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A B S T R A C T

To overcome the low antigenicity of insulin due to its relatively low molecular weight, its close resemblance among different species and its hypoglycemic effect, insulin was coupled - as if it were a hapten - to bovine serum albumin via tolylene-2,4-diisocyanate. The resulting insulin-BSA conjugates were immunogenic in all rabbits used in this study, and the corresponding antibodies were readily demonstrated by precipitin, passive hemagglutination, immunoelectrophoresis and immunodiffusion procedures.

For the unequivocal demonstration that the antibodies had been produced against the determinant groups of the insulin molecule, the immunological reactions were inhibited with a free, highly soluble insulin derivative produced by coupling to sodium 4-fluoro-3-nitrobenzene sulphonate.

The disulphide bonds were reduced electrolytically at a constant cathode voltage, the extent of the reduction being determined amperometrically. It was shown by paper electrophoresis, ion-exchange chromatography, amino acid analysis and analytical ultracentrifugation that the two interchain S-S bonds were preferentially reduced and that the intrachain S-S bond was reduced at a more negative cathode voltage. The products of reduction were purified by chromatography on Sephadex G-75. Neither the B chain nor the totally reduced A chain was immunologically active, as demonstrated by their inability to combine in vitro with anti-insulin antibodies. However, the A chain with the intrachain S-S bond intact was shown to retain its combining capacity with antibodies to insulin and to be univalent, and its antigenic determinant was located at A6 - A11, i.e. in a region encompassing the intrachain S-S bond.

ANTIGENICITY OF INSULIN

by

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Chapter 1

GENERAL INTRODUCTION

Immunology grew out of the common knowledge that those who survived an infectious disease seldom contracted the same disease again during their lifetime. As early as the 15th century, the practice of prophylaxis for smallpox by sniffing dried powders of smallpox crusts was recognized. However, the idea of artificial immunization was not introduced until the end of the 18th century, when Edward Jenner embarked methodically on experiments to test the popular view of the prophylactic power of cowpox (vaccination) in preventing subsequently smallpox. This concept was further extended by the work of Pasteur (1) and Kraus (2) who showed that the resistance to certain diseases could be developed not only by exposure to the intact bacteria but also to their 'attenuated' cultures, extracts or toxins.

The search for the explanation of this remarkable phenomenon led to the discovery of a peculiar sort of globulin in the blood serum of the animals which had been exposed to an infectious organism (3). These globular proteins were termed antibodies and were shown to possess the unique property of combining specifically with the bacterium which elicited their production. Similarly, it was shown that the formation of specific antibodies could be elicited by the injection of high

molecular weight substances which were foreign to the body. The foreign materials which stimulate antibody formation are termed antigens.

PROPERTIES OF ANTIGEN

Besides the intact bacteria and their soluble extracts or toxins, innocuous substances such as blood cells, serum proteins or tissue extracts of one species are capable of stimulating antibody formation on injection into an animal of a different species. In addition, there are many other substances such as protein conjugates (4), polysaccharides (5), lipids (6), and nucleic acids (7), which can act as antigens. More recently, it has been shown that synthetic polymers such as polyvinylpyrrolidone (8) and polypeptides (9) are also antigenic. Although these antigens are diverse in their chemical nature, they share the distinct feature of being macromolecules.

In addition to relatively high molecular weight, it seems that for a molecule to be antigenic it must have a stable structure. In the study of the antigenicity of gelatine, Sela and Arnon (10-12) have demonstrated that gelatine by itself was only weakly antigenic; however, conjugation of gelatine with tyrosine, tryptophan or phenylalanine was found to enhance greatly its antigenicity. On the other hand, the specificity of a native protein was found to be completely abolished when the protein was substituted to near saturation. Thus, the introduction of more than 100 groups of phenyl arsonate per

molecule of globulin (13), or saturation of a globulin molecule with 2,4-dinitrophenyl residues (14) destroyed the antigenic specificity of the globulin.

Low molecular weight substances with well-defined chemical structures are incapable of stimulating antibody formation by themselves, but become immunogenic after covalent binding to suitable carrier macromolecules. To distinguish these compounds from true antigens, Landsteiner (15) coined the term 'hapten' (from the Greek = to bind) for these substances. The use of haptens in immunology has in fact provided the molecular basis for the understanding of the specificity of antigen-antibody reactions and the concept that antibody combining sites were configurationally complementary to small chemical groups of the antigen molecule and not to the antigen molecule as a whole.

As already mentioned, for a substance to be antigenic, it has to be foreign to the host. However, an artificial antigen can be also made by coupling a compound normally found in the host's organism to a carrier protein, which upon injection into the host is capable of eliciting the formation of antibodies to both the 'auto-hapten' and to the determinant groups of the carrier macromolecule.

PROPERTIES OF ANTIBODY

Classification of antibodies

Antibodies in man and several mammalian species can be subdivided into three main classes of Ig (immunoglobulin)

which are known as IgG, IgA and IgM. While the classes of Ig differ considerably in physical and chemical properties (table I), they all appear to have a basically similar four-chain structure (i.e. two heavy chains and two light chains), which is polymerized to a different extent to form IgA and IgM. The heavy chains of these three main classes of immunoglobulin are structurally distinct and are referred to as γ, α and μ, respectively. In contrast, the light chains from all classes of human Ig fall into two antigenically distinct and chemically different forms known as κ and λ. A normal human serum contains about 60% κ- and 30% λ-chains with a smaller amount of light chains possessing neither antigenic determinant.

A fourth class in human serum (IgD), discovered by Rowe and Fahey (16), represents less than one per cent of immunoglobulins. Recently, a new class of immunoglobulin present in extremely low concentration, designated as IgE, was proposed to include skin-sensitizing antibodies found in various atopic patients. Originally, it was demonstrated that reagins did not belong to any of the known classes of immunoglobulins on the basis of their antigenic properties (17-21). Moreover, within the last year it was shown that IgE had antigenic determinants in common with those of a new class of myeloma globulins, tentatively termed Ig-ND (22).

General configuration of antibody molecules

The multichain structure of antibodies was established by Edelman (24) who showed that on reduction of human IgG in the

Table I

SOME PROPERTIES OF THE MAIN CLASSES OF HUMAN Ig (23)

Physicochemical properties	IgG	IgA	IgM
% N	15.6	16.2	14.5
% carbohydrate	3.0	10.0	10.0
elect. mobility pH 8.6 (-10^{-5} cm^2 V^{-1} s^{-1})	-0.6 to +3.0	+1.2 to +3.6	about +2
molecular weight	150,000	150,000 and 400,000	900,000
range of salting out $(NH_4)_2SO_4$	1.49 - 1.64 M		1.64 - 2.0 M

Biological properties	IgG	IgA	IgM
antibody activity	+	+	+
antigenic determinants:			
specific	γ	α	μ
common	κ, λ	κ, λ	κ, λ

presence of 6 M urea, approximately 15 disulphide bonds were split and the molecular weight decreased from about 150,000 to 50,000. The products obtained were separable by chromatography but were biologically inactive and insoluble in aqueous solutions (25). Reduction with 0.2 M mercaptoethanol at pH 8.2 in the absence of urea led to splitting of 5 of the 20 disulphide bonds present in immunoglobulin (26), but without alkylation the apparent molecular weight of the protein remained unchanged, and the capacity of the antibody to precipitate with the antigen was not altered. However, if the partially reduced and alkylated protein was dialysed against N-acetic or N-propionic acid, it dissociated into two fractions now referred to as heavy and light chains, which could be separated with 100 percent recovery by gel filtration on Sephadex G-75 columns. The heavy chains, having a molecular weight of 50,000, constituted three-fourths of the original molecule and the light chains with a molecular weight of 20,000 made up the rest. Complete reduction of all the disulphide bonds of either H or L chains in 6 M guanidine caused no further reduction in molecular weight. Conditions expected to split esterlike bonds also had no effect and, hence, it is probable that IgG consists of only two types of peptide chains (27). These results thus suggested that the molecule was made up of two heavy chains and two light chains. In addition, the carbohydrate appeared to be associated almost entirely with the heavy chain.

Amino acid analysis revealed that there was one S-carboxymethylcysteine residue per mole of light chain. Since no dissociation of chains occurred without reduction, it was concluded that there was one disulphide bond between heavy and light chains (26) and that this was located at the C-terminal end of the human light chain (28). The heavy chain contained four S-carboxymethylcysteine residues per mole, indicating that these chains must be linked to each other by three of the disulphide bonds, the fourth joining onto a light chain (26). However, subsequent investigations suggested that there might be only one disulphide bond joining the two heavy chains, and the other two disulphide bonds might be of the intra-chain type (29). At present the number and position of inter-heavy chain disulphide bonds remain uncertain.

Enzymic fragmentation of immunoglobulin

It was shown that digestion of rabbit immunoglobulins with crystalline papain, activated by cysteine, resulted in their degradation into three fragments, which were separable by chromatography on carboxymethyl cellulose (30-32). These fragments represented over 90 percent of the original protein and were non-dialysable through Visking tubing. The largest of these three fragments (Fc) had a molecular weight of 50,000; this fragment lacked antibody-combining activity, but it carried most of the carbohydrate of the parent molecules and also the major antigenic determinants unique to this class of

immunoglobulins (33). The other two fragments (Fab), each having a molecular weight of 45,000, appeared to be almost identical in physical, chemical and immunological properties. The relative yields of these three fractions were found to be almost identical and, therefore, it seemed likely that a rabbit antibody molecule consisted of two identical portions joined together to a third portion of a quite different structure. It was demonstrated by Nisonoff et al. (34,35) that each of the Fab fragments had only one antibody combining site.

Nisonoff et al. (34,36) showed that immunoglobulins could be degraded by the combined or successive actions of pepsin and reducing agents (such as cysteine, thioglycolate, and 2-mercaptoethylamine) into fragments essentially similar to those obtained by papain digestion. Treatment with pepsin alone caused the reduction in the molecular weight of the antibody from 160,000 to 106,000 (37). The latter fragment, $[F(ab')_2]$, was shown to be still bivalent and, on subsequent treatment with mercaptoethanol, was reduced into univalent fragments with a molecular weight of about 56,000. Thus, it was conceived that an antibody molecule consisted of two identical fragments, joined through a disulphide bridge, each having one antibody site and that a third fragment was linked to one or both of these fragments through peptide bonds.

Antibody combining site

The forces participating in the specific reaction

between an antibody and antigen are non-covalent and have been shown to be weak. These forces result from the co-operative interactions of electrostatic attractions between charges of opposite sign, Van der Waal's forces, hydrogen bonds (38,39), and hydrophobic bonds (40). The co-operative interaction of all these forces is accompanied with a change in free energy (ΔF) ranging from -7 to -11 Kcal $mole^{-1}$ (41). The change in enthalpy has been found to be small or almost zero (within $\pm$ 2 Kcal/mole) and, therefore, the main contribution to the free energy term, ΔF, is represented by the term $-T\Delta S$, i.e. the reaction is primarily entropy driven. This corresponds to a positive value (of about 20 e.u.) for the change in entropy for these association reactions. This has been interpreted as being due to the release of the water molecules, originally 'frozen' on the surface of the combining sites, into the bulk of the solution. Only a few typical examples illustrating the participation of these different forces will be cited here. In some of the earlier studies, Pressman and Siegel (42) pointed out that an appropriate steric configuration of the hapten was the most important factor favouring binding of the hapten with homologous antibodies, and that as a result of this, the maximum interplay of Van der Waal's forces could take place. Thus, 4-chlorophthalic acid was shown to be bound more strongly and 4-iodophthalic acid less strongly to antibodies homologous to the 4-azophthalate ion than phthalate ion itself. In the former case, it was postulated that the Van der Waal's

attraction was larger than steric hindrance due to the chlorine atom; conversely, the latter case was explained by assuming that the larger steric hindrance of the iodine atom had outweighed the Van der Waal's forces which favoured binding.

Evidence indicating the presence of a charged group in the antibody combining site of opposite sign to that of the determinant group in the antigen molecule was provided by Epstein and Singer (43). Using antibodies to the para-azo-benzene arsonate group and a bivalent benzene arsonic acid hapten, they showed that $-NH_3^+$ group of lysine had to be ionized for association to occur.

On the other hand, formation of antibodies can be directed against non-ionizable haptens, such as the dinitro-phenyl group (44), the free energy term (ΔF) being of the same order of magnitude, i.e. -11 Kcal $mole^{-1}$; it is likely that in these associations the participation of hydrophobic bonds is the predominant factor driving these reactions. These bonds would result from the tendency of the hydrophobic portions of the combining molecules to aggregate with one another in water and, thus, reduce the surface area exposed to the polar environment.

Karush (45) studied the binding of various Lacdyes*

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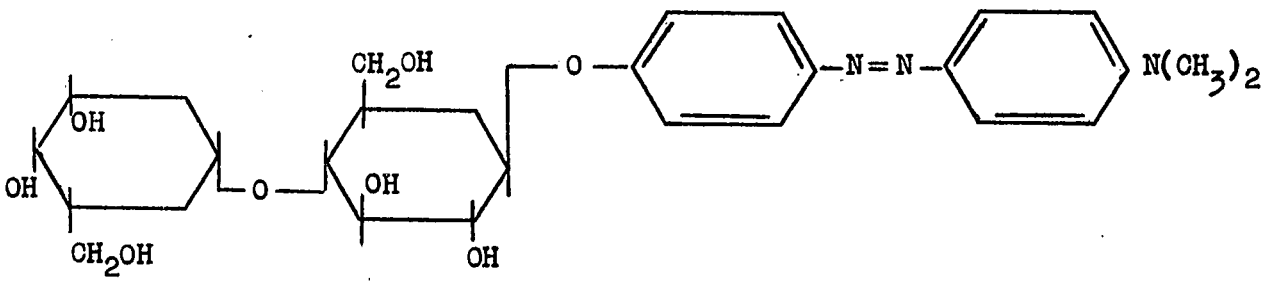
*e.g. p-(p-dimethylaminobenzeneazo)phenyl-β-lactoside



and lactosides with anti-p-azophenyl-β-lactoside antibodies and showed that the dominant contribution to the stability of the antibody-Lacdye complex stemmed from the interaction between the disaccharide portion and the protein, and concluded that in this case hydrogen bonding provided the main intermolecular cohesive force.

Pressman et al. (46) were able to show that antibodies specific to the para-azobenzene arsonate group did not react with apparently similar molecules in which the arsonate group was substituted by carboxylate, sulphonate, methyl arsonate or stibonate group, yet did show the same affinity for phosphoric acid derivatives. This demonstrated that the only hapten which could fit the antibody site produced against the p-azobenzene arsonate ion was the corresponding azobenzene phosphonate derivative, which could be fitted into the combining site capable of accommodating the larger but otherwise similar, p-azobenzene arsonate ion.

Pauling postulated in 1940 (47), on the basis of rather limited data available at the time, that all antibody molecules possessed polypeptide chains identical to those of normal globulins and differed only in the configuration of these chains; he ascribed the observed versatility of antibodies, with respect to their complementariness to different antigens, to the existence of an extremely large number of accessible configurations. Furthermore, his postulate that an antibody molecule had at most two combining sites was verified

several years later by a number of investigators (48-51). Moreover, the isolation of two antibody fragments, each with a single combining site, also indicated very convincingly that antibody molecules were bivalent (52). In addition, these two sites on a single molecule always appeared to have the same specificity (53-55).

The combining site is visualized as the three-dimensional surface area of the antibody molecule which is directly involved in combination with the antigen. For example, there is evidence that antibodies consist of heterogeneous populations of molecules having combining sites of various sizes (56). From studies of the inhibition of precipitation of dextran-antidextran complexes with a series of 1-6 linked oligosaccharides of increasing chain length, it was shown that the limit of inhibition was reached with a hexasaccharide (57-59). Although the most extended form of the determinant measures 34 x 12 x 8 Å, the exact shape of the antibody combining site is not known, since one can also visualize that the antibody may be directed against some partially folded and compact form of the hexasaccharide. Moreover, it is likely that the antibody combining sites complementary to the hexasaccharide may have different shapes directed against the many possible configurations of the determinant. Similar inhibition studies have been done with poly-L-alanyl-anti-poly-L-alanyl and poly-D-alanyl-anti-poly-D-alanyl systems (60). In both cases pentapeptides were not better inhibitors than tetrapeptides,

suggesting that the size of the combining sites of the antibodies studied corresponded to a peptide composed of not more than four alanine residues. From such studies it can be inferred that the antibody combining site represents only a small portion of the antibody molecule, i.e. approximately 1% of the total surface of the immunoglobulin molecules. Very little is known about the composition of antibody sites, although there is some evidence to show that the combining sites of anti-hapten antibodies contain the tyrosyl residue (61) and that it may contain a charge opposite to that of the hapten (62).

As already mentioned, the enzymatic cleavage of antibody molecules has led to the isolation of active, univalent fragments having a molecular weight of about 50,000. These fragments consist of the L chain joined onto a portion of the H chain. Thus, the antibody activity must reside either in the L chain, or in the portion of the H chain, or must be jointly made up by portions of both chains. Fleischman et al. (27) demonstrated with a horse anti-diphtheria toxoid system that the inhibiting activity of the H chain was equivalent to that of the original antibody; this activity was specific, as the heavy chains from anti-rabbit γ-globulin or inert γ-globulin had no inhibitory power. Utsumi and Karush (63) used purified rabbit anti-Lac (p-azophenyl-β-lactoside) antibody, reduced in aqueous solution, and separated the chains on a Sephadex column in 0.03 M sodium decyl sulphate.

After removal of the detergent, the heavy chain retained 87% of the affinity. These experiments provide a clear, quantitative demonstration that the isolated heavy chain contains combining specificity for antigen.

Isolated light chains had never been shown to retain activity; however, there is some evidence for the participation of the light chains at the active site. Metzger and Singer (64), in their study of rabbit antibodies to the hapten dinitrophenyl-lysine, observed that the separation of the chains of the reduced antibody was less effective when hapten was present. This result suggested that the hapten stabilized the non-covalent association of the two chains. Furthermore, Franěk and Nezlin (65) demonstrated that, while H chains isolated from horse antibodies to diphtheria and tetanus toxoid were active, their activity was increased by the addition of either non-specific or specific L chain. Similar observations were made on guinea pig antibodies to two bacteriophages and a hapten (66).

