyl glycol sebacate (NPGS; 0.75 g) was dissolved in methylene chloride. Acid-washed Chromosorb G (150 g) was placed into a ribbed round-bottom flask (1 liter). The solution of NPGS was added to the flask, and methylene chloride

was added until the liquid level was ½ inch above the Chromosorb. The slurry was evaporated to dryness by means of a rotary evaporator (50°C).

The column was packed as follows: a plug of silanized glass wool (Applied Science Laboratories, State College, Pa. 14502, U.S.A.) was inserted in one end of the column and this end of the column was connected to a water vacuum pump. The column was then vibrated (Burgess Vibratool) as the packing was poured into the column. The column was filled to a distance six inches from the other end and another plug of silanized glass wool was inserted to hold the column packing in place. The empty region coincided with that part of the column which served as a glass liner in the injection port.

The column was conditioned overnight (12-15 h) at 210°C and the gas flow rates were identical to those described previously in this chapter (Section 2.1.(a)).

(c) Calibration of Gas Flow Rates

Most manufacturers of gas chromatographs supply a set of calibration curves with which it is possible to determine the tank pressure settings and rotameter (if any) settings which will provide a given flow rate. In most instances, these calibration curves are general curves and do not

necessarily apply to a specific instrument. In any case, the curves for the carrier gas will most probably be meaningless because of variation in column packing. Figures 24, 25, 26, and 27 show the calibration curves for "A" column carrier gas, "B" column carrier gas, "A" and "B" detector purge air (scavenger) flow, and "A" and "B" detector hydrogen flow. Since single column operation was employed in these amino acid analyses, the "A" column data are of special importance.

2.2. MISCELLANEOUS APPARATUS AND GLASSWARE

(1) Oil baths

Three deep fryers (Danby Frymaster Cooker-Fryer, Model 400 A, Danby Corporation, Montreal 9, P.Q., Canada) were used as constant-temperature baths. The fryers were filled to a depth of two inches with Fisher Thermally Stablized Bath Oil No. 0-2. One fryer was set at 70°C, one at 100°C, and the other at 150°C.

(2) Ultrasonic mixer

A Branson Sonifier Cell Disruptor was fitted with a micro-tip.

(3) Boiling water bath

(4) Hydrogen chloride generator

The apparatus used was essentially that described by Vogel (299) (p.180, Fig.II,48,I).

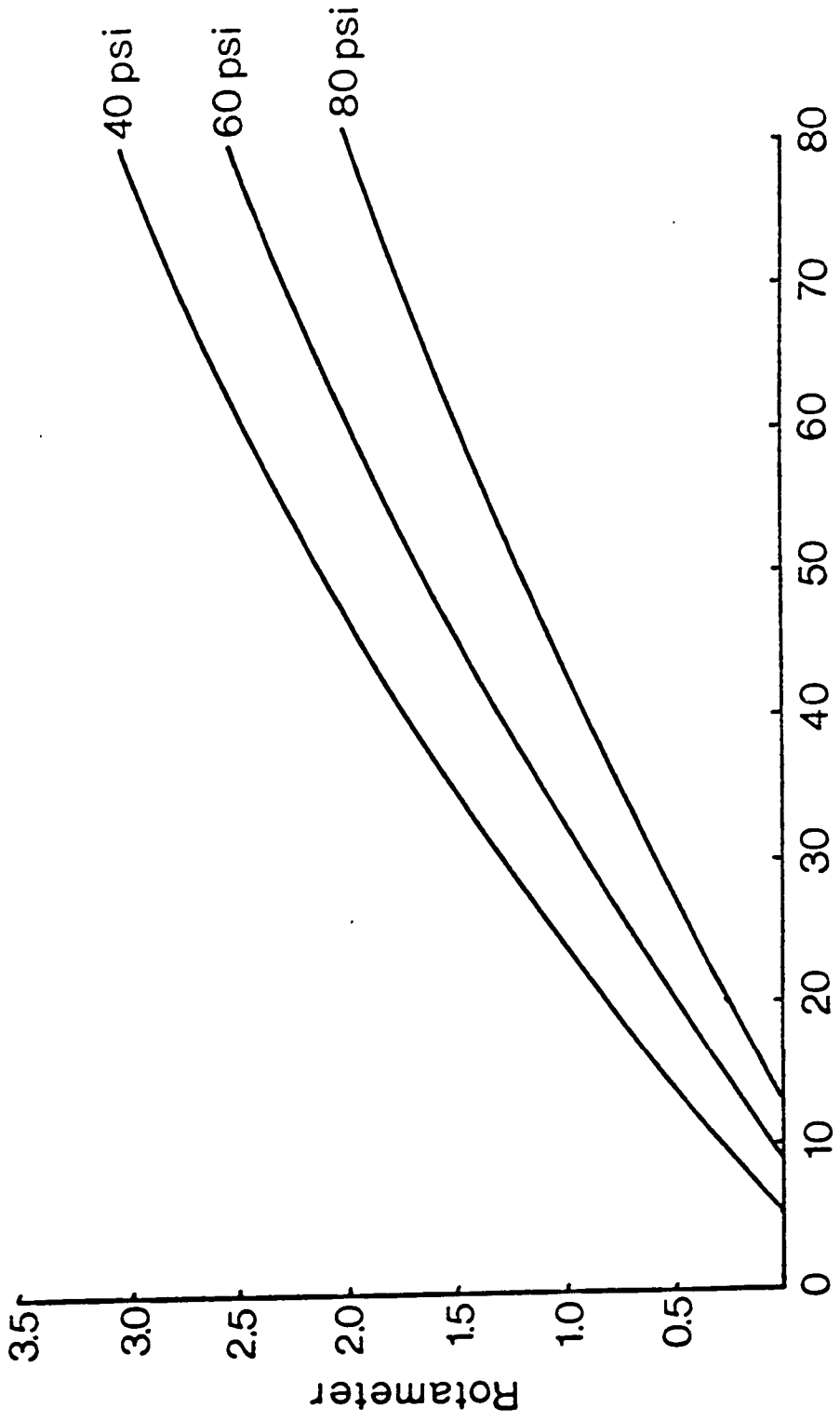


Figure 24. Calibration curves for "A" column carrier gas flow.

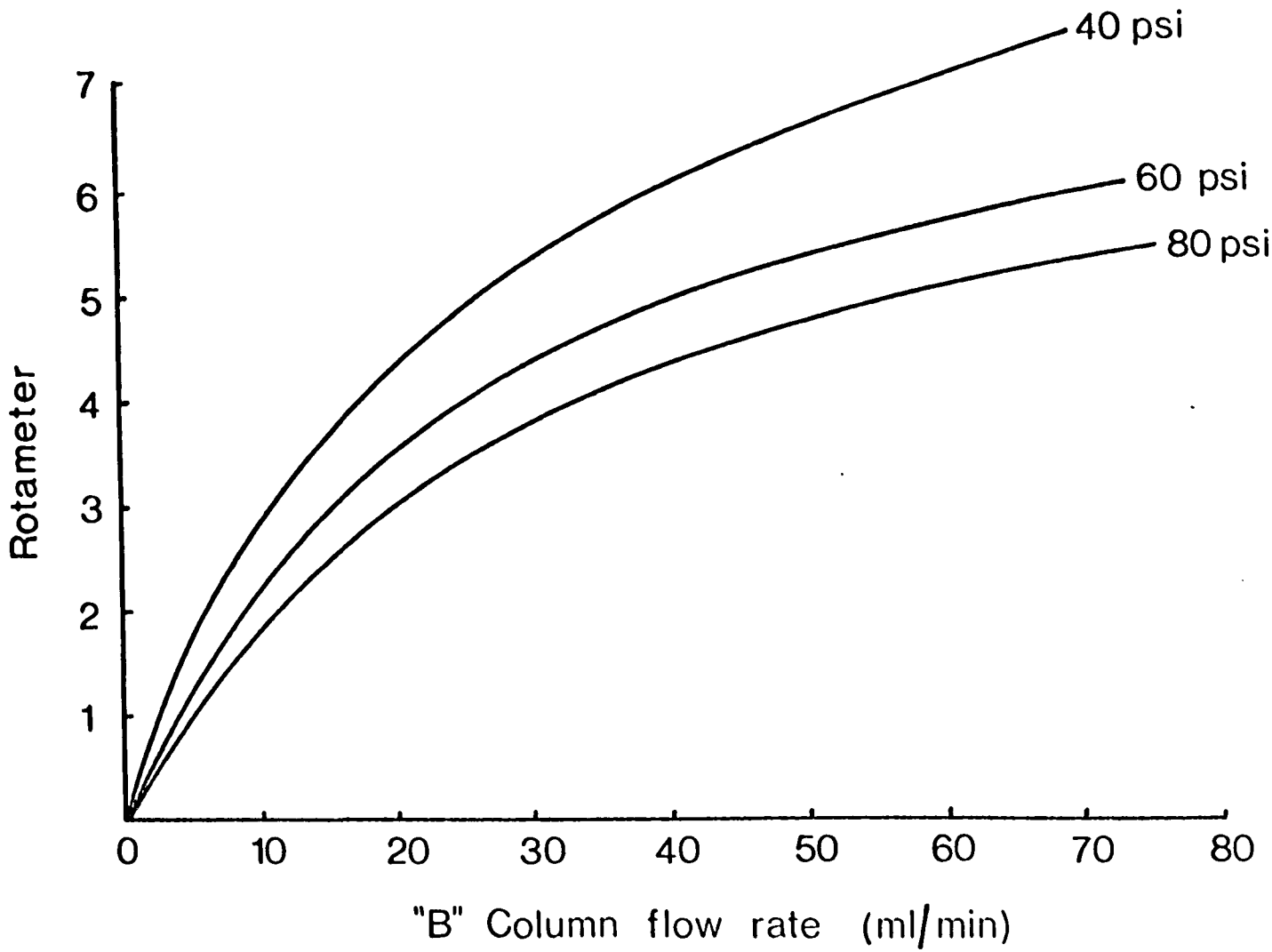


Figure 25. Calibration curves for "B" column carrier gas flow.

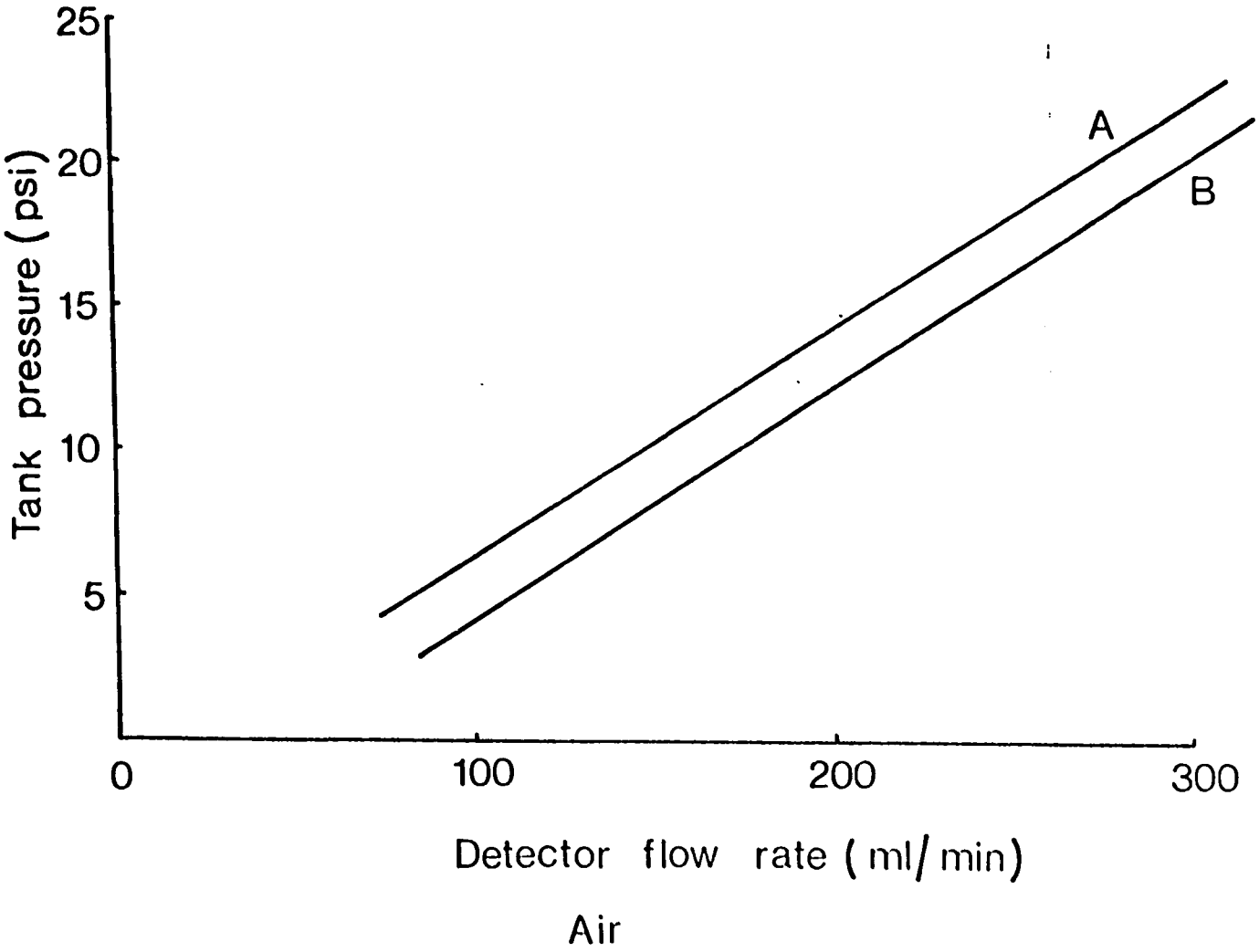


Figure 26. Calibration curves for "A" and "B" detector scavenger flow.

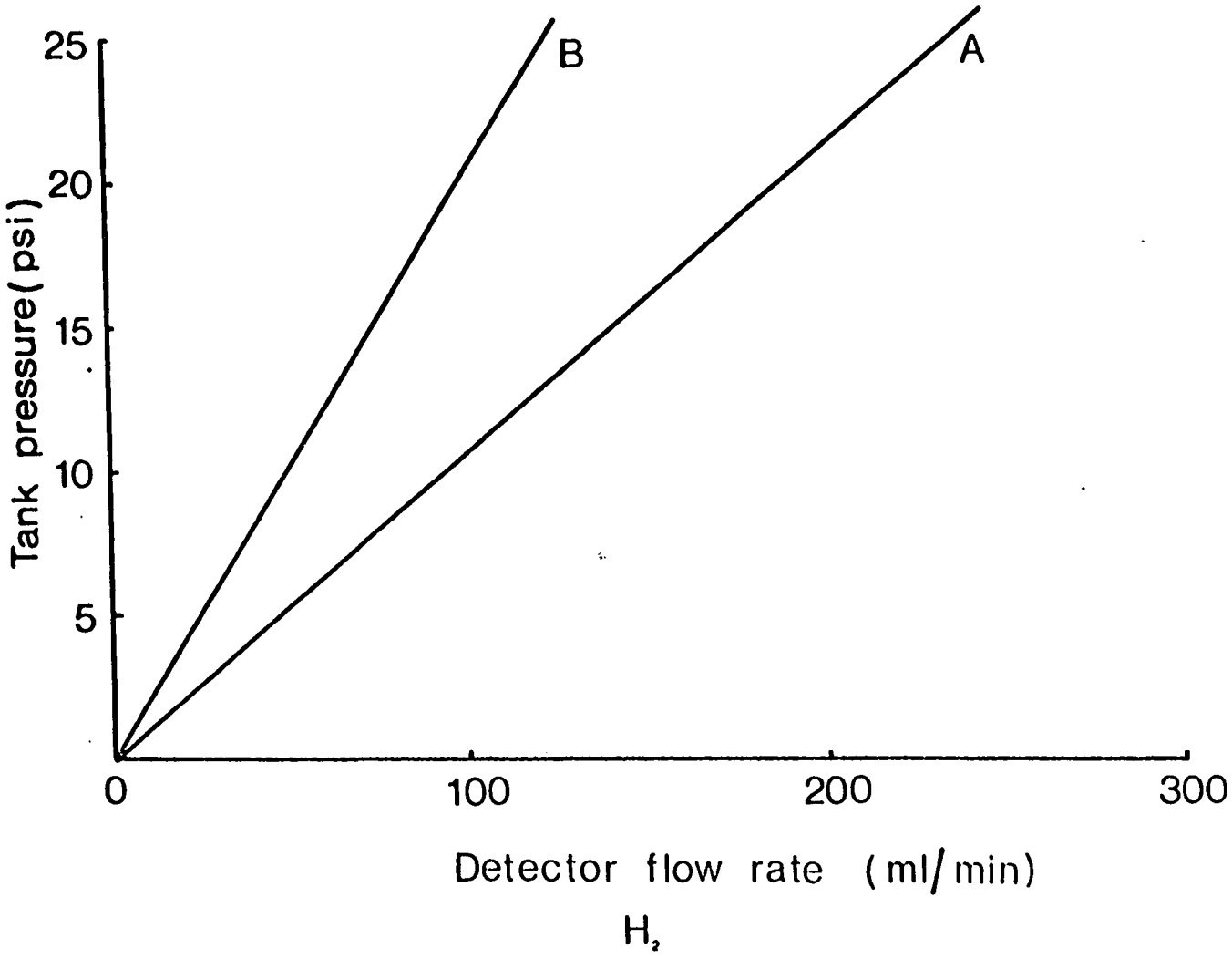


Figure 27. Calibration curves for "A" and "B" detector hydrogen flow.

(5) Nitrogen evaporator

The nitrogen evaporator consisted of six Pasteur disposable pipettes (Fisher, Cat. No. 13-678-5A) which were joined to the outlets of a aquarium gang valve by means of pieces of rubber tubing. The inlet of the gang valve was connected to a nitrogen tank by a piece of rubber tubing. A drying tube half-filled with "tell-tale" silica gel and half-filled with Linde molecular sieve 5A (Hewlett-Packard, Avondale Division, Avondale, Pa., Cat. No. 8501-5208) was inserted between the gang valve and the tank.

(6) Reagent dispensers

Three "Repipets" (Labindustries Ltd., Berkeley, Calif 94710, U.S.A.; 10 ml dispensers) were fitted with drying tubes ($CaCl_2$). These were used to dispense dry esterification reagents.

(7) Chromatographic columns

Columns (o.d.=½ in; length=12 in) containing sintered-glass retainers at one end were used for ion-exchange clean-up of hydrolyzed biological materials.

(8) Vacuum pump and large desiccator

These were required to facilitate removal of HCl after hydrolysis of biological samples. The bottom of the large desiccator was covered with NaOH flakes.

(9) Reaction tubes

Several tubes (Pyrex #9826, culture; 13 x 100 mm) fitted with Teflon-lined caps were used to perform the esterification reactions. Care was taken to choose tubes which had tightly-fitting caps as slight defects in the thread caused loss of sample.

(10) Test tubes

Pyrex test tubes (15 x 150 mm) were used for hydrolysis of biological samples.

(11) Tube chamber for hydrolysis

The tubes in which the hydrolyses were performed were placed in a vacuum desiccator (diameter = 15 in) such as that described by Keutmann and Potts (146). The top was held in place by two square plywood (½ in) flanges which were clamped at each corner by means of four bolts and wing nuts. The desiccator was heated by means of an air oven.

(12) Syringes

Hamilton syringes (10 µl, Cat.No.701 N; 50 µl, Cat.No.703 N) were used to inject the samples into the gas chromatograph.

2.3. REAGENTS AND MATERIALS

(1) Anhydrous methanol

Anhydrous methanol was prepared according to the

method of Vogel (299). Magnesium turnings (2.5 g) and iodine (0.5 g) were placed in a round-bottom Pyrex flask (2 liter) which was then fitted with an Allihn condenser. Methanol (50-75 ml) was added through the condenser and the mixture was warmed until all the magnesium was converted to the methoxide. Methyl alcohol (900 ml) was then added, the mixture was refluxed (30 min), and the final product was separated from the mixture by distillation. The first 25 ml of distillate was discarded and the product was collected at 65°C (760 mm Hg).

(2) Anhydrous n-butanol

Anhydrous n-butanol was prepared according to the method of Vogel (299). Butanol (1 liter) and sodium (7 g) were placed in a round-bottom Pyrex flask (2 liter) fitted with an Allihn condenser and the reaction was allowed to continue until the sodium disappeared. Ethyl phthalate (27.5 g, purified, Fisher Scientific Co., Cat.No.E-162) was introduced into the flask and the mixture was gently refluxed (2 h). The butanol was separated from the mixture by distillation. The first 25 ml of distillate was discarded. The fraction which boiled at 116.5-118°C (760 mm Hg) was collected.

(3) Anhydrous methylene chloride

Reagent grade methylene chloride was dried over

anhydrous calcium chloride and was then redistilled. The fraction which boiled at 40-41°C (760 mm Hg) was collected.

(4) Methanolic-HCl (1.25 N)

Dry hydrogen chloride gas (45.63 g) was bubbled into anhydrous methanol (800 ml) and the volume of the resultant solution was adjusted to 1000 ml with anhydrous methanol. The reagent was stored in a "Repipet".

(5) Butanolic-HCl (1.25 N)

Dry hydrogen chloride gas (45.63 g) was bubbled into anhydrous n-butanol (800 ml) and the volume of the resultant solution was adjusted to 1000 ml with anhydrous n-butanol. The reagent was stored in a "Repipet".

(6) Butanolic-HCl (3 N)

Dry hydrogen chloride gas (109.5 g) was bubbled into anhydrous n-butanol (700 ml) and the volume of the resultant solution was adjusted to 1000 ml with anhydrous n-butanol. The reagent was stored in a "Repipet".

(7) Acylation reagent

Trifluoroacetic anhydride (20 ml; Eastman Cat.No. 7386, Eastman Kodak Co., Rochester, N.Y. 14650, U.S.A.) was added to anhydrous methylene chloride (60 ml).

(8) Internal standard solution (1 mg/ml)

Stearic acid butyl ester (100 mg; Cat.No.5-5001, Grade 1, Approx 99%, Sigma Chemical Co., St. Louis, Mo.

63118, U.S.A.) was dissolved in anhydrous n-butanol (80 ml) and the volume of the resultant solution was adjusted to 100 ml with anhydrous n-butanol.

(9) Amino acid standard solutions

Standard N-trifluoroacetyl n-butyl amino acid solutions were purchased from Regis Chemical Co. (1101 N. Franklin St., Chicago, Ill. 60610, U.S.A.). The kit (Cat. No.TK-1) consists of 20 amino solutions of individual amino acids (0.05 M, 5 ml each) in chloroform. The solutions are packed under nitrogen and come in vials which are stoppered with inert septums.

(10) Amino acids

Amino acids were purchased from Nutritional Biochemicals Company, Cleveland, Ohio, 44128, U.S.A.

(11) Standard proteins

The following proteins were obtained from Sigma Chemical Company, St. Louis, Mo. 63118, U.S.A., except for the albumin which was obtained from Nutritional Biochemicals Company, Cleveland, Ohio 44128, U.S.A.

Albumin, bovine: 4x crystallized

Albumin, egg: Grade V: salt free, crystallized and lyophilized; Cat. No. A 5503

Zein: Grade II: from corn; Cat. No. Z 3625

Gelatin; Type I: 300 bloom, from swine skin; Cat. No. G 2500

K-Casein: Cat. No. C 3883

Ribonuclease A; Type 1-A: from bovine pancreas, 5x crystallized, protease-free, essentially salt-free; Cat. No. R 4875

Lactalbumin: practical grade; Cat. No. L 7252

(12) Liquid phase

Neopentyl glycol sebacate (NPGS) was obtained from Chromatographic Specialties, Brockville, Ont., Canada.

(13) Solid support

Acid-washed Chromosorb G (80/100 mesh) was obtained from Chromatographic Specialties, Brockville, Ont., Canada.

(14) Ion-exchange resins

Dowex 50 x 12 and Dowex 2 x 8 (200-400 mesh) were obtained from the J.T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.

(15) Hydrochloric acid for hydrolysis

Mercaptoethanol was added to constant-boiling hydrochloric acid (5.7 N) such that the ratio of mercaptoethanol:hydrochloric acid was 1:2000.

(16) Hydrochloric acid (10% w/v)

(17) Sodium hydroxide (10% w/v)

(18) Ammonium hydroxide (10% w/v)

2.4. PRELIMINARY WORK

(a) Introduction

The object of this work was to find out whether or not it was possible to use gas chromatography for the determination of amino acids in casein hydrolysates. Stated below are several reasons why it would be advantageous to use a gas chromatograph rather than an amino acid analyzer for amino acid analysis: (a) the cost of a gas chromatograph is much less than that of an amino acid analyzer; (b) the time required for the amino acid analysis of a protein using gas chromatography should be less than that required for the same analysis using an amino acid analyzer; and (c) when a gas chromatographic run is completed, column regenerative procedures are not necessary as they are with the amino acid analyzer. The main disadvantage of the gas chromatographic method is that it is necessary to convert the amino acids into appropriate derivatives and this may lead to losses of amino acids.

Several workers have suggested procedures for the preparation of derivatives and their separation by gas chromatography. However, the methods developed by Gehrke and his coworkers at the University of Missouri seemed to be promising

for routine laboratory work. Gehrke's first method (97) involved the preparation of the methyl esters of the amino acids, interesterification of the methyl esters to form the n-butyl esters, and acylation of the butyl esters with trifluoroacetic anhydride to form the N-trifluoroacetyl n-butyl amino acid esters. The preparation of the methyl esters was necessary because some amino acids, notably cystine, lysine, and histidine, dissolved in butanolic-HCl (1.25 N) only with great difficulty (155), but they were readily soluble in methanolic-HCl (1.25 N). Gehrke's second method (240) involved "direct" esterification of the amino acids to form the butyl esters followed by acylation of the butyl esters with trifluoroacetic anhydride to form the N-trifluoroacetyl n-butyl amino acid esters. It should be pointed out that during the course of this work, a change was also made in the conditions (time and temperature) of acylation. An increase in the acylation temperature from 100° (1 h) to 150° (5 min) caused increased yields of arginine.

The preliminary experiments which follow involve comparisons of the recoveries obtained with Gehrke's two methods. These preliminary experiments led to the development of a procedure which was applied to the analysis of casein.

(b) Precision of Measurements

An experiment was conducted to establish the reproducibility of retention times and of peak area measurements for each of the amino acid N-trifluoroacetyl n-butyl esters.

Equal volumes (10 µl) of each of the standard N-trifluoroacetyl n-butyl ester solutions (0.05 M) were mixed to yield an equimolar mixture of the 20 amino acid derivatives. Five separate samples (3 µl, each) of this solution were injected into the gas chromatograph and each analysis was performed using the conditions described in Section 2.1. (b). The "retention distance" (i.e. the distance in inches of a peak from the origin on the chart paper) was determined for each amino acid derivative in each run and the average distance, standard deviation, and coefficient of variation were determined for each amino acid. Since the chart paper moves at a known rate (¼ in/min), the "retention distance" can easily be converted to "retention time". Table 22 shows the retention distances, averages, standard deviations, and coefficients of variation for 17 of the 20 amino acid derivatives (the author was unable to detect arginine, histidine, and cystine). Two peaks (valine and isoleucine) had a coefficient of variation greater than 1.00% (1.22 and 1.36%, respectively), and aspartic acid had the lowest coefficient

Table 22

Retention distances* of amino acid N-trifluoroacetyl n-butyl esters

N-TFA** n-butyl ester of	Trial No. 1	2	3	4	5	Average ($\bar{X}$)	Standard deviation ($\pm$ s)	Coefficient of variation ($\pm$ %)
Alanine	2.13	2.17	2.11	2.11	2.13	2.13	0.02	0.93
Valine	2.44	2.50	2.45	2.42	2.45	2.45	0.03	1.22
Glycine	2.85	2.77	2.81	2.80	2.80	2.80	0.02	0.71
Isoleucine	2.95	3.00	2.92	2.90	2.93	2.94	0.04	1.36
Threonine	3.14	3.10	3.10	3.10	3.12	3.11	0.02	0.64
Leucine	3.30	3.30	3.28	3.25	3.26	3.28	0.02	0.60
Proline	3.60	3.61	3.58	3.56	3.54	3.57	0.03	0.84
Serine	3.79	3.80	3.78	3.85	3.77	3.80	0.03	0.78
Cysteine	4.70	4.71	4.70	4.66	4.69	4.69	0.02	0.42
Hydroxyproline	4.80	4.80	4.79	4.75	4.79	4.79	0.02	0.41
Methionine	5.30	5.30	5.29	5.25	5.29	5.29	0.02	0.37
Aspartic acid	5.50	5.50	5.49	5.46	5.49	5.49	0.01	0.18
Phenylalanine	5.64	5.67	5.64	5.60	5.62	5.63	0.03	0.53
Glutamic acid	6.45	6.50	6.45	6.44	6.46	6.46	0.02	0.30
Tyrosine	6.80	6.95	6.83	6.81	6.82	6.84	0.06	0.87
Lysine	8.00	8.05	8.00	8.00	8.00	8.01	0.02	0.24
Tryptophan	8.50	8.58	8.56	8.50	8.48	8.52	0.04	0.47

*Retention distance = distance in inches on chromatogram from origin to peak of amino acid derivative. Chromatographic conditions are those described in Section 2.1.(b). Chart speed = ¼ in/min. Therefore, Retention time = Retention distance x 4.

**N-TFA = N-trifluoroacetyl.

of variation (0.18%).

Three separate samples (5 μl, each) of the standard mixture were injected into the gas chromatograph and each analysis was performed using the conditions described in Section 2.1.(b). The area for each amino acid peak in each of the three runs was determined and the slope factor (97) (238) for each amino acid (area/μM) was calculated. The slope factors, averages, standard deviations, and coefficients of variation for 17 of the 20 amino acid derivatives are tabulated in Table 23. A typical chromatogram which shows the separation of an amino acid standard mixture is shown in Figure 28.

(c) Recovery Experiments: Methods

i) Experiment No. 1

The procedure which was employed was essentially that of Gehrke and Stalling (97) except that the ultrasonic mixing steps were omitted.

(1) Samples (25 mg) of each of 20 amino acids were placed in a volumetric flask (100 ml). Distilled water was added and the volume of the resultant solution was adjusted to 100 ml.

(2) An aliquot (1 ml = 5 mg total amino acids) was

Table 23

Slope factors* of amino acid N-trifluoroacetyl n-butyl esters

N-TFA** n-butyl ester of	Trial No. 1	2	3	Average ($\bar{X}$)	Standard deviation ($\pm$ s)	Coefficient of variation ($\pm$ %)
Alanine	7,200	7,560	7,020	7,260	275	3.78
Valine	9,840	9,960	9,120	9,520	421	4.42
Glycine	5,760	5,940	5,520	5,740	211	3.67
Isoleucine	11,400	11,760	10,980	11,380	390	3.42
Threonine	8,580	8,940	8,220	8,580	360	4.19
Leucine	11,100	11,640	10,860	11,200	399	3.56
Proline	8,760	9,300	8,460	8,840	426	4.81
Serine	4,980	5,220	4,980	5,060	139	2.74
Cysteine	6,240	6,600	5,760	6,200	421	6.79
Hydroxyproline	10,560	10,920	9,300	10,260	851	8.29
Methionine	9,360	9,720	8,700	9,260	517	5.58
Aspartic acid	11,160	11,400	10,200	10,920	635	5.81
Phenylalanine	14,100	14,700	13,140	13,980	787	5.62
Glutamic acid	10,980	11,280	10,260	10,840	524	4.83
Tyrosine	7,260	7,560	7,140	7,320	216	2.95
Lysine	11,640	12,360	10,980	11,660	690	5.91
Tryptophan	16,560	17,340	15,420	16,440	966	5.87

*Slope factor = area (mm^2)/μM amino acid injected. Chromatographic conditions are those described in Section 2.1.(b). Sample volume: 5 μl, consisting of 0.0125 μM of each of the 20 common amino acid N-TFA n-butyl esters.