Pressman and Roholt (67,68) iodinated rabbit antihapten antibody with I^{131} in the presence of the hapten and with I^{125} in the absence of the hapten. The two samples were pooled, digested and separated. The ratio of I^{125}/I^{131} was determined for each fraction. Peptide fractions at or near the active site, presumably blocked by the hapten during iodination with I^{131}, were expected to have higher I^{125}/I^{131} ratios than those obtained from the other fractions. Such

fractions were indeed located in the L chain, this result suggesting that the light chain participated in the active site. However, configurational changes elsewhere in the antibody molecule may occur while the hapten is bound, causing decreased iodination of residues at positions other than at the active site.

In summary, it seems that heavy chains provide the main contribution for the specificity for antigen. In addition, the presence of L chains enhances its activity. However, the exact function of the L chains at the active site is still not certain.

IN VITRO MANIFESTATION OF ANTIGEN-ANTIBODY REACTION

The precipitin reaction

One of the most common in vitro reactions between (rabbit) antibodies and antigens is the formation of a flocculent precipitate. The quantitative relationship between the amount of precipitate formed and the amount of antigen added to an antiserum is represented by a typical curve (figure 1), known as the precipitin curve (69,70). It is evident from this curve that the amount of precipitate consisting of both antigen and antibody increases at first in the antibody excess zone, reaches a maximum in the equivalence zone where both reactants are precipitated quantitatively, and then decreases in the antigen excess zone where precipitation is progressively inhibited. The features of this curve can be

Figure 1

PRECIPITIN CURVE

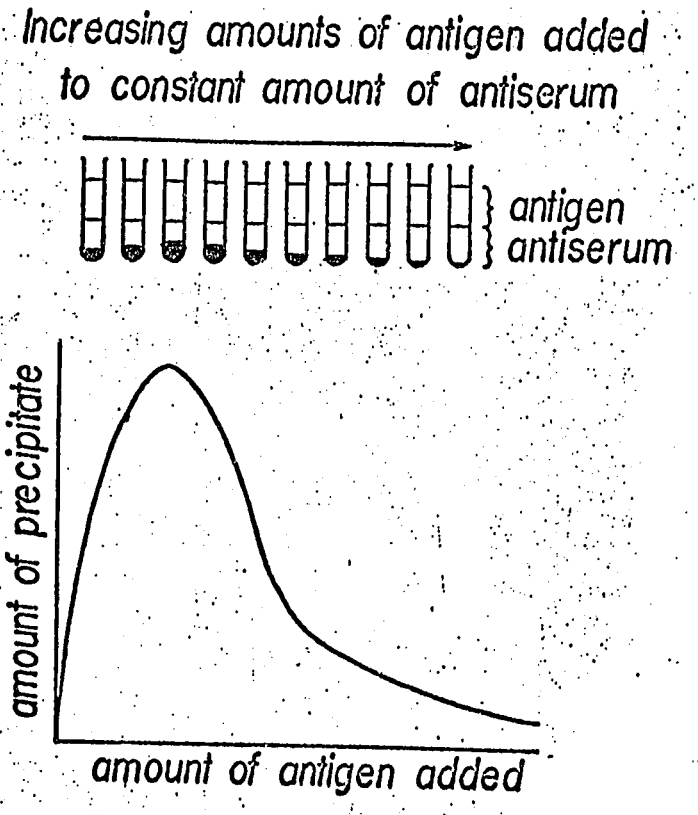


Fig. 1. Precipitin curve.

explained in terms of the framework (or lattice) theory, which is based on the postulate that all antibody and antigen molecules are polyvalent, i.e. that each of these molecules has more than one combining site (47). Accordingly, in the region of antibody excess the precipitate would consist of small aggregates composed primarily of antibody molecules cross-linked by a small number of antigen molecules; in the region of maximum precipitation the antigen-antibody complexes would be cross-linked into larger and more compact aggregates consisting of an alternating and recurring antigen-antibody pattern. Addition of more antigen than that required to combine with all antibody sites would result in the disruption and loosening of this compact regular framework, and in the formation of smaller aggregates. In the limit, in antigen excess, only small complexes would be formed, consisting of one antibody molecule combined with the number of antigen molecules equivalent to the valence of the former; no cross-linking of these complexes could occur and these complexes would remain in solution. As a corollary, with univalent antigens or hapten, or with antibody fragments possessing only one combining site, multimolecular aggregates cannot be formed and the corresponding antibody-antigen or antibody-hapten complexes are soluble.

On the other hand, precipitation can be inhibited or the precipitate formed can be redissolved if the free univalent hapten is added prior to, or after, the addition of a polymeric hapten molecule or of a hapten-protein conjugate to the

anti-serum (71). Pauling et al. (72) developed a theory which quantitatively described this reaction. The application of the technique and theory of hapten inhibition to systems containing chemically well-defined haptens had led to an understanding of the concept and of the main features of configurational complementariness of the combining sites on a molecular basis.

Agglutination reactions

The principle of the passive agglutination technique consists of the adsorption of an antigen to some particulate matter, such as collodion particles (73) or red blood cells (pretreated with tannic acid) (74) and in the cross-linking of these 'sensitized' particles by homologous antibodies. Fundamentally, the mechanism of agglutination reaction has been shown to be similar to that of the precipitin test inasmuch as the antigen molecules are linked together by divalent antibodies (75). To prevent desorption of the antigen from red cells, a number of procedures have been devised by which the antigen is covalently coupled to the erythrocytes, using bis-diazotized benzidine (76-78) or tolylene-2,4-diisocyanate (79) for conjugation. With this method antibodies can be detected in concentrations as low as 0.001 μg/ml (80).

CHAPTER 2

CHEMISTRY OF INSULIN

INSULIN STRUCTURE

(i) Primary structure

It was shown by Chibnall (81) that insulin was peculiar in having a high content of free α-amino groups. This indicated that it was composed of a relatively short polypeptide chain, since free α-amino groups would be found only on those residues (the N-terminal residues) which were present at one end of the chain. In fact, Jensen and Evans (82) had demonstrated that phenylalanine was at the N-terminal of one of the chains.

In order to elucidate the nature of the free amino groups of insulin, Sanger (83) worked out a general method for labelling the N-terminal groups with the help of 1,2,4-fluoro-dinitrobenzene (FDNB), which reacts with the free amino groups of a protein or peptide to form the corresponding DNP-derivatives. The reaction takes place under mildly alkaline conditions under which, normally, dissociation of the peptide bonds does not occur. The DNP-protein derivative is then subjected to hydrolysis with acid which splits the peptide bonds in the chain, leaving the N-terminal residue in the form of its DNP-derivative. The DNP-derivatives of amino acids are

bright yellow substances and can be separated from the unsubstituted amino acids by extraction with ether. With this method the free amino groups in insulin were identified.

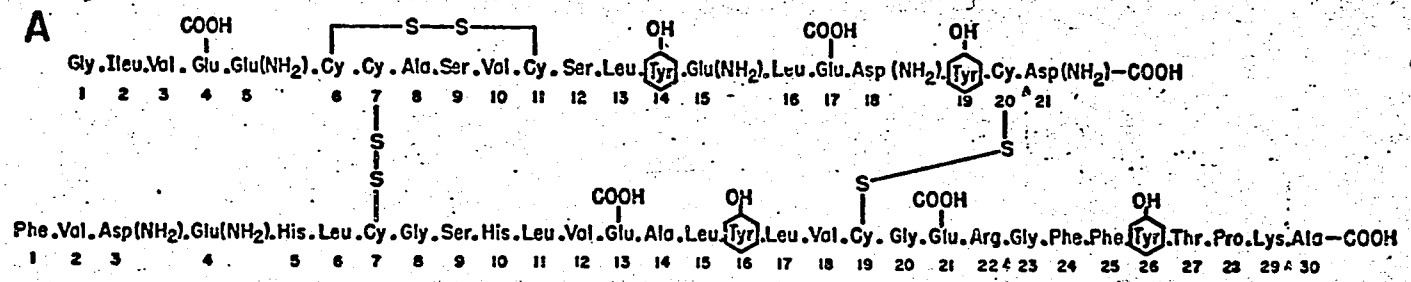
The determination of an amino acid sequence involves the degradation of each peptide chain by partial hydrolysis into a number of shorter peptide chains, separating them from one another by chromatography or by migration in an electric field, analysing each of the shorter chains for its amino acid composition and subsequently determining the amino acid sequence within it by a repetition of the procedure. Finally, all the data on the breakdown products are filed together, as a jigsaw puzzle, in an attempt to represent the sequence in the original chain.

Two methods of partial hydrolysis were employed: (i) acid hydrolysis, which gives a more or less random splitting of the various peptide linkages; (ii) hydrolysis with enzymes, in which only certain specific peptide linkages were split. With the use of these methods, amino acid sequences of a few mammalian insulins were first determined (84-90).

Insulins of the ungulate species consist of an acidic A chain and a basic B chain, containing 21 and 30 amino acid residues, respectively (figure 2). These two chains are joined together by two disulphide bonds, i.e. one disulphide bond between A7 and B7 and the other between A20 and B19. A third disulphide bond is an intrachain bond, connecting A6 to A11 in chain A. In addition, there are three free amino

Figure 2

THE AMINO ACID SEQUENCE OF CATTLE INSULIN


A
COOH
S—S
OH
COOH
OH
Gly.Ileu.Val.Glu.Glu(NH2).Cy.Cy.Ala.Ser.Val.Cy.Ser.Leu.Tyr.Glu(NH2).Leu.Glu.Asp(NH2).Tyr.Cy.Asp(NH2)—COOH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
S
S
S
S
COOH
OH
COOH
OH
Phe.Val.Asp(NH2).Glu(NH2).His.Leu.Cy.Gly.Ser.His.Leu.Val.Glu.Ala.Leu.Tyr.Leu.Val.Cy.Gly.Glu.Arg.Gly.Phe.Phe.Tyr.Thr.Pro.Lys.Ala—COOH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
B

groups in the molecule: the N-terminal of glycine in A chain, the N-terminal of phenylalanine of B chain, and ε-amino group of the lysyl residue in B29. In recent years, the amino acid sequences of insulins from sperm whale (90), sei whale (91), human (92), dog, rabbit, rat (93), cod (94) and other fish insulins (94-98) have been established. It is interesting to note that insulin molecules of most mammalian species differ among each other in positions A8, A9, A10 and in B30 (table II). Moreover, it is worth pointing out that there are two different rat insulins which differ from each other in the amino acid at position B29: one having lysine and the other having methionine.

(ii) Secondary structure

The secondary structure of insulin can be defined as the way in which amino acids are oriented with respect to each other within a turn of helix or within a short stretch of a polypeptide. Several theoretical models for the spatial structure of insulin have been advanced. Each makes use of the essential physical characteristics of insulin, but also involves unproved assumptions. By far the most widely accepted one is that of Lindley and Rollet (99). In this model, the B chain is in the form of a right-handed α-helix. The A chain is also in the form of an α-helix, but with a change in the screw sense, being left-handed from residues A1 to A9, and right-handed from residues A9 to A21. This change of sense of the helix causes the α-β carbon-carbon bonds of the two halves

Table II

COMPARISON OF AMINO ACID SEQUENCE* OF PIG INSULIN WITH OTHER MAMMALIAN AND CHICKEN INSULINS

Source	A chain				B chain					
	4	8	9	10	1	2	3	27	29	30
Pig	Glu	Thr	Ser	Ilu	Phe	Val	Asn	Thr	Lys	Ala
Cattle	-	Ala	-	Val	-	-	-	-	-	-
Sheep	-	Ala	Gly	Val	-	-	-	-	-	-
Horse	-	Thr	Gly	Val	-	-	-	-	-	-
Dog	-	-	-	-	-	-	-	-	-	-
Sperm	-	-	-	-	-	-	-	-	-	-
Fin whale	-	-	-	-	-	-	-	-	-	-
Sei whale	-	Ala	-	Thr	-	-	-	-	-	-
Human	-	-	-	-	-	-	-	-	-	Thr
Elephant	-	-	Gly	Val	-	-	-	-	-	Thr
Rabbit	-	-	-	-	-	-	-	-	-	Ser
Goat	-	Ala	Gly	Val	-	-	-	-	-	-
Rat 1	Asp	-	-	-	-	-	Lys	-	-	Ser
Rat	Asp	-	-	-	-	-	Lys	-	Met	Ser
Chicken	-	His	Asn	Thr	Ala	Ala	-	Ser	-	-

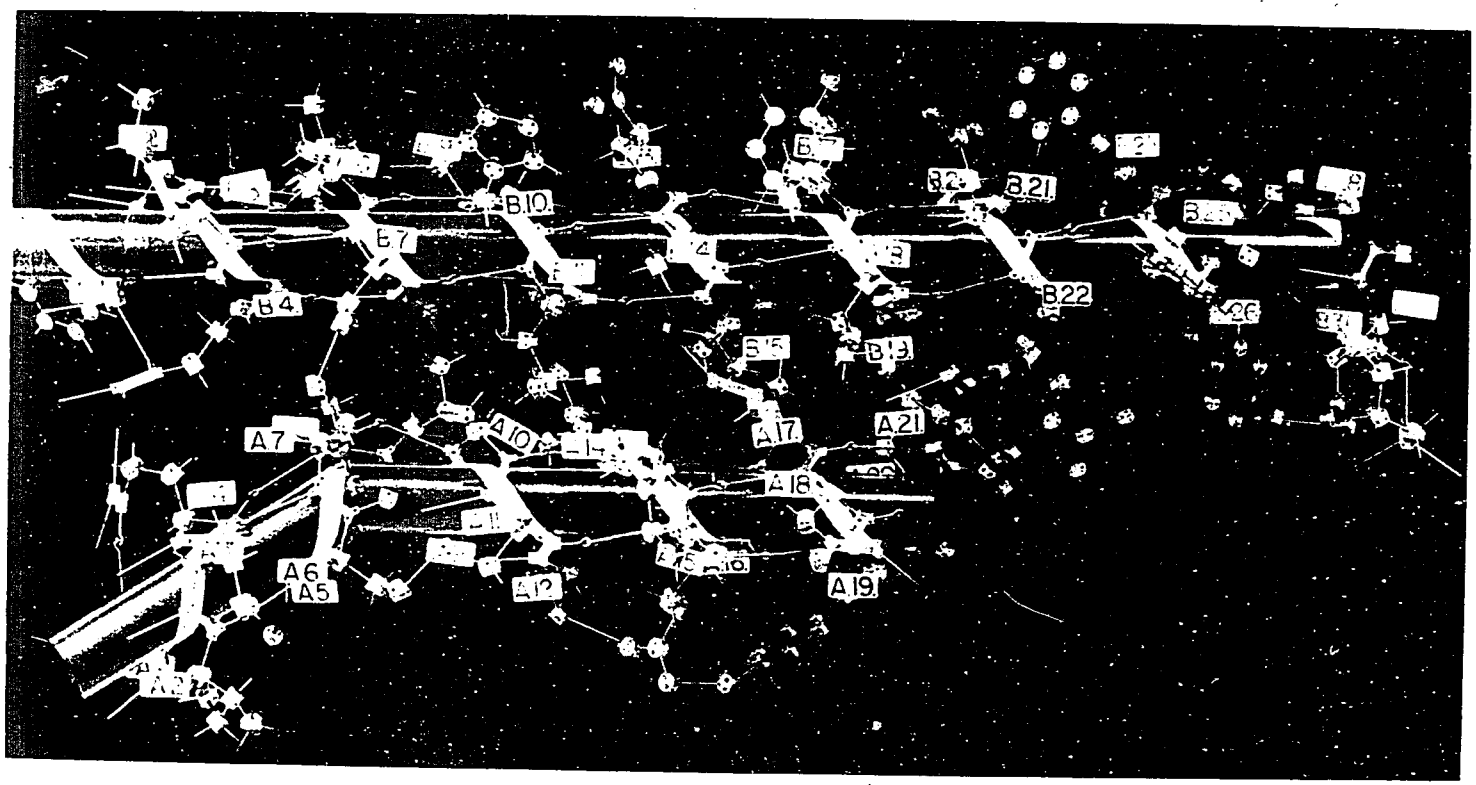
*Sequence is identical except in position indicated.

of the L-cystine molecule to point towards each other so that a disulphide bond can be formed with an acceptable dihedral angle of 90° (figure 3).

The evidence that the peptide chains of the insulin molecule have the form of an α-helix comes from X-ray studies of Low (100) on crystals of acid insulin sulphate, which are orthorhombic. The X-ray data suggest a rod-like chain structure running parallel to the α-axis of the unit cell, which is 44 - 44.5 Å in length. Another elegant method of study is that of determining the rate of deuterium exchange between a protein molecule and the surrounding aqueous medium. In the A and B chains of beef insulin, there are 91 potentially exchangeable hydrogens. Morrison (101) noted that 81 atoms were exchangeable with deuterium, but that four distinct rates of exchange appeared to be present. It seems probable that the less readily exchangeable hydrogen atoms are those in the peptide links located in the central portion of the A and B chains, between the two interchain disulphide bonds. The loose ends of these chains would probably exchange their hydrogens more readily, as would the peptide hydrogens in the loop of the A chain produced by the intrachain disulphide link between A6 and A11. These findings indicate the existence of a hydrogen-bonded structure in a portion of the insulin molecule. As for the four tyrosyl residues, Springell (102) observed that under conditions in which iodination of insulin was carefully controlled, the A chain could be iodinated

Figure 3

MODEL OF INSULIN PROPOSED BY LINDLEY AND ROLLETT


B.10
B7
B4
B.21
B.22
B15
B19
A7
A10
A17
A21
A18
A6
A5
A12
A19

exclusively, suggesting that the tyrosyl groups of the A chain were exposed and that those of the B chain were not.

Moreover, Laskowski and co-workers (103), using ultraviolet difference spectra for the detection of the hydrogen-bond rupture, demonstrated that the B26 tyrosyl residue was hydrogen bonded to a non-ionizable acceptor, and the B16 tyrosyl group was hydrogen bonded to an ionizable acceptor, possibly to B13 glutamic acid residue of a second insulin molecule in the formation of a dimer. Gruen et al. (104) demonstrated that the apparent pK of the ε-amino group in insulin was similar to that of the ε-amino group of a simple lysine, thus indicating that the B29 lysyl residue was not hydrogen bonded.

(iii) Tertiary structure

The tertiary structure of insulin refers to the way in which each of the peptide chains is folded in space. At present, this three-dimensional configuration of crystalline insulin cannot be described. Various kinds of allomorphic crystals of insulin can be obtained depending on the conditions of crystallization. The unit cell dimensions of insulin crystals from various species and grown in various ways appear to be similar.

(iv) Quaternary structure

Quaternary structure refers to the spatial organization of the molecule as a whole, resulting from interactions of all

the chains. Before the elucidation of the amino acid sequences of insulin by Sanger (83-90) confusion reigned as to the minimum molecular weight of insulin, the estimates ranging from 6,000 to 48,000 (105-109). The decisive counter-current distribution studies of Harfenist and Craig (107) established that insulin in its monomeric form has a molecular weight of 6,000. However, there is yet no way of determining the functional unit under physiological conditions in vivo.

It has long been known that insulin exists in a polymeric form in aqueous solution. From numerous investigations, it was demonstrated that the polymerization depends upon the presence of metal ions, especially zinc. Crystalline zinc-insulin containing two atoms of zinc per unit cell has been shown to be monodisperse in solutions at basic and neutral pH, having a molecular weight of 36,000 (110), which corresponds to six monomer units. However, Fredericq (111) has established the conditions in which the molecular weight of insulin can vary from the monomeric form of 6,000 to 36,000 in a solution containing 0.25% zinc, and to 300,000 in a solution containing 1.0% zinc. It has been suggested that the site of binding of the zinc is through the co-ordination of histidine residue with the zinc ion (112-113).

By contrast, the behaviour of zinc-free insulin is quite different. Over the entire pH range, zinc-free insulin is not homogeneous with respect to molecular weight, which depends on pH and insulin concentration (114). Using the

ultracentrifuge, Miller and Andersson (115) found a molecular weight of 46,000 for insulin at neutral pH and at a concentration of 1% (sedimentation coefficient of 3.55S* and diffusion coefficient of 7.53×10^{-7} $cm^2/sec.$). Later, determinations by osmotic pressure (116) confirmed this value, the calculated molecular weight being 47,000 - 48,000 for 0.5% - 1.0% solution of insulin at pH 7. In the presence of detergent, aggregates of insulin dissociate into smaller molecular weight complexes of 12,500 (117). Furthermore, the complexes of insulin dissociate in acid and alkaline solutions; the minimal molecular weight was found to be 12,000 at an acid pH and 6,000 at a basic pH (114).

When insulin is heated in weakly acidic solutions it forms an insoluble precipitate which is physiologically inactive (118). The precipitate formed in aqueous solutions is re-solubilized at high pH to insulin with full biological activity (119). Formation of insulin spherites takes place in two stages (119-121). The first stage is characterized by the formation of fibrils and the second by the aggregation of these fibrils to form spherites. These spherites constitute the visible 'heat precipitate'. By heating insulin at pH 2.5, a gel is produced which consists of fibrils having lengths of about 20,000 Å and widths of about 150 Å. In stronger acid

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*Svedberg unit = 10^{-13} sec.

(0.1 N HCl) these fibrils aggregate to form spherites in which the fibrils are statistically oriented radially. These changes are summarized diagrammatically in figure 4.

ANTIGENICITY OF INSULIN

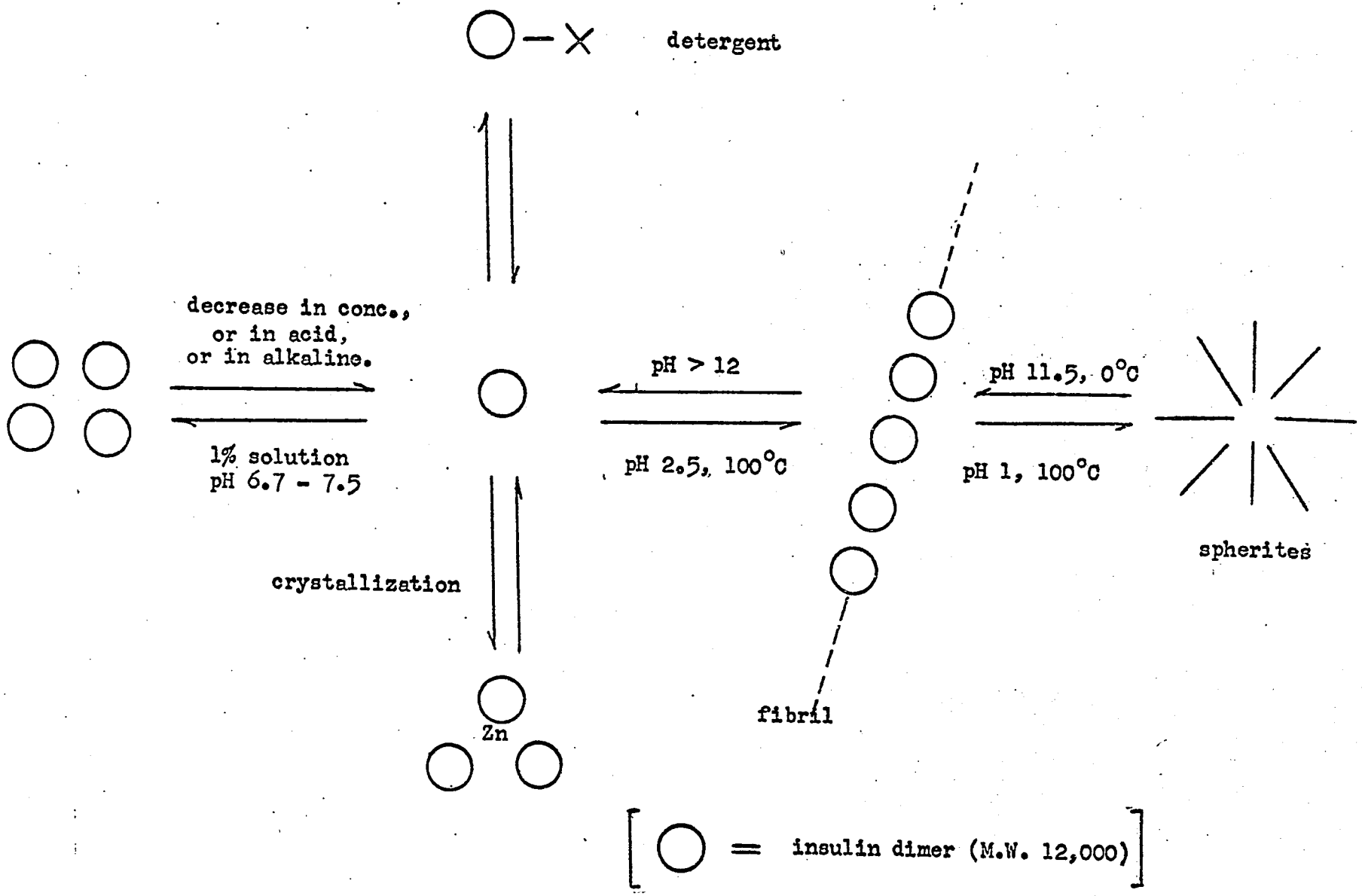
General aspect

Since insulin has been used therapeutically, it has been considered as a potential antigen, and antibodies to it have been sought in the serum of animals and men submitted to insulin treatment. At first, many of the immunological reactions observed in diabetic patients were attributed to impurities in the insulin preparations, but later it became obvious that the hormone itself was capable of inducing antibody production. Depending upon the technique used for the detection of anti-insulin antibodies, the acquired antibodies have been described by different terms.

Tuft (122) was the first to show that often patients allergic to crude insulin were also allergic to re-crystallized insulin but not necessarily to the protein extracts of the animal from which the insulin had been obtained. The serum of such a patient contained two types of antibodies with distinct immunological manifestations. (i) a skin-sensitizing or reaginic activity demonstrated by the Prausnitz-Küstner test, and (ii) a precipitating antibody demonstrated with the capillary tube technique by reaction between the serum and insulin. Banting et al. (123) observed that the serum from an

Figure 4

DIFFERENT FORM OF AGGREGATION OF INSULIN
IN SOLUTION UNDER DIFFERENT CONDITIONS


detergent
decrease in conc.,
or in acid,
or in alkaline.
1% solution
pH 6.7 - 7.5
pH > 12
pH 2.5, 100°C
pH 11.5, 0°C
pH 1, 100°C
spherites
crystallization
Zn
fibril
[○ = insulin dimer (M.W. 12,000)]

insulin resistant patient could neutralize the hypoglycemic effect of insulin as demonstrated by the mouse-convulsion test.

The observations of Tuft (122) and of Banting et al. (123) were further extended by Lowell (124); the serum of an insulin allergic and resistant patient was capable of protecting mice against the hypoglycemic effect of insulin and of passively sensitizing the skin of the normal subject. The reagin was destroyed by heating the serum at 57°C for 2 hours, whereas the insulin neutralizing effect persisted after heating. This study together with others clearly indicated that insulin allergy and insulin resistance were due to different antibodies. Fractional precipitation of the proteins of such active sera showed that the insulin neutralizing component was associated with globulins (123,125), and by starch-block electrophoresis it was shown to be localized in the slow gamma-globulin region (126). In fact, Loveless and Cann (127) separated the skin sensitizing antibody from insulin-neutralizing antibody in different serum fractions obtained by electrophoresis-convection. The reagin was found predominantly in the beta-globulin, whereas the insulin neutralizing antibody was associated with gamma-globulin. The reaginic activity was heat labile, whereas the insulin-neutralizing activity was heat stable. In addition, the passive transfer of insulin sensitivity to the skin of a normal subject by intradermal injection of reagins could be blocked by addition of the heat-inactivated serum from an insulin resistant patient to the insulin prior to injection. This

acquired activity was also described as 'blocking antibody'.

Berson et al. (128,129) demonstrated that anti-insulin antibodies were produced by insulin-resistant patients as well as by most diabetic patients treated with insulin. Thus, in their study of the turnover rate and disappearance of I^{131}-insulin in normal and diabetic subjects, they observed that the disappearance of insulin was rapid in all diabetic or nondiabetic subjects never treated with insulin or treated for a few weeks. In contrast, the labelled insulin showed a prolonged retention in the circulation of patients under prolonged insulin therapy. Using I^{131}-labelled insulin, it was shown by paper and starch block electrophoresis (128,129), paper chromatography and density gradient centrifugation that insulin was bound to antibody globulins in the plasma of treated subjects. Moreover, it was inferred that in treated patients insulin was restricted from easy transcapillary passage, whereas in non-treated persons and animals the small unbound insulin molecule could readily escape from the blood stream to be rapidly metabolized in extravascular sites, notably in liver and kidney; consequently in normal recipients the half-life of I^{131}-labelled insulin was short, i.e. only thirty-five minutes in man and twenty-five minutes in the rabbit (129).

The presence of insulin-binding antibodies in all insulin-treated human subjects was subsequently confirmed by Skom and Talmage (130) who showed that rabbit anti-human serum

globulin uniformly precipitated I^{131}-labelled insulin from the sera of treated subjects only. Similarly, Gordis (131) demonstrated that cold alkaline ethanol-salt solutions could precipitate insulin-I^{131} together with the serum globulins from the plasma of patients treated with insulin but not of control subjects. Furthermore, Welsh et al. (132) found that the insulin neutralizing capacity of plasma was correlated with marked retention of insulin-I^{131} in the circulation of the plasma donor and confirmed that significant retention of insulin-I^{131} occurred only in treated patients.

Antibodies to insulin in insulin-treated diabetic patients were also demonstrated by agglutination of insulin-erythrocyte conjugates (133).

By electrophoresis of serum from insulin-resistant patients, it was shown that insulin-neutralizing antibody (134), also referred to as blocking antibody (127) or insulin-binding antibody (129), was associated with globulins with a wide range of electrophoretic mobilities, i.e. gamma-globulins and β-γ globulins.

The insulin-neutralizing antibodies delay but do not prevent the hypoglycemic effect (127,129,135,136) of insulin, by binding the insulin in a reversible fashion. Insulin added to serum having insulin-neutralizing properties can be precipitated in a bound form and active insulin can be retrieved from the precipitate (137). Serum capable of binding large quantities of insulin in vivo and in vitro also neutralizes the

hypoglycemic effect of insulin in mice (132,138). On the basis of this overall evidence, it may be suggested that the neutralizing, binding, precipitating, blocking and hemagglutinating capacities of anti-insulin anti-sera are due to the same antibody, i.e. different manifestations of the same antibody.

Insulin antibodies have been repeatedly induced in rabbits, guinea pigs and other animals. The first observation of an insulin-precipitating antibody produced in a horse, immunized subcutaneously with crystalline ox insulin in Freund's adjuvant containing phenol, light paraffin oil and mannide mono-oleate, was reported by Moloney and Aprile (139). On the other hand, these authors were unable to obtain precipitating antibodies in anti-sera of guinea pigs, rabbits, sheep or other horses. However, more recently precipitating antibodies against insulin have been observed in guinea pig (140-144), rabbit (141,142,145) and chicken (143). Thus, it appears that the ability to form antibodies varies with the species and probably with the method and length of immunization.

Two types of 7S gamma-globulin antibodies, different in terms of their electrophoretic mobilities, i.e. gamma-1 and gamma-2 globulins, against insulin in guinea pig have been reported (146). While both types of antibodies showed very similar binding characteristics, they differed in their biological activity. The slow moving gamma-2 antibodies provoked hemagglutination and hemolysis in the presence of

complement, whereas the faster moving gamma-1 antibodies gave PCA reactions in the guinea pig (144).

Valence of insulin molecule

From the evaluation of the dissociation curves obtained in the study of the insulin-antibody system, Berson and Yalow (147) concluded that there may be two antibodies having insulin-binding capacity, i.e. one leading to a rapidly dissociating and the other to a slowly dissociating insulin-antibody complex. Further analysis of the steady state and transient-state kinetics of this system suggested that the insulin molecule was univalent and that it combined with two different orders of antibody combining sites (148). To supplement the kinetic data, ultracentrifugal analysis of labelled insulin antibody complexes in the region of marked antibody excess (at least 200-fold over insulin) was carried out (149). The sedimentation velocities of the insulin-antibody complexes was determined to be 1.054 times that of gamma globulin. The ratios of the molecular weights of two particles of identical partial specific volume ($\bar{v}$) sedimenting in the same medium, of density ρ and viscosity η, may be expressed (149) as a function of the relative sedimentation constants and frictional ratios by a relation readily derived from the Svedberg equation:

$$\frac{S_c}{S_\gamma} = \frac{1.49}{(176,000)^{2/3}} \cdot \frac{M_c^{2/3}}{(f/f_o)_c}$$

where the subscripts c and γ refer to the insulin-antibody complex and gamma globulin respectively, M is molecular weight and f/f_o is the frictional ratio. To be compatible with the ratio of sedimentation velocities obtained, it is required that the frictional ratio of the complex be 1.49 and the molecular weight of the complex be 182,000, i.e. an insulin molecule of molecular weight of 6,000 combining with an antibody of molecular weight of 176,000 under experimental conditions used. On the basis of these studies, it was suggested that insulin had two antigenic sites, and that for each of these sites there was a single species of antibodies and that each had a different dissociation constant.

Precipitating antibodies to insulin have been produced in guinea pigs and rabbits (141,142); the ratio of antibody to antigen at the point of maximum precipitation was 20.1 (for rabbit) and 27.9 (for guinea pig) immunized with crystalline beef insulin in complete Freund's adjuvant. Furthermore, it was demonstrated by Arquilla and Coblence (150) that rabbit antibodies against beef insulin can be dissociated to varying degree from an immuno-adsorbent prepared by coupling insulin to stroma, dispersed in cellulose, over a wide range of pH, temperature, and salt concentration. It, therefore, appeared likely that insulin, like other proteins, was an antigen with multiple antigenic sites eliciting the formation of multiple antibodies with varying binding affinities for each of these sites.

The differences observed with human and animal antisera would indicate that results obtained with sera of one species cannot be unreservedly considered to hold for another species. Beef insulin differs from human insulin in the C-terminal amino acid of the B chain and in positions 8 and 10 of the A chain, whereas pork insulin differs only in the C-terminal. The mixture of beef and pork insulin, which was commercially available, was used in the past for diabetic therapy. The weak antigenicity of beef and pork insulins in human subjects may be attributed to the small differences in the amino acid sequences between these insulins and human insulin. On the other hand, guinea pig insulin differs from beef insulin in eighteen amino acids and from pork in seventeen amino acids out of fifty-one amino acids (figure 5). It has been demonstrated (151) that the highly inbred Hartley strain II guinea pigs can produce antibodies to portions of the beef insulin molecule to which strain XIII guinea pigs cannot produce antibodies. The marked difference between guinea pig insulin and insulin of the other species, therefore, results in the recognition of a greater number of the antigenic determinants of beef or pork insulin by the immuno-competent cells of guinea pigs; consequently one would expect to find a multi-specific spectrum of the corresponding guinea pig antibodies.

Nature of the antigenic determinants of insulin

The initial differences in amino acid sequences of some mammalian insulins as determined by Sanger and his

Figure 5

COMPARISON OF THE AMINO ACID SEQUENCE OF BEEF AND GUINEA PIG INSULINS

A chain

	4	8	9	10	12	13	14	18
Guinea pig	Asp.	Thr.	Gly.	Thr.	Thr.	Arg.	His.	Ser.
Beef	Glu.	Ala.	Ser.	Val.	Ser.	Leu.	Tyr.	Asn.

B chain

	3	4	10	14	17	20	21	22	27	30
Guinea pig	Ser.	Arg.	Asn.	Thr.	Ser.	Glu.	Asp.	Asp.	Ilu.	Asp.
Beef	Asn.	Glu.	His.	Ala.	Leu.	Gly.	Glu.	Arg.	Thr.	Ala.

co-workers were confined to positions 8, 9, and 10 of the A chain and to position 30 of the B chain of the molecule only (figure 2). Despite the relatively small differences in amino acid sequences, human antisera specific to beef and pork insulins have been obtained. If the antigenicity of the protein molecule depends on some distinct amino acid sequence, only the 8-10 region of the A chain and the carboxyl end of the B chain should represent potential sites of antigenicity. Berson and Yallow (152) have shown that insulin-binding antibodies in the sera of subjects immunized with beef and pork insulins react with four ungulate species to a different extent, the order of reactivity being beef $\cong$ sheep > pork > horse. The insulins differing in only one of the A8-10 residues reacted more alike than insulins differing in two to more residues (table II). These results suggested that the antigenic site was located within the intra-chain disulphide region.