**N-TFA = N-trifluoroacetyl.

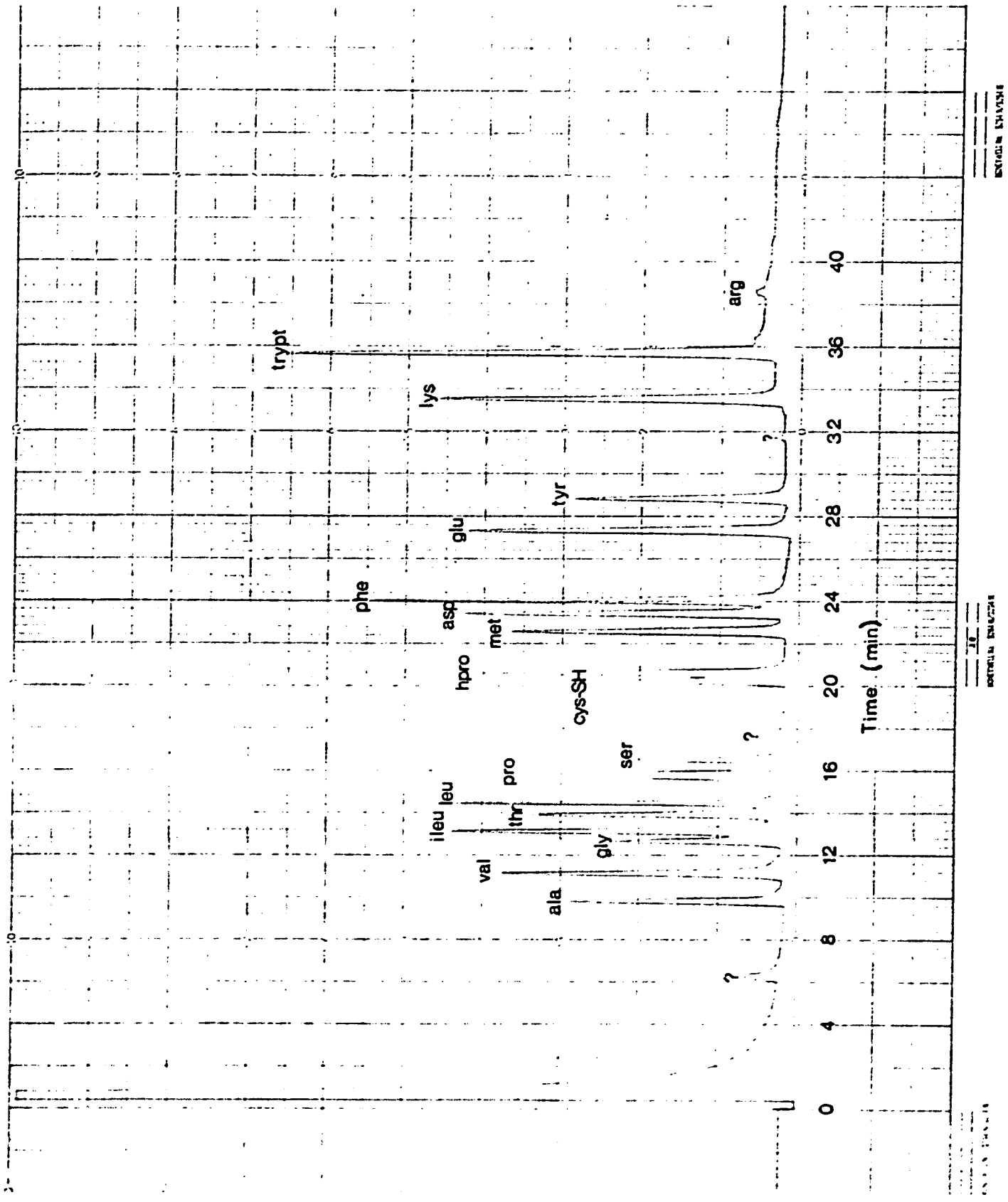


Figure 28. Typical chromatogram of equimolar mixture of N-TFA n-butyl standard amino acid derivatives.

transferred to a culture tube (Pyrex No. 9826; screw-top; Teflon-lined cap) and the tube was placed in a boiling water bath (100°C). A stream of nitrogen was directed to the surface of the solution to aid in the evaporation. The solution was evaporated just to dryness.

(3) Azeotropic water removal was effected by successive addition and evaporation of three portions (150 µl, each) of anhydrous redistilled methylene chloride.

(4) Methanolic-HCl (1.25 N; 2.0 ml) was added to the tube containing the residue of dry amino acids and the tube was capped, shaken, and allowed to stand for 30 min (room temperature).

(5) Excess methanolic-HCl was removed by nitrogen evaporation (100°C) as described in Step (2). The water of esterification was removed by azeotropic distillation as described in Step (3).

(6) Butanolic-HCl (1.25 N; 2.0 ml) and n-butyl stearate dissolved in butanol (1 mg/ml; 0.1 ml) were added and the tube was capped, shaken, placed in a water bath (100°C), and allowed to stand for 2.5 h.

(7) Excess butanolic-HCl was removed by nitrogen evaporation (100°C)(see Step (2) above). Moisture that might have been present was removed by azeotropic distillation as described in Step (3).

(8) Methylene chloride (anhydrous, redistilled; 1.8 ml) and trifluoroacetic anhydride (0.2 ml) were added and the tube was capped, shaken, placed in a water bath (100°C), and allowed to stand for 1 h.

(9) Excess acylation reagents were removed by nitrogen evaporation (100°C)(see Step (2) above).

(10) The residual oil-like substance was taken up in a suitable volume (approx 50-100 µl) of chloroform, and an aliquot (1 µl) was injected into the gas chromatograph.

ii) Experiment No. 2

The procedure which was employed for this experiment was exactly the same as that employed in Experiment No. 1, except that the sample was subjected to short ultrasonic mixing periods (15 sec) after addition of the methanolic-HCl, butanolic-HCl, and the acylation reagents.

iii) Experiment No. 3

The procedure which was employed in this experiment incorporated the direct esterification as was reported by Roach and Gehrke (240). Thus, Steps (4) and (5) of Experiment No. 1 were omitted and the concentration of HCl in butanol in Step (6) was increased from 1.25 to 3.0 N. The samples were subjected to short ultrasonic mixing periods (15 sec) after the addition of butanolic-HCl and the acylation

reagents. The initial dissolution of the amino acids was effected using 6 N HCl rather than distilled water.

iv) Experiment No. 4

An experiment was performed to investigate the effect of ultrasonic mixing on the recoveries of different amino acid derivatives. The procedure which was employed in this experiment was essentially that employed for Experiment No. 3, except that four different ultrasonic mixing times were used: 0, 15, 30, and 60 seconds. Three samples were subjected to the derivatization procedure for each ultrasonic mixing period. The same mixing period was used for the acylation as was used for the esterification. The sample tubes were flushed with nitrogen for 10 seconds before and after ultrasonic mixing.

v) Experiment No. 5

One final recovery experiment was performed with a view to obtaining higher recoveries and with a view to obtaining information on the precision of the derivatization method. The experiment incorporated another change, namely, the use of three oil baths; one at 70°C for the evaporation steps, one at 100°C for esterification, and the other at 150°C for high-temperature acylation. The procedure which

was employed was essentially that described by Roach and Gehrke (240) and is outlined below:

(1) A mixture of amino acids (250 mg, each) was placed in a volumetric flask (1000 ml) along with hydrochloric acid (6 N). When the amino acids were dissolved, the volume of the resultant solution was adjusted to 1000 ml with the same acid. The concentration of amino acids in this solution was 5 mg/ml. Aliquots (0.5 ml=2.5 mg total amino acids) of this solution were pipetted into each of 5 culture tubes (Pyrex No. 9826; screw-top; Teflon-lined caps).

(2) The tubes were placed in an oil bath (100°C) and the solutions were evaporated with the aid of a stream of dry nitrogen.

(3) Azeotropic water removal was effected by successive addition and evaporation (70°C) of 3 portions (150 µl, each) of anhydrous redistilled methylene chloride.

(4) Butanolic-HCl (3 N; 3.75 ml) and n-butyl stearate in butanol (1 mg/ml; 0.1 ml) was added to the dried amino acid mixtures, the tubes were flushed with nitrogen, and the contents were subjected to ultrasonic mixing (45 Watts, 20 sec). The tubes were again flushed with nitrogen, capped, and placed in an oil bath at 100°C (35 min).

(5) The tubes were cooled and opened and the contents

were evaporated to dryness at 70°C with the aid of a stream of nitrogen.

(6) Water of esterification was removed by azeotropic distillation (see Step (3) above).

(7) Acylation reagent (25% v/v trifluoroacetic anhydride in anhydrous methylene chloride; 2.0 ml) was added to the dried butyl esters, the tubes were flushed with nitrogen, and the contents were subjected to ultrasonic mixing (45 Watts, 20 sec). The tubes were again flushed with nitrogen, capped, and placed in an oil bath at 150°C (5 min).

(8) The tubes were stored at -5°C until the analyses were to be performed. The tubes were opened and the solutions were evaporated to dryness at 40°C with the aid of a stream of nitrogen. Methylene chloride (100 µl) was then added to each tube and aliquots of the resultant solutions (1.0 µl) were injected into the gas chromatograph.

vi) Calculation of Recoveries

The following computations were performed to obtain the percent recovery of each amino acid.

(1) Determination of slope factor for each amino acid

The slope factor is the peak area per micromole of amino acid.

$$\text{Slope Factor (SF)} = \frac{\text{Area } (mm^2)}{\mu M \text{ injected}}$$

A standard mixture which contained a known number of micromoles of each of the 20 amino acid N-TFA n-butyl derivatives was injected into the gas chromatograph and the areas (mm^2) of the corresponding peaks were measured by triangulation. The slope factor for each amino acid was calculated by use of the above formula. The calculation was facilitated by the injection of an equimolar mixture of the standard amino acid derivatives. This meant that the denominator of the expression above became a constant. The slope factors reflect the unique response of the hydrogen flame detectors to each different amino acid derivative.

(2) Determination of concentration factor

The concentration factor is the ratio formed by dividing the amount of sample injected on the column by the amount of total sample. Since an internal standard is used, the concentration factor is the ratio of the amount of internal standard found, to the amount of internal standard added to the sample.

$$\text{Concentration Factor (CF)} = \frac{\text{amount of internal standard found}}{\text{amount of internal standard added}}$$

$$= \frac{\frac{AIS}{AS}}{m}$$

where AIS = peak area of the internal standard in the ali-

quot of the analytical sample* which was injected into the gas chromatograph.

AS = peak area per microgram of the internal standard

m = micrograms internal standard added to the analytical sample*

An aliquot of the n-butyl stearate solution (1 mg/ml) was injected into the gas chromatograph and the area (mm^2) of the resultant peak was measured. From this data, AS was calculated. AIS obtained simply by measurement of the peak area (mm^2) of n-butyl stearate in the aliquot of the analytical sample that was injected into the gas chromatograph (the peak of n-butyl stearate just precedes that of lysine). In the present work, 0.1 ml of standard n-butyl stearate solution was added to the analytical samples, thus the "micrograms internal standard added to the analytical sample" (m) had a value of 100.

(3) Determination of amino acid in sample:

The quantity of amino acid (μM) in the analytical sample is given by:

$$A.A. = \frac{A_{AA}}{SF_{AA} \times CF}$$

where A.A. = μM amino acid in the analytical sample

---

*The "analytical sample" is the sample which is subjected to the derivatization procedure.

A_{AA} = peak area (mm^2) of the amino acid

SF_{AA} = slope factor ($mm^2/\mu M$) of the corresponding amino acid as determined in Step (1)

CF = concentration factor as determined in Step (2)

The peak areas (A_{AA}) were determined by triangulation.

(4) Determination of percent recoveries:

The number of micromoles of each amino acid in the culture tube was known from initial weighing, and the number of micromoles found was determined in Step (3).

$$\% \text{ recovery} = \frac{\text{number of micromoles found}}{\text{number of micromoles added}} \times 100$$

(d) Recovery Experiments: Results and Discussion

The recoveries obtained for amino acids in Experiment Nos. 1, 2, 3, 4, and 5 are tabulated in Tables 24, 25, 26, 27, and 28, respectively. Figures 29, 30, and 31 show typical gas chromatograms obtained in Experiment Nos. 1, 2, and 3, respectively; and Figure 32 shows a typical gas chromatogram obtained in Experiment No. 5.

The recoveries obtained in Experiment No. 1 were extremely low, although quite reproducible. The reasons for low recoveries were probably: (a) there was incomplete dissolution of the amino acids in distilled water, (b) there was incomplete dissolution of the amino acids in the esterification reagents (a white precipitate was noted in the samples

after esterification with methanolic-HCl and interesterification with butanolic-HCl), and (c) the esterification and acylation reactions were not performed in a nitrogen atmosphere.

Recoveries were greatly improved in Experiment No. 2 in which ultrasonic mixing was employed. Some amino acids dissolved with difficulty in methanolic-HCl and butanolic-HCl, and ultrasonic mixing aided in the dissolution process. Stalling, Gille, and Gehrke (269) and Roach and Gehrke (240) also observed precipitates after esterification with methanolic-HCl and interesterification with butanolic-HCl. These workers found that ultrasonic mixing was an effective means of aiding dissolution.

The results obtained for Experiment No. 3 indicated that direct esterification with butanolic-HCl (3 N) did not lead to appreciably greater recoveries. Since direct esterification made possible the omission of a step, it was decided to employ this technique in further experiments.

The use of direct esterification in a nitrogen atmosphere with butanolic-HCl (3 N) led to increased recoveries even in the absence of ultrasonic mixing. Comparison of the average recoveries in Table 24 with the average recoveries in Column 1 of Table 27 will verify this statement. The

average recoveries of amino acids in the ultrasonic mixing experiment were 69.4% (0 sec), 75.7% (15 sec), 75.9% (30 sec), 61.2% (60 sec). These results indicate that best recoveries are obtained when esterification is performed employing ultrasonic mixing periods of 15-30 seconds. In subsequent experiments, an ultrasonic mixing period of 20 seconds was employed. Roach and Gehrke (240) found that a 15 second ultrasonic mixing period led to best recoveries.

Experiment No. 5 (Table 28) provides an indication of the precision of the method. The lowest recovery was obtained for tryptophan (37.1%). Tyrosine had the lowest recovery of those amino acids that can be determined in acid hydrolysates. The recoveries for this amino acid were extremely variable. The highest recovery was obtained with hydroxyproline. High coefficients of variation were obtained for cysteine (11.7%) and serine (9.7%). In general, hydroxy- and sulfur-containing amino acids exhibit low recoveries and high coefficients of variation. This is because the N-TFA n-butyl derivatives of these amino acids are extremely susceptible to hydrolysis by residual moisture. Moisture may be introduced inadvertently as follows: (a) by reagents which are not completely anhydrous, (b) by water formed in the esterification process, (c) by moisture present in the nitrogen gas used for evaporation, (d) by the use of hot

water baths for evaporation (steam condenses on the glass disposable pipette used to introduce nitrogen into the culture tubes), (e) by moisture present in the nitrogen carrier gas used for chromatography, and (f) by moisture present in the solid support used in the chromatographic columns. For these reasons, the following precautions were taken: (a) the reagents were thoroughly dried, (b) the water of esterification which was formed during the derivatization procedure was removed by azeotropic means, (c) the nitrogen gas used for chromatography and evaporation procedures was filtered to remove moisture, (d) oil baths were used in later experiments to esterify, acylate, and evaporate samples, and (e) the solid support was dried in an oven (15 h, 450°C) to remove traces of moisture.

Despite all these precautions, it was not possible to obtain yields such as those obtained by Gehrke and his coworkers (93)(97)(156)(240)(269). The average yield obtained for the 17 amino acids in Experiment No. 5 was about 70%. Apart from losses encountered in the procedure, the final percent recoveries obtained in these analyses depend on the values obtained for the slope factors and concentration factors, which, in turn, ultimately depend on the availability of high quality N-TFA n-butyl amino acid standard solutions and n-butyl stearate solutions. The standard

solutions employed in this work were of the highest quality that were commercially available. The standards were purchased in 1967, however, and were used over a three-year period and changes in concentration of these standards could have occurred, either through evaporation of the solvent, or due to hydrolysis of the esters.

2.5. A COMPUTER PROGRAM FOR AMINO ACID ANALYSIS

(a) Introduction

Several workers have designed programs for computer processing of the results of amino acid analyses. The most important programs have been written by Ozawa and Tanaka (225); Starbuck, Mauritzen, McClimans, and Busch (270); and Exss, Hill, and Summer (82).

Starbuck, Mauritzen, McClimans, and Busch (270) determined amino acids by means of an amino acid analyzer which was accelerated so that an analysis could be completed in 2½ hours. The results were then processed by an IBM 360 computer. The programs which the authors reported were for use on an IBM 7094 or on an IBM 1410 computer. The programs (FORTRAN) were divided into two parts; the first part was designed to process the analytical data obtained in an amino acid analysis of a protein hydrolysate, and the second part

Table 24

Recoveries of amino acids (Experiment No. 1)

Amino acid	µM Added	µM Found			Average	Average Recovery (%)
		1	2	3		
Alanine	13.16	0.029	0.019	0.029	0.026	0.19
Valine	8.33	0.643	0.633	0.647	0.641	7.69
Glycine	10.52	0.807	0.810	0.799	0.805	7.65
Isoleucine	8.66	0.743	0.728	0.763	0.745	8.60
Threonine	8.33	0.936	0.918	0.971	0.942	11.3
Leucine	7.81	1.81	1.94	2.09	1.95	25.0
Proline	10.63	1.43	1.43	1.45	1.44	13.5
Serine	10.24	1.42	1.43	1.45	1.43	14.0
Cysteine	8.16	0.749	0.722	0.669	0.713	8.73
Hydroxyproline	7.29	1.29	1.26	1.30	1.28	17.6
Methionine	6.89	0.977	0.968	0.978	0.974	14.1
Aspartic acid	7.18	1.25	1.23	1.28	1.25	17.4
Phenylalanine	6.08	1.01	0.994	1.04	1.01	16.6
Glutamic acid	6.55	1.71	1.69	1.78	1.73	26.4
Tyrosine	6.60	1.08	1.03	0.698	0.936	14.2
Lysine	4.68	0.988	0.911	0.957	0.952	20.3
Tryptophan	4.78	0.398	0.386	0.417	0.400	8.36

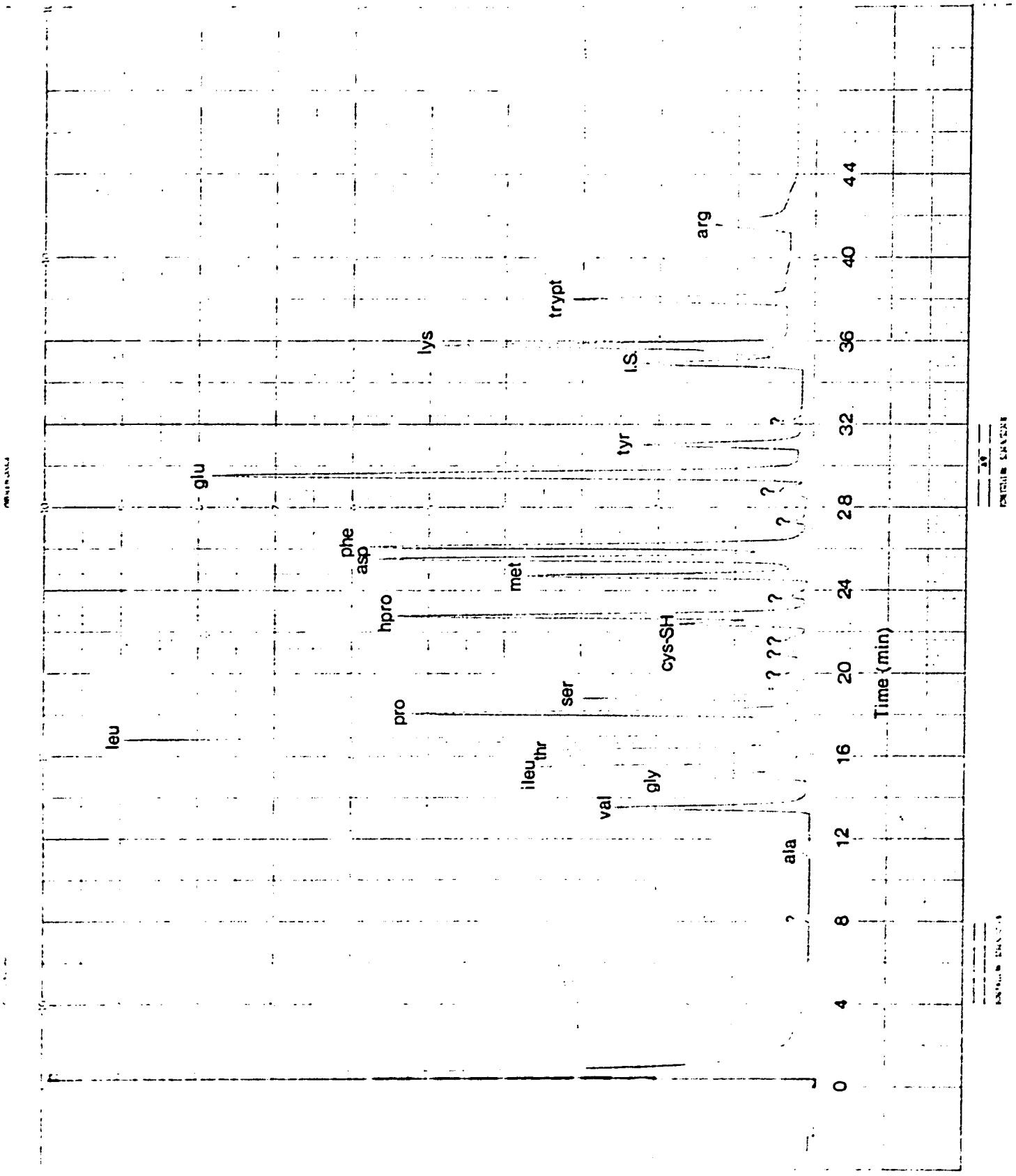


Figure 29. Typical chromatogram of amino acid derivatives obtained in Experiment No. 1.

Table 25

Recoveries of amino acids (Experiment No. 2)

Amino acid	µM Added	µM Found		Average	Average Recovery (%)
		1	2		
Alanine	11.67	-	0.040	0.020	0.17
Valine	7.38	3.76	2.89	3.33	45.1
Glycine	11.50	8.15	7.82	7.99	69.5
Isoleucine	7.04	3.94	3.13	3.54	50.3
Threonine	7.39	3.21	3.21	3.21	43.4
Leucine	6.43	7.26	5.65	6.46	100.5
Proline	9.73	6.98	5.71	6.35	65.3
Serine	8.94	3.25	3.92	3.59	40.2
Cysteine	8.35	2.66	1.71	2.19	26.2
Hydroxyproline	8.63	2.90	5.42	4.16	48.2
Methionine	7.16	5.15	4.36	4.76	66.5
Aspartic acid	7.63	6.39	5.30	5.85	76.7
Phenylalanine	6.05	4.98	4.20	4.59	75.9
Glutamic acid	6.31	6.50	6.25	6.38	101.1
Tyrosine	6.03	1.38	0.120	0.750	12.4
Lysine	5.34	4.11	3.48	3.80	71.2
Tryptophan	4.92	1.42	1.61	1.52	30.9

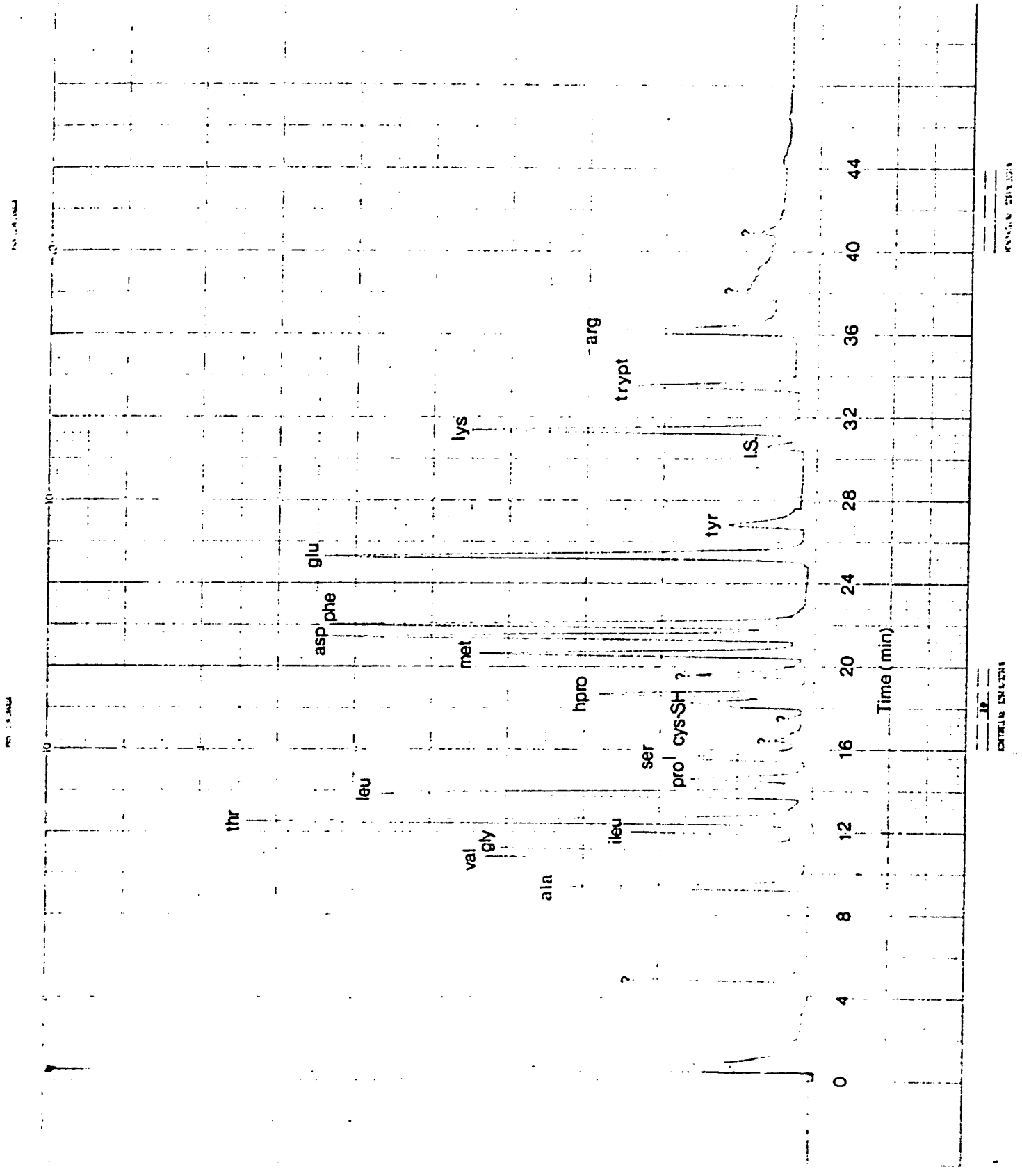


Figure 30. Typical chromatogram of amino acid derivatives obtained in Experiment No. 2.

Table 26

Recoveries of amino acids (Experiment No. 3)

Amino acid	µM Added	µM Found			Average	Average Recovery (%)
		1	2	3		
Alanine	11.67	-	-	-	-	-
Valine	7.38	5.26	3.58	2.73	3.85	52.2
Glycine	11.50	11.74	9.30	6.09	9.04	78.6
Isoleucine	7.04	4.61	3.80	2.91	3.77	53.6
Threonine	7.39	1.32	4.23	2.45	2.67	36.1
Leucine	6.43	7.39	6.73	5.39	6.50	101.1
Proline	9.73	6.55	6.40	5.39	6.11	62.8
Serine	8.94	-	5.90	3.85	3.25	36.4
Cysteine	8.35	3.39	4.43	2.55	3.46	41.4
Hydroxyproline	8.63	0.32	6.70	3.81	3.61	41.8
Methionine	7.16	4.35	4.53	3.79	4.22	58.9
Aspartic acid	7.63	5.42	5.97	4.76	5.38	70.5
Phenylalanine	6.05	4.29	4.70	3.73	4.24	70.1
Glutamic acid	6.31	5.84	6.80	5.00	5.88	93.2
Tyrosine	6.03	2.29	2.73	0.52	1.85	30.7
Lysine	5.34	3.55	4.20	3.27	3.67	68.7
Tryptophan	4.92	0.71	1.07	1.21	1.00	20.3

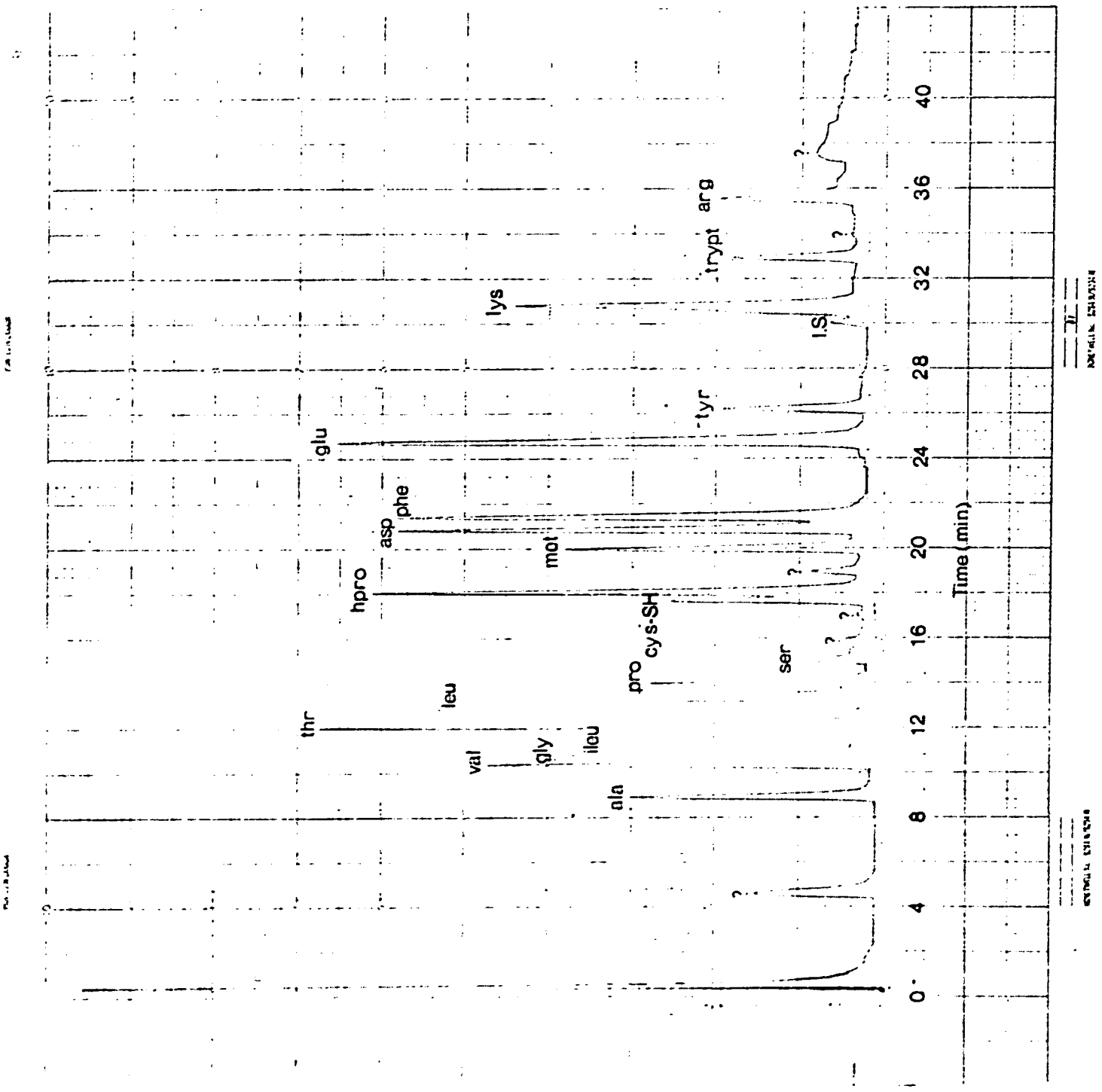


Figure 31. Typical chromatogram of amino acid derivatives obtained in Experiment No. 3.

Table 27

Recoveries* of amino acids for different ultrasonic mixing periods (Experiment No. 4)

Amino acid	Time of ultrasonic mixing (sec)			
	0**	15**	30**	60**
Alanine	-	-	-	-
Valine	59.8	68.2	65.6	70.3
Glycine	68.6	79.3	76.3	74.9
Isoleucine	66.2	72.1	72.8	69.7
Threonine	68.2	71.3	80.0	54.7
Leucine	84.2	94.2	94.5	88.8
Proline	70.1	80.9	77.0	87.9
Serine	103.0	110.6	119.0	67.2
Cysteine	70.2	76.4	80.2	51.3
Hydroxyproline	76.5	84.0	83.2	60.3
Methionine	70.4	77.9	80.5	69.9
Aspartic acid	65.6	72.3	71.8	64.7
Phenylalanine	72.7	79.4	80.3	70.3
Glutamic acid	87.3	95.6	83.5	81.1
Tyrosine	86.3	91.2	91.4	34.8
Lysine	88.9	95.9	96.6	78.9
Tryptophan	20.7	20.9	17.8	10.4
Arginine	20.3	17.4	19.0	4.6

*Values in this table are expressed in % recovery.

**Each value represents the average of three separate determinations.

Table 28

Recoveries of amino acids (Experiment No. 5)

Amino acid	Trial No. 1	2	3	4	5	$\bar{X} \pm s$[b] (% Recovery)	c.v.[c]
	(% Recovery)[a]						
Alanine	65.6	73.3	66.9	72.2	76.5	70.9 ± 4.5	6.4
Valine	64.3	71.0	67.7	67.7	73.3	68.8 ± 3.5	5.1
Glycine	69.2	76.3	73.0	73.4	76.7	73.7 ± 3.1	4.1
Isoleucine	66.2	75.1	73.0	72.6	78.2	73.0 ± 4.4	6.1
Threonine	73.8	80.3	76.6	70.5	84.2	77.1 ± 5.4	7.0
Leucine	61.5	67.6	64.3	65.2	66.7	65.1 ± 2.4	3.6
Proline	68.3	69.0	67.3	67.5	70.2	68.5 ± 1.2	1.7
Serine	77.4	94.0	87.0	76.5	93.1	85.6 ± 8.3	9.7
Cysteine	60.8	82.8	67.5	68.5	67.5	69.4 ± 8.1	11.7
Hydroxyproline	84.2	90.8	83.5	83.2	87.6	85.9 ± 3.3	3.8
Methionine	68.1	72.6	70.0	66.9	73.8	70.3 ± 2.9	4.2
Aspartic acid	72.6	79.1	71.0	72.8	80.6	75.2 ± 4.3	5.7
Phenylalanine	75.2	79.8	78.2	75.4	84.1	78.5 ± 3.7	4.7
Glutamic acid	86.4	88.9	85.6	87.1	88.7	87.3 ± 1.4	1.6
Tyrosine	21.9	91.4	23.5	46.6	25.8	41.8 ±29.4	70.4
Lysine	69.9	71.4	71.0	69.1	75.6	71.4 ± 2.5	3.5
Tryptophan	37.8	39.4	37.6	33.7	36.8	37.1 ± 2.1	5.6
Arginine	ND[d]	ND	ND	ND	ND	-	-
Histidine	ND	ND	ND	ND	ND	-	-
Cystine	ND	ND	ND	ND	ND	-	-

[a]Ultrasonic mixing period: 20 sec.

[b]Average ± standard deviation.

[c]c.v. = coefficient of variation.

[d]ND = abbreviation for "not detected".

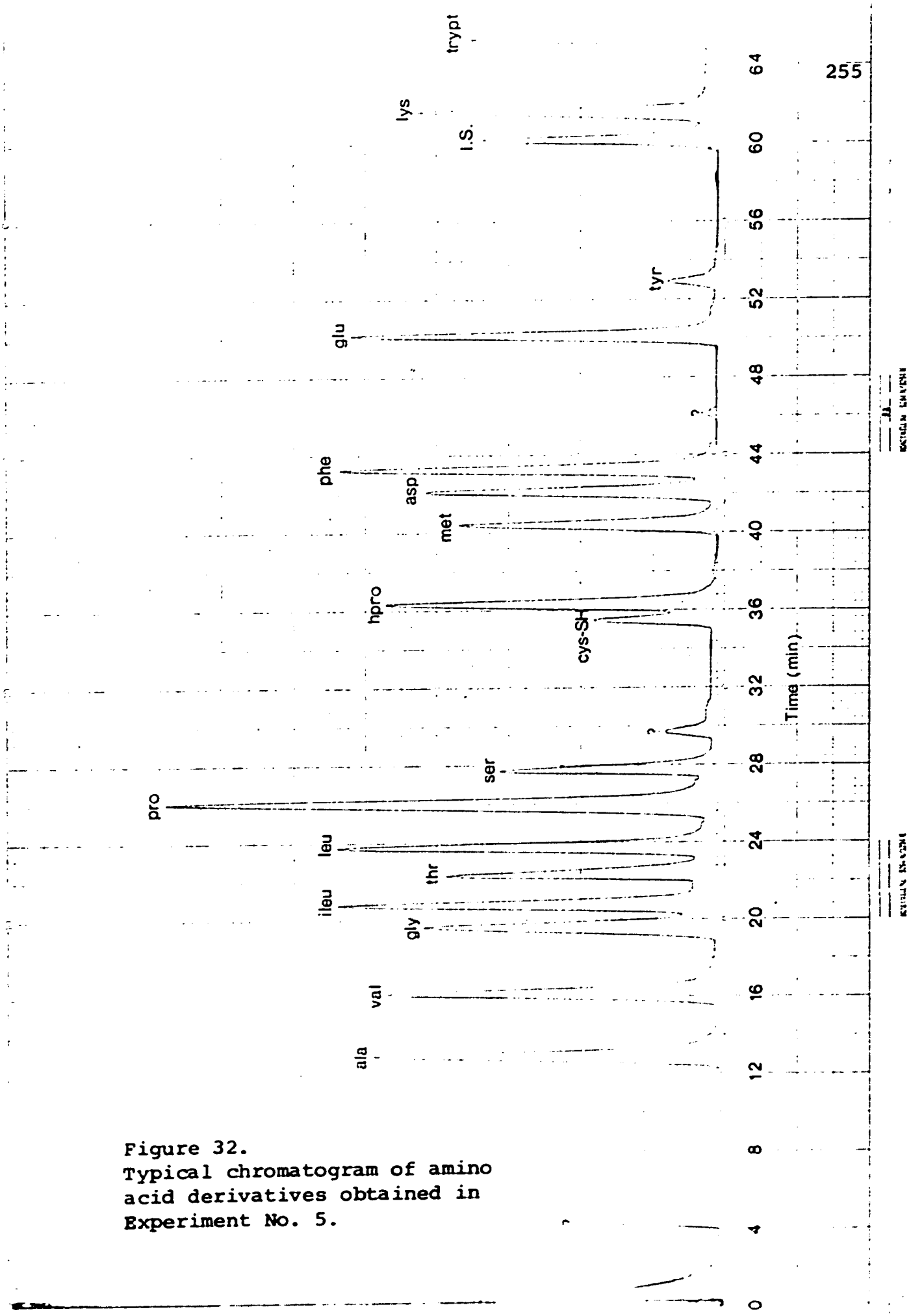


Figure 32.
Typical chromatogram of amino acid derivatives obtained in Experiment No. 5.

was designed to calculate the composition of peptides.

Exss, Hill, and Summer (82) described a program (FORTRAN IV) by which the computer (IBM 1130) could identify individual amino acids and calculate their concentrations from the data provided by the integrator attached to the automatic amino acid analyzer.

As far as the author is aware, there are no published papers which deal with computer programs designed to process the data obtained from the analysis of amino acids by gas-liquid chromatography. The computer program described in the present work has been designed to process the data of one or more determinations of amino acids by gas chromatography. The program (FORTRAN IV) has been written specifically for use on an IBM 360 computer.

The detailed calculation which is involved in the method of Gehrke and Stalling (97)(238) has already been presented in a previous section of this chapter (Section 2.4. (c) vi) and thus the discussion below will be confined to the computer program which is presented in Figure 33.

(b) Definition of Terms

The following terms have been used in the computer program which appears in Figure 33:

N = the number of analyses to be performed.

M = the number of the analysis that is being performed at any one given time.

C = the concentration (μM/μl) of each standard solution of amino acid derivative (N-TFA n-butyl ester) which was injected into the gas chromatograph.

V = the volume (μl) of each standard solution of amino acid derivative (N-TFA n-butyl ester) which was injected into the gas chromatograph.

VC = the product (μM) of V and C, and refers to the number of micromoles of each N-TFA n-butyl ester injected onto the gas chromatographic column. Rather than inject a volume, V, of each amino acid standard solution of concentration, C, a standard mixture is made from each individual solution, and an aliquot of the resultant solution is injected into the gas chromatograph. Since an equimolar solution of standard amino acid derivatives is made, the product, VC, is a constant for a given set of analyses. For the present set of analyses, a standard mixture of amino acid derivatives was made by taking 10 μl of each of the 20 individual solutions (0.05 M) of amino derivatives, and placing them in a septum-stoppered mixing vial. Therefore,

the concentration of this solution was: 0.5 μM/200 μl = 0.0025 μM/μl = 0.0025 M with respect to each amino acid derivative. An aliquot (2.5 μl) of this solution was injected into the gas chromatograph. Thus, for the purpose of these analyses:

V = 2.5 μl

C = 0.0025 μM/μl

and VC = 2.5 μl x 0.0025 μM/μl = 0.00625 μM of each amino acid derivative injected

AREA (subscripted variable) = the peak area (mm^2) obtained for each of the 20 amino acids by injection of a given number of micromoles (0.00625) of standard N-TFA n-butyl ester. The 20 values obtained for AREA must be punched on a data card.

SF (subscripted variable) = the slope factor ($mm^2/\mu M$) for each amino acid derivative. The slope factor for each amino acid is computed from the AREA of the corresponding amino acid and VC, i.e.

$$SF(J) = \frac{AREA(J)}{VC}$$

AIS = peak area (mm^2) of the internal standard in the aliquot of the analytical sample which was injected into the gas chromatograph*. The value of AIS must be

*Note: The "analytical sample" refers to the weight of that portion of the hydrolysate which is subjected to the derivation procedure. After derivatization, an "aliquot" of the analytical sample is analyzed by gas chromatography.

punched on a data card.

AS = peak area per microgram ($mm^2/\mu g$) of the internal standard. The value for AS must be punched on a data card.

SAMIS= micrograms (μg) internal standard added to the analytical sample. This value must be punched on a data card.

CF = the concentration factor. This value is computed from AIS, AS, and SAMIS, i.e.

$$CF = \frac{\frac{AIS}{AS}}{SAMIS}$$

X = the weight (mg) of sample which was subjected to the derivatization procedure; also known as the "analytical sample". The value for X must be punched on a data card.

Y = the nitrogen content (%) of the protein sample that was subjected to hydrolysis.

J = the subscript which defines the number assigned to each amino acid. The numbers assigned to the amino acids are indicated in the print-out of data.

AAA (subscripted variable) = the peak area (mm^2) obtained for each of the 20 amino acids after injection of an aliquot of the derivatized analytical sample. The 20 values for AAA must be punched on a data card.

AAUM (subscripted variable) = the number of micromoles (μM) of each amino acid present in the analytical sample. The values for AAUM are computed from the corresponding values of AAA and SF, and from CF, i.e.

$$AAUM(J) = \frac{AAA(J)}{SF(J) \times CF}$$

AAMW = the molecular weight (mg/mM) of an amino acid.

AAMG (subscripted variable) = the number of milligrams (mg) of each amino acid present in the analytical sample. The values for AAMG are computed from the corresponding values of AAUM and AAMW, i.e.

$$AAMG(J) = \frac{AAUM(J) \times AAMW}{1000}$$

WTPC (subscripted variable) = the weight percent (%) of each amino acid present in the analytical sample. The values for WTPC are computed from the corresponding values of AAMG and from X, i.e.

$$WTPC(J) = \frac{AAMG(J)}{X} \times 100$$

SUM = the total (%) obtained by summing the individual values for WTPC.

(c) Discussion

The program begins with a suitable identification statement.

Figure 33. A computer program for the processing of data obtained from the analysis of amino acids by gas chromatography.

```
$WATFOR MAS805-PC-AA-C-23-LAUER,XP=20
      DIMENSION AREA(20),SF(20),AAA(20),AAUM(20),AAMG(20),MTPC(20)
      READ 101,N
  101 FORMAT(I3)
      DO 100 M=1,N
      PRINT 2,M
    2 FORMAT(1H1,25HAMINO ACID COMPOSITION OF CASEIN IN MILKS OF ARCTIC
     1MAMMALS ANALYSIS NUMBER,1X,I3)
      PRINT 4
    4 FORMAT(1H0,30HNUMBERS ASSIGNED TO VARIOUS AMINO ACIDS//5X,17HALANI
     1NE          1//5X,17HVALINE          2//5X,17HISOLEUCINE       3//5X
     2,17HGLYCINE          4//5X,17HLEUCINE          5//5X,17HTHREONINE
     3     6//5X,17HPROLINE          7//5X,17HSERINE          8//5X,17HCYS
     4TEINE          9//5X,17HHYDROXYPROLINE 10//5X,17HMETHIONINE      11)
      PRINT 88
   88 FORMAT(1H0,4X,17HPHENYLALANINE  12//5X,17HASPARTIC ACID  13//5X,17
     1HGLUTAMIC ACID  14//5X,17HTYROSINE        15//5X,17HLYSINE
     2 16//5X,17HHISTIDINE       17//5X,17HARGININE        18//5X,17HCYSTIN
     3E         19//5X,17HTRYPTOPHANE     20)
      READ 10,(AREA(J),J=1,20)
   10 FORMAT(20F4.0)
      DO 15 J=1,20
      VC=0.00025
      SF(J)=AREA(J)/VC
      IF(AREA(J).EQ.0)SF(J)=1
   15 CONTINUE
      READ 25,A1S,AS
   25 FORMAT(F4.0,5X,F4.0)
      SAMIS=100.0
      CF=(A1S/AS)/SAMIS
      READ 30,T,Y
   30 FORMAT(F4.1,5X,F4.1)
      PRINT 97,T,Y
   97 FORMAT(1H0,35HTHE WEIGHT OF SAMPLE DERIVATIZED IS,1X,F4.1,1X,42HAN
     1D THE NITROGEN CONTENT OF THIS SAMPLE IS,1X,F4.1)
      PRINT 31
   31 FORMAT(1H0,30HTABLE OF RESULTS OF AMINO ACID ANALYSIS)
      PRINT 32
   32 FORMAT(1H0,5X,5HAA NO,6X,4HAREA,6X,7HSL FACT,6X,11HAMT AA (UM)6X,5
     1HMW AA,6X,11HAMT AA (MG),6X,4HMTPC)
      READ 35,(AAA(J),J=1,20)
   35 FORMAT(20F4.0)
      SUM=0.0
      DO 40 J=1,20
      AAUM(J)=AAA(J)/(SF(J)*CF)
      GO TO (36,37,38,39,40,41,42,43,44,45,46,47,48,49,50,51,52,53,54,55
     1),J
   36 AAMW=89.09
      GO TO 56
   37 AAMW=117.15
      GO TO 56
   38 AAMW=131.17
      GO TO 56
   39 AAMW=75.07
      GO TO 56
   40 AAMW=131.17
      GO TO 56
   41 AAMW=119.12
      GO TO 56
   42 AAMW=115.13
```

```
      GO TO 56
   47 AAMW=185.89
      GO TO 56
   44 AAMW=121.16
      GO TO 56
   45 AAMW=131.13
      GO TO 56
   4A AAMW=149.21
      GO TO 56
   47 AAMW=145.19
      GO TO 56
   48 AAMW=133.18
      GO TO 56
   49 AAMW=147.13
      GO TO 56
   5A AAMW=191.19
      GO TO 56
   51 AAMW=144.19
      GO TO 56
   52 AAMW=155.16
      GO TO 56
   53 AAMW=174.28
      GO TO 56
   54 AAMW=248.38
      GO TO 56
   55 AAMW=284.22
      GO TO 56
   56 AAMG(J)=(AAIIN(J)*AAMW)/1000.0
      WTPC(J)=(AAMG(J)*100.0)/X
      SUM=SUM+WTPC(J)
      PRINT 57,J,ARFA(J),SF(J),AAIIN(J),AAMW,AAMG(J),WTPC(J)
   57 FORMAT(1H0,4X,I3,5X,F4.0,4X,F7.0,4X,F8.4,4X,F4.2,4X,F7.4,4X,F5.2)
   40 CONTINUE
      PRINT 58,SUM
   58 FORMAT(1H0,20HTHE SUM OF THE PERCENTAGES IS,F5.1)
  100 CONTINUE
   99 CALL EXIT
      END

$DATA
```

Statement No. 1* is a DIMENSION statement which provides the information necessary to allocate storage for arrays in the object program. All subscripted variables are dimensioned.

Statement Nos. 2 & 3 define N, which is the number of separate amino acid analyses that are being performed. N may vary from 1 to 999.

Statement No. 4 defines a DO loop, which is a command to execute repeatedly the statements which follow the DO statement, up to and including Statement No. 84 (indexed 100). This DO loop will be executed N times (as many times as the number of analyses).

Statement Nos. 5-10 are PRINT and FORMAT statements which are involved in the print-out of the title and analysis number, and the numbers which are assigned to the various amino acids for identification.

Statement Nos. 13-17 comprise a DO loop in which the calculations necessary to obtain the slope factors for each amino acid, are computed from the values of AREA. The values

---

*Note: In this discussion, "Statement Numbers" refer to the numbers in the column which is located near the margin in Figure 33. An indexed statement will be referred to in the following manner, for example:

Statement No. 68 (indexed 52)

for AREA are read into core storage by means of Statement Nos. 11 & 12. Note that the AREA's are punched on the data card in the order in which numbers were assigned to individual amino acids. Statement No. 16 ensures that, in the event that AREA = 0 (see histidine, for example, which was not detected) and consequently SF = 0, a zero will not appear in the divisor of Statement No. 34.

Statement Nos. 18-21 involve the computation of the concentration factor, CF.

Statement Nos. 22-29 comprise statements involved in the format of data print-out, and are concerned with construction of a suitable table in which the results will be printed.

Statement No. 30 is a READ statement in which the values of AAA are read into core storage. Again, it is important that the values for AAA are punched on the data card in the order in which numbers were assigned to individual amino acids.

For discussion of Statement Nos. 33-81, let us assume that J = 2, that is, the computations for valine are being performed. The value for AAA(2) was read from the data card (Statement No. 30). Control now reaches a DO loop commencing at Statement No. 33. The value for AAUM(2) is computed from

AAA(2), SF(2), and CF (Statement No. 34). Control is then transferred to Statement No. 38 (indexed 37) by the computed GO TO statement (Statement No. 35). Statement No. 38 defines the molecular weight of valine (117.15) and Statement No. 39 represents an unconditional GO TO statement which transfers control to Statement No. 76 (indexed 56). Statement Nos. 76 & 77 are involved with the computation of AAMG(2) and WTPC(2). Statement Nos. 79 & 80 are concerned with the print-out of data, and the results for valine are then printed in the following order: J (the amino acid number; 2), AREA(2), SF(2), AAUM(2), AAMW, AAMG(2), and WTPC(2). As soon as control reached Statement No. 81 (indexed 60), the value of J is incremented by 1, and control reverts to Statement No. 33. Now, J = 3, and the same procedure is performed for isoleucine, and so on. After the DO loop beginning with Statement No. 33 has been satisfied (J = 20), and the values for tryptophan have been computed and printed, the final sum (Statement No. 78) of the percentages of the individual amino acids is printed (Statement Nos. 82 & 83).

When Statement No. 84 (indexed 100) is reached, control is transferred back to Statement No. 4, and the value for M (the analysis number) is incremented by 1. Calculations for the next analysis are then performed. When this DO loop is

satisfied (M = N), the program is ended.

The order of the data cards which follow the END statement is:

(1) $DATA

(2) N (the total number of analyses; 2 in this case)

(3) AREA's for Analysis No. 1

(4) AIS and AS for Analysis No. 1

(5) X and Y for Analysis No. 1

(6) AAA's for Analysis No. 1

(7) AREA's for Analysis No. 2

(8) AIS and AS for Analysis No. 2

(9) X and Y for Analysis No. 2

(10) AAA's for Analysis No. 2

(11) $

The data which were used to demonstrate the use of the computer program are presented in Figure 34.

The print-out is shown in Figure 35.

2.6. APPLICATION OF METHODS TO AMINO ACID ANALYSIS OF CASEINS ISOLATED FROM MILKS OF DIFFERENT SPECIES

(a) Introduction

Hagen and Black (116) subjected samples which contained different weights of each amino acid to the same

Figure 34. Data to be punched on cards for computer processing.

IBM

FORTRAN Coding Form

FORM 3262-1

Program: Data for Amino Acid Analyses Nos. 1 & 2 | Punching Instructions | Graphic | Punch | Page 1 of 1

Programmer: B.H. Lauer | Date: June 27, 1971 | Card Form # IBM 888157

Card No.	FORTRAN STATEMENT	Identification Sequence
1	$DATA	
2	002	
3	101 104 130 61 139 87 91 84 61 144 90 224 131 135 136 149 0 41	65 130
4	63 68	
5	14 143	
6	64 83 91 52 93 84 74 86 44 98 91 134 105 200 132 99 0 10	10 78
7	101 104 130 61 139 87 91 84 61 144 90 224 131 135 136 149 0 41	65 130
8	58 68	
9	14 143	
10	59 73 82 48 82 72 66 77 41 90 84 127 96 200 122 96 0 10	10 85
11	$	

Figure 35. Computer print-out of amino acid results.

AMINO ACID COMPOSITION OF CASEIN IN MILKS OF ARCTIC MAMMALS ANALYSIS NUMBER 1

NUMBERS ASSIGNED TO VARIOUS AMINO ACIDS

ALANINE	1
VALINE	2
ISOLEUCINE	3
GLYCINE	4
LEUCINE	5
THREONINE	6
PROLINE	7
SERINE	8
CYSTEINE	9
HYDROXYPROLINE	10
METHIONINE	11
PHENYLALANINE	12
ASPARTIC ACID	13
GLUTAMIC ACID	14
TYROSINE	15
LYSINE	16
HISTIDINE	17
ARGININE	18
CYSTINE	19
TRYPTOPHANE	20

THE WEIGHT OF SAMPLE DERIVATIZED IS 1.4 AND THE NITROGEN CONTENT OF THIS SAMPLE IS 14.3

TABLE OF RESULTS OF AMINO ACID ANALYSIS

AA NO	AREA	SL FACT	AMT AA (UM)	MW AA	AMT AA (MG)	WTPC
1	191.	14140.	0.4275	89.09	0.0381	2.72
2	196.	14440.	0.5384	117.15	0.0631	4.51
3	139.	29999.	0.4722	131.17	0.0619	4.42
4	81.	9760.	0.5751	75.07	0.0432	3.08
5	119.	22240.	0.4514	131.17	0.0592	4.23

6	87.	11920.	0.6511	119.12	0.0776	5.54
7	91.	14540.	0.5686	115.13	0.0632	4.51
8	84.	13440.	0.6987	105.80	0.0726	5.18
9	41.	9760.	0.6866	121.16	0.0590	4.21
10	144.	23840.	0.6591	111.13	0.0602	4.30
11	98.	14480.	0.6821	149.21	0.1018	7.27
12	224.	35840.	0.6836	145.19	0.0667	4.76
13	131.	20960.	0.5607	111.18	0.0720	5.14
14	135.	21600.	0.0004	147.13	0.1470	10.50
15	110.	21760.	0.6560	101.10	0.1106	0.67
16	140.	23840.	0.6602	144.10	0.0655	6.60
17	0.	1.	0.0000	155.16	0.0000	0.00
18	41.	6560.	0.1645	174.20	0.0207	2.05
19	65.	10400.	0.1030	240.30	0.0240	1.70
20	170.	28000.	0.6060	204.22	0.0027	5.90

THE SUM OF THE PERCENTAGES IS 93.7

AMINO ACID COMPOSITION OF CASEIN IN MILKS OF ARCTIC MAMMALS ANALYSIS NUMBER 2

NUMBERS ASSIGNED TO VARIOUS AMINO ACIDS

ALANINE	1
VALINE	2
ISOLEUCINE	3
GLYCINE	4
LEUCINE	5
THREONINE	6
PROLINE	7
SERINE	8
CYSTEINE	9
HYDROXYPROLINE	10
METHIONINE	11
PHENYLALANINE	12
ASPARTIC ACID	13
GLUTAMIC ACID	14
TYROSINE	15
LYSINE	16
HISTIDINE	17
ARGININE	18
CYSTINE	19
TRYPTOPHANE	20

THE WEIGHT OF SAMPLE DERIVATIZED IS 1.6 AND THE NITROGEN CONTENT OF THIS SAMPLE IS 14.7

TABLE OF RESULTS OF AMINO ACID ANALYSIS

AA NO	AREA	SL FACT	AMT AA (UM)	MW AA	AMT AA (MG)	WTPC
1	181.	14140.	0.6288	89.09	0.0781	2.72
2	184.	14440.	0.5143	117.15	0.0603	4.38
3	178.	28900.	0.6622	131.17	0.0486	4.33
4	61.	9760.	0.5766	75.07	0.0433	3.89
5	179.	22240.	0.6323	131.17	0.0547	4.85

6	87.	17920.	0.4844	119.12	0.0722	5.16
7	91.	14540.	0.5315	115.13	0.0412	4.37
8	84.	17440.	0.4717	105.80	0.0786	5.84
9	41.	9760.	0.4025	121.14	0.0597	4.24
10	144.	27840.	0.4588	111.13	0.0681	4.29
11	98.	14480.	0.6830	140.21	0.1024	7.29
12	224.	35960.	0.4154	145.19	0.0686	4.98
13	131.	20960.	0.5378	133.18	0.0715	5.11
14	135.	21600.	1.0856	147.13	0.1597	11.41
15	134.	21760.	0.6573	181.19	0.1191	8.51
16	149.	23840.	0.4721	144.19	0.0698	4.93
17	0.	1.	0.0000	155.16	0.0000	0.00
18	41.	6560.	0.1797	174.28	0.0311	2.22
19	65.	10400.	0.1127	248.38	0.0271	1.93
20	138.	20000.	0.4791	204.22	0.0978	6.99

THE SUM OF THE PERCENTAGES IS 94.9

CORE USAGE OBJECT CODE= 1856 BYTES,ARRAY AREA= 488 BYTES,TOTAL AREA AVAILABLE= 28491 BYTES

COMPILE TIME= 0.28 SEC,EXECUTION TIME= 0.29 SEC. WATFIV - VERSION 1 LEVEL 1 JANUARY 1970 DATE= 71/149

esterification procedure and made the N-trifluoroacetyl methyl amino acid esters. These esters were dissolved in standard volumes of methanol and equal volumes of each solution were injected onto the column of a gas chromatograph. These workers found a linear relationship between the amount of amino acid injected and the corresponding peak area (as expressed in disc-integrator units). The authors postulated that the reason for this linear relationship was either that the conversion of the amino acid to its volatile derivative was complete, or that a constant proportion of each amino acid was converted to its volatile derivative, regardless of the amount of amino acid.

The present author has shown that, at best, the average recovery of 17 amino acids as their N-trifluoroacetyl n-butyl esters is about 70% when the procedures described in the previous section were employed. The fact that the hydrogen flame detector gives a unique response for each amino acid derivative and that the conversion of amino acids to their corresponding N-trifluoroacetyl n-butyl derivatives is unique and incomplete for each amino acid, means that complicated calculations are necessary. Thus, it was decided to test the hypothesis of Hagen and Black and determine whether the proportion of an amino acid converted to its N-trifluoroacetyl n-butyl ester was constant within a specified

concentration range. This fact would be evident if there were a linear relationship between concentration and peak area. It was also decided to attempt to simplify the method of Hagen and Black by use of an internal standard, which eliminates the need to inject a specified amount of a final standard ester solution. Thus, steps are eliminated where error is likely to occur. Dilution, concentration, or spillage of the final solution, or of intermediate solutions containing the amino acid derivatives, will not affect the final result, since this method makes use of the ratios formed by dividing the peak areas of the amino acid derivatives by the peak area of the internal standard.

(b) Procedure

i) Preparation of ion-exchange resins

Dowex-50 x 12 (H^+ form; 100 g) was placed in a large sintered-glass funnel which was connected to a suction flask. Sodium hydroxide (10% w/v) was added to the funnel and the resultant suspension was stirred periodically for a period of 15 min. A gentle vacuum was applied to the suction flask and the NaOH was allowed to drain. Several portions of distilled water were added to the resin in the funnel and care was taken to ensure that the surface of the liquid did not sink below that of the resin. The process of addition of

portions of distilled water was repeated until the washings were neutral to red litmus paper. The above process was repeated, this time using hydrochloric acid (10% w/v), and the resin was rinsed with several portions of distilled water until the washings were neutral to blue litmus. The recycling of the resin was repeated again with NaOH, and finally with HCl. This process ensured that the resin was completely converted to the H^+ form.

ii) Preparation of standard calibration curves

(1) A standard solution was made which was millimolar with respect to each of the 20 amino acids. One millimole of each amino acid was placed in a volumetric flask (1000 ml), HCl (6 N) was added, and the volume of the resultant solution was adjusted to 1000 ml with the same acid.

(2) Samples (0.25, 0.50, 0.75, 1.0, 2.0, 3.0, and 4.0 ml) of this solution were pipetted into seven separate culture tubes (Pyrex 9826; screw-top; Teflon-lined caps) and each of these solutions was evaporated (100°C; oil bath) with the aid of a stream of dry nitrogen.

(3) Water was removed by azeotropic distillation (anhydrous methylene chloride; 150 µl) and by subsequent evaporation (70°C; oil bath) with the aid of a stream of dry nitrogen. This process was performed three times.

(4) Butanolic-HCl (3 N, 3.75 µl) and internal standard solution (n-butyl stearate in n-butanol, 1 mg/ml; 0.1 ml) was added to each of the seven tubes. The tubes were then flushed with nitrogen and the contents were subjected to ultrasonic mixing (45 watts; 20 sec). The tubes were flushed with nitrogen, capped tightly, and placed in an oil bath (100°C) for 35 min.