Support for the conclusion that the A chain is the principal site of the antigenicity comes from the studies of Wilson, Dixon, and Wardlaw (153). Cod and beef insulins were split into their respective A and B chains and the corresponding hybrid insulin, i.e. cod A chain-beef B chain and beef A chain-cod B chain, were synthesized. Cod A chain-beef B chain, like pure cod insulin, reacted weakly with guinea pig antibodies to beef insulin, whereas beef A chain-cod B chain reacted as strongly as beef insulin.

On the other hand, highly purified oxidized A chain

and B chain did not react with insulin antibody. As has already been discussed, the principal site of the antigenicity resides in the A chain; the non-reactivity of the totally reduced A chain may be attributed to the conformation changes resulting from the rupture of the disulphide bond. In fact, rotatory dispersion measurements on the separated, reduced, and alkylated A and B chains indicated that both are present as random coils (154).

Changes in the original secondary and the tertiary structures of the polypeptide chains might lead to the masking of the original determinant groups present on the surface of the molecule, at the same time exposing new antigenic groups on the surface of the molecule. Thus, in the studies by Yagi et al. (155) antibodies were produced in guinea pigs against A and B chains of bovine insulin. It was observed that antibodies against A chain reacted with A chain, but not with B chain or native insulin. Antibodies against B chain reacted strongly with both B chain and insulin, but not with A chain. Antibodies against insulin reacted strongly with insulin, weakly with B chain, and not at all with A chain. From these findings it was concluded that the anti-insulin antibodies in antisera to the B chain and to insulin differed in their specificities inasmuch as they were directed against different individual antigenic regions of the insulin molecule.

Concerning the importance of the tertiary structure of insulin for its antigenicity, it is worth pointing out that

while pork and sperm whale insulins have identical amino acid sequences (table II), anti-beef insulin sera produced in guinea pigs or in man reacted in an identical fashion with these insulins. However, with some human antisera it was found that the insulin from sperm whale consistently failed to inhibit competitively the binding between I^{131}-labelled beef insulin and homologous antibody as strongly as did pork insulin (156). Similarly, it was noted (157) that dog plasma insulin inhibited the binding between I^{131}-beef insulin and homologous antibody as strongly as crystalline dog insulin, but that the inhibition was weaker than that produced by crystalline pork insulin or plasma pork insulin, which in turn inhibited to the same extent. Thus, it appears that dog plasma insulin, like crystalline dog insulin is distinguishable from pig plasma insulin and crystalline pork insulin using certain human antiserum. As already shown, dog insulin has the same amino acid sequences as pork insulin; it would seem, therefore, that these two insulins differ in their conformations despite their identical amino acid sequences and that their distinct structures are due to the specificity of the biosynthetic processes, which are genetically controlled.

Moloney and Coval (136) discovered a noteworthy variability in the reactivity of anti-insulin sera with preparations of insulins from homologous species. These authors immunized guinea pigs with crystalline pig and ox insulins in Freund's adjuvant. The general health of the

guinea pigs which were producing antibodies to insulin was excellent. The unimpaired health and normal blood-glucose levels observed support the inference that the antibodies present in the animals did not neutralize endogeneous insulin. In addition, the neutralizing activity of serum from guinea pigs which had been inoculated with crystalline pig or ox insulin was tested by the mouse-convulsion assay procedure. It was found that these sera neutralized not only crystalline pig and ox insulin, but also crude extracts of pig, ox, sheep and rabbit insulins. No neutralizing activity was ever observed with normal guinea pig, rabbit or sheep sera. Furthermore, six batches of guinea pig insulin were prepared. Five of these could not be neutralized by the immune serum but one could. This particular batch was prepared from glands which had been stored in the cold for six months. All the other batches were prepared from fresh glands. Similar observations were reported for the horse (139). On the basis of these observations, Moloney and Coval (136) suggested that two forms of insulin could be distinguished by studying their immunological reactivity with homologous antisera. They have called the form of endogeneous insulin that does not react with homologous antiserum 'native' and have suggested that this type can be converted, for example, by certain storage or purification procedures, into an 'altered' form that would react.

SCOPE OF THE PRESENT STUDY

The present study is divided into three main parts.

(I) (a) The preparation of insulin-BSA conjugates for use as antigen, with tolylene-2,4-diisocyanate as coupling agent, and the analysis of the insulin-BSA conjugates.

(b) To increase the solubility of the monomeric unit of insulin, insulin was coupled to the sodium salt of 4-fluoro-3-nitrobenzene sulphonic acid (FNBSNa).

(II) The production of precipitating antibodies specific to insulin by immunization of rabbits with insulin-BSA conjugates.

(III) The preparation, by electrolytic reduction of insulin, of (i) B chain, (ii) A chain with the intra-chain disulphide bond intact, and (iii) the totally reduced A chain; the antigenic properties of these chains were subsequently established.

CHAPTER 3

PREPARATION OF INSULIN-BOVINE ALBUMIN CONJUGATE

Introduction

As described in the previous chapter, antibodies of the non-precipitating type against insulin have been demonstrated in the serum of immunized animals and of human individuals subjected to insulin treatment (146,148,158,159). It is not clear from these studies if the failure to precipitate antibodies in some of the anti-insulin antisera was due to the low concentration of these antibodies or to some special physical-chemical features of the antibodies or of antibody-antigen complexes. Only in recent years have a few investigators succeeded in producing precipitating anti-insulin antibodies in the horse (139), rabbit (141,142), and guinea pig (140). The difficulty in producing precipitating antibodies against insulin in animals may be attributed to the following factors: (i) insulin is weakly antigenic, presumably because of its low molecular weight, (ii) the injection of large doses of insulin causes often the death of the animals due to the hypoglycemic effect of insulin, and (iii) structural similarity to autologous insulin to which the host is normally tolerant.

To overcome these limitations, in the present study

insulin was coupled, as if it were a simple hapten, to a large protein carrier, i.e. bovine serum albumin (BSA). The coupling agent used was tolylene-2,4-diisocyanate (referred to hereafter as TDIC), which previously had been successfully used for conjugating protein to protein and protein to red cells (79, 160). Thus, insulin was incorporated into a significantly larger molecular weight compound, which would be expected on general grounds to render the determinant groups of insulin more immunogenic; in addition, it was expected that the hormonal activity of insulin would be abolished by coupling it covalently to a carrier protein.

Materials and Methods

Crystalline beef zinc-insulin (IN, M.W. 36,000), lot No. 975, having a potency of 23 units per mg was purchased from Connaught Medical Research Laboratory, Toronto, Ontario. Radioactive labelling of insulin was performed with I^{131} by Charles E. Frosst & Co., Montreal. This batch had a specific activity of 4 mc/mg. Bovine serum albumin (BSA), fraction V powder, was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. Tolylene-2,4-diisocyanate (TDIC) was purchased from Eastman Organic Chemicals, Rochester, New York. This reagent is easily decomposed when exposed to the moisture in the air; hence, the necessary amount of TDIC was removed for each experiment, and the reagent bottle was hermetically closed immediately afterwards.

Preparation of IN-TDIC-BSA conjugate

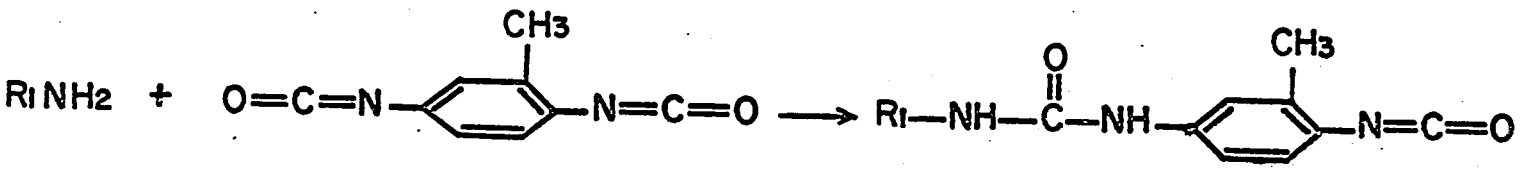
The conjugation procedure involved two stages as shown in the flowsheet in figure 6. Thus, TDIC was first added at 0°C to the insulin solution, represented here as R_1NH_2. At this temperature the sterically less hindered isocyanate group in the para-position reacted readily with the free amino groups. Then the TDIC-insulin conjugate was added to the BSA solution at 37°C, when the second isocyanate group in the ortho-position reacted with the amino groups present on the BSA molecules. Thus, in the first stage, to 5 ml of a 1% solution of insulin in borate buffer, pH 9.5, ionic strength 0.1* was added 0.1 ml of TDIC at 0°C. The latter is mostly insoluble in the aqueous phase and was solidified upon addition at this temperature. After vigorous stirring in an ice bath at 0°C for 35 minutes, the mixture was centrifuged in a Spinco Model L preparative ultracentrifuge at 0°C for 30 minutes at a speed of 15,000 r.p.m. The supernatent was decanted and was allowed to stand at 0°C for an additional hour. In the second stage, this solution of the modified insulin (IN-TDIC) was added to an equal volume of a 1% solution BSA in the same buffer at 37°C; no precipitate was formed when the two solutions were mixed together. After one hour of incubation

*The required amount of insulin was first dissolved in 2.5 ml of 0.04 N NaOH; the solution was then diluted to 5 ml with borate buffer to give the required pH and ionic strength.

Figure 6

FLOWSHEET FOR THE PREPARATION OF IN-TDIC-BSA CONJUGATION

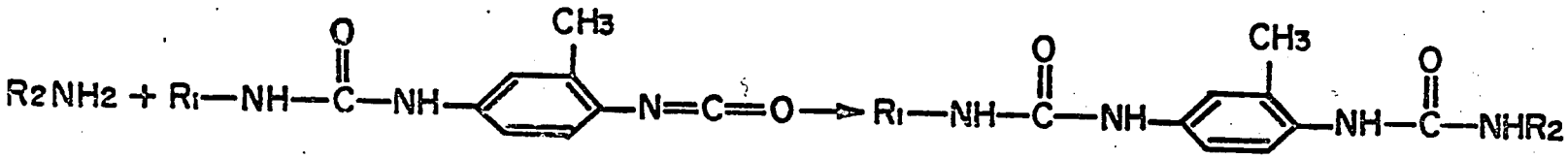
STAGE 1 : TEMPERATURE at 0°C



Tolyiene-2,4-diisocyanate (TDIC)

Protein-TDIC conjugate

STAGE 2 : TEMPERATURE at 37°C



Protein-TDIC conjugate

at 37°C, the solution was dialysed against 0.1 M $(NH_4)_2CO_3$, pH 8.8, to destroy any unreacted isocyanate groups and then dialysed exhaustively against the buffer required for further operations.

For preparative purposes, the conjugate was separated from the uncoupled proteins by electrophoresis on a thick block of Schleicher & Schüll, No. 2695, paper (30 cm x 14 cm), which was saturated with 100 ml of phosphate buffer, pH 7.5, ionic strength 0.025, and placed between two Whatman No. 1 filter papers (55 cm x 14 cm). This block was then equilibrated while passing current through it for 30 minutes in a conventional trough system. Then 100 mg of the reaction mixture was applied on this paper block along the central line. Electrophoresis was carried out at 4°C for 24 hours at 250 V, 17 mA current. At the end of the electrophoresis an imprint of the paper block was taken on Whatman No. 1 filter paper, which was then stained with Brilliant Blue. The conjugate was found to move with a mobility between that of the modified insulin and of the BSA. The portion of the paper block which contained the conjugate was eluted with 0.9% saline; the eluate was dialysed exhaustively against distilled water and freeze-dried. Attempts to separate the conjugates from the unreacted proteins by molecular sieve chromatography with Sephadex G-75, G-100 and G-200 were unsuccessful.

For the preparation of I^{131}-IN-TDIC-BSA conjugate, the I^{131}-labelled insulin was dialysed against four or five

changes of borate buffer, pH 9.5, ionic strength 0.1, until there was no detectable radioactivity in the dialysate. The I^{131}-insulin was further purified by passing through a column of Sephadex G-75 (1 cm x 40 cm). Approximately 1 mc of labelled insulin was added to the solution of unlabelled insulin and the conjugation was performed as described above.

Preparation of the control proteins (IN-TDIC + BSA)

The procedure was repeated up to the end of the first stage. The modified insulin solution was incubated at 37°C for an additional hour in the absence of BSA and then dialysed against 0.1 M $(NH_4)_2CO_3$ and exhaustively against the borate buffer. An equal volume of 1% BSA solution in the same buffer was added to it. The preparation thus obtained is referred to hereafter as (IN-TDIC + BSA).

CHEMICAL ANALYSES OF MODIFIED INSULIN IN-TDIC

The reaction of various aromatic isocyanates with insulin had been studied by several workers in the past. Phenylisocyanate was reported to react only with the free ε-amino groups of egg albumin at pH 8.0 (161). On the other hand, it was demonstrated by Fraenkel-Conrat (162) that even under the mildest conditions (pH 7.3) insulin combines with more m-chlorophenyl isocyanate than could be accounted for by its amino groups alone. However, Anderson (163) reported that

the phenolic groups of insulin are less reactive than the amino groups and, with proper control of the experimental conditions, the extent of the reaction could be limited to the amino groups only. Furthermore, with the bulky fluorescein isothiocyanate, in spite of the reactive isothiocyanate group (164), only the N-terminal free amino groups reacted at pH 9.0 even with prolonged reaction time. Thus, it appeared that the specificity of the reaction of aromatic isocyanates or isothiocyanates depended on the particular reagent used.

In the present study insulin was coupled to BSA, using TDIC as coupling agent. In view of the possible lack of selectivity of TDIC in the coupling reaction, insulin-TDIC conjugates were subjected to chemical analyses for a better characterization and for the elucidation of the mechanism for the coupling reaction.

(A) Determination of decrease in phenolic groups

To determine the relative phenolic group content of the intact insulin and modified insulin preparations, the Folin-Ciocalteu method was used (165). Generally, 0.5 -3.0 mg of protein was used in these analyses. To 9.0 ml of the sample in borate buffer, pH 9.5, ionic strength 0.1, 5 ml of a 20% Na_2CO_3 solution was added slowly with constant mixing. Then 1 ml of phenol reagent was added to it with vigorous stirring. The resulting solution was incubated in a 37°C

water bath for five minutes followed by 30 minutes of incubation at room temperature. The intensity of the colour developed was determined in a Beckman spectrophotometer (Model DU) at 750 mμ against a reagent blank which contained 9 ml of buffer in place of the sample. The ratio of the number of free phenolic groups of the modified insulin molecule (IN-TDIC) to the corresponding number of the intact insulin (i.e. 18) was denoted by the symbol f in the equation below (160)

$$f = \left[\frac{C_{IN}}{C_{IN\text{-}TDIC}} \right] O.D.$$

where C_{IN} and $C_{IN\text{-}TDIC}$ are the concentrations of insulin and modified insulin (IN-TDIC), respectively, required to give the same optical density (O.D.) in the Folin-Ciocalteu analysis.

(B) Determination of free amino groups

The relative number of free amino groups was determined by the ninhydrin colorimetric method as described by Fraenkel-Conrat (166). To 1.0 ml of protein solution was added 1.0 ml of 10% pyridine and 1.0 ml of 2% ninhydrin solutions. The mixture was loosely stoppered and was placed in a boiling water bath for 20 minutes, then cooled and diluted to 50 ml. The optical density of the solution was measured at 570 mμ against a reagent blank. The relative number of free amino groups was determined using the same method as that for the

determination of the phenolic groups.

Results and Discussion

From the curves obtained with the Folin-Ciocalteu and ninhydrin methods for insulin and modified insulin (IN-TDIC), presented in figures 7 and 8, it is evident that all amino groups of insulin were substituted on reaction with TDIC, whereas none of the phenolic groups reacted under the present experimental conditions. According to Sanger et al. (167), the insulin monomer (M.W. 6,000) contains three primary amino groups, the N-terminal α-amino groups of glycine and phenylalanine of the A and B chains, respectively, and the ϵ-amino groups of the lysine residue in the position next to the C-terminal of B chain. The pK values of the two N-terminal α-amino groups may be taken approximately as 7.5 and the pK of the ϵ-amino group of the lysine residue as 11.9. Because of the relatively high pK value of the ϵ-amino group of lysine, this group is expected to be less reactive at neutral or slightly alkaline pH than the N-terminal α-amino groups. In fact, it was reported by Fraenkel-Conrat (162) that in most cases only 65% of the amino groups of insulin had reacted with phenylisocyanate and m-chlorophenylisocyanate; this observation might be attributed to reaction occurring only with the two N-terminal α-amino groups. Furthermore, Tietze et al. (164) reported that no reaction had taken place between the fluorescein isothiocyanate and the ϵ-amino group of lysine at

Figure 7

STANDARD CURVES FOR INSULIN AND MODIFIED INSULIN

(Folin-Ciocalteu method)