(5) Excess esterification reagent was removed by evaporation (70°C; oil bath) with the aid of a stream of dry nitrogen.

(6) Water of esterification was removed as described in Step (3).

(7) Acylation reagent (trifluoroacetic anhydride in methylene chloride, 25% v/v; 2.0 ml) was added to each of the seven tubes. The tubes were then flushed with nitrogen and the contents were subjected to ultrasonic mixing (45 watts; 20 sec). The tubes were flushed with nitrogen, capped tightly, and placed in an oil bath (150°C) for 5 min.

(8) The samples were stored in the freezer until ready for use. When the gas chromatographic analyses were to be performed, the caps were removed and excess acylating reagent was removed (140°C) with the aid of a stream of dry nitrogen. Anhydrous methylene chloride (100 µl) was then added to the

dry N-trifluoroacetyl n-butyl esters and aliquots (0.5-1.0 µl) were injected into the gas chromatograph.

(9) The ratios formed by dividing the peak area (AAA) of each amino acid by the peak area (AIS) of the internal standard were calculated. A standard curve was constructed for each amino acid by plotting the ratio obtained for that amino acid in each tube, versus the corresponding amount of that amino acid in that tube.

Figures 36, 37, 38, and 39 show the standard calibration curves for alanine, valine, glycine, isoleucine, threonine, leucine, proline, serine, cysteine, hydroxyproline, methionine, aspartic acid, phenylalanine, glutamic acid, tyrosine, and lysine.

Arginine, histidine, and cystine could not be detected on the columns employed. Since tryptophan was estimated by other means, no standard calibration curve was constructed for this amino acid.

iii) Preparation of sample (hydrolysis and ion-exchange clean-up)

The procedure used for the hydrolysis of the protein samples was that described by Keutmann and Potts (146). The samples of protein to be hydrolyzed (10 mg) were placed in clean, dry test tubes (13 x 150 mm). Constant-boiling HCl (5.7 N; 1.0 ml) containing mercaptoethanol (1:2000 v/v) was

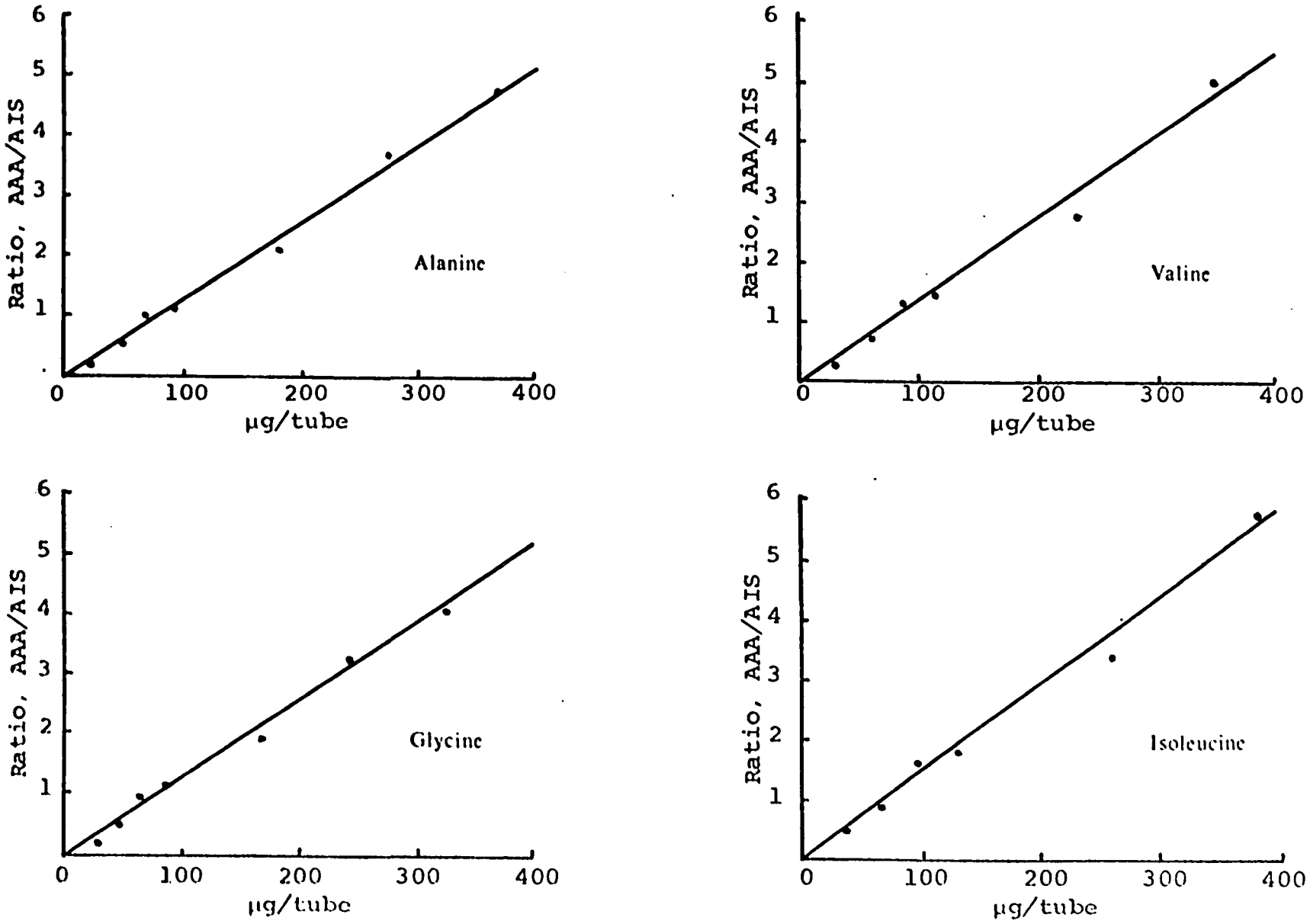
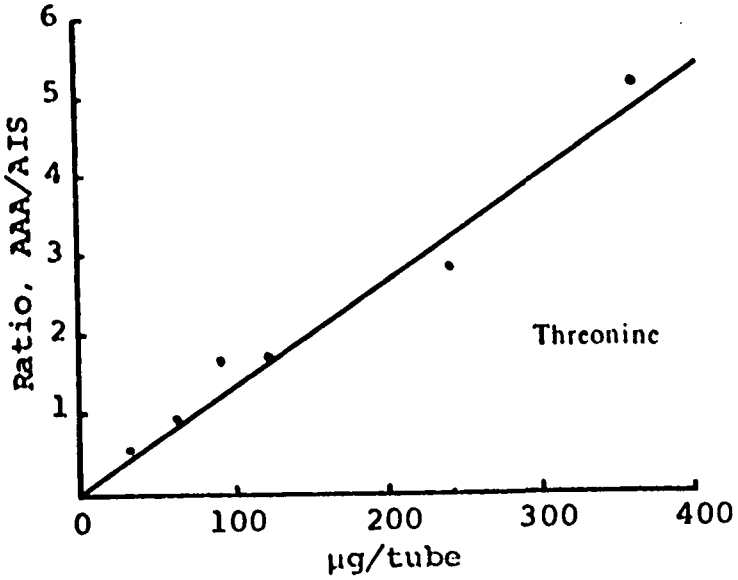
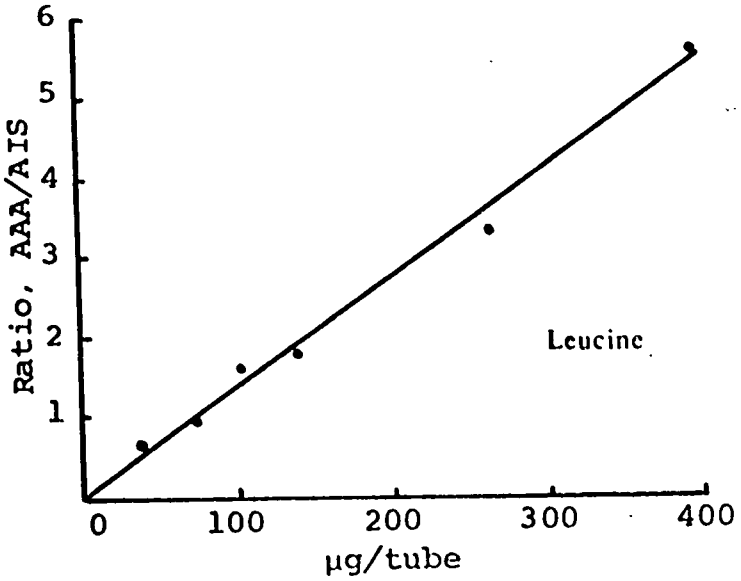
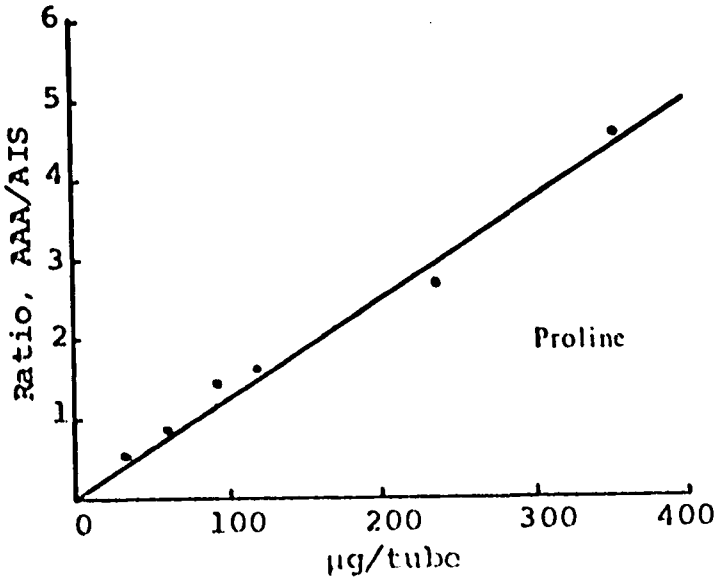


Figure 36. Standard calibration curves for alanine, valine, glycine, and isoleucine.







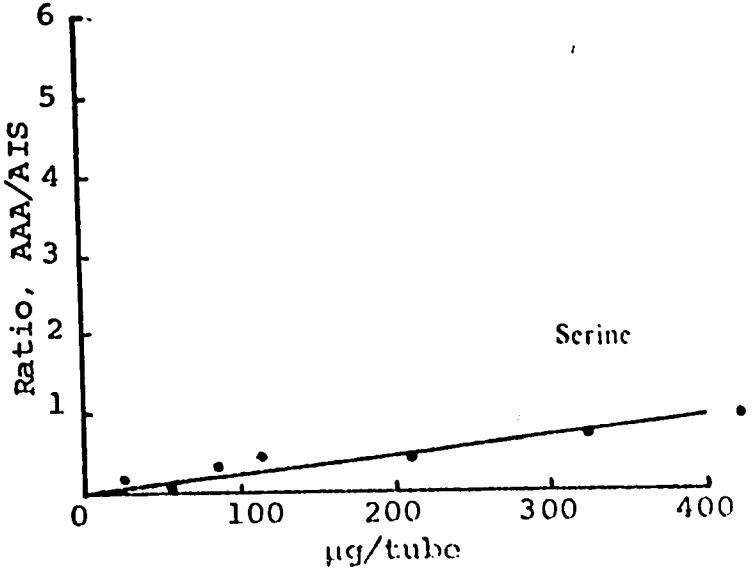


Figure 37. Standard calibration curves for threonine, leucine, proline, and serine.

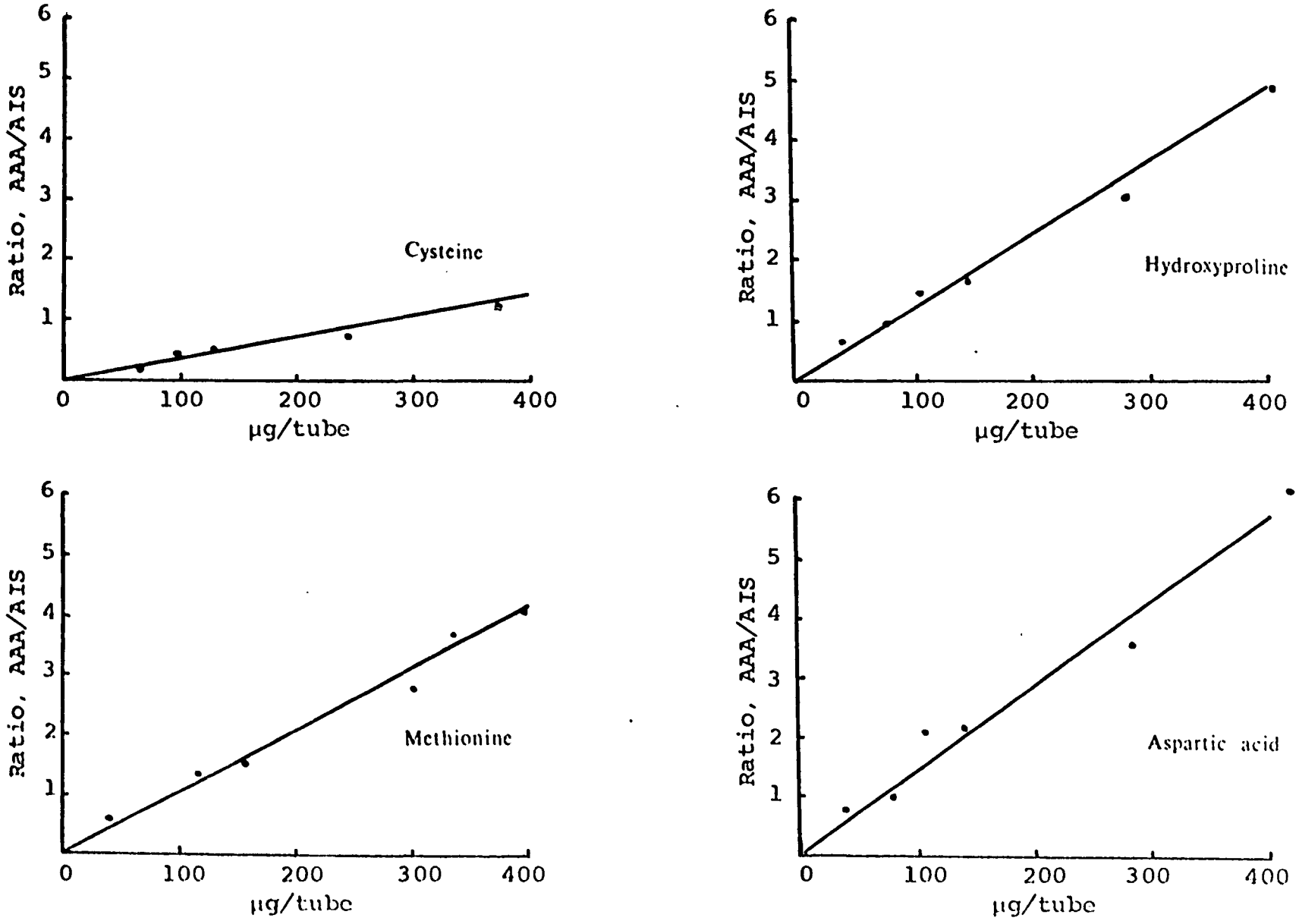
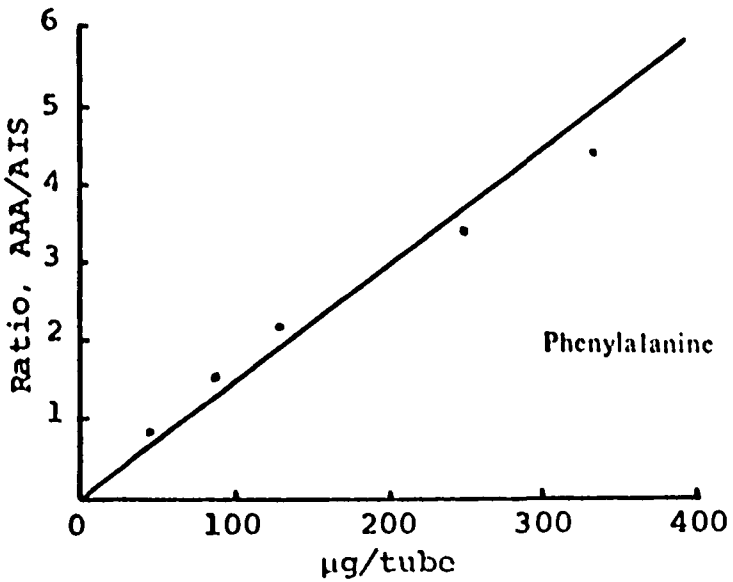
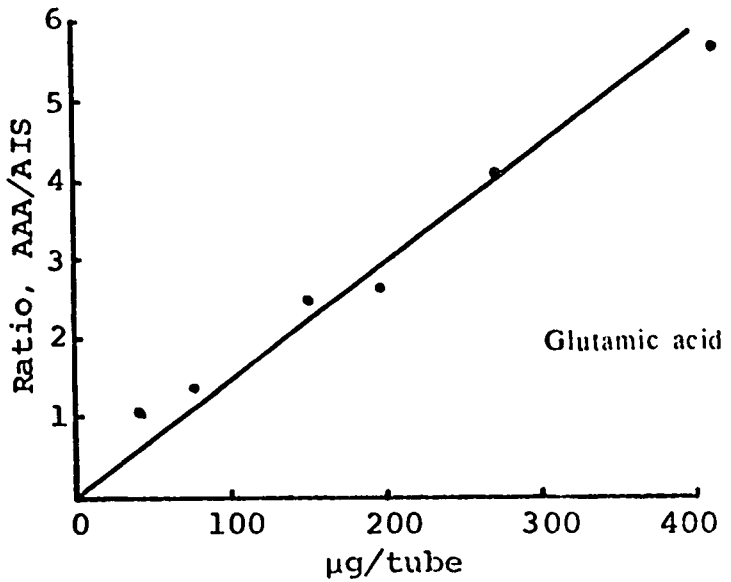
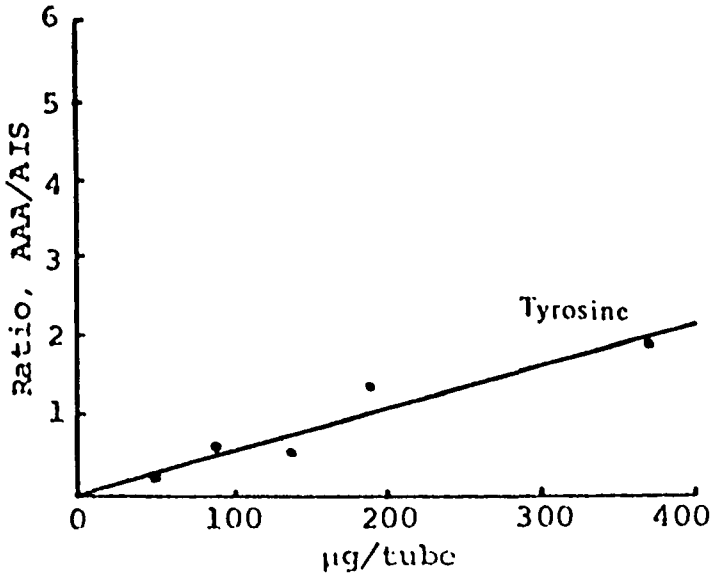


Figure 38. Standard calibration curves for cysteine, hydroxyproline, methionine, and aspartic acid.







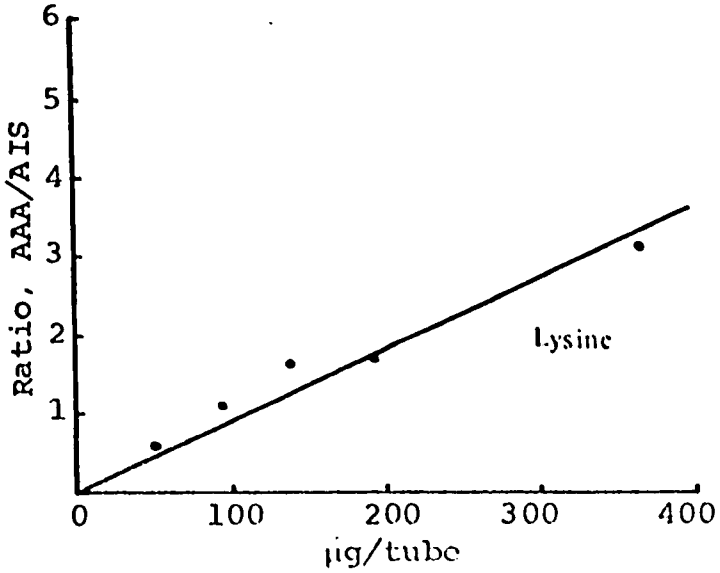


Figure 39. Standard calibration curves for phenylalanine, glutamic acid, tyrosine, and lysine.

added to the tubes which were then placed in the bottom of a glass desiccator (diameter=15 cm). Constant-boiling HCl containing mercaptoethanol was poured into the desiccator until the liquid level was even with that in the tubes. The desiccator was then sealed and clamped by means of two plywood flanges which were bolted together. The desiccator was alternately evacuated and flushed three times with nitrogen. After a final evacuation, the desiccator was heated in a forced-draft oven (110^{o}C) for 22 h. The tubes were opened and the hydrolysates were extracted with two portions (1 ml, each) of diethyl ether to remove fatty acids. The samples were then dried under vacuum in a desiccator containing sodium hydroxide flakes. This process took about 8 h.

Distilled water (1 ml) was added to each tube to dissolve the dried hydrolysates. The contents of each tube were then poured on separate columns (diameter = ½ in) which were filled to a depth of about ½ inch with Dowex 50 x 12 (H^{+} form). The tubes were rinsed four times (1 ml, each time) with distilled water to ensure that the contents were quantitatively transferred to the column. The columns were washed with five portions (1 ml, each) of distilled water to remove sugars and carboxylic acids, and the washings were discarded. A volumetric flask (10 ml) was placed under each column and the amino acids were eluted with five portions (1 ml, each)

of ammonium hydroxide (10% w/v). The column was then washed with portions (1 ml, each) of distilled water until the volume of the liquid collected was 10 ml.

iv) Amino acid determination

Aliquots (2.0 ml) from each volumetric flask were placed in separate culture tubes (Pyrex No. 9826) and were evaporated to dryness (100°C) with the aid of a stream of dry nitrogen. The samples were then subjected to exactly the same procedure that was described in (ii). The chromatographic peak areas were determined for the individual amino acids in each sample. The ratio of the peak area for each amino acid to that of n-butyl stearate in each chromatogram was determined and the number of micrograms of each amino acid present in that tube was determined by reference to the standard calibration curves (Figures 36-39). Since the weight of protein material present in the tube was known (20% of the weight of protein originally taken for hydrolysis; approximately 2 mg), the amino acid composition of each hydrolysate could be easily calculated.

v) Determination of tryptophan

Tryptophan was determined by the method of Shaw and McFarlane (254). The standard curve was prepared as follows: Aliquots (0-2.00 ml) of a standard tryptophan solution (0.075

mg/ml) were placed in separate test tubes. The volume in each tube was adjusted to 2.00 ml with distilled water and glyoxylic acid reagent* (0.5 ml) and copper sulfate solution (M/25; 0.5 ml) were added. Concentrated sulfuric acid (5.0 ml) was then added from a burette in portions of 0.5, 1.0, 1.5, and 2.0 ml and the tube was shaken (Vortex-Genie mixer) and placed in an ice bath after the addition of each portion of acid. The tube was allowed to cool in the ice bath (10 min) after the addition of the last portion and it was then placed in boiling water (5 min). The tubes were cooled to room temperature and the volume of the solution was adjusted to 10 ml with sulfuric acid solution (H_2SO_4:H_2O = 5:3 v/v). The solution was transferred to a clean dry colorimeter tube and after 15 min, the absorbance was read at 540 mμ. Figure 40 shows the standard calibration curve for tryptophan.

Tryptophan was determined in protein samples as follows: casein or other protein (25 mg) was dissolved in a solution of sodium hydroxide (10% w/v; 1 ml) and the volume of the resultant solution was adjusted to 5 ml with distilled water. An aliquot (1.0 ml) of this solution was placed in a test tube, the volume was adjusted to 2.0 ml with distilled

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*See Shaw and McFarlane (254).

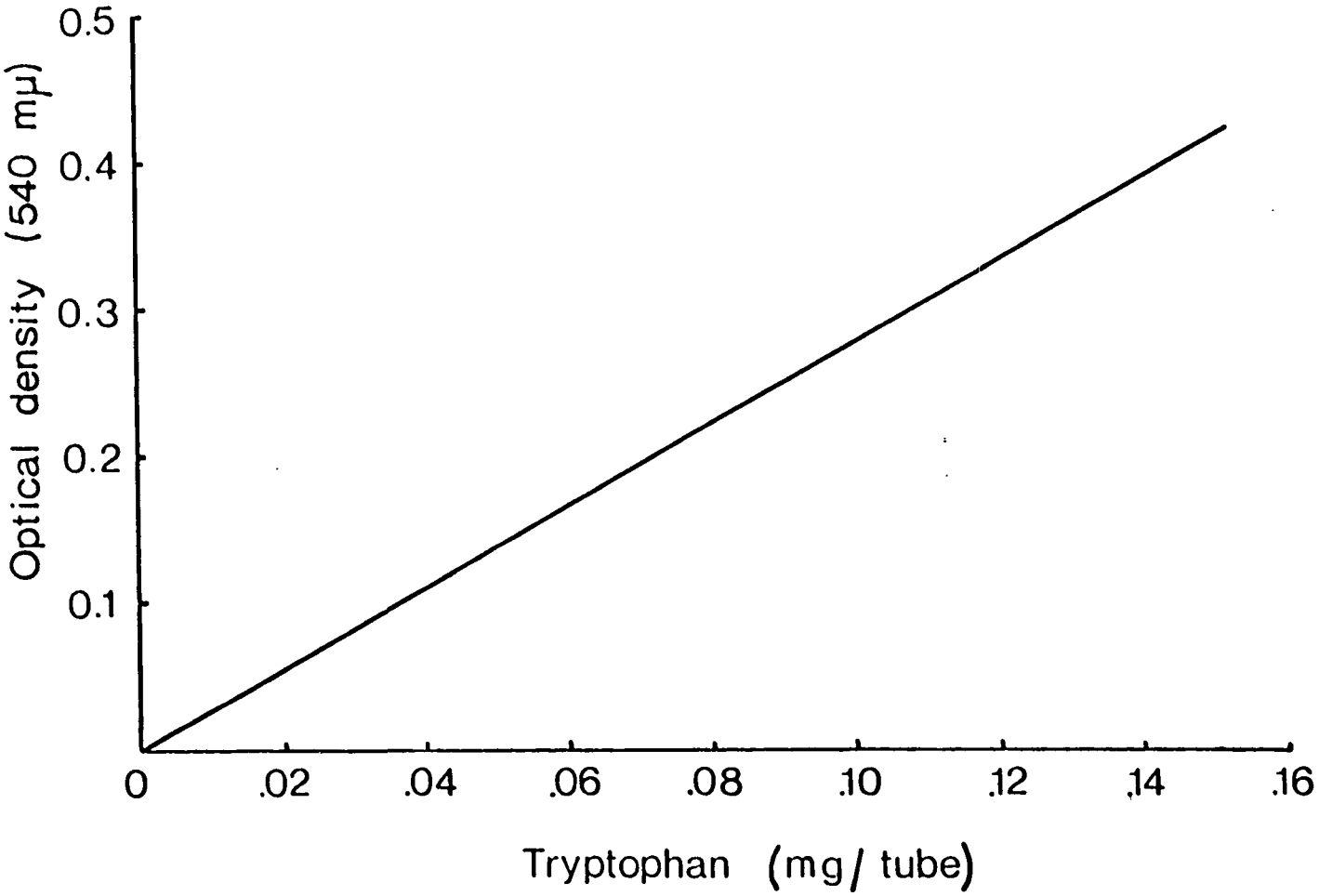

Figure 40. Standard calibration curve for tryptophan.

water and the same procedure as described for the preparation of the standard curve was followed. The amount of tryptophan in each sample was then ascertained by reference to the standard calibration curve.

(c) Results and Discussion

A linear relation was found between the amount of each amino acid injected and the corresponding peak areas obtained for that amino acid. In construction of the standard curves, the method of least squares was applied so that the curve would be the best possible fit for the data obtained. Differences in the slopes of the various lines reflect differences in the response of the hydrogen flame detector to the derivatives of each amino acid and/or differences in yields obtained in the derivatization procedure. For instance, the relatively low slope of the curves obtained for serine, cysteine, and tyrosine are probably due to a combination of low yields and low recorder response to the esters of these amino acids.

Tables 29, 30, 31, 32, 33, 34, and 35 show the results which were obtained in the analyses of ribonuclease, egg albumin (ovalbumin), gelatin, zein, α-casein, α-lactalbumin, and bovine serum albumin, respectively. The tables

also list the results obtained for these proteins by other workers. Reproductions of the chromatograms which were obtained for these proteins are shown in Figures 41, 42, 43, 44, 45, 46, and 47, respectively.

In most instances, there was fairly close agreement between the values which were obtained in this work and those which were obtained by other workers. The discrepancies might not be due only to the analytical methods, but could be due also to the fact that the samples were not the same protein preparations. The chromatograms of the standard proteins, in most instances, showed very few extraneous peaks, and the retention times of the peaks obtained in these separations corresponded to the peaks which were obtained with the standard amino acid mixtures.

The amino acid composition of the casein samples and some precipitates* are tabulated as follows: bovine casein, Table 36; horse and pig casein, Table 37; reindeer casein, Table 38; caribou casein, Table 39; moose casein, Table 40; harp seal and musk-ox casein, Table 41; polar bear casein (and precipitate), Table 42; dall sheep casein (and precipitate), Table 43; and fin whale casein (and precipitate), Table 44.

It is interesting to note that horse casein contained

*Precipitates obtained on initial centrifugation of certain milk samples.

relatively high levels of glycine, and harp seal and polar bear casein contained relatively low levels of glycine. Horse, pig, reindeer, caribou and harp seal caseins contained relatively high levels of glutamic acid. Harp seal, musk-ox, and polar bear caseins contained relatively low levels of lysine.

The results of the amino acid composition of the casein isolated from reindeer milk were in good agreement with those obtained by Luhtala, Rautiainen, and Antila (174) for reindeer milk "protein", except that the present author found over twice the amount of serine, 0.80 times the amount of lysine, and about 1.25 times the amount of glutamic acid as did the former authors.

The values obtained for the amino acid composition of harp seal casein differed widely from those obtained by Ashworth, Ramaiah, and Keyes (25) for Northern fur seal casein. The most striking differences were for the amino acids serine (7.91%; 2.4%, Ashworth *et al.*) and glutamic acid (25.26%; 16.9%, Ashworth *et al.*).

The amino acid composition of reindeer and caribou caseins were very similar, as were the electrophoretic patterns shown in Chapter IV. Since the electrophoretic properties of caseins at pH 8.6 are probably dependent in part, on

the concentrations of aspartic and glutamic acids, it is not surprising to note that these two caseins, which have roughly equal amounts of each of these two amino acids, are similar electrophoretically. One would also expect two animals of the same species (Rangifer tarandus) to produce milks that are similar in composition. There could be some correlation between the amino acid composition and electrophoretic properties of casein obtained from animals within the same species.

In general, the amino acid compositions of casein isolated from milks of different animals in the same species were similar. In some instances, there was a wide variability in the amount of hydroxy-containing amino acids in different samples of the same species. It is difficult to say whether this variability was due to actual variation within the species, or whether it was due to the derivatization procedure.

The fact that histidine, arginine, and cystine were not determined makes it difficult to determine the limiting amino acids by the chemical scoring technique of Block and Mitchell (43)(205). However, the limiting amino acids are likely to be cystine and methionine as they are in bovine casein.

Table 29

Amino acid composition of ribonuclease

Amino Acid	1 (g/100 g protein)	2[a] (g/100 g protein)	3[b] (g/100 g protein)
Alanine	7.61	7.37	6.12
Valine	7.38	6.95	6.33
Glycine	1.62	1.69	1.25
Isoleucine	2.07	1.64	2.30
Threonine	3.06	7.86	7.55
Leucine	2.16	2.02	1.74
Proline	2.97	2.98	3.32
Serine	13.96	9.74	9.45
Cysteine	ND[d]	-	ND[d]
Hydroxyproline	ND[d]	-	0
Methionine	3.37	2.63	3.52
Aspartic Acid	13.24	13.73	12.97
Phenylalanine	2.65	3.25	3.13
Glutamic Acid	12.02	12.06	10.88
Tyrosine	6.93	6.36	6.84
Lysine	13.06	10.21	9.20
Tryptophan	ND[c,d]	-	ND[d]
Arginine	-	4.93	4.43
Histidine	-	3.40	3.73
Cystine	-	6.23	5.95
Total	92.64	103.05	98.8

[a]Results of Gehrke, Roach, Zumwalt, Stalling, and Wall (9).

[b]Results of Hirs, Stein, and Moore (128).

[c]Determined by the method of Shaw and McFarlane (254).

[d]Not detected.

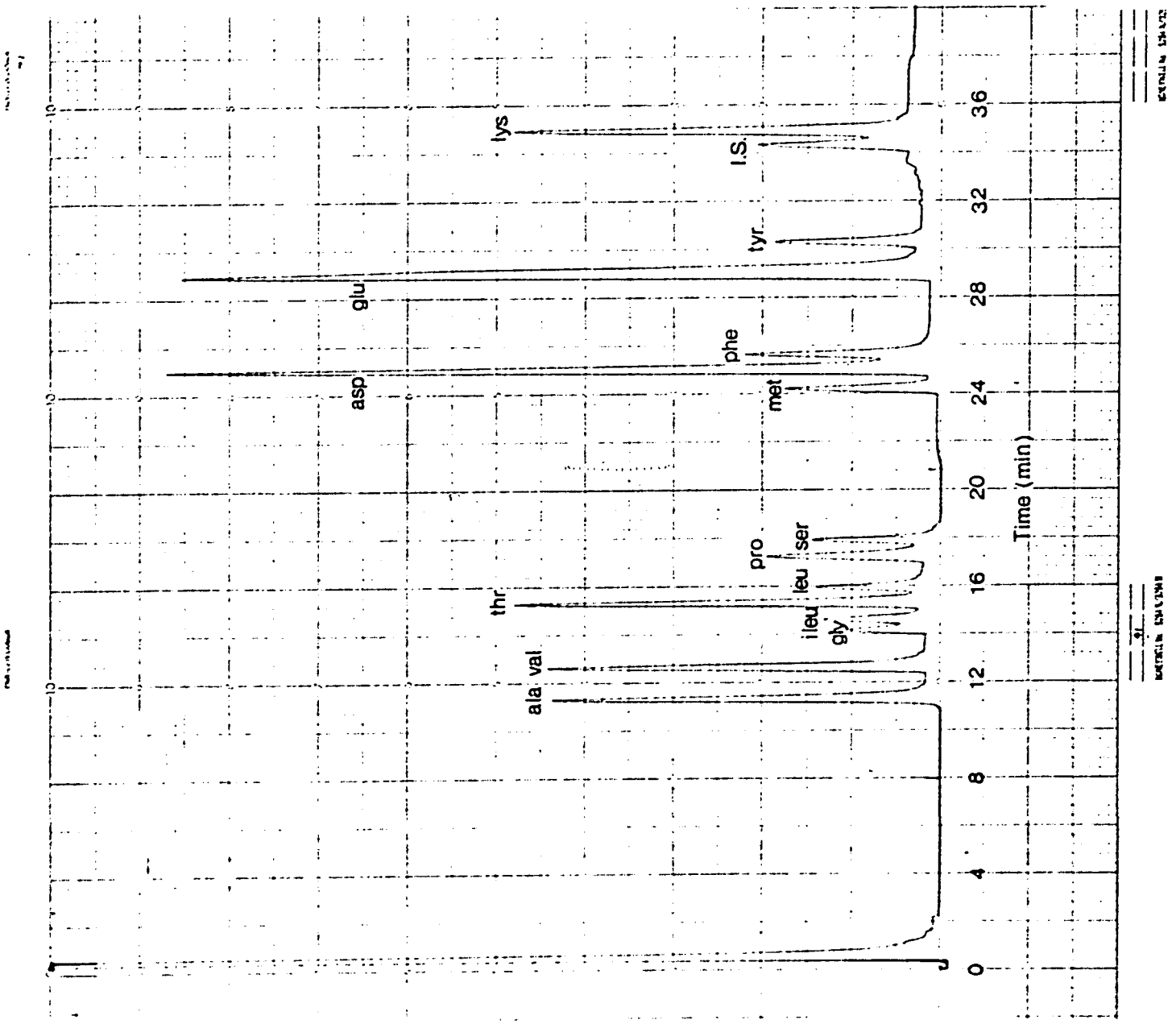

Figure 41. Gas chromatogram of N-TFA n-butyl amino acid esters of ribonuclease hydrolysate.

Table 30

Amino acid composition of egg albumin

Amino Acid	1 (g/100 g protein)	2[a] (g/100 g protein)	3[b] (g/100 g protein)
Alanine	5.95	6.32	5.36
Valine	6.96	7.33	5.96
Glycine	2.92	2.84	2.32
Isoleucine	5.35	5.61	6.04
Threonine	3.43	3.33	3.42
Leucine	8.48	8.30	7.94
Proline	2.92	2.86	3.04
Serine	5.75	6.23	6.75
Cysteine	ND[d]	-	1.15
Hydroxyproline	ND[d]	-	0
Methionine	3.73	4.51	1.57
Aspartic Acid	8.98	8.16	8.04
Phenylalanine	4.94	6.25	6.86
Glutamic Acid	13.63	14.41	14.48
Tyrosine	2.42	3.84	3.33
Lysine	6.06	5.48	5.52
Tryptophan	1.14[c]	-	1.09
Arginine	-	5.06	5.13
Histidine	-	2.10	2.08
Cystine	-	1.17	0.43
Total	82.96	93.74	93.5

[a]Results of Gehrke, Roach, Zumwalt, Stalling, and Wall (9).

[b]Results of Lewis, Snell, Hirschmann, and Frankel-Conrat (167).

[c]Determined by the method of Shaw and McFarlane (254).

[d]Not detected.

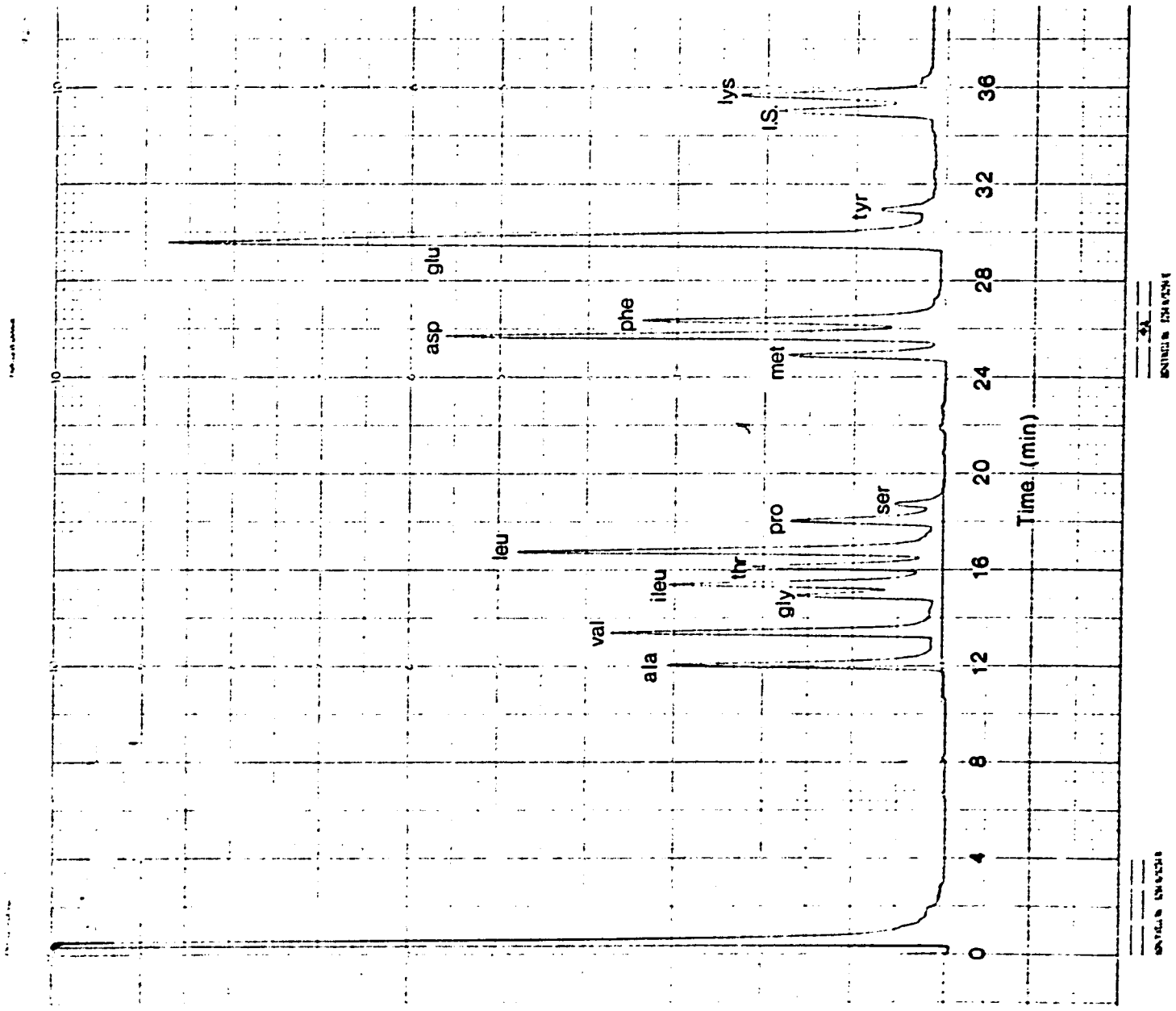

Figure 42. Gas chromatogram of N-TFA n-butyl amino acid esters of egg albumin hydrolysate.

Table 31

Amino acid composition of gelatin

Amino Acid	1 (g/100 g protein)	2[a] (g/100 g protein)	3[b] (g/100 g protein)
Alanine	10.30	8.81	8.54
Valine	2.87	2.05	2.34
Glycine	26.36	26.60	20.10
Isoleucine	2.27	1.08	1.17
Threonine	1.41	1.32	1.86
Leucine	3.33	2.57	2.88
Proline	14.74	13.85	13.66
Serine	4.44	2.86	3.42
Cysteine	ND[d]	-	0
Hydroxyproline	11.91	10.44	11.65
Methionine	0.20	T[e]	0.77
Aspartic Acid	5.35	5.33	5.79
Phenylalanine	1.31	2.03	2.28
Glutamic Acid	10.10	9.60	9.92
Tyrosine	0.40	0.42	0.54
Lysine	3.83	3.30	3.63
Tryptophan	ND[c,d]	-	0
Arginine	-	7.90	8.16
Histidine	-	0.79	0.89
Cystine	-	-	0
Total	98.82	98.95	98.5

[a]Results of Gehrke, Roach, Zumwalt, Stalling, and Wall (9).

[b]Results of Eastoe (76).

[c]Determined by the method of Shaw and McFarlane (254).

[d]Not detected.

[e]Trace

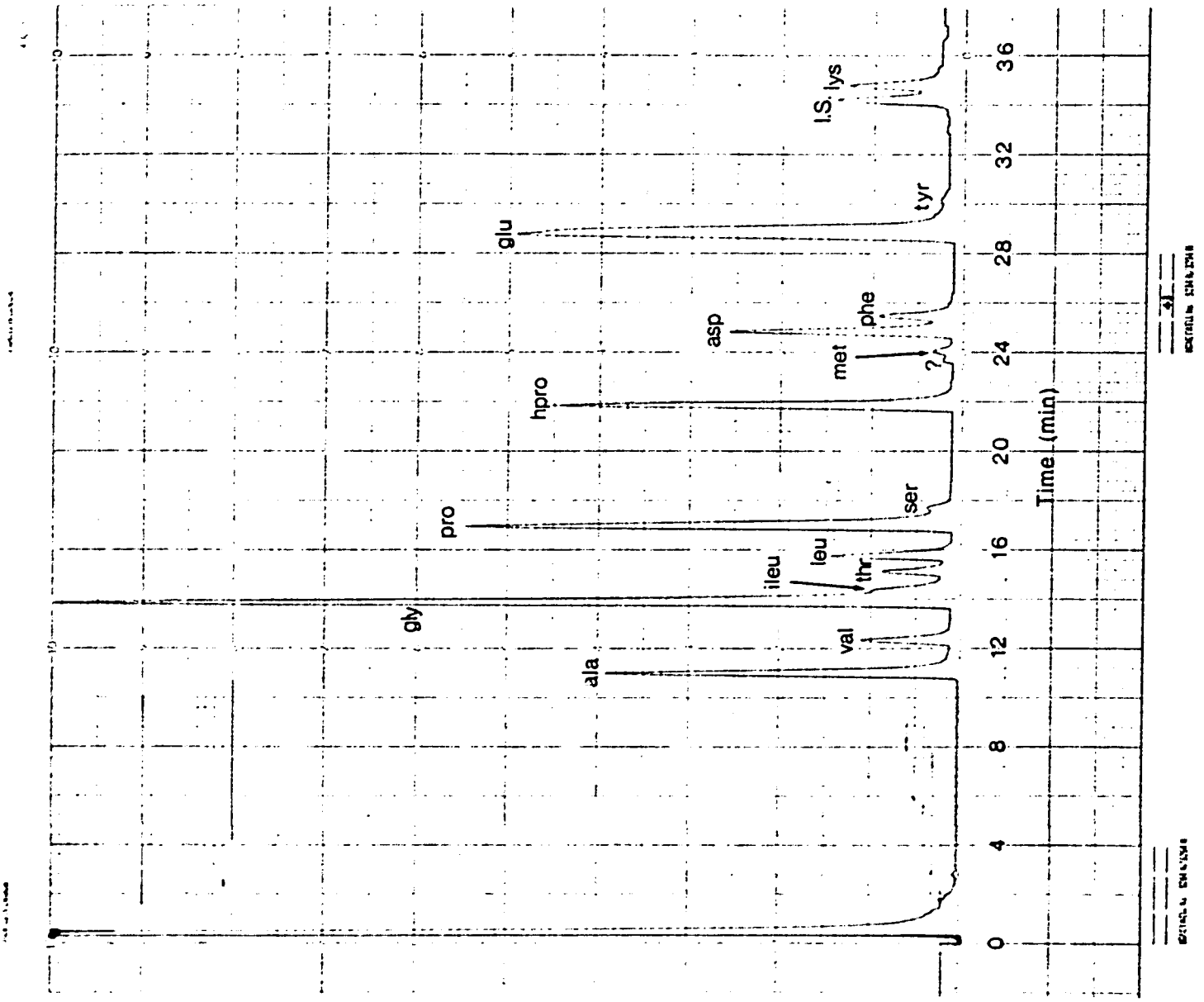

Figure 43. Gas chromatogram of N-TFA n-butyl amino acid esters of gelatin hydrolysate.

Table 32

Amino acid composition of zein

Amino Acid	1 (g/100 g protein)	2[a] (g/100 g protein)
Alanine	9.51	8.39
Valine	3.59	3.37
Glycine	0.92	ND
Isoleucine	3.88	4.31
Threonine	2.33	2.93
Leucine	20.43	18.21
Proline	10.38	8.88
Serine	5.82	5.84
Cysteine	ND[b]	0.70[c]
Hydroxyproline	ND[b]	0
Methionine	0.97	2.12
Aspartic Acid	5.33	3.98
Phenylalanine	6.40	6.50
Glutamic Acid	26.89	23.61
Tyrosine	3.88	4.72
Lysine	0.09	0
Tryptophan	-	0.14
Arginine	-	1.53
Histidine	-	1.17
Cystine	-	-
Total	100.42	96.4

[a]Results of Tristram (292).

[b]Not detected.

[c]Cysteine + cystine.

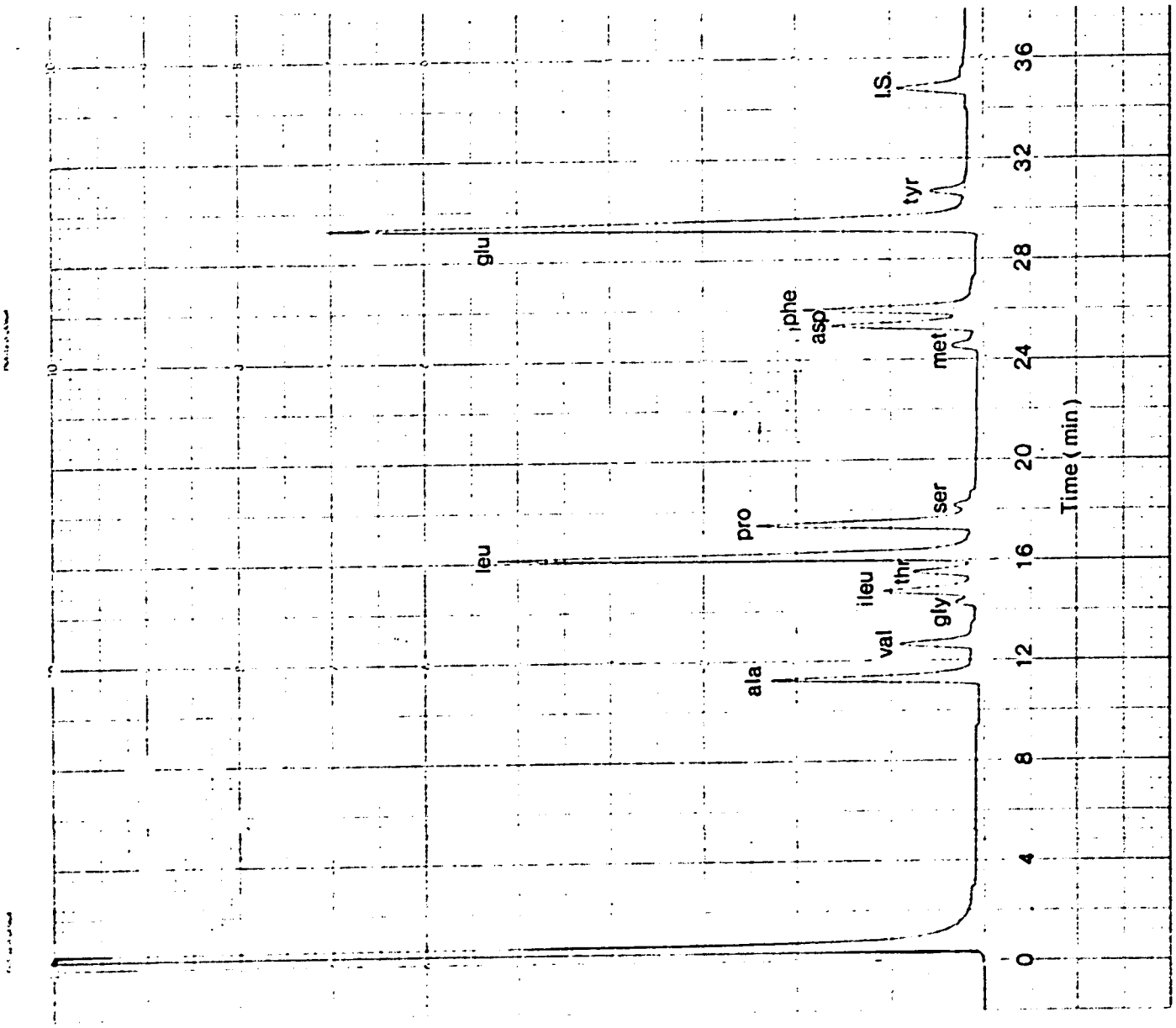

Figure 44. Gas chromatogram of N-TFA n-butyl amino acid esters of zein hydrolysate.

Table 33

Amino acid composition of α-casein

Amino acid	1 (g/100 g protein)	2[a] (g/100 g protein)
Alanine	3.91	2.78
Valine	6.18	4.82
Glycine	1.95	1.53
Isoleucine	5.82	4.96
Threonine	3.91	3.56
Leucine	9.74	6.89
Proline	9.17	6.95
Serine	6.70	5.30
Cysteine	ND[c]	ND[c]
Hydroxyproline	ND[c]	0
Methionine	2.26	2.35
Aspartic Acid	8.14	6.96
Phenylalanine	4.12	4.15
Glutamic Acid	22.26	18.43
Tyrosine	4.84	6.57
Lysine	6.90	8.16
Tryptophan	1.80[b]	1.82
Arginine	-	3.51
Histidine	-	2.58
Cystine	-	0.37
Total	97.90	91.7

[a]Results of Hipp, Basch, and Gordon (124).

[b]Determined by the method of Shaw and McParlane (254).

[c]Not detected.

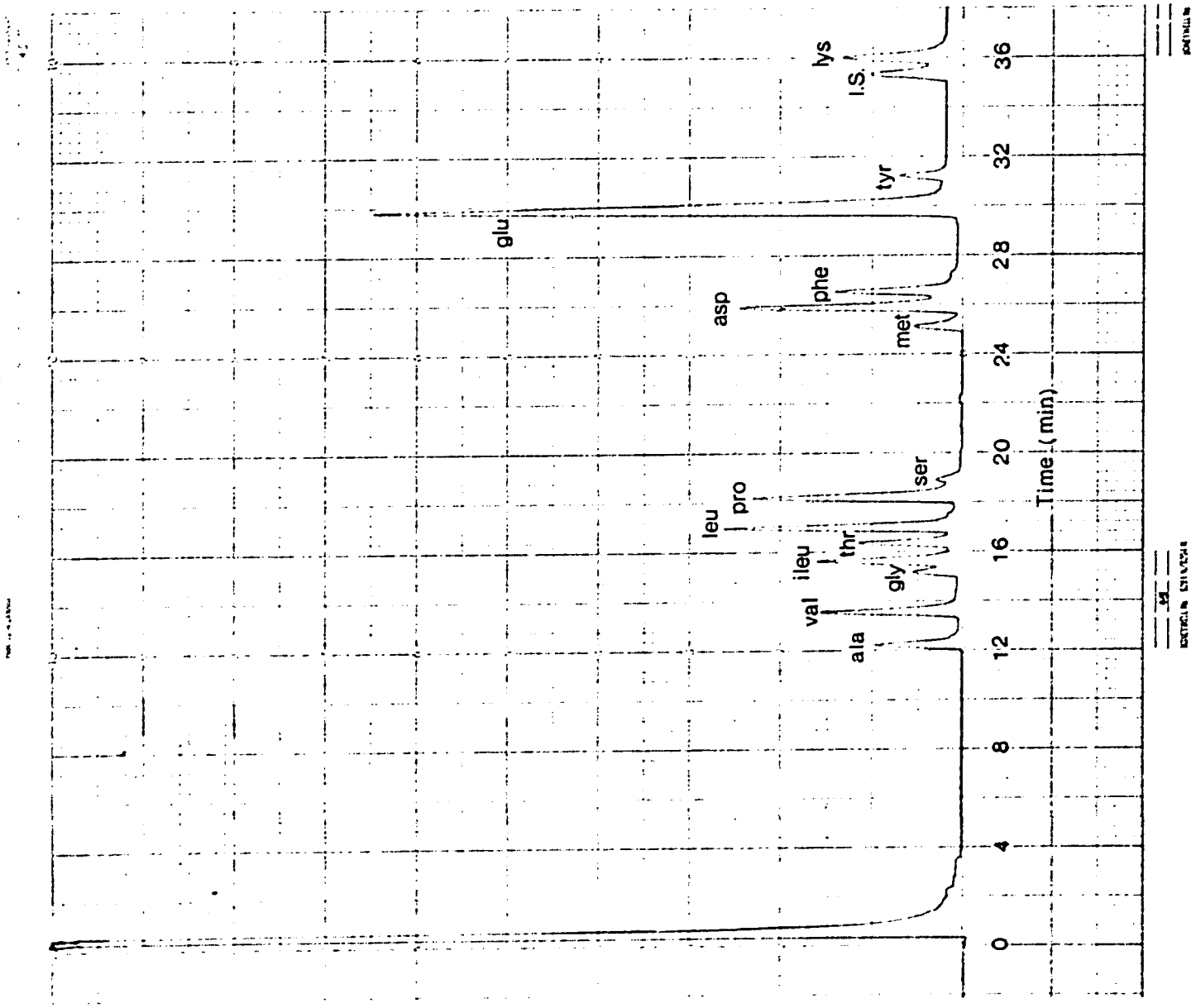

Figure 45. Gas chromatogram of N-TFA n-butyl amino acid esters of α-casein hydrolysate.

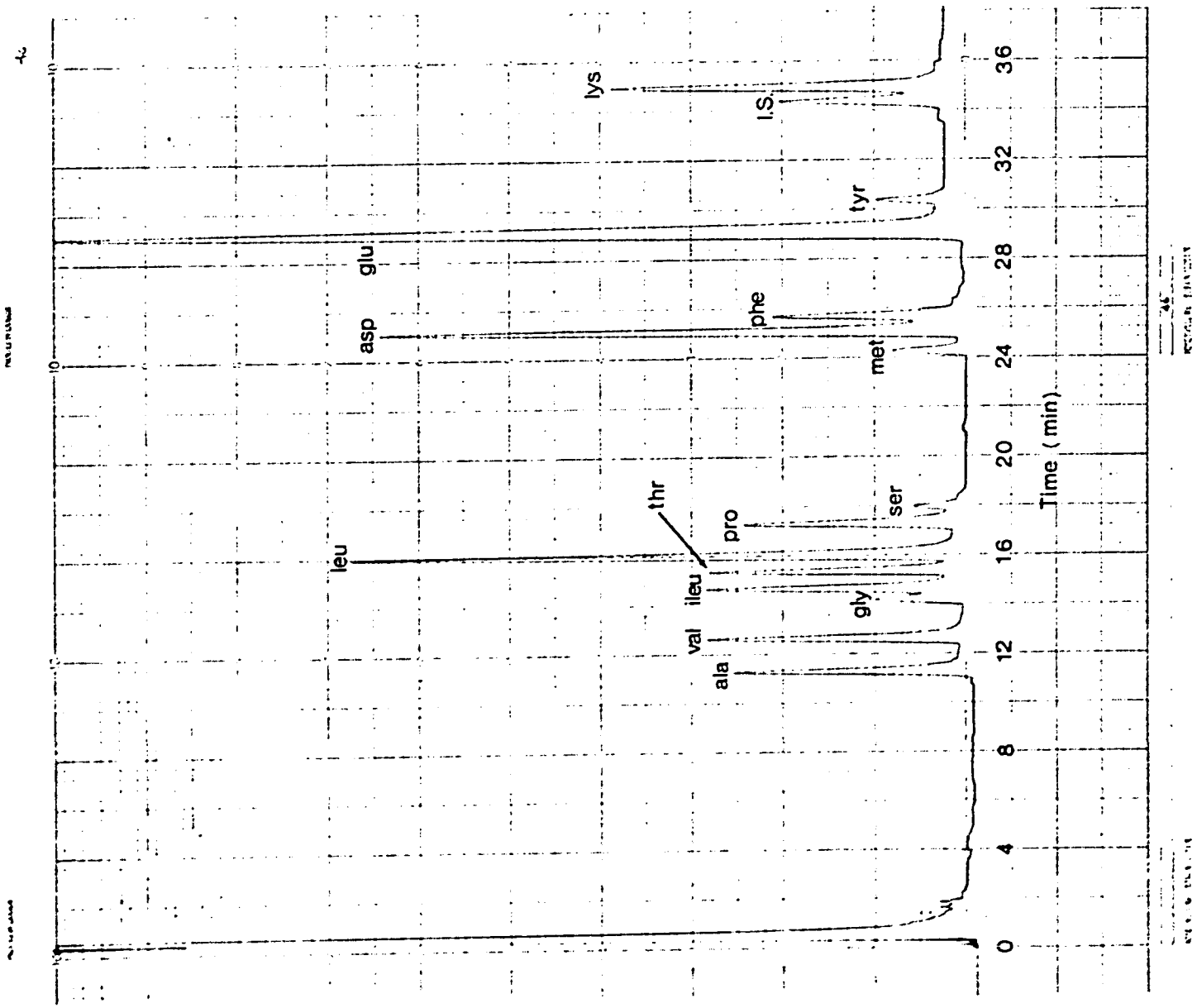

Figure 46. Gas chromatogram of N-TFA n-butyl amino acid esters of α-lactalbumin hydrolysate.

Table 35

Amino acid composition of bovine serum albumin

Amino Acid	1 (g/100 g protein)	2[a] (g/100 g protein)
Alanine	5.57	4.99
Valine	5.76	5.01
Glycine	1.49	1.49
Isoleucine	2.54	2.25
Threonine	5.19	4.95
Leucine	12.11	10.59
Proline	4.51	4.01
Serine	4.13	3.50
Cysteine	ND[c]	ND[c]
Hydroxyproline	ND[c]	0
Methionine	0.24	0.71
Aspartic Acid	10.19	9.43
Phenylalanine	5.09	5.87
Glutamic Acid	16.34	14.48
Tyrosine	3.26	4.56
Lysine	15.38	11.25
Tryptophan	0.44[b]	0.53
Arginine	-	5.29
Histidine	-	3.54
Cystine	-	5.02
Total	92.61	97.36

[a]Results of Stein and Moore (275).

[b]Determined by the method of Shaw and McFarlane (254).

[c]Not detected.