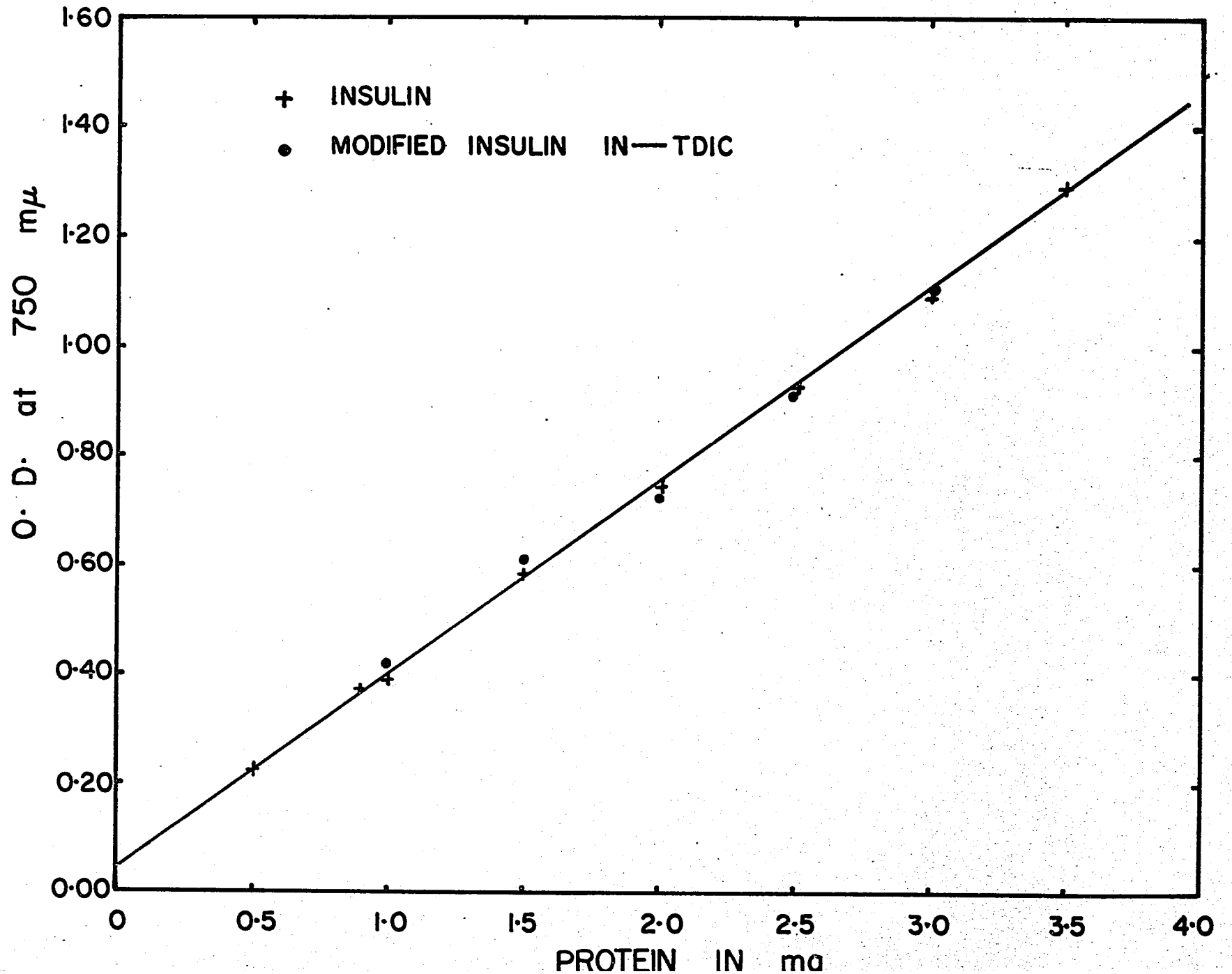
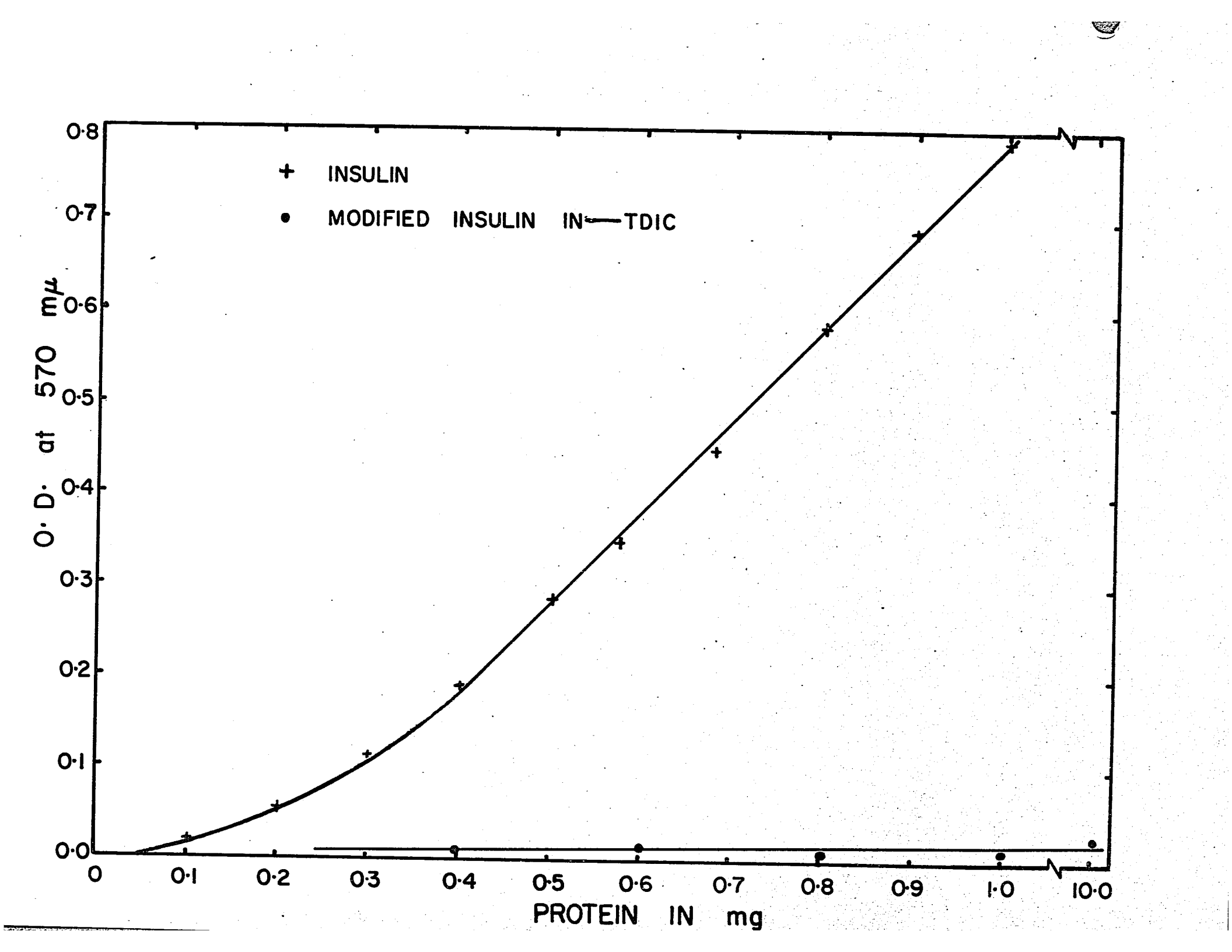

+ INSULIN
● MODIFIED INSULIN IN—TDIC
O·D· at 750 mμ
1·60
1·40
1·20
1·00
0·80
0·60
0·40
0·20
0·00
0
0·5
1·0
1·5
2·0
2·5
3·0
3·5
4·0
PROTEIN IN mg

Figure 8

STANDARD CURVES FOR INSULIN AND MODIFIED INSULIN

(ninhydrin method)


+ INSULIN
• MODIFIED INSULIN IN—TDIC
O·D· at 570 mμ
0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
10·0
PROTEIN IN mg

pH 9.0, when the reaction was carried out for a prolonged time. On the other hand, the analytical data of the present study indicate that the reaction of all amino groups with TDIC was complete within 35 minutes. The increase in reactivity of the ε-amino group under the present experimental conditions was probably brought about by the ionization of the ε-amino group at pH 9.5, which enables its reaction with the isocyanate groups of the TDIC. It is known that the phenolic groups are generally less reactive with respect to the isocyanates than to the free amino groups (163), and therefore it is not surprising that within a short period of incubation used in this study the coupling did not involve the phenolic groups of insulin.

In contrast to insulin, the modified insulin (IN-TDIC), in spite of having three additional aromatic residues coupled to it per monomer unit, dissolved readily in the buffers used (pH 7.5, ionic strength 0.1) up to concentrations of 0.5%. This unexpected increase in solubility reflects a change in the structure of the molecule or in the degree of aggregation. The latter, however, was not substantiated by ultracentrifugal analyses discussed later. Therefore, it would seem that this change in solubility was caused by some intramolecular alteration of the tertiary structure leading to an overall increase in the hydrophylic nature of the molecule.

BIOLOGICAL ACTIVITY OF MODIFIED INSULIN IN-TDIC

IN-TDIC was injected into two rabbits at a dose of 2 mg/kg intraveneously. Blood glucose was determined over a six-hour period*. IN-TDIC produced a 30 to 40 mg percent fall in blood glucose which was maximal at 30 minutes following injection and which remained at this depressed level for the entire period of six hours. The effect was equal to 0.016 mg/kg of the starting insulin. Moreover, the effect of IN-TDIC was somewhat different from insulin inasmuch as the hypoglycemic response due to insulin was essentially back to the base line values after four hours, while the response with IN-TDIC after six hours remained at the level measured after 30 minutes.

The biological activity of IN-TDIC was significantly lower than that of crystalline insulin. As the coupling of insulin with TDIC was carried out under very mild conditions, this loss of activity was attributed to the alteration of the insulin molecule on conjugation with TDIC and not to the method of conjugation per se. Furthermore, this result indicates that the small residual hypoglycemic activity associated with IN-TDIC was not due to contamination with unaltered insulin, which may have remained in the preparation, since the hypoglycemic effect caused by the unaltered insulin would have

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* The author is indebted to Dr. J.D. McColl of F.W. Horner Limited, Montreal, for these physiological determinations.

decreased to the base line value after four hours. Consequently, it is inferred that the prolonged depression of blood glucose after the injection of IN-TDIC reflected the slower turn-over rate of IN-TDIC than that of insulin.

Previously, Stern and White (168) showed that acetylation, which blocked only the free amino groups of insulin, caused no change in hormonal activity as measured by the mouse convulsion test; these results suggested that free amino groups were not essential constituents of the insulin molecule for its biological activity. On the other hand, Hopkins and Wormall (169,170), who had studied the immunological and chemical properties of proteins treated with phenylisocyanate, stated that insulin was inactivated by phenylisocyanate and p-bromophenylisocyanate. In the present study, insulin was coupled to TDIC and the biological activity was significantly reduced. Thus, it would appear that blocking of the free amino groups with large molecules results in depression of the hormonal activity.

EVIDENCE FOR CONJUGATION

(A) Paper electrophoresis

Method

A strip of Whatman No. 3 filter paper (14 cm x 56 cm) was used. After the paper strip had been moistened in phosphate buffer of pH 6.0 (or 7.5 or 9.5) and ionic strength

0.025, and gently blotted, approximately 0.05 ml of the corresponding buffer solution, containing 1% of the control proteins and of a sample of the conjugation product, was applied centrally on a line of about 2 cm. The paper was then placed on a glass tray in a horizontal position, serving as a bridge between two buffer chambers, which in turn were connected with the corresponding outer electrode chambers with paper bridges. A power supply, provided with a voltage regulator, was used to supply stable D.C. voltage. Optimal separations were generally obtained at 110 V and 7.5 mA for 24 hours at 4^{o}C. At the end of the run, the paper was removed from the apparatus, and the excess moisture was immediately blotted from the buffer-soaked ends of the paper. It was then dried in an oven at 100^{o}C for 30 minutes, and immersed for 5 minutes in a solution of 0.1% Brilliant Blue in 5% acetic acid. Removal of the background stain from the filter paper was achieved by repeated washings in 5% acetic acid.

The radioactivity of the components of the electrophoretogram was detected in an automatic recording apparatus* shown in figure 9. After electrophoresis the filter paper was cut into separate strips containing either control proteins (I^{131}-IN-TDIC + BSA) or the conjugate. Each paper strip (A) was mounted on a motor driven pulley (M) in

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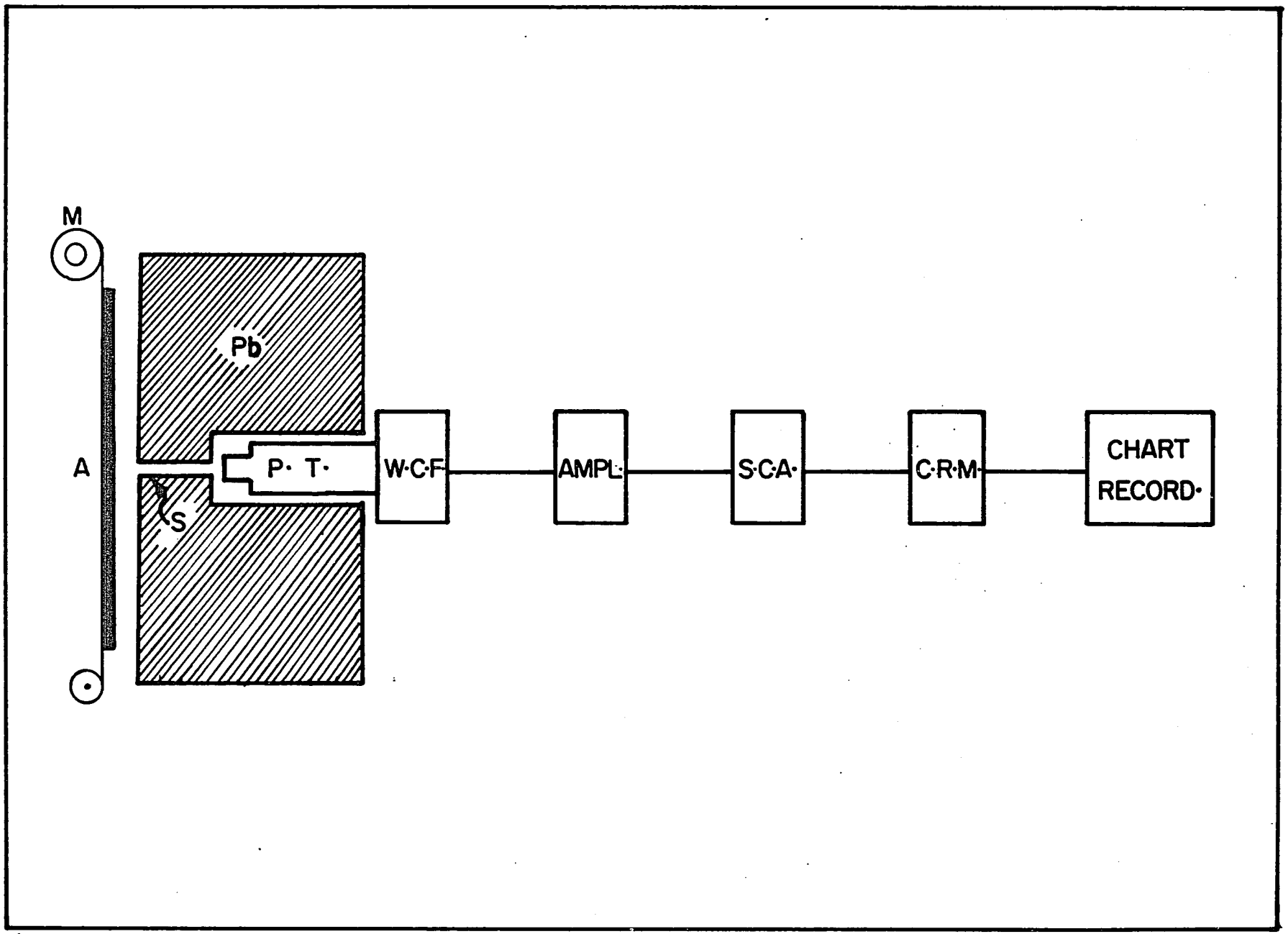
*The author is indebted to Dr. S.K. Mark for his help in building up this apparatus.

Figure 9

BLOCK DIAGRAM OF THE AUTOMATIC RADIOACTIVE RECORDING UNIT

Legend:

A.	- paper strip
M.	- motor driven pulley
S.	- slit
Pb.	- lead castle
P.T.	- the radiation detector which consists of a NaI scintillator, which has been optically coupled to a R.C.A. 6342 photomultiplier tube
W.C.F.	- White Cathode Follower
Ampl.	- linear amplifier
S.C.A.	- single channel analyser
C.R.M.	- counting rate meter


M
A
Pb
P· T·
S
W·C·F·
AMPL·
S·C·A·
C·R·M·
CHART
RECORD·

front of the slit, S (0.1 cm x 3 cm) of the lead castle (Pb)* with a wall thickness of about 5 cm. The radiation detector consisted of a 3.65 cm x 3.65 cm NaI scintillator (containing Tl), which had been optically coupled to a R.C.A. 6342 photomultiplier tube; this whole unit, labelled as P.T., was placed inside the lead castle.

Radiation which passed through the slit was detected by the scintillator which converted the radiation energy into light quanta, which were in turn transformed into electrical signals by the photomultiplier. The output of the photomultiplier was fed to a White Cathode Follower circuit (W.C.F.) and then to a linear amplifier (Ampl.). The White Cathode Follower was used to isolate the linear amplifier from interfering with the operation of the photomultiplier tube. To achieve better reproducibility, a single channel analyser (S.C.A.), which had one lower and one upper level settings, was used to select the output signals. In a typical experiment, the S.C.A. settings were such as to include only signals corresponding to the most intense group of rays (364 KeV) resulting from the decay of I^{131}. The output from the S.C.A. was monitored by a counting rate meter (C.R.M.), which in turn drove the chart recorder whose speed was synchronized with that

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*The lead castle was used to shield the radiation detector from detecting excessive amounts of low energy background radiation in the experimental area.

of the rate of movement of the paper strip. Although this arrangement could have yielded both analog and digital counting rates, only the analog output was recorded on the chart. Thus, it was possible to record the radioactivity along the paper strip.