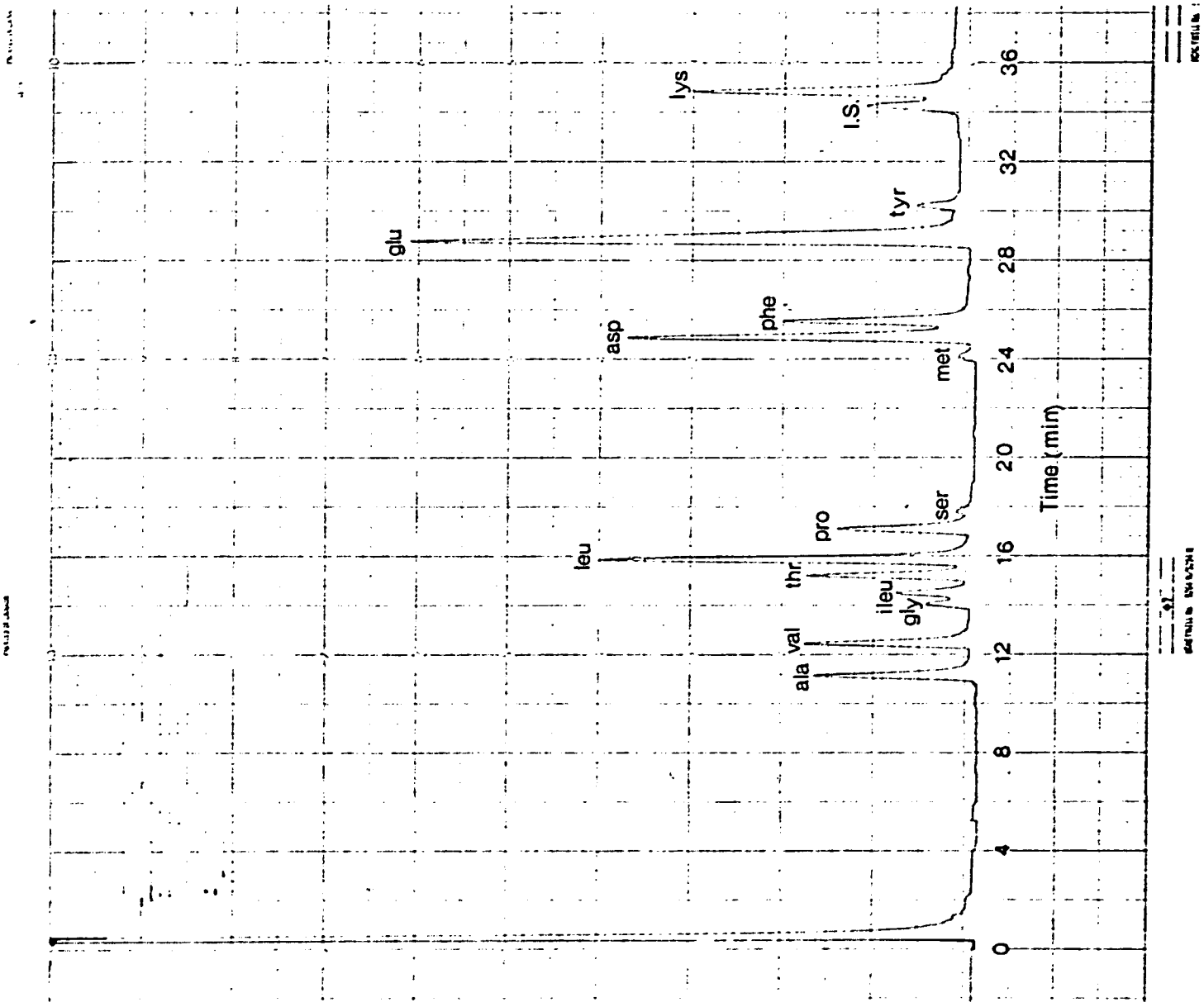

Figure 47. Gas chromatogram of N-TFA n-butyl amino acid esters of bovine serum albumin hydrolysate.

Table 36

Amino acid composition of bovine casein

Amino acid	Cow No. 1 (15.11% N) (g/100 g protein)	Cow No. 2 (13.16% N) (g/100 g protein)
Alanine	3.79	3.03
Valine	8.89	6.63
Glycine	2.52	1.80
Isoleucine	6.97	4.95
Threonine	5.20	3.60
Leucine	13.47	9.45
Proline	15.39	11.35
Serine	10.10	6.60
Cysteine	ND[b]	0.60
Hydroxyproline	ND[b]	0.35
Methionine	3.26	2.35
Aspartic Acid	8.82	6.92
Phenylalanine	5.94	4.05
Glutamic Acid	26.68	23.70
Tyrosine	4.42	3.65
Lysine	8.73	8.20
Tryptophan[a]	1.45	1.46

[a]Determined by the method of Shaw and McFarlane (254).

[b]Not detected.

Table 37

Amino acid composition of horse and pig caseins

Amino Acid	Horse Casein (15.86% N) (g/100 g protein)	Pig Casein (15.41% N) (g/100 g protein)
Alanine	3.40	3.61
Valine	6.90	6.58
Glycine	6.67	1.43
Isoleucine	5.38	5.89
Threonine	3.67	4.75
Leucine	11.00	10.47
Proline	12.24	12.62
Serine	8.81	8.56
Cysteine	0.10	0.25
Hydroxyproline	0.05	1.00
Methionine	1.61	2.42
Aspartic Acid	7.43	8.11
Pehnylalanine	4.28	5.04
Glutamic Acid	26.91	27.82
Tyrosine	4.38	5.59
Lysine	7.23	9.60
Tryptophan[a]	0.87	1.38

[a]Determined by the method of Shaw and McFarlane (254).

Table 38

Amino acid composition of reindeer caseins

Amino Acid	Reindeer No.1 (15.32% N) (g/100 g protein)	Reindeer No.2 (16.16% N) (g/100 g protein)	Reindeer No.3 (15.18% N) (g/100 g protein)
Alanine	2.71	2.43	2.89
Valine	6.22	5.93	6.14
Glycine	2.19	2.14	2.20
Isoleucine	4.81	4.20	4.54
Threonine	4.74	4.11	4.81
Leucine	10.37	9.72	10.14
Proline	9.43	11.51	11.42
Serine	11.79	11.16	13.53
Cysteine	ND[b]	ND[b]	ND[b]
Hydroxyproline	ND[b]	ND[b]	ND[b]
Methionine	2.83	2.71	2.71
Aspartic Acid	6.93	7.85	7.11
Phenylalanine	4.64	4.62	4.70
Glutamic Acid	26.51	25.74	25.84
Tyrosine	6.88	6.26	6.97
Lysine	7.88	8.80	8.07
Tryptophan[a]	1.37	1.47	1.61

[a]Determined by the method of Shaw and McFarlane (254).

[b]Not detected.

Table 39

Amino acid composition of caribou casein

Amino Acid	Caribou No. 2 (12.71% N) (g/100 g protein)	Caribou No. 3 (11.52% N) (g/100 g protein)
Alanine	2.83	2.58
Valine	5.59	5.41
Glycine	2.01	2.01
Isoleucine	4.10	4.38
Threonine	4.62	4.48
Leucine	9.29	9.28
Proline	9.40	9.97
Serine	12.01	10.93
Cysteine	ND[b]	ND[b]
Hydroxyproline	ND[b]	ND[b]
Methionine	2.17	2.16
Aspartic Acid	7.82	7.06
Phenylalanine	4.24	4.02
Glutamic Acid	25.43	24.28
Tyrosine	5.59	5.87
Lysine	9.07	9.28
Tryptophan[a]	1.52	1.48

[a]Determined by the method of Shaw and McFarlane (254).

[b]Not detected.

Table 40

Amino acid composition of moose casein

Amino Acid	Moose No.1 (14.61% N) (g/100 g protein)	Moose No.2 (15.32% N) (g/100 g protein)	Moose No.3 (15.60% N) (g/100 g protein)
Alanine	2.52	2.38	2.45
Valine	5.32	5.27	5.27
Glycine	1.77	1.77	1.69
Isoleucine	3.83	3.88	4.05
Threonine	3.45	3.73	3.98
Leucine	8.31	8.55	8.79
Proline	7.94	8.74	9.21
Serine	6.63	9.16	10.23
Cysteine	ND[b]	ND[b]	ND[b]
Hydroxyproline	ND[b]	ND[b]	ND[b]
Methionine	1.91	2.08	2.40
Aspartic Acid	6.07	7.38	7.36
Phenylalanine	3.50	4.18	4.07
Glutamic Acid	19.71	23.52	23.06
Tyrosine	4.57	6.30	6.52
Lysine	9.71	9.06	9.30
Tryptophan[a]	1.64	1.95	1.97

[a]Determined by the method of Shaw and McFarlane (254).

[b]Not detected.

Table 41

Amino acid composition of harp seal and musk-ox caseins

Amino Acid	Harp Seal Casein (16.00% N) (g/100 g protein)	Musk-ox Casein (13.74% N) (g/100 g protein)
Alanine	2.69	3.23
Valine	4.46	5.23
Glycine	0.63	1.52
Isoleucine	3.54	3.76
Threonine	2.72	3.62
Leucine	10.73	9.11
Proline	8.81	9.78
Serine	7.91	8.98
Cysteine	ND[b]	ND[b]
Hydroxyproline	ND[b]	0.13
Methionine	0.87	2.03
Aspartic Acid	9.71	8.03
Phenylalanine	3.47	3.93
Glutamic Acid	25.26	21.50
Tyrosine	5.87	7.08
Lysine	6.29	6.77
Tryptophan[a]	2.00	1.85

[a]Determined by the method of Shaw and McFarlane (254).

[b]Not detected.

Table 42

Amino acid composition of polar bear caseins and precipitate

Amino Acid	Polar Bear No.1 (11.55% N) (g/100 g protein)	Polar Bear No.3 (12.49% N) (g/100 g protein)	Polar Bear Precipitate[a] (13.28% N) (g/100 g protein)
Alanine	2.23	2.48	3.24
Valine	5.04	5.34	6.11
Glycine	0.72	0.55	1.20
Isoleucine	4.41	4.23	4.72
Threonine	3.75	3.63	3.61
Leucine	9.19	9.44	10.55
Proline	10.09	10.90	10.83
Serine	9.28	9.57	8.88
Cysteine	0.18	ND[c]	ND[c]
Hydroxyproline	ND[c]	ND[c]	ND[c]
Methionine	1.22	1.32	1.75
Aspartic Acid	8.55	8.20	9.72
Phenylalanine	3.89	3.85	4.44
Glutamic Acid	24.62	24.70	23.33
Tyrosine	4.59	5.13	3.79
Lysine	5.36	6.32	4.81
Tryptophan[b]	1.45	-	1.39

[a]Precipitate "D" of Baker, Hatcher, and Harington (29).

[b]Determined by the method of Shaw and McFarlane (254).

[c]Not detected.

Table 43

Amino acid composition of dall sheep casein and precipitate

Amino Acid	Dall Sheep Casein (10.95% N) (g/100 g protein)	Dall Sheep Precip. (10.14% N) (g/100 g protein)
Alanine	2.47	2.44
Valine	3.06	3.46
Glycine	1.08	1.02
Isoleucine	2.90	3.36
Threonine	1.76	1.93
Leucine	5.14	5.51
Proline	3.97	4.69
Serine	6.19	4.38
Cysteine	0.80	ND[b]
Hydroxyproline	0.06	ND[b]
Methionine	0.75	0.81
Aspartic Acid	6.19	5.40
Phenylalanine	2.04	2.14
Glutamic Acid	15.48	14.28
Tyrosine	5.34	3.46
Lysine	2.33	6.12
Tryptophan[a]	-	1.96

[a]Determined by the method of Shaw and McFarlane (254).

[b]Not detected.

Table 44

Amino acid composition of fin whale casein and precipitate

Amino Acid	Fin Whale No.1 (9.58% N) (g/100 g protein)	Fin Whale No.5 (15.95% N) (g/100 g protein)	Fin Whale Precipitate[a] (13.24% N) (g/100 g protein)
Alanine	2.50	3.00	2.68
Valine	3.38	4.17	4.53
Glycine	1.25	1.94	1.54
Isoleucine	2.80	3.30	4.22
Threonine	2.52	3.39	3.81
Leucine	5.69	6.60	8.24
Proline	3.79	4.95	7.21
Serine	6.48	6.31	7.21
Cysteine	ND[c]	ND[c]	ND[c]
Hydroxyproline	ND[c]	ND[c]	0.10
Methionine	1.08	1.11	1.75
Aspartic Acid	5.64	7.28	6.80
Phenylalanine	2.47	3.20	3.50
Glutamic Acid	13.89	16.31	22.42
Tyrosine	3.05	3.20	4.02
Lysine	5.23	7.57	8.76
Tryptophan[b]	1.73	-	1.46

[a]Data pertaining to milk sample collection is unavailable.

[b]Determined by the method of Shaw and McFarlane (254).

[c]Not detected.

SUMMARY

1. Casein was precipitated (acid-precipitation, pH 4.6) from the milks of the following Arctic, sub-Arctic, and domestic mammals: cow (Bos taurus), horse (Equus caballus), pig (Sus scrofa), Arctic wolf (Canis lupus arctos), barren-ground caribou (Rangifer tarandus groenlandicus), dall sheep (Ovis dalli dalli), fin whale (Balaenoptera physalus), harp seal (Pagophilus groenlandicus), moose (Alces alces), musk-ox (Ovibos moschatus), and reindeer (Rangifer tarandus); the whey solids were also isolated from these milks. The precipitates (P) obtained on initial centrifugation of certain samples of fin whale, dall sheep, and polar bear milk, and fractions obtained in the preparation of casein from Arctic wolf and fin whale milk were isolated.

2. The levels of hexose, hexosamine, and sialic acid were determined (colorimetric methods) in all of the samples of casein and precipitates (P). Caribou and fin whale caseins contained the highest concentrations of hexose (3.93%, 4.24%, respectively); caribou and polar bear caseins contained the highest concentrations of hexosamine (0.58%, 0.59%, respectively); and pig and polar

bear caseins contained the highest concentrations of sialic acid (2.26%, 1.17%, respectively). The levels of hexose, hexosamine, and sialic acid in the precipitates (P) were lower than those in the corresponding caseins.

3. The caseins were analyzed by polyacrylamide-disc electrophoresis (pH 8.6; gels 4 M in urea). The patterns obtained with reindeer casein were similar to those obtained with caribou casein. The electropherograms produced evidence for the existence of genetic polymorphism in the β- and α_{s_1}-caseins of reindeer, caribou, and moose milks. Electrophoretic analysis of the precipitates (P) did not indicate a large amount of α_{s_1}-casein to β-casein present, as was suggested by the high P/N ratios obtained with these precipitates.

4. The whey proteins were analyzed by polyacrylamide-disc electrophoresis (pH 8.6). The bovine serum albumin and α-lactalbumin components were unresolved. The patterns obtained for the whey proteins were: (a) extremely complex, (b) similar for animals within the same species, and (c) contained high concentrations of a component with a mobility similar to that of bovine

β-lactoglobulin.

5. The average recovery, based on gas-chromatographic analyses, which was obtained in the conversion of amino acids to their N-trifluoroacetyl n-butyl ester derivatives was about 70%.

6. A method was developed for the quantitative gas-chromatographic analysis of N-trifluoroacetyl n-butyl derivatives of amino acids in protein hydrolysates. In this method, the ratios of the peak areas (AAA) given by a suitable range of amounts of each amino acid, to the peak area (AIS) given by a set amount of internal standard (n-butyl stearate), were plotted against the amounts of that amino acid. The standard calibration curve which was obtained for each amino acid indicated a linear relationship between the ratio, AAA/AIS, and the amount of each given amino acid.

7. The amino acid composition of the caseins, the precipitates (P), and certain standard proteins (ribonuclease, gelatin, zein, α-casein, α-lactalbumin, bovine serum albumin, and ovalbumin) was determined by gas chromatography using the method described above

(No. 6).

8. A computer program (FORTRAN IV) was written for processing the data obtained in the determination of amino acids by gas-liquid chromatography. An IBM 360 computer was used to demonstrate the practical application of the program.

CLAIMS TO ORIGINAL RESEARCH

Note: Claims to original research based on the studies in the Appendix are presented on p. 369.

1. Preparation of casein from the milks of the following Arctic or sub-Arctic mammals: Arctic wolf (*Canis lupus arctos*), barren-ground caribou (*Rangifer tarandus groenlandicus*), dall sheep (*Ovis dalli dalli*), fin whale (*Balaenoptera physalus*), harp seal (*Pagophilus groenlandicus*), moose (*Alces alces*), and reindeer (*Rangifer tarandus*).

2. Determination of the hexose, hexosamine, and sialic acid contents of the caseins isolated from the milks designated in (1), as well as the determination of the hexose, hexosamine, and sialic acid contents of the caseins isolated from the milks of the following mammals: pig (*Sus scrofa*), musk-ox (*Ovibos moschatus*), polar bear (*Thalarctos maritimus*).

3. Determination of the phosphorus:nitrogen ratios in the caseins isolated from the milks of the following animals: caribou, fin whale, harp seal, moose, musk-ox, pig, polar bear, and musk-ox.

4. Electrophoretic (polyacrylamide-disc) analysis of the casein isolated from the milks of the following mammals: caribou, dall sheep, fin whale, harp seal, horse, moose, musk-ox, pig, polar bear, and reindeer. Provision of evidence which suggests the existence of genetic polymorphism in the α_{s_1}- and β-fractions of the caseins isolated from the milks of reindeer, moose, and caribou.

5. Electrophoretic (polyacrylamide-disc) analysis of the whey proteins isolated from the milks of the following mammals: caribou, dall sheep, fin whale, harp seal, horse, moose, musk-ox, pig, polar bear, Arctic wolf, and reindeer.

6. Development of a method for the quantitative gas chromatographic analysis of appropriate derivatives of amino acids in protein hydrolysates, whereby the ratios of the peak areas given by a suitable range of amounts of each amino acid to the peak area given by an appropriate set amount of a suitable internal standard, are plotted against the amounts of the amino acid so as to provide a standard curve for that amino acid. The ratio of the peak area for this amino acid to the peak area for the same set amount of internal standard on the chromatogram

for the protein hydrolysate is then converted into terms of the amount of the amino acid by reference to the standard curve.

7. Determination by gas-liquid chromatography of the amino acid composition of the caseins isolated from the milks of the following mammals: cow, horse, pig, dall sheep, fin whale, harp seal, caribou, moose, musk-ox, polar bear, and reindeer.

8. Development of a computer program (FORTRAN IV) for processing the data obtained in the determination of amino acids by gas-liquid chromatography.

REFERENCES

1. Akroyd, P. 1968. Separation of milk proteins. From: Acrylamide gel disc electrophoresis (Chapter X). Section 3. Chromatographic and Electrophoretic Techniques. Edited by I. Smith. Volume II. Zone electrophoresis. William Heinemann Medical Books Ltd., London.

2. Alais, C. 1956. Étude des substances azotées non-protéiques (NPN) séparées de la caséine du lait de vache sous l'action de la présure. XIVth Intern. Dairy Congr. Proc.(Rome), 2 (Part 2): 823. Cited from: Kalan, E.B., and Woychik, J.H. 1965. Action of rennin on K-casein, the amino acid compositions of the para-K-casein, and glycomacropeptide fractions. J. Dairy Sci., 48: 1423.

3. Alais, C., and Jollès, P. 1961. Étude comparée des caséinoglycopeptides formés par action de la présure sur les caséines de vache, de brebis et de chèvre. II. Étude de la partie non-peptidique. Biochim. Biophys. Acta, 51: 315.

4. Alais, C., and Jollès, P. 1962. Human casein and its caseino-glycopeptide. Nature, 196: 1098.

5. Alais, C., and Jollès, P. 1970. Comparative electrophoretical studies of human and rabbit caseins. Int. J. Biochem., 1: 546.

6. Altaner, C. 1962. Starch gel electrophoresis. Chemické Listy, 56: 334. (National Research Council of Canada: Technical Translation 1046; Ottawa, 1962).

7. Ambrosino, C., Liberatori, J., LaVecchia, L., Sarra, C., and Ubertalle, A. 1967. Milk serum proteins. VII. Comparative studies on the immuno-electrophoretic patterns of cow, buffalo, sheep, and goat wheys. Ric. Sci., 37: 873. Cited from: Chem. Abstr., 68: 85734u (1968).

8. American Dairy Science Association (A.D.S.A.). 1966. International Symposium: Milk handling and utilization in developing countries. 61st Annual Meeting. Cited from Grindrod, J., and Nick, T.A. 1967. Changes in milk proteins treated with hydrogen peroxide. J. Dairy Sci., 50: 142.

9. Analytical BioChemistry Laboratories. 1968. Quantitative Gas-Liquid Chromatography of Amino Acids in Proteins and Biological Substances. Macro, Semimicro, and Micro Methods. Edited by: C.W. Gehrke, D. Roach, R.W. Zumwalt, D.L. Stalling, and L.L. Wall. Columbia, Missouri (LCC #68-57507).

10. Anastassiadis, P.A., and Common, R.H. 1953. Studies on the glycoproteins of the domestic fowl. I. A modification of the method of Elson and Morgan for the determination of hexosamine, and its applicability to tissue hydrolysates. Can. J. Chem., 31: 1093.

11. Anastassiadis, P.A., and Common, R.H. 1955. The use of an ion-exchange resin for tissue hydrolysis in the determination of hexosamine. J. Sci. Food Agric., 6: 229.

12. Anastassiadis, P.A., and Common, R.H. 1958. Liberation of hexosamine, hexuronic acid, and hydroxyproline from tissues by resin hydrolysis. Can. J. Biochem. Physiol., 36: 413.

13. Aschaffenburg, R. 1961. Inherited casein variants in cow's milk. Nature, Lond., 192: 431.

14. Aschaffenburg, R. 1963. Inherited casein variants in cow's milk. II. Breed differences in the occurrence of β-casein variants. J. Dairy Res., 30: 251.

15. Aschaffenburg, R. 1963. Milk Protein Polymorphisms in 'Man and Cattle'. Edited by A.E. Mourant and F.E. Zeuner. Royal Anthropological Institute, London (pp. 50-54).

16. Aschaffenburg, R. 1964. Protein phenotyping by direct polyacryamide-gel electrophoresis of whole milk. Biochim. Biophys. Acta, 82: 188.

17. Aschaffenburg, R. 1965. Variants of milk proteins and their pattern of inheritance. J. Dairy Sci., 48: 128.

18. Aschaffenburg, R. 1966. Modified procedure of starch gel electrophoresis for β-casein phenotyping. J. Dairy Sci., 49: 1284.

19. Aschaffenburg, R. 1968. Genetic variants of milk proteins: their breed distribution. J. Dairy Res., 35: 447.

20. Aschaffenburg, R., and Drewry, J. 1955. Occurrence of different beta-lactoglobulins in cow's milk. Nature, 176: 218.

21. Aschaffenburg, R., and Drewry, J. 1957. Genetics of the β-lactoglobulins of cow's milk. Nature, 180: 376.

22. Aschaffenburg, R., Gregory, M.E., Kon, S.K., Rowland, S.J., and Thompson, S.Y. 1962. The composition of the milk of the reindeer. J. Dairy Res., 29: 324.

23. Aschaffenburg, R., Sen, A., and Thompson, M.P. 1968. Genetic variants of casein in Indian and African Zebu cattle. Comp. Biochem. Physiol., 25: 177.

24. Aschaffenburg, R., and Thymann, M. 1965. Simultaneous phenotyping procedure for the principal proteins of cow's milk. J. Dairy Sci., 48: 1524.

25. Ashworth, U.S., Ramaiah, G.D., and Keyes, M.C. 1966. Species difference in the composition of milk with special reference to the Northern fur seal. J. Dairy Sci., 49: 1206.

26. Association of Official Agricultural Chemists. 1965. Official Methods of Analysis. 10th Edition. Washington, D.C.

27. Baker, B.E., Bertok, E.I., and Symes, A.L. 1963. The protein and lipid constitution of guinea pig milk. Can. J. Zool., 41: 1041.

28. Baker, B.E., Blood, D.A., and Chen, E.C.H. 1967. Rocky Mountain bighorn sheep (Ovis canadensis canadensis) milk. II. Electrophoretic analyses of proteins; carbohydrate content of casein. Can. J. Zool., 45: 369.

29. Baker, B.E., Hatcher, V.B., and Harington, C.R. 1967. Polar bear milk. III. Gel-electrophoretic studies of protein fractions isolated from polar bear milk and human milk. Can. J. Zool., 45: 1205.

30. Baker, B.E., Huang, F.Y.Y., and Harington, C.R. 1963. The carbohydrate content of polar bear milk casein. Biochem. Biophys. Res. Comm., 13: 227.

31. Baker, B.E., and Khan, N.A. 1957. Quantitative determinations of amino-acids in undesalted hydrolysates by buffer filter-paper chromatography. J. Sci. Food Agr., 8: 217.

32. Barbiroli, G. 1964. Determination of amino acids by circular paper chromatography. Rass. Chim., 16: 79. Cited from: Chem. Abstr., 61: 8898c (1964).

33. Bayer, E. 1958. Separation of derivatives of amino acids using gas-liquid chromatography. From: Gas Chromatography, 1958. Edited by: D.H. Desty. Butterworths Scientific Publication, London (p. 333).

34. Bell, K. 1962. One-dimensional starch-gel electrophoresis of bovine skimmilk. Nature, 195: 705.

35. Bell, K., and McKenzie, H.A. 1964. β-Lactoglobulins. Nature, 204: 1275.

36. Ben Shaul, D.M. 1962. The composition of the milk of wild animals. Int. Zoo Yrbk., 4: 333.

37. Bentley, R., Sweeley, C.C., Makita, M., and Wells, W.W. 1963. Gas chromatography of sugar and other polyhydroxy compounds. Biochem. Biophys. Res. Comm., 11: 14.

38. Bhattacharya, S.D., Roychoudbury, A.K., Sinha, N.K., and Sen, A. 1963. Inherited α-lactalbumin and β-lactoglobulin polymorphism in Indian Zebu cattle. Comparison of Zebu and buffalo α-lactalbumins. Nature, 197: 797.

39. Bier, M., and Teitelbaum, P. 1959. Gas chromatography in amino-acid analysis. Ann. N.Y. Acad. Sci., 72: 641.

40. Blau, K. 1968. Analysis of amino acids by gas chromatography. From: Biomedical Applications of Gas Chromatography. Vol. 2. Edited by Herman A. Szymanski. Plenum Press, New York.

41. Blau, K., and Darbre, A. 1965. Gas chromatography of volatile amino acid derivatives. II. Leucine, cysteine, proline, hydroxyproline, methionine, phenylalanine, aspartic acid and glutamic acid. J. Chromatog., 17: 445.

42. Blau, K., and Darbre, A. 1967. Gas chromatography of volatile amino acid derivatives. III. Aspartic acid, lysine, ornithine, tryptophan and tyrosine. J. Chromatog., 26: 35.

43. Block, R.J., and Mitchell, H.H. 1946. The correlation of the amino-acid composition of proteins with their nutritive value. Nutr. Abstr. Rev., 16: 249.

44. Blumberg, B.S., and Tombs, M.P. 1958. Possible polymorphism of bovine α-lactalbumin. Nature, 181: 683.

45. Bolcato, P., Spettoli, P., and Cagliari, A. 1970. Composition of the bound lipids in caseins and in ripening cheeses. J. Dairy Res., 37: 431.

46. Bourne, E.J., Tatlow, C.E.M., and Tatlow, J.C. 1950. Studies of trifluoroacetic acid. Part III. Preparation and properties of some trifluoroacetyl esters. J. Chem. Soc., p. 1367.

47. Brew, K., and Campbell, P.N. 1967. The characterization of the whey proteins of guinea-pig milk. Biochem. J., 102: 258.

48. Brieskorn, C.H., and Berg, H.W. 1958. Suitability of the anthrone reagent for quantitative determination of protein-bound carbohydrates. Z. Lebensm.-Untersuch. u.-Forsch. 108: 170. Cited from: Chem. Abstr., 53: 491b (1958).

49. Brown, J.W., Aurand, L.W., and Roberts, W.M. 1961. The influence of different methods of heating on the electrophoretic patterns of whey proteins. Food Tech., 15: 480.

50. Brunner, J.R., Ernstrom, C.A., Hollis, R.A., Larson, B.L., Whitney, R.McL., and Zittle, C.A. 1960. Nomenclature of the proteins of bovine milk - first revision. J. Dairy Sci., 43: 901.

51. Carlström, G. 1968. A method for gas chromatographic determination of 11 amino acids in protein hydrolysate. Acta Vet. Scand., 9: 71.

52. Cayen, M.N., Henneberry, G.O., and Baker, B.E. 1962. Studies on casein. IV. The sialic acid content of casein. J. Dairy Sci., 45: 706.

53. Cerbulis, J. 1967. Distribution of lipids in various fractions of cow's milk. J. Agr. Food Chem., 15: 784.

54. Cerbulis, J., and Zittle, C.A. 1965. Lipids associated with acid-precipitated casein. J. Dairy Sci., 48: 1154.

55. Cessi, C., and Piliego, F. 1960. The determination of amino sugars in the presence of amino acids and glucose. Biochem. J., 77: 508.

56. Code of Federal Regulation. 1966. 21 CFR 19,500 E3: 165. Cited from: Grindrod, J., and Nick, T.A. 1967. Changes in milk proteins treated with hydrogen peroxide. J. Dairy Sci., 50: 142.

57. Cole, E.G., and Mecham, D.K. 1966. Cyanate formation and electrophoretic behavior of proteins in gels containing urea. Anal. Biochem., 14: 215.

58. Connaught Medical Research Laboratories. 1967. Starch-Gel Electrophoresis. Second Edition. Willowdale, Ontario.

59. Coulter, J.R., and Hann, C.S. 1968. A practical quantitative gas chromatographic analysis of amino acids using the n-propyl N-acetyl esters. J. Chromatog., 36: 42.

60. Cruickshank, P.A., and Sheehan, J.C. 1964. Gas chromatographic analysis of amino acids as N-trifluoroacetylamino acid methyl esters. Anal. Chem., 36: 1191.

61. Darbre, A., and Blau, K. 1965. Gas chromatography of volatile amino acid derivatives. I. Alanine, glycine, valine, leucine, isoleucine, serine and threonine. J. Chromatog., 17: 31.

62. Darbre, A., and Blau, K. 1965. Trifluoroacetylated amino acid esters: the stability of the derivatives of cysteine, hydroxyproline, serine, threonine and tyrosine. Biochim. Biophys. Acta, 100: 298.

63. Darbre, A., and Blau, K. 1966. Breakdown of trifluoroacetylated esters of cysteine and hydroxyl amino acids during gas chromatography: effects caused by some polar stationary phases. Biochim. Biophys. Acta, 126: 591.

64. Darbre, A., and Blau, K. 1967. Gas chromatography of volatile amino acid derivatives. IV. Mixed stationary phases for the separation of N-trifluoroacetylated amino acid n-amyl esters. J. Chromatog., 29: 49.

65. Darbre, A., and Islam, A. 1968. Gas-liquid chromatography of trifluoroacetylated amino acid methyl esters. Biochem. J., 106: 923.

66. Davies, W.L. 1936. The Chemistry of Milk. Chapman & Hall Ltd., London.

67. Davis, B.J. 1964. Disc electrophoresis-II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci., 121: 404.

68. Deutsch, H.F. 1947. A study of whey proteins from the milk of various animals. J. Biol. Chem., 169: 437.

69. Dewar, R.A. 1961. The flame ionization detector. A theoretical approach. J. Chromatog., 6: 312.

70. Doherty, D.G., Stein, W.H., and Bergmann, M. 1940. Aromatic sulfonic acids as reagents for amino acids. J. Biol. Chem., 135: 487.

71. Dovey, A., and Campbell, P.N. 1952. A comparison of the electrophoretic patterns of cow, goat and rabbit casein. Nature, 169: 1014.

72. Dreywood, R. 1946. Qualitative test for carbohydrate material. Ind. Eng. Chem., Anal. Edition, 18: 499.

73. Dubois, M., Gilles, K., Hamilton, J.K., Rebers, P.A., and Smith, J. 1951. A colorimetric method for the determination of sugars. Nature, 168: 167.

74. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, J. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem., 28: 350.

75. d'Yachenko, P.F. 1959. Research on milk proteins. Proceedings of the Federative Scientific Research Institute of the Dairy Industry. No. 19. Moscow.

76. Eastoe, J.E. 1955. The amino acid composition of mammalian collagen and gelatin. Biochem. J., 61: 589.

77. El-Negoumy, A.M. 1966. Rapid recovery, preservation, and phenotyping of milk proteins by a modified starch gel technique of superior resolving power. Anal. Biochem., 15: 437.

78. El-Negoumy, A.M. 1967. Polymorphism in Y-casein fractions from the milk of individual cows. Biochim. Biophys. Acta, 140: 503.

79. El-Negoumy, A.M. 1968. Starch gel electrophoresis of products of action of crystalline rennin on casein and its components. J. Dairy Sci., 51: 1013.

80. Elson, L.A., and Morgan, W.T.J. 1933. A colorimetric method for the determination of glucosamine and chondrosamine. Biochem. J., 27: 1824.

81. Evans, D.E. 1959. Milk composition of mammals whose milk is not normally used for human consumption. Dairy Sci. Abstr., 21: 277 (Review Article No. 80).

82. Exss, R.E., Hill, H.D., and Summer, G.K. 1969. Computer analysis of amino acid chromatograms. J. Chromatog., 42: 442.

83. Fairbairn, N.J. 1953. A modified anthrone reagent. Chem. and Ind., January 24 issue, p. 86.

84. Fales, H.M., and Pisano, J.J. 1964. The gas chromatography of amines, alkaloids, and amino acids. From: Biomedical Applications of Gas Chromatography. Edited by H.A. Szymanski. Plenum Press, New York.

85. Felszeghy, E., and Abraham, A. 1961. Paper chromatographic determination of amino acids. Studia Univ. Babes-Bolyai, Ser. Chem. 1: 119. Cited from: Chem. Abstr., 62: 30c (1965).

86. Foster, J.F., Friedell, R.W., Catron, D., and Dieckmann, M.R. 1951. Electrophoretic studies on swine. II. Composition of baby pig plasma and sow's whey during lactation. Arch. Biochem. Biophys., 31: 104.

87. Fox, K.K. 1958. Separation of a calcium-soluble fraction of casein from isoelectric casein. J. Dairy Sci., 41: 715.

88. Fox, S.F., and Foster, J.F. 1957. Introduction to Protein Chemistry. John Wiley & Sons, Inc., New York.

89. Ganguli, N.C., and Bhalerao, V.R. 1964. Comparative study of the caseins of buffalo and cow milks by paper-disk electrophoresis. Milchwissenschaft, 19: 535.

90. Ganguli, N.C., and Majumder, G.C. 1967. A simple petri dish device for resolving milk proteins by starch gel electrophoresis. Ind. J. Biochem., 4: 232.

91. Ganguli, N.C., Prabhakaran, R.J.V., and Iya, K.K. Compositions of the caseins of buffalo and cow milk. J. Dairy Sci., 47: 13.

92. Gehrke, C.W., Freeark, C.W., Oh, Y.H., and Chun, P.W. 1964. Isolation of electrophoretically pure β-caseins. Anal. Biochem., 9: 423.

93. Gehrke, C.W., Lamkin, Wm.M., Stalling, D.L., and Shahrokhi, F. 1965. Quantitative Gas Chromatography of Amino Acids. Biochem. Biophys. Res. Comm., 19: 328.

94. Gehrke, C.W., and Leimer, K. 1970. The effect of salts on the derivatization and chromatography of amino acids. J. Chromatog., 53: 195.

95. Gehrke, C.W., Nakamoto, H., and Zumwalt, R.W. 1969. Gas-liquid chromatography of protein amino acid trimethylsilyl derivatives. J. Chromatog., 45: 24.

96. Gehrke, C.W., and Shahrokhi, F. 1966. Chromatographic separation of n-butyl N-trifluoroacetyl esters of amino acids. Anal. Biochem., 15: 97.

97. Gehrke, C.W., and Stalling, D.L. 1967. Quantitative analysis of the twenty national protein amino acids by gas-liquid Chromatography. Separation Sci., 2: 101.

98. Gehrke, C.W., and Zumwalt, R.W. Sept. 14-18, 1970. Quantitative amino acid analysis by gas-liquid chromatography. 160th A.C.S. National Meeting, Chicago, Illinois. Division of Agricultural and Food Chemistry, Symposium on Characterization of Proteins. (Abstr. #36).

99. Gehrke, C.W., Zumwalt, R.W., Aue, W.A., Stalling, D.L., and Rash, J.J. 1971. A search for organics in hydrolysates of lunar fines. J. Chromatog., 54: 169.

100. Gehrke, C.W., Zumwalt, R.W., and Wall, L.L. 1968. Gas-liquid chromatography of protein amino acids. Separation factors. J. Chromatog., 37: 398.

101. Glass, R.L. 1957. Chemical, physical and biological studies of rats' milk and its components. Diss. Abstr., 17: 494.

102. Glass, R.L., and Jenness, R. Comparative biochemical studies of milk.- VI. Constituent fatty acids of milk fats of additional species. Comp. Biochem. Physiol. (In Press).

103. Glass, R.L., Troolin, H.A., and Jenness, R. 1967. Comparative biochemical studies of milks.- IV. Constituent fatty acids of milk fats. Comp. Biochem. Physiol., 22: 415.

104. Gordon, W.G., Semmett, W.F., Cable, R.S., and Morris, M. 1949. Amino acid composition of α-casein and β-casein. J. Am. Chem. Soc., 71: 3293.

105. Gordon, W.G., and Whittier, E.O. 1965. Proteins of milk. From: Fundamentals of Dairy Chemistry. Edited by: B.H. Webb and A.H. Johnson. AVI Publishing Company, Inc.; Westport, Connecticut.

106. Gordon, W.G., and Ziegler, J. 1955. Amino acid composition of crystalline α-lactalbumin. Arch. Biochem. Biophys., 57: 80.

107. Gottschalk, A. 1966. Glycoproteins. Elsevier Publishing Co., Amsterdam.

108. Graff, J., Wein, J.P., and Winitz, M. 1963. Quantitative determination of α-acids by gas-liquid chromatography. Fed. Proc., 22: 499 (Abstr. # 500).

109. Grindrod, J., and Nick, T.A. 1967. Changes in milk proteins treated with hydrogen peroxide. J. Dairy Sci., 50: 142.

110. Grosclaude, F., Pujolle, J., Garnier, J., and Ribadeau-Dumas, B. 1966. Mise en évidence de deux variants supplémentaires des protéines du lait de vache: α_{s_1}-Cn^D et Lg^D. Ann. Biol. Anim. Biochem. Biophys., 6: 215.

111. Groves, M.L. 1969. Some minor components of casein and other phosphoproteins in milk. A review. J. Dairy Sci., 52: 1155.

112. Groves, M., Gordon, W.G., and Kiddy, C.A. 1968. Polymorphism of electrophoretically slow-moving caseins and their relationship to the Y-casein and β-casein variants. J. Dairy Sci., 51: 946.

113. Groves, M.L., and Kiddy, C.A. 1968. Polymorphism of Y-casein in cow's milk. Arch. Biochem. Biophys., 126: 188.

114. Groves, M.L., McMeekin, T.L., Hipp, N.J., and Gordon, W.G. 1962. Preparation of β- and Y-caseins by column chromatography. Biochim. Biophys. Acta, 57: 197.

115. Gupta, S.K., and Ganguli, N.C. 1965. Sialic acid content of casein preparations from cow and buffalo milks. Milchioissenschaft, 20: 10.

116. Hagen, P.B., and Black, W. 1965. Gas chromatographic method for the separation and estimation of amino acid derivatives. Can. J. Biochem., 43: 309.

117. Hartman, G.H. Jr., and Swanson, A.M. 1965. Changes in mixtures of whey protein and K-casein due to heat treatments. J. Dairy Sci., 48: 1161.

118. Haurowitz, F. 1963. The Chemistry and Function of Proteins. Second Edition. Academic Press, New York.

119. Heathcote, J.G., and Haworth, C. 1969. The direct determination of amino acids on thin-layer chromatograms by densitometry. Biochem. J., 114: 667.

120. Henneberry, G.O., and Baker, B.E. 1961. Interference by chloride ion in the determination of tryptophan by Shaw and McFarlane's method. Analyst, 86: 416.

121. Hill, R.D. 1963. The preparation of K-casein. J. Dairy Res., 30: 101.

122. Hill, R.L. 1965. Hydrolysis of proteins. Adv. Prot. Chem., 20: 37.

123. Hilpert, H., and Enkelmann, D. 1963. Paper electrophoretic characterization of whey proteins originating from various species of animals. Milchwissenschaft, 18: 26. Cited from: Chem. Abstr., 59: 13182h (1964).

124. Hipp, N.J., Basch, J.J., and Gordon, W.G. 1961. Amino acid composition of α_1-, α_2-, and α_3-caseins. Arch. Biochem. Biophys., 94: 35.

125. Hipp, N.J., Groves, M.L., Custer, J.H., and McMeeking, T.L. 1950. Separation of Y-casein. J. Am. Chem. Soc., 72: 4928.

126. Hipp, N.J., Groves, M.L., Custer, J.H., and McMeekin, T.L. 1952. Separation of α-, β-, and Y-casein. J. Dairy Sci., 35: 272.

127. Hipp, N.J., Groves, M.L., and McMeekin, T.L. Sept., 1959. Separation of the components of α-casein. The preparation of α_3-casein. Abstr. No. 12. 136th Meeting Am. Chem. Soc.

128. Hirs, C.H.W., Stein, W.H., and Moore, S. 1952. The amino acid composition of ribonuclease. J. Biol. Chem., 211: 941.

129. Hoeller, H. 1962. Composition of amino acids in goat milk and its casein. Milchwissenschaft, 17: 485. Cited from: Chem. Abstr., 59: 15855g (1963).

130. Hofman, T. 1958. Inhomogeneity of α-casein from goat milk. Nature, 181: 633.

131. Huang, F.Y.Y., and Baker, B.E. 1964. Casein. VI. Determination of sialic acid in casein. J. Sci. Food Agr., 15: 312.

132. Hunter, I.R., Dimick, K.P., and Corse, J.W. 1956. Determination of amino acids by ninhydrin oxidation and gas chromatography. Chem. and Ind., 294: (1956).

133. Islam, A., and Darbre, A. 1969. Gas-liquid chromatography of trifluoroacetylated amino acid methyl esters. Development of a mixed stationary phase for their separation. J. Chromatog., 43: 11.

134. Jenness, R., Erickson, A.W., and Graighead, J.J. 1971. Some comparative aspects of milks of the Ursidae. J. Mammal. (In Press).

135. Jenness, R., and Koops, J. 1962. Preparation and properties of a salt solution which simulates milk ultrafiltrate. Netherlands Milk Dairy J., 16: 153.

136. Jenness, R., Larson, B.L., McMeekin, T.L., Swanson, A.M., Whitnah, C.H., and Whitney, R.McL. 1956. Nomenclature of the proteins of bovine milk. J. Dairy Sci., 39: 536.

137. Jenness, R., and Patton, S. 1959. Principles of Dairy Chemistry. John Wiley & Sons, Inc., New York.

138. Jenness, R., Regehr, E.A., and Sloan, R.E. 1964. Comparative biochemical studies of milks-II. Dialyzable carbohydrates. Comp. Biochem. Physiol., 13: 339.

139. Jenness, R., and Sloan, R.E. 1970. The composition of milks of various species: a review. Dairy Sci. Abstr., 32: 599 (Review Article No. 158).

140. Jollès, P., Alais, C., and Jollès, J. 1962. Amino acid composition of K-casein and terminal amino acids of K- and para-K-casein. Arch. Biochem. Biophys., 98: 56.

141. Johansson, B., and Svennerholm, L. 1956. The content of carbohydrates in caseins from different species. Acta Physiol. Scand., 37: 324.

142. Johnson, D.E., Scott, S.J., and Meister, A. 1961. Gas-liquid chromatography of amino acid derivatives. Anal. Chem., 33: 669.

143. Jordan, J., and Loehr, H. 1962. The occurrence of neuraminic acid in human and cow milk and in dairy products. Milchwissenschaft, 17: 61.

144. Kalan, E.B., and Woychik, J.H. 1965. Action of rennin on K-casein, the amino acid compositions of the para-K-casein, and glycomacropeptide fractions. J. Dairy Sci., 48: 496.

145. Kanomata, K., and Mashiko, Y. 1966. Pyrolysis gas chromatography of amino acids and proteins. Nippon Kagaku Zasshi, 87: 57. Cited from Chem. Abstr., 65: 12542g (1966).

146. Keutmann, H.T., and Potts, J.T., Jr. 1969. Improved recovery of methionine after acid hydrolysis using mercaptoethanol. Anal. Biochem., 29: 175.

147. Khan, N.A., Baker, B.E., and Van Horn, W.F. 1955. Paper chromatography in routine determination of glutamic acid in production. Agr. Food Chem., 3: 853.

148. Kiddy, C.A. 1964. Inherited differences in specific blood and milk proteins in cattle. J. Dairy Sci., 47: 510.

149. Kiddy, C.A., Johnston, J.O., and Thompson, M.P. 1964. Genetic polymorphism in caseins of cow's milk. I. Genetic control of α_s-casein variation. J. Dairy Sci., 47: 147.

150. Kiddy, C.A., Townend, R.E., Thatcher, W.W., and Timasheff, S.N. 1965. β-Lactoglobulin variation in milk from individual cows. J. Dairy Res., 32: 209.

151. Kim, Y.K., Yaguchi, M., and Rose, D. 1969. Isolation and amino acid composition of para-kappa-casein. J. Dairy Sci., 52: 316.

152. Kon, S.K., and Cowie, A.T. 1961. Milk: the Mammary Gland and Its Secretion. Vols. I and II. Academic Press, London.

153. Kraeling, R.R., and Gerrits, R.J. 1969. Polymorphism of a protein of sows' whey. J. Dairy Sci., 52: 2036.

154. Kugenev, P.V., and Medvedeva, M.N. 1960. Comparative level of amino acids in the casein of milk (produced) by some species of animals. Vopr. Pitaniya, 19: 43. Cited from: Chem. Abstr., 57: 9-049f (1962).

155. Lachovitzki, N., and Björklund, B. 1970. Determination of amino acids by gas chromatography of trifluoroacetylated n-butyl ester derivatives. Anal. Biochem., 38: 446.

156. Lamkin, Wm.M., and Gehrke, C.W. 1965. Quantitative gas chromatography of amino acids. Separation of n-butyl N-trifluoroacetyl esters. Anal. Chem., 37: 383.

157. Larson, B.L., and Hagerman, E.C. 1965. Apparent immunological similarity of bovine β-lactoglobulins A, B, and C. J. Dairy Sci., 48: 1111.

158. Larson, B.L., and Jenness, R. 1955. Identification of α-lactalbumin in the electrophoretic pattern of milk serum proteins. J. Dairy Sci., 38: 313.

159. Lauer, B.H. 1968. Comparative Studies on the Compositions of Milks of Different Species. M.Sc. Thesis. McGill University.

160. Lauer, B.H., and Baker, B.E. 1969. Mineral constituents of the milks of some Arctic species. Can. J. Zool., 47: 185.

161. Lauer, B.H., and Baker, B.E. 1969. Whale milk. I. Fin whale (Balaenoptera physalus) and beluga whale (Delphinapterus lencas) milk: gross composition and fatty acid constitution. Can. J. Zool., 47: 95.

162. Lauer, B.H., Blood, D.A., Pearson, A.M., and Baker, B.E. 1969. Goat milk. I. Mountain goat (Oreamnos americanus) milk. Gross composition and fatty acid constitution. Can. J. Zool., 47: 5.

163. Lauer, B.H., Kuyt, E., and Baker, B.E. 1969. Wolf milk. I. Arctic wolf (Canis lupus arctos) and husky milk: gross composition and fatty acid constitution. Can. J. Zool., 47: 99.

164. Lee, M., Mehta, R., and Lucia, S.P. 1962. Amino acid composition and nutritive value of goat milk casein. Proc. Soc. Expt. Biol. Med., 110: 115.

165. Lee, Y.C., and Montgomery, R. 1961. Determination of hexosamines. Arch. Biochem. Biophys., 93: 292.

166. Lemon, M., and Poole, W.E. 1969. Specific proteins in the whey from milk of the grey kangaroo. Aust. J. Exp. Biol. Med. Sci., 47: 283.

167. Lewis, J.C., Snell, N.S., Hirschmann, D.J., and Fraenkel-Conrat, H. 1950. Amino acid composition of egg proteins. J. Biol. Chem., 186: 23.

168. Libbey, L.M., and Ashworth, U.S. 1957. Paper electrophoresis of casein. Proc. 38th Ann. Meeting Western Div. Am. Dairy Sci. Assoc.

169. Liberti, A. 1958. Gas Chromatography, 1958. Edited by: D.H. Desty. Academic Press, New York (p.341).

170. Lindner, R.C., and Harley, C.P. 1942. A rapid method for the determination of nitrogen in plant tissue. Science, 96: 565.

171. Lindqvist, B. 1963. Casein and the action of rennin. Part I. Dairy Sci. Abst., 25: 257; Part II. Dairy Sci. Abst., 25: 299.

172. Long, J., Van Winkle, Q., and Gould, I.R. 1958. Isolation and identification of λ-casein. J. Dairy Sci., 41: 317.

173. Losse, G., Losse, A., and Stoeck, J. 1962. Separation of N-formylamino acid methyl esters by gas chromatography. Z. Naturforsch., 17b: 785. Cited from: Chem. Abstr., 58: 9221b (1963).

174. Luhtala, A., Rautiainen, A., and Antila, M. 1968. Die Zusammensetzung der Finnischen Rentiermilch. Suomen Kemistilehti B 41: 6.

175. Lunsford, L. Jr., and Deutsch, H.F. 1957. Human milk whey proteins. Proc. Soc. Exp. Biol. Med., 96: 742.

176. MacKinlay, A.G., Hill, R.J., and Wake, R.G. 1966. The action of rennin on K-casein. The heterogeneity and origin of the insoluble products. Biochim. Biophys. Acta, 115: 103.

177. MacKinlay, A.G., and Wake, R.G. 1965. Fractionation of S-carboxymethyl-K-casein and characterization of the components. Biochim. Biophys. Acta, 104: 167.

178. MacRae, H.F., and Baker, B.E. 1958. Application of electrophoresis on paper to the estimation of alpha-, beta-, and gamma-casein. J. Dairy Sci., 41: 233.

179. Macy, I.G., Kelly, H.J., Sloan, R.E. 1953. The composition of milks. A compilation of the comparative composition and properties of human, cow, and goat milk, colostrum and transitional milk. Natl. Res. Council, Natl. Acad. Sci.(U.S.) Publication 254, Washington, D.C.

180. Maeno, M., and Kiyosawa, I. 1963. Proteins of human milk. V. Changes on the electrophoretic properties at various times after parturition. Nippon Nogei Kagaku Kaishi, 37: 362. Cited from: Chem. Abstr., 63: 2187a (1965).

181. Makisumi, S., Nicholls, C.H., and Saroff, H.A. 1963. The influence of esterifying and acetylating groups on the retention times of amino acid derivatives in gas chromatography. J. Chromatog., 12: 106.

182. Makisumi, S., and Saroff, H.A. 1965. Preparation, properties and gas chromatography of the N-trifluoroacetyl esters of the amino acids. J. Gas Chrom., January, 1965, p. 21.

183. Malpress, F.H. 1962. Sialic acid in casein preparations from human milk. Biochem. J., 85: 33P.

184. Manson, W. 1962. The effect upon casein of aqueous solutions of urea. Biochim. Biophys. Acta, 63: 515.

185. Marier, J.R., Tessier, H., and Rose, D. 1963. Sialic acid as an index of the K-casein content of bovine skimmilk. J. Dairy Sci., 46: 373.

186. Martin, A.J.P., and Synge, R.L.M. 1941. Some applications of periodic acid to the study of the hydroxyamino acids of protein hydrolysates. Biochem. J., 35: 294.

187. Mason, P.S., and Smith, E.D. 1966. A quantitative study of reagents and procedures for the synthesis of trimethyl silyl derivatives. J. Gas Chromatog., Nov., 1966, p. 398.

188. McBride, W.J., and Klingman, J.D. 1968. Single-column gas chromatographic separation of nanomolar quantities of amino acids. Anal. Biochem., 25: 109.

189. McCready, R.M., Guggolz, J., Silviera, V., and Owens, H.S. 1950. Determination of starch and amylose in vegetables. Anal. Chem., 22: 1156.

190. McGregor, W.H., and Carpenter, F.H. 1962. Alkaline bromine oxidation of amino acids and peptides: formation of α-ketoacyl peptides and their cleavage by hydrogen peroxide. Biochemistry, 1: 53.

191. McGugan, W.A., Zehren, V., Zehren, V.C., and Swanson, A.M. 1954. Interaction between α-casein and β-lactoglobulin. Science, 120: 145.

192. McKenzie, H.A., and Sawyer, W.H. 1966. Zone electrophoresis of β-lactoglobulins. Nature, 212: 161.

193. McKenzie, H.A., and Wake, R.G. 1961. An improved method for the isolation of K-casein. Biochim. Biophys. Acta, 47: 240.

194. McMeekin, T.L., Groves, M.L., and Hipp, N.J. April, 1957. The separation of a new component of casein. Abstr. No. 143. 131st Meeting Am. Chem. Soc.

195. McMeekin, T.L., Hipp, N.J., and Groves, M.L. 1959. The separation of the components of α-casein. I. The preparation of α_1-casein. Arch. Biochem. Biophys., 83: 35.

196. McMeekin, T.L., and Polis, B.D. 1949. Milk proteins. Adv. Prot. Chem., 5: 201.

197. Meister, A. 1965. Biochemistry of the Amino Acids. Volume I. Second Edition. Academic Press, Inc., New York.

198. Melachouris, N. 1969. Discontinuous gel electrophoresis of whey proteins, casein, and clotting enzymes. J. Dairy Sci., 52: 456.

199. Melachouris, N.P., and Tuckey, S.L. 1966. Changes of the proteins in cheddar cheese made from milk heated at different temperatures. J. Dairy Sci., 49: 800.

200. Melachouris, N.P., and Tuckey, S.L. 1966. Denaturation of whey proteins in milk heated at high temperatures for short times. J. Dairy Sci., 49: 1154.

201. Melamed, N., and Renard, M. 1960. Analyse de mélanges d'acides aminés par chromatographie gazeuse. J. Chromatog., 4: 339.

202. Mellander, O. 1939. Electrophoretische Untersuchung von Casein. Biochem. Z., 300: 240.

203. Mellander, O. 1945. Electrophoretic and enzymatic fractionation of casein from human milk. Nature, 155: 604.

204. Merck & Co., Inc. 1968. The Merck Index. Eigth Edition. Paul G. Stecher, Editor. Merck & Co., Inc., Rahway, N.J.

205. Mitchell, H.H., and Block, R.J. 1946. Some relationships between the amino acid contents of proteins and their nutritive values for the rat. J. Biol. Chem., 163: 599.

206. Montgomery, R. 1961. Further studies of the phenol-sulphuric acid reagent with carbohydrates. Biochim. Biophys. Acta, 48: 591.

207. Moore, S., Spackman, D.H., and Stein, Wm.H. 1958. Chromatography of amino acids on sulfonated polystyrene resins. An improved system. Anal. Chem., 30: 1185.

208. Moore, S., and Stein, Wm.H. 1949. Chromatography of amino acids on starch columns. Solvent mixtures for the fractionation of protein hydrolysates. J. Biol. Chem., 178: 53.

209. Moore, S., and Stein, W.H. 1951. Chromatography of amino acids on sulfonated polystyrene resins. J. Biol. Chem., 192: 663.

210. Moore, S., and Stein, W.H. 1954. Procedures for the chromatographic determination of amino acids on 4% cross-linked polystyrene resins. J. Biol. Chem., 211: 893.

211. Moore, S., and Stein, W.H. 1963. Chromatographic determination of amino acids by the use of automatic recording equipment. Methods Enzymol., 6: 819.

212. Morr, C.V. 1959. Investigating β-Lactoglobulin and K-Casein Solutions and Their Interaction by Means of Fluorescence Polarization. Ph.D. Thesis, Ohio State University. Dissert. Abstr., 20: 3943 (1960).

213. Morr, C.V., and Kenkare, D.B. 1964. On the heterogeneity of β-lactoglobulin. J. Dairy Sci., 47: 294.

214. Morris, D.L. 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science, 107: 254.

215. Morse, E.E. 1947. Anthrone in estimating low concentrations of sucrose. Ind. Eng. Chem. Anal. Ed., 19: 1012.

216. Nagasawa, T., Kiyosawa, I., and Kuwahara. 1970. Acrylamide gel electrophoresis and amino acid compositions of human colostral casein. J. Dairy Sci., 53: 92.

217. Neelin, J.M. 1962. Identification of K-casein in zone electrophoresis. Can. J. Biochem. Physiol., 40: 693.

218. Neelin, J.M. 1964. Variants of K-casein revealed by improved starch gel electrophoresis. J. Dairy Sci., 47: 506.

219. Neelin, J.M., Rose, D., and Tessier, H. 1962. Starch-gel electrophoresis of various fractions of casein. J. Dairy Sci., 45: 153.

220. Nicholls, C.H., Makisumi, S., and Saroff, H.A. 1963. Gas chromatography of the methyl esters of the amino acids as the free base and by dissociation of their acid salts. J. Chromatog., 11: 327.

221. Nitschmann, Hs., and Henzi, R. 1959. Das Lab und seine Wirkung auf das Casein der Milch (XIII). Untersuchung der bei der Labung in Freiheit gesetzten Peptide. Helv. Chim. Acta, 42: 1985. Cited from: Kalan, E.B., and Woychik, J.H. 1965. Action of rennin on K-casein, the amino acid compositions of the para-K-casein, and glycomacropeptide fractions. J. Dairy Sci., 48: 1423.

222. Noble, R.W. Jr., and Waugh, D.F. 1965. Casein micelles. Formation and structure. I. J. Am. Chem. Soc., 87: 2236.

223. Ohta, K., Watarai, J., Oishi, J., Ueshiba, Y., Hirose, S., Yoshizawa, J., Akikusa, Y., Sato, M., and Okano, H. 1953. Composition of fin whale milk. Proc. Imp. Acad. Japan, 29: 329.

224. Ornstein, L. 1964. Disc electrophoresis-I. Background and theory. Ann. N.Y. Acad. Sci., 121: 321.

225. Ozawa, K., and Tanaka, S. 1968. Computer-aided calculation of amino acid composition of proteins. Anal. Biochem., 24: 270.

226. Payens, T.A. 1961. Electrophoresis of casein in urea-buffer mixtures. Biochim. Biophys. Acta, 46: 441.

227. Perlmann, G.E. 1955. The nature of phosphorus linkages in phosphoproteins. Adv. Prot. Chem., 10: 1.

228. Peterson, R.F. 1963. High resolution of milk proteins obtained by gel electrophoresis. J. Dairy Sci., 46: 1136.

229. Peterson, R.F., and Kopfler, F.C. 1966. Detection of new types of β-casein by polyacrylamide gel electrophoresis at acid pH: a proposed nomenclature. Biochem. Biophys. Res. Comm., 22: 388.

230. Pilson, M.E.Q., and Kelly, A.L. 1962. Composition of the milk from Zalophus californianus, the California sea lion. Science, 135: 104.

231. Poulik, M.D. 1957. Starch gel electrophoresis in a discontinuous system of buffers. Nature, 180: 1477.

232. Prodanski, P., and Petrov, P. 1962. Comparative electrophoretic and chromatographic studies on the protein composition of different kinds of milk. Deut. Milchwirtsch., 9: 290. Cited from: Chem. Abstr., 58: 4883h (1963).

233. Purkayastha, R., and Rose, D. 1965. Location of the carbohydrate-containing fraction of K-casein after gel electrophoresis. J. Dairy Sci., 48: 1419.

234. Rao, M.B. 1964. Electrophoretic properties of the non-micellular proteins of buffalo milk as compared with those of cow milk. Nature, 201: 1217.

235. Raymond, S. 1962. A convenient apparatus for vertical gel electrophoresis. Clin. Chem., 8: 455.

236. Raymond, S., and Nakamichi, M. 1962. Electrophoresis in synthetic gels. I. Relation of gel structure to resolution. Anal. Biochem., 3: 23.

237. Raymond, S., and Wang, Y.J. 1960. Preparation and properties of acrylamide gel for use in electrophoresis. Anal. Biochem., 1: 391.

238. Regis Chemical Company. 1967. TAB Operational Manual. Chicago, Illinois.

239. Reynolds, L.M., Henneberry, G.O., and Baker, B.E. 1959. Studies on casein. II. The carbohydrate moiety of casein. J. Dairy Sci., 42: 1463.

240. Roach, D., and Gehrke, C.W. 1969. Direct esterification of the protein amino acids. Gas-liquid chromatography of N-TFA n-butyl esters. J. Chromatog., 44: 269.

241. Roach, D., and Gehrke, C.W. 1969. The gas-liquid chromatography of amino acids. J. Chromatog., 43: 303.

242. Roach, D., Gehrke, C.W., and Zumwalt, R.W. 1969. Quantitative gas-liquid chromatography of histidine. J. Chromatog., 43: 311.

243. Roberts, H.R., Kolor, M.G., and Bucek, W. 1958. Rapid paper chromatographic method for quantitative determination of tryptophan. Anal. Chem., 30: 1626.

244. Roberts, H.R., Pettinati, J.D., and Bucek, W. 1954. A comparative study of human, cow, sow, and rat milk using paper chromatography. J. Dairy Sci., 37: 538.

245. Rose, D., Brunner, J.R., Kalan, E.B., Larson, B.L., Melnychyn, P., Swaisgood, H.E., and Waugh, D.F. 1970. Nomenclature of the proteins of cow's milk: Third revision. J. Dairy Sci., 53: 1.

246. Ross, V., and Moore, D.H. 1955. Some properties of the casein of mouse milk (RIII). Biochim. Biophys. Acta, 16: 293.

247. Rozhanskii, M.O., Sergeeva, A.V., and Kudryashov, A.G. 1962. Protein composition of mare milk. Dokl. Mosk. Sel'skokhoz. Akad., 78: 188. Cited from: Chem. Abstr., 59: 11983a (1963).

248. Saroff, H.A., and Karmen, A. 1960. Gas chromatography of the N-trifluoroacetylmethyl esters of the amino acids. Anal. Biochem., 1: 344.

249. Saroff, H.A., Karmen, A., and Healy, J.W. 1962. Gas chromatography of the amino acid esters in ammonia. J. Chromatog., 9: 122.

250. Schmidt, D.G. 1964. Starch-gel electrophoresis of K-casein. Biochem. Biophys. Acta, 90: 411.

251. Schmidt, D.G., Both, P., and de Koning, P.J. 1966. Fractionation and some properties of K-casein variants. J. Dairy Sci., 49: 776.

252. Schulte, K.E., and Müller, F. 1955. Electrophoretic studies on milk proteins. I. Paper electrophoretic resolution of milk and corresponding whey proteins. Milchwissenschaft, 10: 90. Cited from: Chem. Abstr., 50: 4257 (1956).

253. Schulte, K.E., and Müller, F. 1955. Electrophoretic studies on milk proteins. III. The paper electrophoretic separation of proteins from the milk and whey of goats and ewes. Milchwissenschaft, 10: 228. Cited from: Chem. Abstr., 50: 11547d (1956).

254. Shaw, J.L.D., and McFarlane, W.D. 1938. The determination of tryptophan by a modified glyoxylic acid method employing photoelectric colorimetry. Can. J. Res., 16B: 362.

255. Sloan, R.E., Jenness, R., Kenyon, A.L., and Regehr, E.A. 1961. Comparative biochemical studies of milks-I. Electrophoretic analysis of milk proteins. Comp. Biochem. Physiol., 4: 47.

256. Smith, E.D., and Shewbart, K.L. 1969. A quantitative comparison of trimethylsilylating reagents for protein amino acids. J. Chromatog. Sci., 7: 704.

257. Smith, I. 1968. Chromatographic and Electrophoretic Techniques. Volume II. Zone Electrophoresis. Second Edition. William Heinemann Medical Books Ltd., London.

258. Smith, I. 1969. Aminoacids, amines, and related compounds. From: Chromatographic and Electrophoretic Techniques. Volume I. Edited by: I. Smith. p. 104. Wiliam Heinemann, London.

259. Smithies, O. 1955. Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. Biochem. J., 61: 629.

260. Smithies, O. 1959. An improved procedure for starch gel electrophoresis: further variations in the serum proteins of normal individuals. Biochem. J., 71: 585.