Results and Discussion

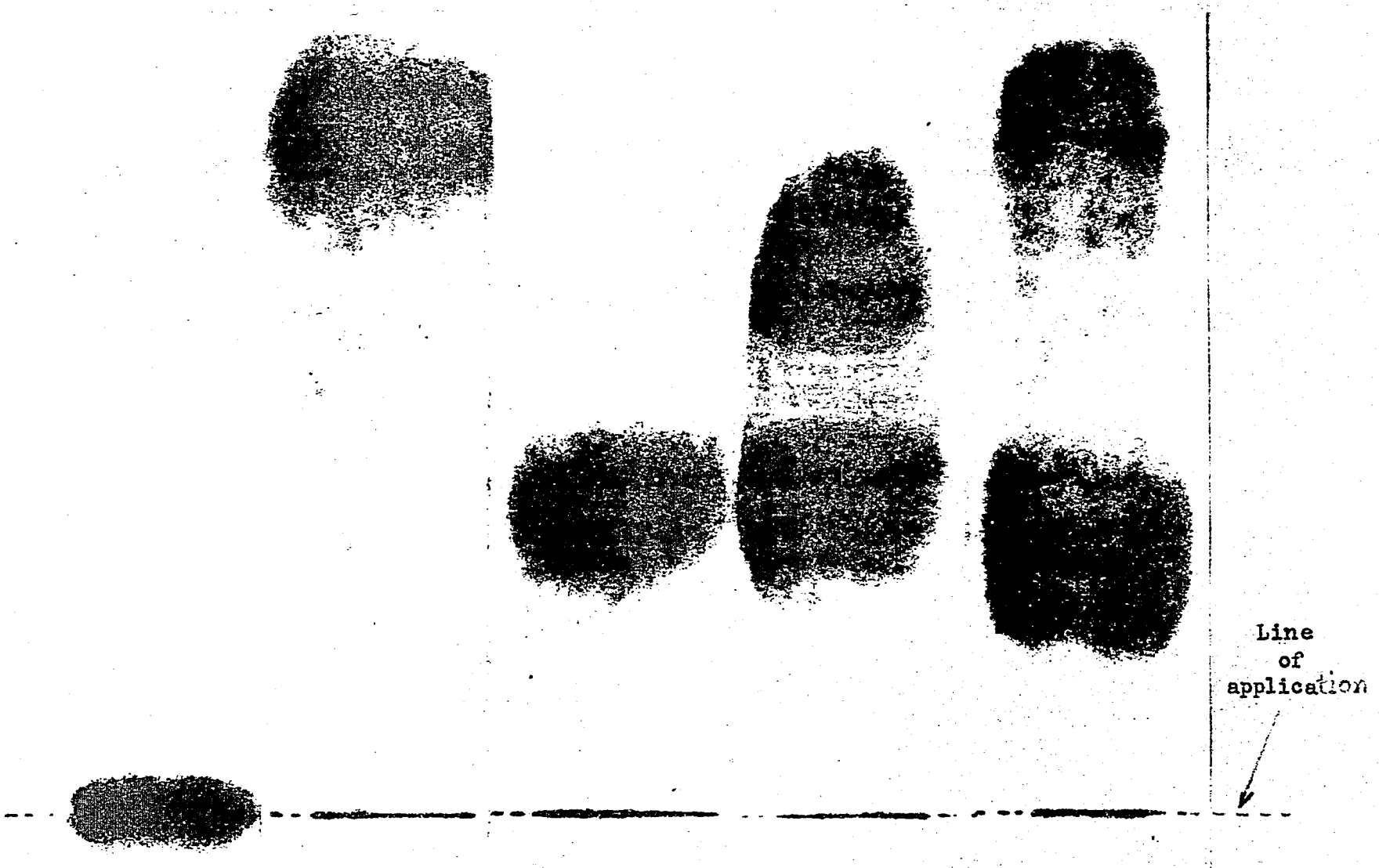
Paper electrophoresis of insulin, modified insulin (IN-TDIC), BSA, the control proteins (IN-TDIC + BSA) and a sample of the products of the conjugation were carried out simultaneously on the same paper. It is seen from figure 10(a) that insulin at pH 6.0 remained at the line of application, whereas the anodic mobility of the modified insulin was greatly increased at the end of stage 1 of the reaction [figure 10(b)]. The increase in anodic mobility in itself does not prove unequivocally that the cationic groups of insulin were blocked as there was the possibility that the electrokinetic properties of the insulin might have been changed through polymerization of the molecule during the conjugation procedure. However, the results of the ultracentrifugal studies unambiguously eliminate this latter possibility. Furthermore, at the end of stage 1 of the reaction, the unreacted isocyanate group in the ortho-position relative to the methyl group of the original TDIC molecule was destroyed with 0.1 M $(NH_4)_2CO_3$ to form an aromatic NH_2 group. Since this aromatic amine is a much weaker base than an aliphatic amine, protonation would not be

Figure 10

Paper electrophoretic patterns of insulin (IN), modified insulin (IN-TDIC), BSA, control proteins (IN-TDIC + BSA) and a sample of the products of the conjugation in phosphate buffer pH 6.0, ionic strength 0.025.

Voltage	-	110 V.
Current	-	7.5 mA.
Time	-	24 hours
Temperature	-	4°C.

ANODE



IN (a) | IN-TDIC (b) | BSA (c) | Sample (d) | IN-TDIC + BSA (e)

CATHODE

expected to occur under the experimental conditions used. The increase in anodic mobility of the modified insulin (IN-TDIC) was, therefore, considered to be entirely due to the reduction of the positive charge on the molecule. In addition, the fact that the zone corresponding to IN-TDIC was not resolved into more than one band was taken as additional evidence (c.f.p. 52) that the modified insulin consisted of a single component, i.e. an insulin molecule of M.W. 6,000 coupled to three TDIC molecules. Figure 10(c) shows the electrophoretic pattern of free BSA, which had a low mobility. On the other hand, the products of the conjugation [figure 10(d)] were separated into two distinct bands: a slow moving band corresponding to the uncoupled free BSA and a faster one - with a mobility between those of free BSA and IN-TDIC - representing the insulin-BSA conjugate (IN-TDIC-BSA). However, on the basis of the electrophoretic behaviour at a single pH value as illustrated in figure 10(d), one could not have established unequivocally whether conjugation through the formation of covalent bonds had occurred or whether IN-TDIC had been complexed to BSA. The possible suggestion that complexes might have been formed as a result of protein-protein interaction may be refuted by the observation that the (IN-TDIC + BSA) [figure 10(e)] was resolved into two distinct bands. Moreover, the persistence of the electrophoretic patterns of the products of conjugation and the control proteins (IN-TDIC + BSA) throughout the pH range 6.0 to 9.5 (figures 11 and 12) indicates that the bond

Figure 11

Paper electrophoretic patterns of a sample of the products of the conjugation and control proteins (IN-TDIC + BSA) in phosphate buffer pH 7.5, ionic strength 0.025.

Voltage	-	110 V.
Current	-	7.5 mA.
Time	-	24 hours
Temperature	-	4°C.

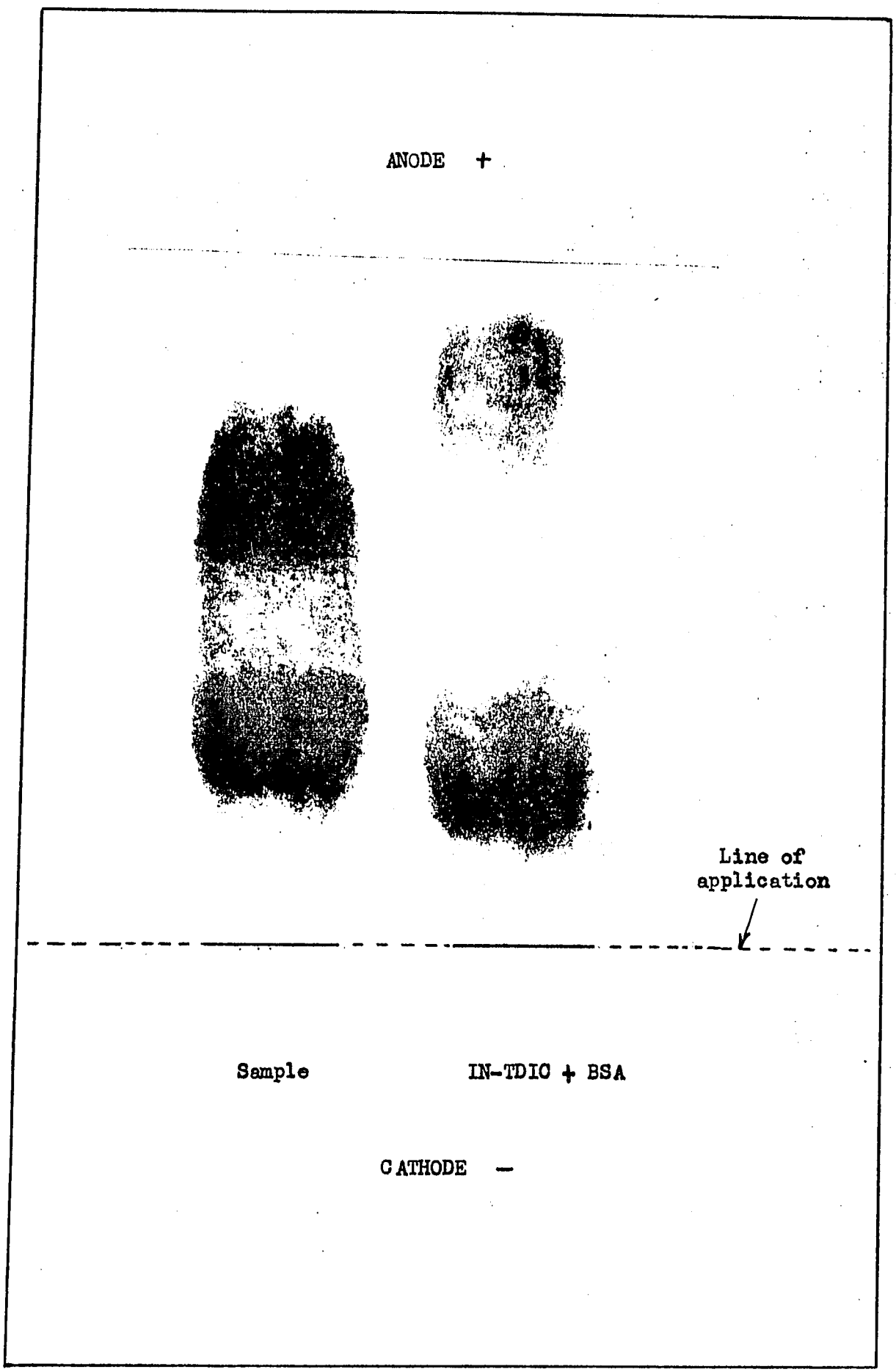

ANODE +
Line of application
Sample
IN-TDIC + BSA
CATHODE –

Figure 12

Paper electrophoretic patterns of a sample of the products of the conjugation and control proteins (IN-TDIC + BSA) in phosphate buffer pH 9.5, ionic strength 0.025.

Voltage	-	110 V.
Current	-	7.5 mA.
Time	-	24 hours
Temperature	-	4^{o}C.

ANODE +

Line of application

Sample IN-TDIC + BSA

CATHODE -

was not of a non-covalent, as this should have dissociated readily at the higher pH when both IN-TDIC and BSA are expected to be highly negatively charged. Further confirmation for this interpretation was provided by the results obtained with I^{131}-labelled insulin (figure 13), inasmuch as the radioactivity of the IN-TDIC-BSA sample was localized in a region distinct from that occupied by the more anodic IN-TDIC.

As a further check on the nature of the linkage, the IN-TDIC-BSA conjugate, separated by paper block electrophoresis, was analysed by paper electrophoresis. As can be seen from figure 14, this preparation had a single electrophoretic component with a mobility characteristic of the conjugate, i.e. between those of BSA and the modified insulin (IN-TDIC). From all this evidence it seems reasonable to conclude that the modified insulin had been coupled to BSA covalently to form the IN-TDIC-BSA conjugate.

As already shown in figure 10(d), the products of conjugation separated into two distinct bands only, namely, the slow moving BSA band and the fast moving IN-TDIC-BSA band. The absence of the band corresponding to that of IN-TDIC would indicate that either during conjugation, all IN-TDIC was used up in the reaction or it was not observed on paper electrophoresis because of low concentration. From the radio-electrophoretogram of the control proteins (I^{131}-IN-TDIC + BSA), it can be seen that a substantial amount of radioactivity was detected along the track travelled by the I^{131}-IN-TDIC (figure 13); this

Figure 13

Radio-electrophoretogram of the sample of IN-TDIC-BSA conjugate and of the control proteins (IN-TDIC + BSA) in phosphate buffer pH 7.5, ionic strength 0.025.

Voltage	-	110 V.
Current	-	7.5 mA.
Time	-	24 hours
Temperature	-	4°C.

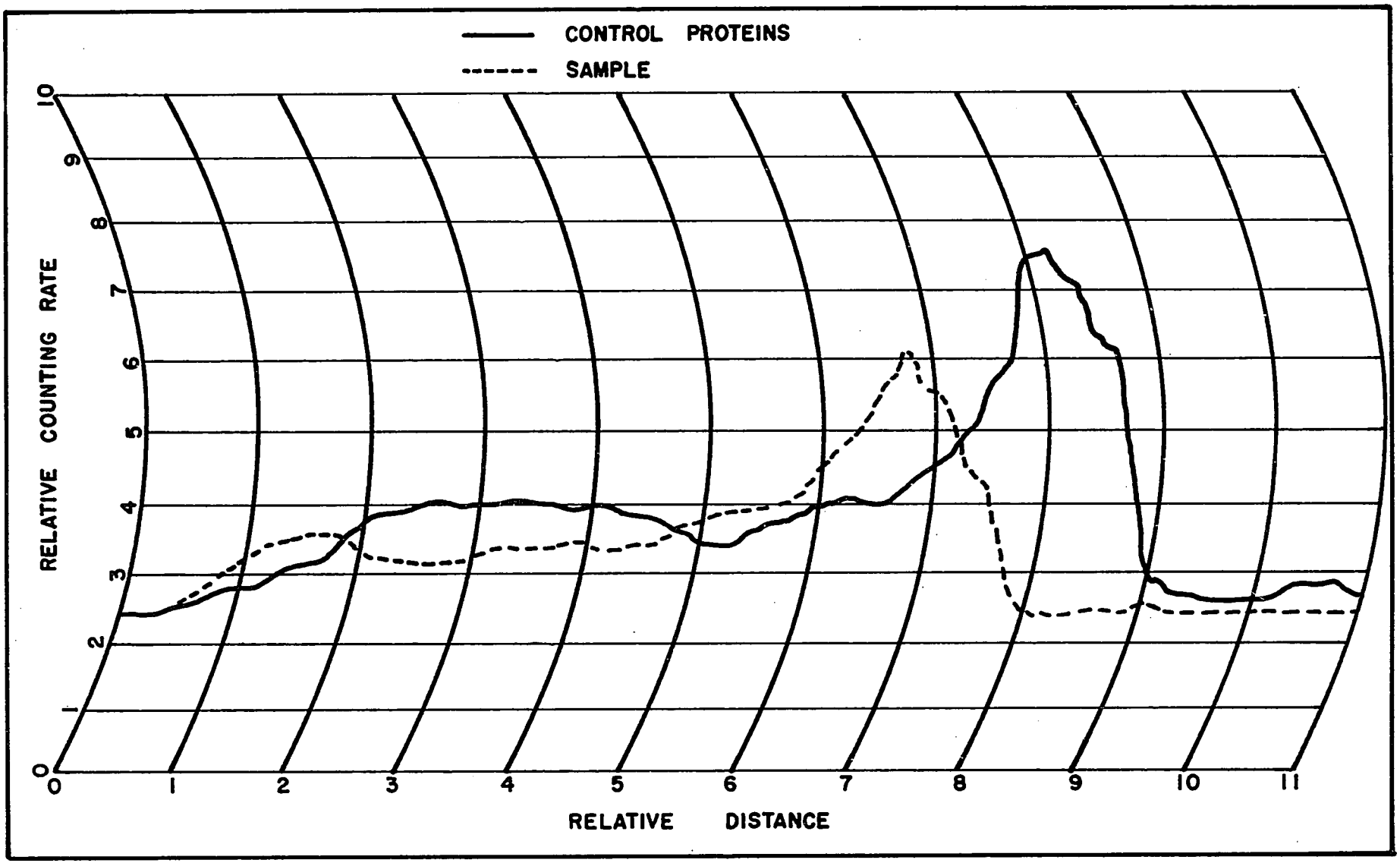
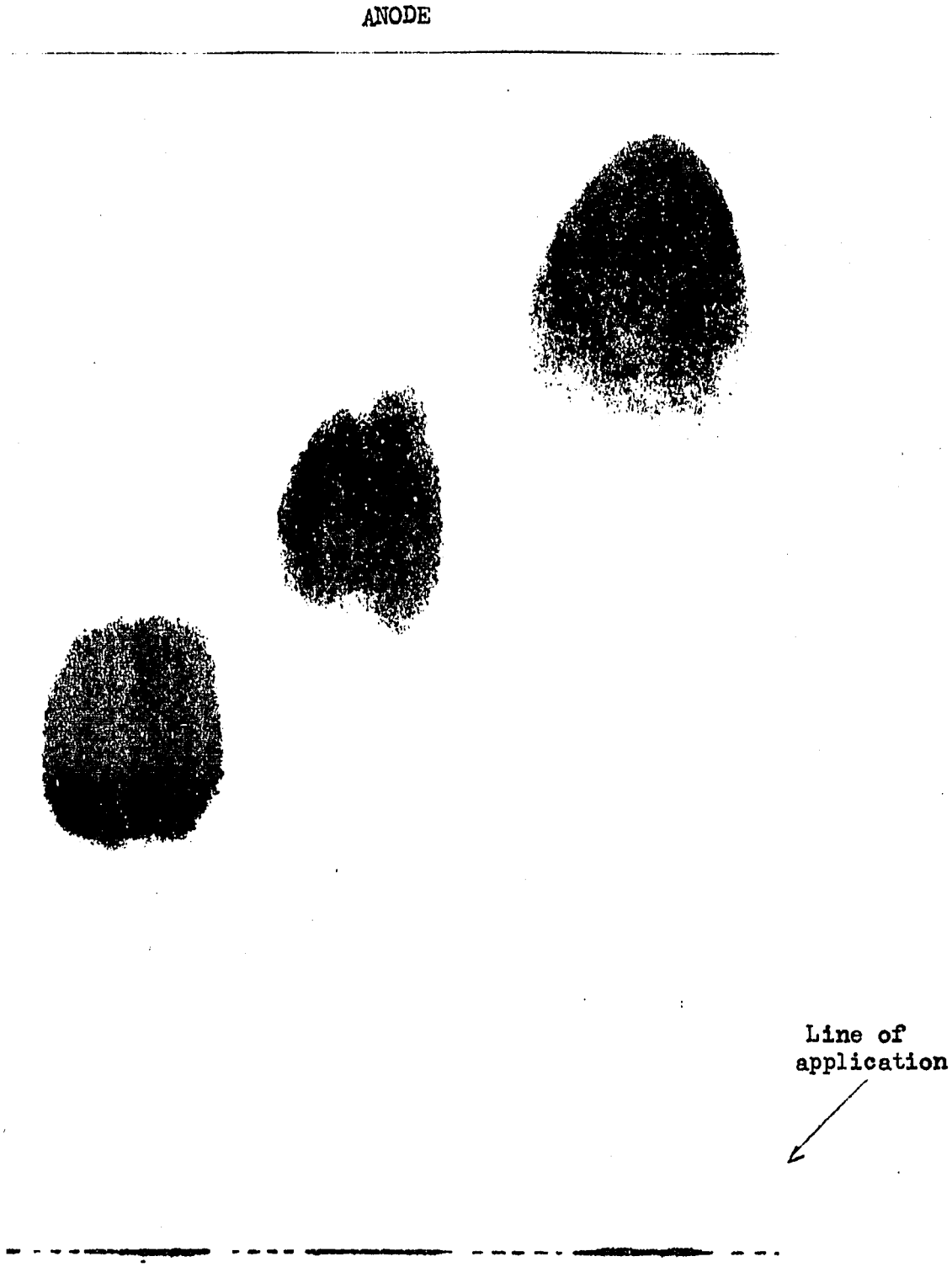

CONTROL PROTEINS
SAMPLE
RELATIVE COUNTING RATE
0
1
2
3
4
5
6
7
8
9
10
0
1
2
3
4
5
6
7
8
9
10
11
RELATIVE DISTANCE

Figure 14

Paper electrophoretic patterns of the control proteins, IN-TDIC and BSA, and a sample of IN-TDIC-BSA conjugate separated by paper block electrophoresis in phosphate buffer pH 7.5, ionic strength 0.025.

Voltage	- 110V.
Current	- 7.5 mA.
Time	- 24 hours
Temperature	- 4°C.


ANODE
Line of application
BSA
IN-TDIC-BSA
IN-TDIC
CATHODE

tailing can be attributed to the adsorption of I^{131}-IN-TDIC by the paper. In addition, the band corresponding to IN-TDIC in the products of conjugation was observed in free electrophoresis (figure 15). Hence, it would seem that the amount of IN-TDIC, which may have remained uncoupled to BSA during conjugation, was small and the adsorptive power of the filter paper further reduced its concentration, thus rendering its absence in paper electrophoresis.

Various di- or polyfunctional coupling reagents such as carbodiimides, dihalogenated dinitrobenzene, the copolymer of ethylene and maleic anhydride ... etc. have been used for protein-protein conjugation. However, most of these reagents have two functional groups of identical reactivity, and the reactions are usually carried out in a single step. In the present study, insulin has been coupled to a large protein carrier with a coupling reagent, which has two functional groups of different reactivity. The conjugation has been carried out in two stages, which permits the detailed analysis of the intermediate products.

The isocyanate group N=C=O contains a very electrophilic carbon atom joined by double bonds to two electronegative atoms. The aliphatic isocyanates decompose too rapidly by reaction with water to form carbamic acids, which decarboxylate almost immediately. The commonly used coupling reagents are, therefore, the aromatic isocyanates, which are less reactive than the corresponding aliphatic one;

yet their reactivities are sufficiently great so as to function under mild conditions. In the present study, TDIC was chosen as a coupling agent. As mentioned earlier, the NCO group at the ortho-position with respect to the CH_3 group in TDIC is somewhat hindered sterically and less reactive than the NCO group at the para-position. Consequently the isocyanate group in the para-position combined preferentially with all available free amino groups of insulin in the first stage of the reaction. The formation of covalently linked IN-TDIC-BSA conjugate indicates that the ortho NCO group of the TDIC, already coupled to insulin, did not react until BSA was added for the second stage of the reaction. The reactivity of the NCO group in the ortho-position of the TDIC was obviously suppressed at $0^{\circ}C$, and was enhanced sufficiently at $37^{\circ}C$ so as to combine with the free NH_2 groups of BSA.

(B) Free electrophoresis

Method

Moving boundary electrophoresis analyses were carried out in the Spinco Model H Tiselius apparatus at $0.8^{\circ}C$. Prior to electrophoresis, all samples were made up to a protein concentration of 1 g per cent in phosphate buffer at pH 7.5, ionic strength 0.1 and were dialysed against the same buffer for 24 hours. The electrophoretic mobilities, μ, were calculated with the aid of the equation:

$$= \frac{a \times q \times c}{t \times 60 \times i \times R_s} \times d \times 10^3 \text{ cm}^2/\text{volt/sec.}$$

where a = magnification factor for projection

q = cross section area in cm^2

c = conductivity cell constant in cm^{-1}

t = time in minutes

i = current in milliamperes

R_s = resistance of the solution in ohms

d = distance travelled by the protein boundary in cm.

Previously, Longsworth and MacInnes (171) noted that the descending boundaries, although in general more diffuse, yielded correct values of mobility. The rising boundaries may give results that are greatly in error unless the change of protein concentration is available from which correction may be made, in which case the two types of boundary yield the same value of mobility. Thus, in the present study, the electrophoretic mobilities were calculated from the data obtained from the descending pattern.

For the estimation of the protein composition of the sample from the Schlieren patterns, the assumption was made that each peak had a Gaussian distribution. For peaks which were not fully resolved, the pattern was extended to the base line. The area under each peak was measured planimetrically on a five-fold magnified tracing of the Schlieren photograph.

The average composition of the IN-TDIC-BSA conjugates

was estimated from the electrophoretic patterns of both the control proteins (IN-TDIC + BSA) and of the products of the conjugation (figure 15) as follows:

(i) Area method

The area under the fast moving peak in figure 15(a) is proportional (neglecting electrophoretic anomalies) to the total amount of IN-TDIC originally used in the conjugation experiment. The area under the fastest moving peak in figure 15(b) is proportional to the amount of IN-TDIC left unconjugated at the end of the reaction. After normalizing the two areas, the former minus the latter is proportional to the amount by weight of IN-TDIC in the IN-TDIC-BSA conjugate. Correspondingly, the weight of BSA in the conjugate is proportional to the difference between the normalized areas of the free BSA peaks in figure 15(a) and 15(b). The ratio of the amounts of IN-TDIC and BSA in the conjugate, multiplied by the ratio of the molecular weights of BSA and insulin, i.e. $7 \times 10^4 / 3.6 \times 10^4$, corresponds to the number of insulin molecules conjugated to one BSA molecule, averaged over all of the conjugates present.

In order to compute the average composition of the IN-TDIC-BSA conjugates according to the method mentioned above, the assumption was made that the specific refractive increments of the electrophoretically separable protein components were identical. The uncertainty in the analysis due to this

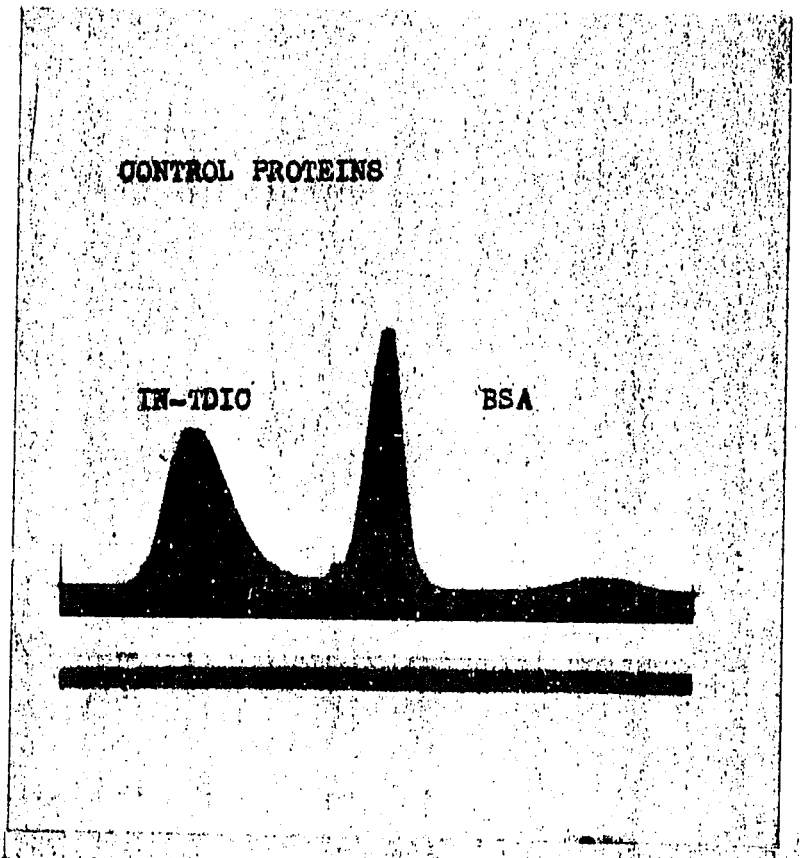
Figure 15

Descending electrophoretic patterns of the products of the coupling reaction of IN and BSA by TDIC, examined in phosphate buffer, pH 7.5, ionic strength 0.1. The direction of migration is indicated by the arrow. The peaks are referred to as IN-TDIC and BSA, respectively, and the intermediate peak in pattern (b) is due to IN-TDIC-BSA conjugate.

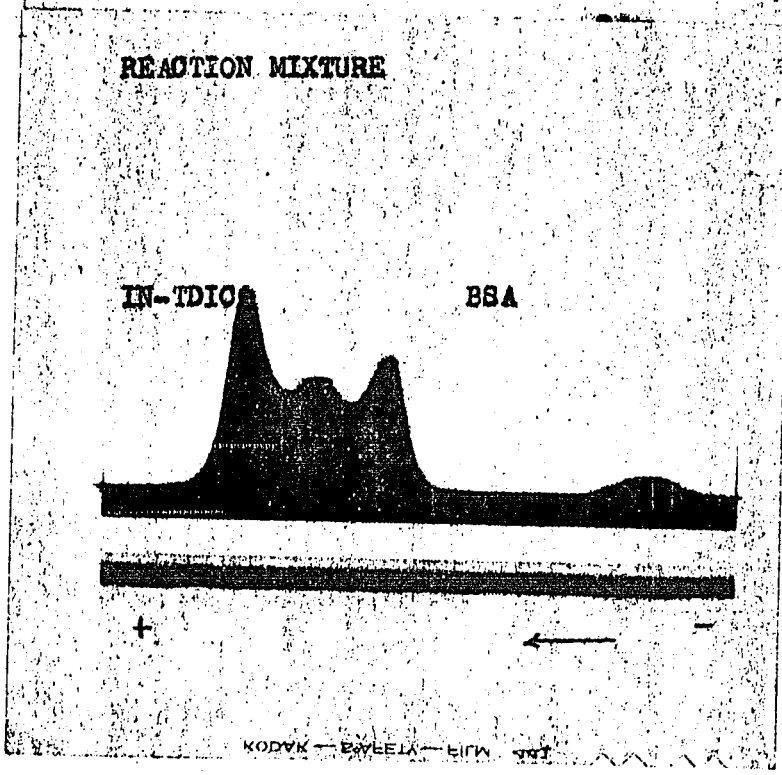
Pattern (a) 'control proteins' (IN-TDIC + BSA).

Pattern (b) reaction mixture (containing IN-TDIC, IN-TDIC-BSA and BSA).

(a)



(b)



assumption depends largely on the nature of the proteins in the mixture. The specific refractive increment, k, of a protein is defined as $k = (\eta_{solution} - \eta_{protein})/p$ where the concentration p is expressed in gram dry weight of protein per 100 ml of solution and η is the refractive index. In fact, the validity of this assumption is justified by the data that the specific refractive increment of bovine serum albumin at 0°C and 578 mμ is 1.90×10^{-3} (172) while that of insulin at 0°C and 546 mμ is 1.82×10^{-3} (173). The error thus made was considered to be within tolerable limits , i.e. within less than 10%.

(ii) Mobility method

Singer and Campbell (174) showed that the electrophoretic mobility of a protein complex, μ_c, could be calculated by the approximate relation:

$$\mu = \sum W_i \cdot \mu_i \qquad (1)$$

where W_i is the weight fraction of the component i in the conjugate and μ_i is the mobility of component i. For the present study, assuming that this equation could be used for the calculation of the mobility of the insulin-BSA conjugate, one could write

$$\mu_c = W_{IN\text{-}TDIC} \cdot \mu_{IN\text{-}TDIC} + W_{BSA} \cdot \mu_{BSA} \qquad (2)$$

Since there are only two components in the conjugate, the W_{BSA}

can be expressed as

$$W_{BSA} = 1 - W_{IN\text{-}TDIC} \qquad (3)$$

Substituting (3) into equation (2) and upon rearrangement, the value of $W_{IN\text{-}TDIC}$ can be given in terms of the mobilities of the components:

$$W_{IN\text{-}TDIC} = (\mu_c - \mu_{BSA})/(\mu_{IN\text{-}TDIC} - \mu_{BSA}) \qquad (4)$$

The weight fraction thus obtained was compared with the theoretical values calculated from the compositions of (IN-TDIC)-BSA, $(IN\text{-}TDIC)_2$-BSA, and $(IN\text{-}TDIC)_3$-BSA conjugates. This method is valid only when the mobility of a conjugate is determined primarily by its constituents rather than by its shape.

Results and Discussion

Upon free electrophoresis at pH 7.5, ionic strength 0.1, of the products of the conjugation reaction of insulin and BSA with the help of TDIC, the pattern shown in figure 15(b) was obtained. Besides the fastest moving peak attributable to the TDIC-insulin adduct, and the slowest moving peak due to free BSA, the intermediate peak is evidently due to the conjugate of the two. On the other hand, the pattern of the control proteins [figure 15(a)] shows two distinct boundaries, indicating no complex formation. Each component was identified

by its electrophoretic mobility listed in table III. It is noted that the mobility of BSA in the products of the reaction (-7.5×10^{-5} cm^2/volt/sec) was higher than that of free BSA (-6.1×10^{-5} cm^2/volt/sec). This increase in mobility is attributed to the reaction of some of the NH_2 groups of BSA with residual TDIC, which remained in solution after coupling of the insulin to TDIC. This explanation was substantiated by the results of experiments in which BSA was reacted directly with TDIC under identical conditions. However, it must be stressed that in these experiments the concentration of TDIC was much higher than that remaining in solution after the first stage of reaction with insulin. The mobility of this BSA-TDIC adduct was indeed very much higher than that of intact BSA, i.e. 10.0×10^{-5} cm^2/volt/sec).

The Schlieren pattern of the products of the conjugation reaction revealed three components instead of two as was the case by paper electrophoresis. The present result supports the earlier conclusion that IN-TDIC was not used up in the conjugation reaction, and that its apparent absence on paper electrophoresis might be ascribed to its being strongly adsorbed to the filter paper.

The contribution of the IN-TDIC-BSA conjugate to the area of the Schlieren patterns was calculated as 40% of the total products of conjugation (figure 15 and table IV). Further information concerning the composition of the conjugates was obtained from a semi-quantitative interpretation of their

Table III

Electrophoretic mobilities obtained by Tiselius electrophoresis for the IN-TDIC-BSA conjugate and control proteins in 1 g per cent solution in phosphate buffer pH 7.5, ionic strength 0.1 and 0°C

Sample		mobility $\mu \times 10^5$ cm^2/volt/sec
IN*		- 4.9
BSA*		- 6.3
Control proteins:	IN-TDIC (fast component)	- 11.1
	BSA (slow component)	- 6.1
Products of conjugation:	IN-TDIC (fastest component)	- 11.0
	IN-TDIC-BSA (intermediate component)	- 9.5
	BSA (slowest component)	- 7.5

*Concentration used for these analyses was 0.5 g per cent.

Table IV

ELECTROPHORESIS OF IN-TDIC-BSA CONJUGATES

	Relative areas in %		
	IN-TDIC	Conjugates IN-TDIC-BSA	BSA
Products of the conjugation	34.9	40.5	24.6
Control proteins	52.5	-	47.5

electrophoretic properties. From the relative areas in the Schlieren pattern, corresponding to insulin and BSA, the molecular ratio of IN/BSA in the conjugate was calculated as 2.4 ± 0.1. This value would indicate that, during conjugation, the major product formed had a molecular composition of $(IN\text{-}TDIC)_2$-BSA and that the higher molecular weight compound corresponding to $(IN\text{-}TDIC)_3$-BSA was formed to a lesser extent. Although the latter conjugate would be expected to have a substantially faster electrophoretic mobility than the former, no separation between their boundaries was observed.

The molecular ratio of insulin to BSA in the conjugate was also estimated from the mobilities of TDIC-insulin and with with the help of equation 4. Thus, the weight fraction of IN-TDIC, i.e. $W_{IN\text{-}TDIC}$, was computed as 0.56. This result is in excellent agreement with the value of 0.54 calculated for the weight fraction of IN-TDIC in a conjugate having the composition of $(IN\text{-}TDIC)_2$-BSA. Therefore, it seems reasonable to conclude that the main product of the reaction was the trimer $(IN\text{-}TDIC)_2$-BSA.

It was demonstrated earlier from the chemical analysis of IN-TDIC that all free amino groups in insulin had been coupled to TDIC; therefore, no free amino group would be expected to be available for the dimerization of IN-TDIC. Consequently it would seem plausible to suggest that the $(IN\text{-}TDIC)_2$-BSA conjugate was formed preferentially as a result of the simultaneous coupling of two IN-TDIC molecules to a

single BSA. Since there are 49 free amino groups on a BSA molecule available for the coupling at the second stage of the reaction, one may have expected a larger number of insulin molecules to be coupled per BSA molecule. However, the free amino groups on the BSA molecule are probably not equally reactive due to steric restrictions. Further evidence could have been obtained by investigating the effect of concentration of BSA in the products of reaction. This aspect, however, was not pursued since the insulin-BSA conjugate described above proved highly immunogenic by themselves.

(C) Ultracentrifugal studies

Method

The modified insulin (IN-TDIC), insulin, BSA, and the conjugate (IN-TDIC-BSA) were examined in a Spinco Model E optical ultracentrifuge. All solutions were prepared in borate buffer, pH 9.5, ionic strength 0.1. For these analyses the synthetic boundary cell (unless otherwise specified) was used. The cells were filled with 0.4 ml of a 1% solution and centrifuged at a speed of 59,780 r.p.m. at 25°C. The sedimentation coefficients, expressed in Svedberg units ($S = 10^{-13}$ sec), were calculated with the aid of the standard equation

$$S = \frac{2.303 \; d\log x/dt}{4\pi^2 \left[\frac{59,780}{60}\right]^2}$$

The term dlog x/dt was evaluated graphically from the photographs of the Schlieren patterns by plotting log x against t, where x is the distance of the boundary from the center of rotation (in cm) and at a time t (in sec). The sedimentation coefficients were not corrected to standard conditions.