261. Smithies, O. 1959. Zone electrophoresis in starch gels and its application to studies of serum proteins. Adv. Prot. Chem., 14: 65.

262. Snell, F.M., Shulman, S., Spencer, R.P., and Moos, C. 1965. Biophysical Principles of Structure and Function. Addison-Wesley Publishing Company, Inc., Reading, Mass.

263. Sode-Morgensen, M.T., and Lahav, E. 1960. Observations on the examination of casein preparations by paper electrophoresis. Lab. Practice, 9: 21. Cited from: Dairy Science Abstr., 22: 202 (1960). Abstr. No. 7083.

264. Sowls, L.K., Smith, V.R., Jenness, R., Sloan, R.E., and Regehr, E. 1961. Chemical composition and physical properties of the milk of the collared peccary. J. Mammalogy, 42: 245.

265. Spackman, D.H., Stein, W.H., and Moore, S. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem., 30: 1190.

266. Stack, M.V. 1965. Pyrolysis and gas chromatography of amino acids and proteins. Biochem. J., 96: 56P.

267. Stalling, D.L., and Gehrke, C.W. 1966. Quantitative analysis of amino acids by gas chromatography: acylation of arginine. Biochem. Biophys. Res. Comm., 22: 329.

268. Stalling, D.L., Gehrke, C.W., and Zumwalt, R.W. 1968. A new silylation reagent for amino acids. Bis (trimethylsilyl) trifluoroacetamide (BSTFA). Biochem. Biophys. Res. Comm., 31: 616.

269. Stalling, D.L., Gille, G., and Gehrke, C.W. 1967. Quantitative gas chromatography of amino acids. Interesterification studies. Anal. Biochem., 18: 118.

270. Starbuck, W.C., Mauritzen, C.M., McClimans, C., and Busch, H. 1967. A computer program for the calculation of amino acid analysis data. Anal. Biochem., 20: 439.

271. Stark, G.R., Stein, W.H., and Moore, S. 1960. Reactions of the cyanate present in aqueous urea with amino acids and proteins. J. Biol. Chem., 235: 3177.

272. Steele, B.E., Sanberlich, H.E., Reynolds, M.S., and Baumann, C.A. 1949. Media for leuconostoc mesenteroides P-60 and leuconostoc citrovorum 8081. J. Biol. Chem., 177: 533.

273. Stefanovic, M., and Walker, B.L. 1967. Effect of stationary phase-support ratio on the gas chromatographic separation of trifluoroacetylamino acid butyl esters. Anal. Chem., 39: 710.

274. Stein, W.H., and Moore, S. 1948. Chromatography of amino acids on starch columns. Separation of phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine. J. Biol. Chem., 176: 337.

275. Stein, W.H., and Moore, S. 1949. Amino acid composition of β-lactoglobulin and bovine serum albumin. J. Biol. Chem., 178: 79.

276. Stevenson, G.W., and Luck, J.M. 1961. The bromodecarboxylation of amino acids: formation of nitriles. J. Biol. Chem., 236: 715.

277. Sullivan, R.A., Fitzpatrick, M.M., Stanton, E.K., Annino, R., Kissel, G., and Palermiti, P. 1955. The influence of temperature and electrolytes upon the apparent size and shape of α- and β-casein. Arch. Biochem. Biophys., 55: 455.

278. Svennerholm, L. 1956. Sialic acids and their quantitative estimation. Biochem. J., 64: 11P.

279. Svennerholm, L. 1957. Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. Biochim. Biophys. Acta, 24: 604.

280. Svennerholm, L. 1958. Quantitative estimation of sialic acids. Acta Chem. Scand., 12: 547.

281. Swaisgood, H.E., and Brunner, J.R. 1960. The isolation of λ-casein from concentrated TCA-urea solutions. J. Dairy Sci., 44: 1163.

282. Swaisgood, H.E., and Brunner, J.R. 1962. Characterization of K-casein obtained by fractionation with trichloroacetic acid in a concentrated urea solution. J. Dairy Sci., 45: 1.

283. Swaisgood, H.E., and Brunner, J.R. 1963. Characteristics of kappa-casein in the presence of various dissociating agents. Biochem. Biophys., Res. Comm., 12: 148.

284. Swaisgood, H.E., Brunner, J.R., and Lillevik, H.A. 1964. Physical parameters of K-casein from cow's milk. Biochemistry, 3: 1616.

285. Sweeley, C.C., Bentley, R., Makita, M., and Wells, W.W. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J. Am. Chem. Soc., 85: 2497.

286. Thompson, M.P. 1964. Phenotyping of caseins of cow's milk: collaborative experiment. J. Dairy Sci., 47: 1261.

287. Thompson, M.P. 1970. Phenotyping milk proteins: a review. J. Dairy Sci., 53: 1341.

288. Thompson, M.P., Kiddy, C.A., Johnston, J.O., and Weinberg, R.M. 1964. Genetic polymorphism in caseins of cow's milk. II. Confirmation of the genetic control of β-casein variation. J. Dairy Sci., 47: 368.

289. Thompson, M.P., Kiddy, C.A., Pepper, L., and Zittle, C.A. 1962. Casein variants in the milk from individual cows. J. Dairy Sci., 45: 650.

290. Thompson, M.P., Kiddy, C.A., Pepper, L., and Zittle, C.A. 1962. Variation in the α_s-casein fraction of individual cow's milk. Nature, 195: 1001.

291. Thompson, M.P., Tarassuk, N.P., Jenness, R., Lillevik, H.A., Ashworth, U.S., and Rose, D. 1965. Nomenclature of the proteins of cow's milk - Second revision. J. Dairy Sci., 48: 159.

292. Tristram, G.R. 1953. Amino acid composition of certain proteins. From: The Proteins. Edited by: H. Neurath and K. Bailey. Vol. 1, Part A, p. 181. Academic Press, New York.

293. Tristram, G.R., and Smith, R.H. 1963. The amino acid composition of some purified proteins. Adv. Prot. Chem., 18: 227.

294. True, L.C., Dooley, S.M., and Mickle, J.B. 1969. Sialic acid content of bovine milk and its relation to lipase activity. J. Dairy Sci., 52: 2046.

295. Úlehla, J. 1960. Pyrolysis and gas chromatography of amino acids. Sborník Československ. akad. zemědél. věd, Živočišna výroba, 5: 567. Cited from: Chem. Abstr., 55: 5242h (1960).

296. United Nations, Food and Agriculture Organization (FAO). September, 1957. Report on the Meeting of Experts on the use of hydrogen peroxide and other preservatives in milk. FAO/57/11/8655. Interbachen. Cited from: Gregory, M.E., Henry, K.M., Kon, S.K., Porter, J.W.G., and Thompson, S.Y. 1961. The effect of hydrogen peroxide on the nutritive value of milk. J. Dairy Res., 28: 177.

297. Vandegaer, J.E., and Miettinen, J.K. 1953. A paper electrophoretic investigation of milk serum proteins. Acta Chem. Scand., 7: 1239.

298. Viles, F.J. Jr., and Silverman, L. 1949. Determination of starch and cellulose with anthrone. Anal. Chem., 21: 950.

299. Vogel, A.I. 1955. A Texbook of Practical Organic Chemistry. 3rd edition. Longmans, Green and Co. Ltd., London, W.I.

300. Von Hippel, P.H., and Waugh, D.F. 1955. Casein: monomers and polymers. J. Am. Chem. Soc., 77: 4311.

301. Wagner, J., and Rausch, G. 1963. Gas-chromatographic separation and determination of amino acids. II. Gas chromatographic separation and determination of amino acids in the form of methyl esters of hydroxy acids. Z. Anal. Chem., 194: 350. Cited from: Chem. Abstr., 59: 3316g (1963).

302. Wagner, J., and Winkler, G. 1961. Gas-chromatographic separation and determination of amino acids. Gas-chromatographic determination of amino acids as N-trifluoroacetyl methyl esters. Z. Anal. Chem., 183: 1. Cited from: Chem. Abstr., 55: 25602a (1961).

303. Wake, R.G., and Baldwin, R.L. 1961. Analysis of casein fractions by zone electrophoresis in concentrated urea. Biochim. Biophys. Acta, 47: 225.

304. Warner, R.C. 1944. Separation of alpha- and beta-casein. J. Am. Chem. Soc., 66: 1725.

305. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem., 234: 1971.

306. Waugh, D.F. 1958. The interaction of α_s-, β- and K-caseins in micelle formation. Discussions Faraday Soc., 25: 186.

307. Waugh, D.F. 1961. Casein interactions and micelle formation. J. Physiol. Chem., 65: 1793.

308. Waugh, D.F., and Gillespie, J.M. 1958. Structure of the stoichiometric complex of α_s- and K-caseins. Abstr. No. 123. 134th Meeting Am. Chem. Soc.

309. Waugh, D.F., Ludwig, M.L., Gillespie, J.M., Melton, B., Foley, M., and Kleiner, E.S. 1962. The α_s-caseins of bovine milk. J. Am. Chem. Soc., 84: 4929.

310. Waugh, D.F., and Noble, R.W. Jr. 1965. Casein micelles. Formation and structure. II. J. Am. Chem. Soc., 87: 2246.

311. Waugh, D.F., and von Hippel, P.H. 1956. K-casein and the stabilization of casein micelles. J. Am. Chem. Soc., 78: 4576.

312. Weigt, U. 1959. Paper electrophoretic investigations on normal bovine whey. Milchwissenschaft, 14: 61.

313. Weinstein, B. 1966. Separation and determination of amino acids and peptides by gas-liquid chromatography methods. Biochem. Anal., 14: 203.

314. West, E.S., Todd, W.R., Mason, H.S., and Van Bruggen, J.T. 1966. Textbook of Biochemistry. Fourth edition. The Macmillan Company, New York.

315. Weygand, F., Kolb, B., Prox, A., Tilak, M.A., and Tomida, I. 1960. N-trifluoroacetylamino acids. XIX. Gas chromatographic separation of N-trifluoroacetyldipeptide methyl esters. Z. Physiol. Chem., 322: 38. Cited from: Chem. Abstr., 55: 8177b (1961).

316. Weygand, F., and Rinno, H. 1959. N-trifluoroacetylamino acids. XIII. Serine and threonine derivatives. Chem. Ber., 92: 517.

317. Williamson, M.B. 1944. The amino acid composition of human milk proteins. J. Biol. Chem., 156: 47.

318. Woychik, J.H. 1964. Investigation of reduced K-casein from cow's milk. Fed. Proc., 23: 474.

319. Woychik, J.H. 1964. Polymorphism in K-casein of cow's milk. Biochem. Biophys. Res. Comm., 16: 267.

320. Woychik, J.H. 1965. Phenotyping of K-caseins. J. Dairy Sci., 48: 496.

321. Woychik, J.H. 1965. Preparation and properties of reduced K-casein. Arch. Biochem. Biophys., 109: 542.

322. Woychik, J.H., Kalan, E.B., and Noelken, M.E. 1966. Chromatographic isolation and partial characterization of reduced K-casein components. Biochemistry, 5: 2276.

323. Yaguchi, M., Davies, D.T., and Kim, Y.K. 1968. Preparation of K-casein by gel filtration. J. Dairy Sci., 51: 473.

324. Yaguchi, M., and Tarassuk, N.P. 1967. Gel filtration of acid casein and skimmilk on Sephadex. J. Dairy Sci., 50: 1985.

325. Youngs, C.G. 1959. Analysis of mixtures of amino acids by gas phase chromatography. Anal. Chem., 31: 1019.

326. Zlatkis, A., Oró, J.F., and Kimball, A.P. 1960. Direct amino acid analysis by gas chromatography. Anal. Chem., 32: 162.

327. Zomzely, C., Marco, G., and Emery, E. 1962. Gas chromatography of the n-butyl N-trifluoroacetyl derivatives of amino acids. Anal. Chem., 34: 1414.

328. Zumwalt, R.W., Kuo, K., and Gehrke, C.W. 1971. Applications of a gas-liquid chromatographic method for amino acid analysis. A system for analysis of nanogram amounts. J. Chromatog., 55: 267.

329. Zumwalt, R.W., Roach, D., and Gehrke, C.W. 1970. Gas-liquid chromatography of amino acids in biological substances. J. Chromatog., 53: 171.

APPENDIX

STRONTIUM-90 AND CESIUM-137 LEVELS IN TISSUES OF FIN WHALE (Balaenoptera physalus) AND HARP SEAL (Pagophilus groenlandicus)*

INTRODUCTION

Radioactive strontium (Sr-90) is one of the high-yield fission products which is released by nuclear bomb detonations and which is an ingestive hazard for weeks and months after an explosion (A-15). Strontium-90 and its short-lived daughter isotope, yttrium-90 (Y-90) are both β-emitters. Strontium-90 is similar to calcium in its chemical and physiological behavior and thus competes with calcium for deposition in the bone.

Marine food products supply man with smaller amounts of strontium-90 than do foods from terrestrial environments

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*Note: In August, 1968, the author collected at the Karl Karlson whale plant (Blandford, N.S.), samples of whale milk for casein precipitation and whale tissues for measurement of radioactivity. Whale meat is flash frozen at this plant and is shipped to Norway. Since whale meat comprises part of the Norwegian diet, it was of interest to know whether or not it was contaminated by radioactive fallout. The seal samples were collected by Dr. B.E. Baker in March, 1968, near the Magdalen Islands. The work reported here represents part of an investigation of radioactivity in foodstuffs being carried out in the Department of Agricultural Chemistry, Macdonald College, and appears in a publication by Samuels, Cawthorn, Lauer, and Baker (A-21).

because strontium-90 is present in lower specific activities. Strontium-90 enters the sea surface from fallout in a soluble form and it is rapidly diluted by the mixing processes in the ocean waters. Since stable strontium is present in sea water (8 mg/litre, approximately), there is also an isotope dilution effect. Strontium is diluted further by calcium which is also present in sea water (400 mg/litre) and which is similar chemically. The small exposure of the human population to strontium-90 derived from the sea results from (a) the low level contamination of the marine environment by this radionuclide, (b) the resultant low specific activity of strontium-90, and (c) the low ratio of Sr-90/g Ca in the water and edible portions (e.g. muscle tissue) of seafood organisms (A-5).

The fallout which comes after the original fallout period may be considered to be a mixture of roughly equal intensities of strontium-90 and cesium-137 (A-15). Cesium-137, which has a half-life similar to that of strontium-90 ($T_{\frac{1}{2}}$ for cesium-137, 30 years; $T_{\frac{1}{2}}$ for strontium-90, 28 years), is a γ- and a β-emitter. Radioactive cesium is distributed throughout the flesh of animals and hence enters the human body when the meat is eaten (A-20). Hood and Comar (A-13) showed that the tissue concentration patterns of injected cesium-137 tracer were remarkably constant from

one species to another and they further demonstrated that muscles had the highest concentrations and that bone and plasma had the lowest concentrations of this element. These workers concluded that the ingestion of cesium-137 into the body of animals may make the animal unfit as a source of meat and milk.

Cesium-137 is deposited on the sea surface by rainfall. Since sea water contains a relatively small concentration of stable cesium (0.5 μg/litre), cesium-137 is present in relatively high specific activity. The potassium in sea water (380 mg/litre) serves as a dilutant (A-5).

The levels of strontium-90 and cesium-137 in the milks of various Arctic species have been reported previously by Baker, Lauer, and Samuels (A-3) and more recently by Baker, Neilson, and Samuels (A-4), and in human milks by other workers (A-1)(A-14)(A-25). The reason that milk has become such a valuable indicator of radioactive fallout is that in the western hemisphere, at least one half of the calcium deposited in the human skeleton through adolescence originates from dairy products. Children who derive their calcium from dairy products will form a skeleton which is in equilibrium with the strontium-90 contamination of the environment from which their food is derived (A-7). Human skeletons formed from milk diets contain 0.5 times the ratio

Sr/Ca of the original milk, indicating a discrimination against strontium (A-6). Anderson (A-2) has shown that the correlation of cesium levels in human beings with cesium levels in milk indicate a discrimination factor of 1.8 in this step of the ecological transfer.

Hawthorn and Duckworth (A-12) measured the total strontium (strontium-89 + strontium-90) activity in a deer's antlers and found it to be 126 μμc/g Ca. Schulert (A-23) has also reported values (for the same year as Hawthorn and Duckworth, 1958) for antlers of California deer (6.81 d.p.m./g ash; 8.05 μμc Sr-90/g Ca) and Alaskan caribou (98.4 d.p.m./g ash; 106.1 μμc Sr-90/g Ca).

A search of the literature has revealed that there are few available data on the strontium-90 and cesium-137 levels in tissues of marine mammals, although considerable work has been done on freshwater and saltwater fishes. Hasanen and Miettinen (A-11) have reported values for the cesium-137 content of freshwater fish in Finland. The values ranged from 0.64 to 5.90 nc Cs-137/kg fresh weight for lake fish caught in Lapland. These workers also showed that the cesium-137 activity decreased when the feed changed from plankton to bottom animals.

Gustafson, Brar, and Muniak (A-10) stated that the cesium-137 concentration in fish is governed by three

factors: (a) the concentration of cesium-137 in the water environment, (b) the level of potassium (the ratio, Cs-137/K), and (c) the position of the fish in the food chain (e.g. the "trophic level"). Analysis of several species of freshwater and saltwater fish revealed that the former contained much higher levels of cesium-137 than did the latter and this reflected the higher Cs-137/K ratio present in freshwater. Carnivorous fish showed higher levels of cesium-137 relative to plankton feeders and other non-carnivorous fish (both saltwater and freshwater).

Various workers have detected both fission products from fallout and neutron-induced radionuclides in marine zooplankton off the coasts of Oregon (A-17)(A-19) and California (A-8).

Osterberg (A-17) stated that euphausids (Euphausia pacificia), which are commonly known as "krill", are good indicators of fallout in the ocean, since they concentrate most radionuclides. The same author points out that these shrimp-like crustaceans, which comprise a large portion of the diet of the fin whale, are important vehicles for the transport of radioactivity in the ocean. These observations were also made by earlier workers (A-24).

Osterberg, Small, and Hubbard (A-19) studied the effect of surface area of an organism on the adsorption of

radionuclides. These workers concluded that surface adsorption played a relatively insignificant part in the accumulation of K-40, Zn-65, Zr-95--Nb-95, Ru-103-106, Cr-51, and Ce-141 by microplankton.

Chipman (A-5) has stated that the uptake of strontium-90 via the digestive tract of fish and other animals is of minor importance and marine plankton does not play a significant role in passing strontium-90 from the sea water to the organism. Rather, calcium and strontium-90 are obtained from the sea water directly via absorptive body surfaces and gill membranes. Chipman has also pointed out that strontium-90 accumulation takes place mainly in the bones and scales, but not in the flesh. Cesium-137 has been observed to accumulate to only a slight extent by marine plankton. Some bottom-dwelling invertebrates, particularly mollusks (mussels, oysters, scallops, and clams), have taken up cesium-137 and concentrated it in their tissues up to about 50 times that of the sea water in their environment. Equilibrium levels were approached more rapidly by shrimp and prawns than by crabs and lobsters, since the former enjoy a more active mode of life. Cesium-137 is concentrated in the internal organs of fish and the rate of uptake in these organs is quite rapid, ½-equilibrium being reached in a few days.

Rosenthal, Eves, and Cochran (A-21) showed that the ratio of Sr/Ca in ossified tissues, to Sr/Ca in water, was essentially constant for marine and freshwater animals within the same class. The ratio, Sr (mg):Ca (g) in tissue to the ratio, Sr (mg):Ca (g) in water indicates the discrimination of strontium relative to that of calcium. These workers found that pelecypods (freshwater clams, oysters, and Quahog clams) and fishes (Wall eye pike, Buffalo carp, Red snapper, and flounder) show the greatest discrimination for strontium relative to calcium, and the Crustacea (crayfish, lobster, and Alaskan king crab) showed the least discrimination for strontium relative to calcium.

Osterberg, Pearcy, and Kujala (A-18) measured the levels of zinc-65 in various tissues of a fin whale (Balaenoptera physalus) and noted that both the muscle tissue and the liver contained traces of cesium-137. These authors pointed out that although cesium is chemically similar to potassium and the two compete for entrance into organisms, the abundance of potassium in the sea reduces the relative uptake of cesium-137 by marine animals compared to terrestrial and freshwater animals. Although the stomach of the whale contained a large quantity of zooplankton ("krill"), cesium-137 was not detected in the stomach contents.

Schulert (A-23) reported that the strontium-90

contents of whale meat and whale rib (bowhead) were <0.07 d.p.m./g ash and <0.12 d.p.m./g ash, respectively. The same author has reported the strontium-90 content of walrus meat (9.38 d.p.m./g ash) and walrus backbone (0.65 d.p.m./g ash taken from an animal which was caught in the St. Lawrence region.

Mohindra and Downs (A-16) reported on the levels of cesium-137 in liver, muscle, and diaphragm tissues of seals located in the District of Franklin, N.W.T.

MATERIALS AND METHODS

Collection of Samples

Samples (50-100 g each) of tissues were obtained from four female and three male fin whales caught (August 15th, 1968) near the Emerald Bank, 100 miles east of the whale factory at Blandford, Nova Scotia. The samples were obtained about 12 h after the death of the animal and were flash frozen and kept in this state until they were analyzed.

Samples (30-125 g each) were obtained from seven female adult harp seals and their respective pups, which were caught (March 9th, 1968) near the Magdalen Islands in the Gulf of St. Lawrence. The samples were frozen and kept in this state until they were analyzed.

Analytical Procedures

The detailed procedures for the determination of cesium-137 and strontium-90 are described in the Manual of Procedures (RPD-OM-2, 1964) which is used in the Radiochemistry Section of the Radiation Protection Division, Department of National Health and Welfare, Ottawa.

The total sample was ashed at a temperature not exceeding 450°C (cesium volatilizes above 450°C). The ash was fused with an alkali fusion mixture and the resultant melt was leached with water. Cesium was separated from the leachate as the silicotungstate. This was then converted to the perchlorate and the cesium was finally isolated as the chloroplatinate. The precipitate was mounted on a planchet for beta-counting and the activity was ascertained by reference to a standard cesium source.

The water-insoluble portion of the fusion melt was treated with nitric acid. The resultant solution which contained strontium and calcium was then treated with fuming nitric acid and the strontium was precipitated as the nitrate. The combined radiostrontium (Sr-89, Sr-90) of the precipitate was then determined by means of a low-background beta counter. Strontium-90 was determined either by extracting and counting the daughter isotope (yttrium-90) or by use of

the simultaneous equation method without the separation of yttrium-90.

Appropriate corrections were made for self-absorption and counter efficiency in all determinations.

RESULTS AND DISCUSSION

The radioactivities of the various samples of tissue are shown in Table A-1. Whale blubber contained the highest concentration (pCi/g ash) of Sr-90. The very low levels of Sr-90 might be subject to relatively high statistical errors (1σ counting error of about 20%) because of the low counting rates from the small samples that were available for analysis. Chipman (A-5) pointed out that, in several species of fish, the levels of Sr-90 resulting from fallout were not detectable. The results given in the present report indicate that the concentration of Sr-90 in marine mammals is also very low.

Muscle tissues of the whales contained more Cs-137 than did the other tissues, except in one animal in which the liver contained slightly more Cs-137. The presence of relatively high concentrations of Cs-137 in these two tissues confirm a similar observation made by Osterberg, Pearcy, and Kujala (A-18). The stomach contents of one whale was

TABLE A-1

Strontium-90 and cesium-137 levels in fin whale and harp seal tissues, animals 1-7 (values in pCi/g ash)

	1		2		3		4		5		6		7	
	Sr-90	Cs-137	Sr-90	Cs-137	Sr-90	Cs-137	Sr-90	Cs-137	Sr-90	Cs-137	Sr-90	Cs-137	Sr-90	Cs-137
Whale[1]														
Blubber	2.9	0.6	3.5	1.1	0.3	0.6	0.0	0.0	0.6	0.0	0.1	1.6	0.3	1.7
Bone[2]	-[5]	-	-	-	-	-	-	-	<0.1	0.0	<0.1	0.3	-	-
Bone[3]	-	-	-	-	<0.1	<0.1	0.0	0.0	-	-	<0.1	0.0	-	-
Kidney	-	-	-	-	0.0	2.9	<0.1	3.9	-	-	1.0	2.8	-	-
Liver	0.0	1.2	<0.1	1.5	0.0	1.0	<0.1	0.5	0.0	4.7	<0.1	1.3	-	-
Lung	-	-	-	-	-	-	<0.1	1.0	-	-	0.0	1.0	-	-
Mammary gland	<0.1	0.5	0.2	0.0	-	-	0.7	1.2	-	-	-	-	-	-
Muscle	0.0	3.1	0.3	5.1	0.0	4.0	0.1	7.0	0.0	6.8	0.0	0.8	0.0	4.7
Pancreas	-	-	-	-	0.1	3.2	<0.1	4.1	-	-	-	-	-	-
Spleen	-	-	-	-	<0.1	1.4	0.0	2.8	-	-	-	-	-	-
Testicle	-	-	-	-	-	-	-	-	<0.1	2.9	0.0	2.2	0.0	1.0
Stomach contents	-	-	-	-	-	-	0.1	0.1	-	-	-	-	-	-
Seal[4]														
Muscle (adult)	<0.1	2.5	0.0	3.2	0.1	0.8	0.0	2.8	0.0	1.7	0.2	4.0	<0.1	1.4
Mammary gland	0.0	1.9	0.0	2.4	<0.1	1.6	0.0	1.7	0.0	1.7	0.0	1.5	1.3	2.9
Muscle (young)	<0.1	3.8	<0.1	3.5	-	-	<0.1	3.9	0.0	3.1	-	-	-	-

[1]Whale Nos. 1-4 were females and 5-7 were males.
[2]Vestigial pelvis and leg bone.
[3]Palatal bone.
[4]All the seals were females. Seal Nos. 1, 2, 4, and 5 were each accompanied by a pup.
[5]The symbol (-) indicates that no sample was available.

examined and was found to consist mainly of krill. The average concentration (pCi/g ash) of Cs-137 in whale muscle was 45 times greater than that of the stomach contents. The concentration of Sr-90 in the bone was less than that found in krill, thus lending weight to the observation of Chipman (A-5) that crustaceans do not accumulate Sr-90 in the flesh, and any Sr-90 that accumulates in the shell, is lost upon moulting. Thus, ingestion of sizable quantities of Sr-90 via the digestive tract of the whale is minimized by a natural biological process. The low level of Cs-137 in krill was probably the result of reduced uptake of Cs-137 by krill in the presence of the potassium in seawater (A-5)(A-10).

Muscle tissues of the young harp seals had a higher concentration of Cs-137 (3.6 pCi/g ash) than did muscle tissue of the adult seals (2.3 pCi/g ash). Fredriksson, Garner, and Russell (A-9) have pointed out that a significant proportion of cesium-137 fed to lactating animals appears in the milk. They also noted that there is a considerable variation in the uptake of Cs-137 by different muscles; those which are most active take up Cs-137 most readily. The average concentration of Cs-137 in the mammary glands was slightly lower than that of the muscle tissue.

Previous work (A-4) showed that seal milk contains about 9 pCi of Cs-137 per gram of ash.

REFERENCES

A-1 Aarkrog, A. 1963. Cesium-137 from fall-out in human milk. Nature, 197: 667.

A-2 Anderson, E.C. 1958. Radioactivity of people and milk: 1957. Science, 128: 882.

A-3 Baker, B.E., Lauer, B.H., and Samuels, E.R. 1968. Strontium-90 and cesium-137 levels in the milks of some Arctic species. J. Dairy Sci., 51: 1508.

A-4 Baker, B.E., Neilson, C.H., and Samuels, E.R. 1970. Strontium-90 and cesium-137 in human and other milks collected in Alaska. J. Dairy Sci., 53: 241.

A-5 Chipman, W.A. 1966. Accumulation of radioactive nuclides by the marine biota. From: Radioactivity and Human Diet. Edited by R.S. Russell. Pergamon Press.

A-6 Comar, C.L., Russell, R.S., and Wasserman, R.H. 1957. Strontium-calcium movement from soil to man. Science, 126: 485.

A-7 Eisenbud, M. 1959. Deposition of strontium-90 through October 1958. Science, 130: 76.

A-8 Folsom, T.R., Young, D.R., Johnson, J.N., and Pillai, K.C. 1963. Manganese-54 and zinc-65 in coastal organisms of California. Nature, 200: 327.

A-9 Fredriksson, L., Garner, R.J., and Russell, R.S. 1966. Radioactivity and Human Diet. Edited by R.S. Russell. Pergamon Press (p. 338).

A-10 Gustafson, P.F., Brar, S.S., and Muniak, S.E. 1966. Cesium-137 in edible freshwater fish. Nature, 211: 843.

A-11 Hasanen, E., and Miettinen, J.K. 1963. Cesium-137 content of freshwater fish in Finland. Nature, 200: 1018.

A-12 Hawthorn, J., and Duckworth, R.B. 1958. Fall-out radioactivity in a deer's antlers. Nature, 182: 1294.

A-13 Hood, S.L., and Comar, C.L. 1953. Metabolism of cesium-137 in rats and farm animals. Arch. Biochem. Biophys., 45: 423.

A-14 Jarvis, A.A., Brown, J.R., and Tiefenbach, B. 1963. Strontium-89 and strontium-90 in breast milk and mineral-supplement preparations. Can. Med. Ass. J., 88: 136.

A-15 Libby, W.F. 1956. Current research findings on radioactive fallout. Proc. Natl. Acad. Sci. U.S., 42: 945.

A-16 Mohindra, V.K., and Downs, A.A. 1966. Cesium-137 activity in seal samples. Data from Radiation Protection Program. Vol. 4, No. 2. Radiation Protection Division, Department of National Health and Welfare, Ottawa.

A-17 Osterberg, C. 1962. Fallout radionuclides in Euphausiids. Science, 138: 529.

A-18 Osterberg, C., Pearcy, W., and Kujala, N. 1964. Gamma emitters in a fin whale. Nature, 204: 1006.

A-19 Osterberg, C., Small, L., and Hubbard, L. 1963. Radioactivity in large marine plankton as a function of surface area. Nature, 197: 883.

A-20 Radiation Protection Division, Department of National Health and Welfare, Ottawa. 1964. Cesium-137 in Northern Canada. Data from Radiation Protection Program. Vol. 2, No. 12, Appendix A (p. 49).

A-21 Rosenthal, H.L., Eves, M.M., and Cochran, O.A. 1970. Common strontium concentration of mineralized tissues from marine and sweet water animals. Comp. Biochem. Physiol., 32: 445.

A-22 Samuels, E.R., Cawthorn, M., Lauer, B.H., and Baker, B.E. 1970. Strontium-90 and cesium-137 levels in tissues of fin whale (Balaenoptera physalus) and harp seal (Pagophilus groenlandicus). Can. J. Zool., 48: 267.

A-23 Schulert, A.R. 1962. Strontium-90 in Alaska. Science, 136: 146.

A-24 Seymour, A.H., Held, E.E., Donaldson, J.R., and South, D.J. 1957. Survey of radioactivity in the sea and in pelagic marine life west of the Marshall Islands, September 1-20, 1956. U.S.A.E.C. Bulletin No. UWFL-47.

A-25 Straub, C.P., and Murthy, G.K. 1965. A comparison of Sr^{90} component of human and cows' milk. Pediatrics, 36: 732.

CLAIM TO ORIGINAL RESEARCH

Note: Claims to original research based on studies in the main body of the thesis are presented on p. 319.

1. Determination of the levels of strontium-90 and cesium-137 in tissues of fin whale (Balaenoptera physalus) and harp seal (Pagophilus groenlandicus).