Results and Discussion

The ultracentrifugal patterns of free insulin, BSA, and the control proteins (IN-TDIC + BSA), as well as of the conjugate, are shown in figure 16, and the sedimentation coefficients are listed in table V. As can be seen, at the end of stage 1 of the coupling reaction the sedimentation coefficient of insulin increased from 3.3 to 3.7 S. Previously, Fredericq (111) reported that a polymeric form of insulin, which had been prepared by crystallizing insulin from a solution containing 1% zinc, had a molecular weight of 72,000; the sedimentation coefficient actually measured in his experiments was $S_{20W} = 5.5$ S. Thus, the slight increase observed in this study is obviously not due to dimerization of insulin preparation, but is considered to be due to some structural changes of insulin. In an earlier study in this laboratory (175,176), it had been demonstrated that proteins did not show a significant increase in their sedimentation coefficient when coupled to n-butyl isocyanate, while the introduction of naphthyl, estronyl or indole residues was associated with a measurable increase in their sedimentaion

Figure 16

ULTRACENTRIFUGAL SCHLIEREN PATTERNS OF THE CONJUGATE AND THE REACTANTS

Speed: 59,780 r.p.m.

Temperature: 25°C

Time: 24 minutes

Solvent: borate buffer pH 9.5, ionic strength 0.1

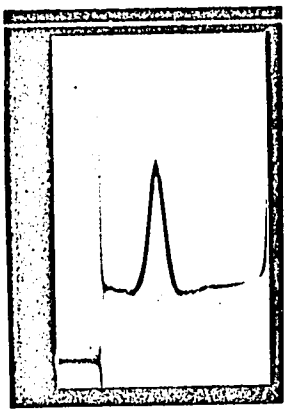
(1) IN 0.5%

(2) BSA 0.5%

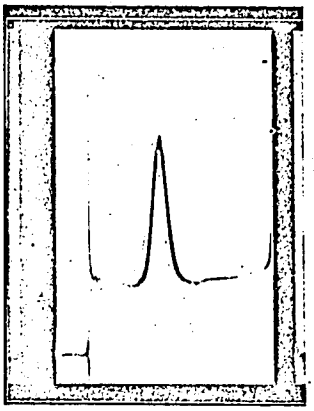
(3) IN-TDIC 0.5%

(4) IN-TDIC-BSA 0.5%

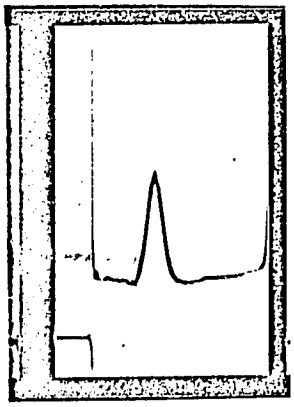
(5) IN-TDIC + BSA 1% (in equal concentration)



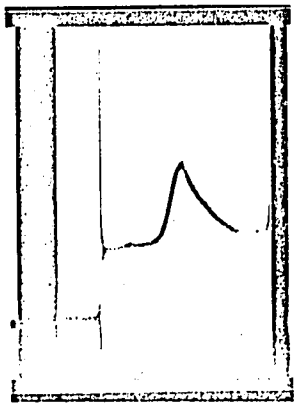
(1) IN



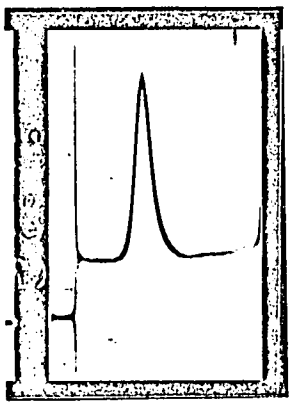
(2) BSA



(3) IN-TDIC



(4) IN-TDIC-BSA



(5) IN-TDIC + BSA

Table V

SEDIMENTATION COEFFICIENTS OF THE CONJUGATE AND REACTANTS

Sample	Concentration	Sedimentation coefficient (S)*
IN	0.5%	3.3
BSA	0.5%	4.6
IN-TDIC	0.5%	3.7
IN-TDIC + BSA	1.0%	4.5
IN-TDIC-BSA	0.5%	6.4

*Svedberg unit = 10^{-13} sec.

coefficients. This finding was attributed to certain changes in the tertiary structure of proteins, which were imposed by the large and rigid hydrophobic aromatic residues but not by flexible aliphatic grouping. Hence, in the present study, the observed change in sedimentation behaviour of insulin which has been coupled to TDIC might be attributed to the incorporation of the aromatic residues into the insulin molecule. The little peak in the descending boundary of figure 16(1) and figure 16(3) was stationary; it represents some imperfection in the optical system.

The Schlieren pattern of the conjugate, IN-TDIC-BSA, showed only a single peak, but somewhat unsymmetrical and broader than that of insulin, thus suggesting the presence of some faster sedimenting compounds. The sedimentation coefficient of the main component of the conjugate was 6.4 S, which is significantly higher than that of IN-TDIC or BSA. On the other hand, the pattern of the control proteins (IN-TDIC + BSA) showed only one symmetrical peak with a sedimentation coefficient of 4.5 S, while the boundary corresponding to that of IN-TDIC was not observed. This might be due to the lack of resolution of these two compounds in the ultracentrifuge, as the sedimentation coefficients of IN-TDIC and BSA differ by only 0.8 S. However, it might also represent a system of components which interact with one another in a reversible manner, the ultracentrifuge patterns being dependent on the rate of re-equilibration as compared to

the rate of centrifugal separation of the component.

Longsworth and MacInnes (177), in a fundamental paper dealing with interacting systems during electrophoresis, discussed various possibilities, which apply also to the process of ultracentrifugation. If the association reaction is fast and the dissociation slow, the system will behave as a mixture of the complex and the component present in excess (if neither of the components is in excess, then only the complex will be observed). Conversely, the system would appear essentially as a mixture of two independent components if the dissociation reaction is rapid and the association is slow. When both reactions are slow, but comparable in velocity, the pattern at equilibrium will indicate the presence of both individual components, as well as of the complex. Finally, there is the system, which is apparently common in biochemistry, in which both the forward and reverse reactions are very rapid and the reaction velocities are comparable in magnitude. Equilibrium is maintained despite the tendency toward separation of the species according to their sedimentation coefficients. This system was analysed by Gilbert and Jenkin (178). If the complex, C, has a sedimentation rate which is appreciably different from the faster sedimenting component, B, in the reaction ($A + B \rightleftharpoons C$), the readjustment to equilibrium may cause depletion of A from the upper region of of the ultracentrifuge cell. This is expected to occur because as C migrates out of the reaction zone, the two

components A and B react immediately to form C, thereby re-establishing the equilibrium. The newly-formed complex would then migrate with its characteristic sedimentation rate, and the equilibrium would again be disturbed only to be re-established by a repetition of the above process. The net effect of this phenomenon would be a continuous depletion of A from the upper region and the lack of separation between the boundaries of B and C, which may appear as a boundary with a sedimentation rate intermediate between those of these two species. However, in the present case, the pattern of the control proteins (IN-TDIC + BSA) which consisted of a single peak, did not fall into any of these catagories as neither an additional boundary corresponding to that of the conjugate (6.4 S) nor a compound boundary intermediate between that of the free, intact BSA (4.6 S) and that of the conjugate (6.4 S) was observed. It would, therefore, seem reasonable to suggest that the observed single peak for the mixture of control proteins was due to lack of resolution in the ultracentrifuge.

To further clarify this point, ultracentrifugal analyses were carried out on mixtures of the control proteins at different IN-TDIC and BSA concentrations. The Schlieren patterns obtained are shown in figure 17. As can be seen, a single peak was observed irrespective of the relative concentrations of IN-TDIC and BSA, and there was no aggregate corresponding to fast sedimentating species. Furthermore, at low IN-TDIC : BSA ratios, the sedimentation coefficients

Figure 17

ULTRACENTRIFUGAL SCHLIEREN PATTERNS OF THE CONJUGATE AND THE REACTANTS

Speed: 59,780 r.p.m.

Temperature: 25°C

Time: 24 minutes

Solvent: borate buffer pH 9.5, ionic strength 0.1

(1) IN-TDIC 0.1% + BSA 0.5%

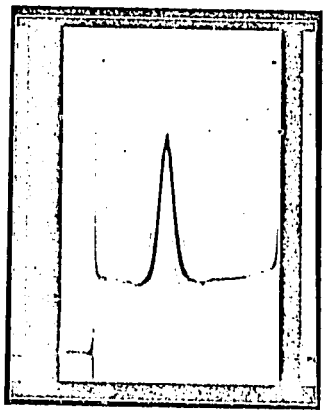
(2) IN-TDIC 0.3% + BSA 0.5%

(3) IN-TDIC 0.5% + BSA 0.5%

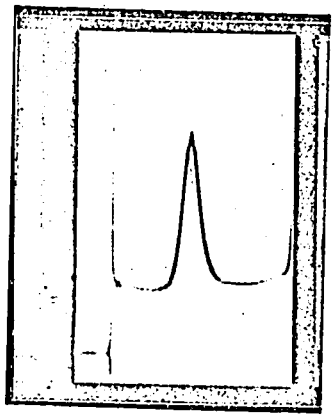
(4) IN-TDIC 0.7% + BSA 0.5%

(5) IN-TDIC 1.1% + BSA 0.5%

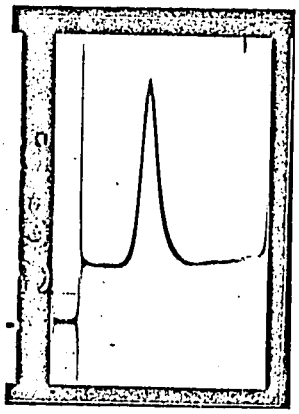
(6) IN-TDIC 1.0% + BSA 1.0%



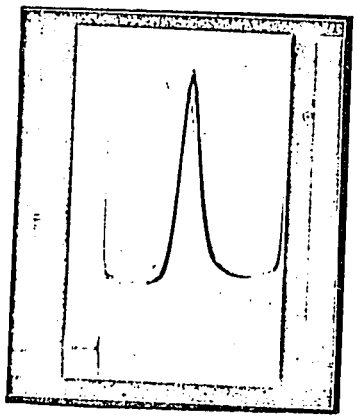
(1)



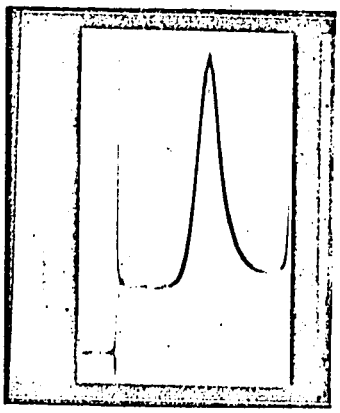
(2)



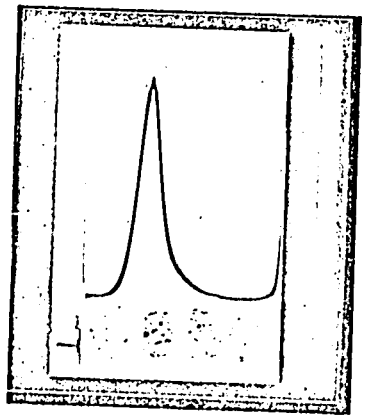
(3)



(4)



(5)



(6)

measured corresponded essentially to the value expected for BSA alone, i.e. 4.6 S (table VI). As the IN-TDIC : BSA ratio increased, the sedimentation coefficients decreased. Consequently, if protein-protein interactions had occurred, a species with a higher sedimentation coefficient should have been observed. Furthermore, the sedimentation coefficient observed for the mixture of control proteins (IN-TDIC + BSA) in equal weight, at a total protein concentration of 2%, was 3.8 S. This further decrease in sedimentation coefficient is not unexpected. As the total protein concentration increases, the medium becomes more viscous and consequently causes the protein molecules to sediment more slowly.

Hence, the results of the ultracentrifugal studies further support the earlier conclusion drawn from paper and free electrophoresis that the insulin had been coupled covalently to the BSA molecule.

Table VI

SEDIMENTATION COEFFICIENTS OF CONTROL PROTEINS (IN-TDIC + BSA) IN DIFFERENT CONCENTRATIONS

% concentration in the control protein		Sedimentation coefficient (S)
IN-TDIC	BSA	
0.5	0.0	3.7
0.0	0.5	4.6
0.1	0.5	4.6
0.3	0.5	4.6
0.5	0.5	4.5
0.7	0.5	4.3
1.1	0.5	4.1
1.0	1.0	3.8*

*Because of high protein concentration, the standard cell was used for this experiment.

Preparation of the 3-nitrobenzene sulphonic acid-insulin conjugate

Introduction

At the outset of this investigation, it had been intended to study both the specificity of the antibodies to insulin as well as the kinetics of the reaction between antibody and insulin. Previously, Farr (179) described a method of measuring the binding of antibody to an albumin antigen which did not require the formation of spontaneously precipitable complexes. The method was based on the differential solubility of albumin and globulin in half-saturated ammonium sulphate. At this salt concentration, 98% of the antibody globulins and the corresponding antibody-antigen complex are precipitated. Thus, if, in such a separation, the albumin antigen has been previously trace-labelled with I^{131}, the fraction of antigen bound may be quantitatively determined from the distribution of the radioactivity between the separated precipitate and supernatant.

The determination of the free and bound forms of the antigen is a prerequisite for the calculation of the equilibrium constant for an antigen-antibody reaction, and this procedure has been commonly used in equilibrium dialysis experiments with small haptens (180-181). Unlike this method, however, the Farr-technique is rapid, the antibody-antigen complexes as well as any unreacted antibodies being precipitated from the

reaction almost 'instantaneously' on addition of ammonium sulphate to half saturation. Consequently, at the beginning of this study, this precipitation process was considered as a possible 'quenching' reaction capable of application to the study of the kinetics of the reaction between I^{131}-labelled insulin and homologous rabbit antibodies. However, since insulin is almost completely insoluble in half-saturated ammonium sulphate, it was conjugated to the highly soluble 4-fluoro-3-nitrobenzene sulphonic acid (figure 18) in the expectation that the resulting conjugate would have sufficient solubility in half-saturated ammonium sulphate so as to render the study of the kinetics possible.

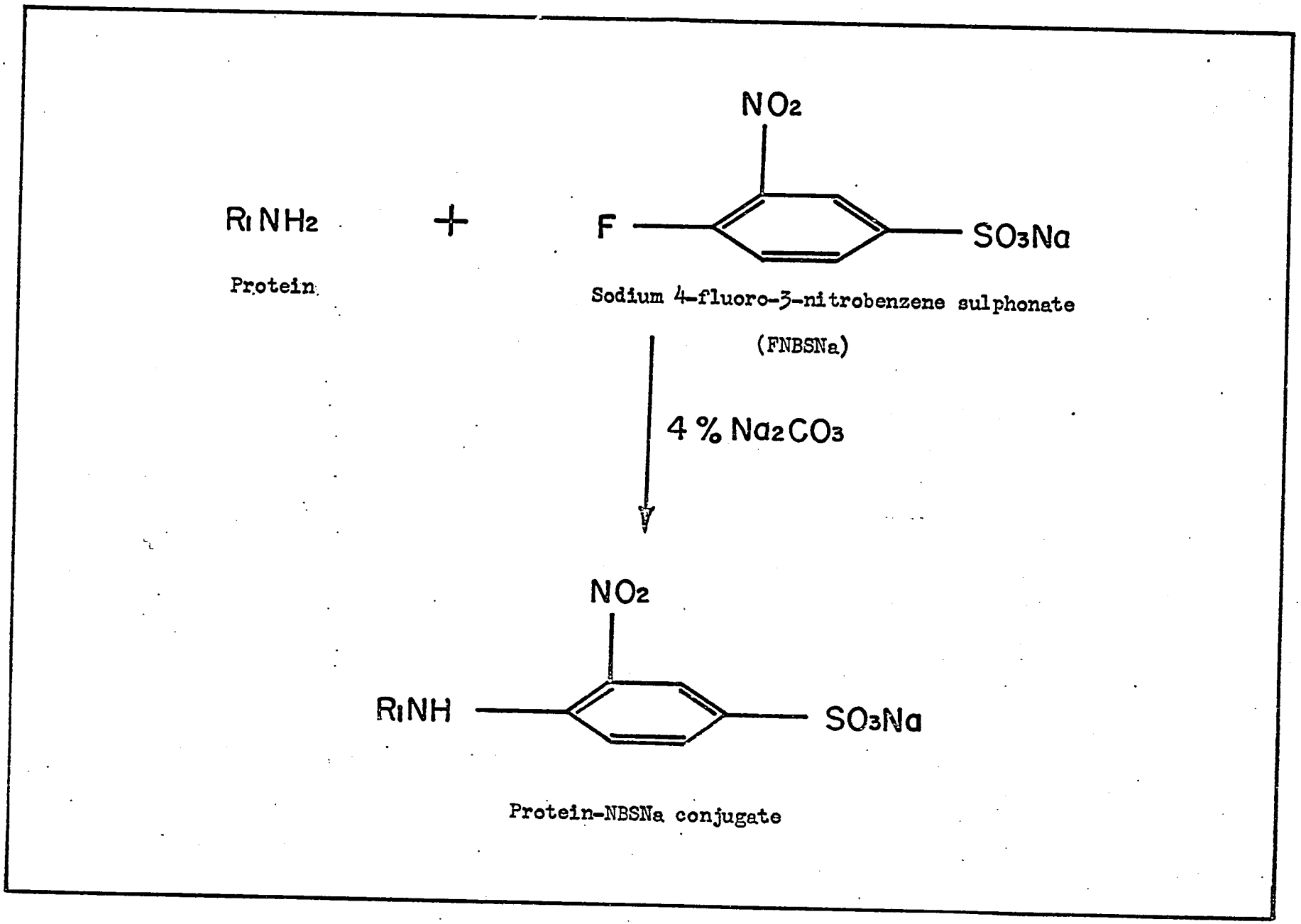
Method and Result

Preparation of 4-fluoro-3-nitrobenzene sulphonic acid

4-fluoro-3-nitrobenzene sulphonic acid (referred to hereafter as FNBS) was prepared according to the method of Plenikowski (182). To 20 g of fuming sulphuric acid (20% SO_3) was added slowly 5 g of fluorobenzene with vigorous stirring. Care was taken to prevent the rise of temperature over 50°C. As soon as all the fluorobenzene had dissolved, 5 g of fuming nitric acid (d = 1.48) was added dropwise with stirring keeping the temperature below 100°C. The solution was then maintained at room temperature for 20 minutes before the addition of 30 g of fuming sulphuric acid (10% SO_3). The resulting solution

Figure 18

FLOWSHEET OF THE PREPARATION OF IN-NBSNa
PROTEIN CONJUGATE

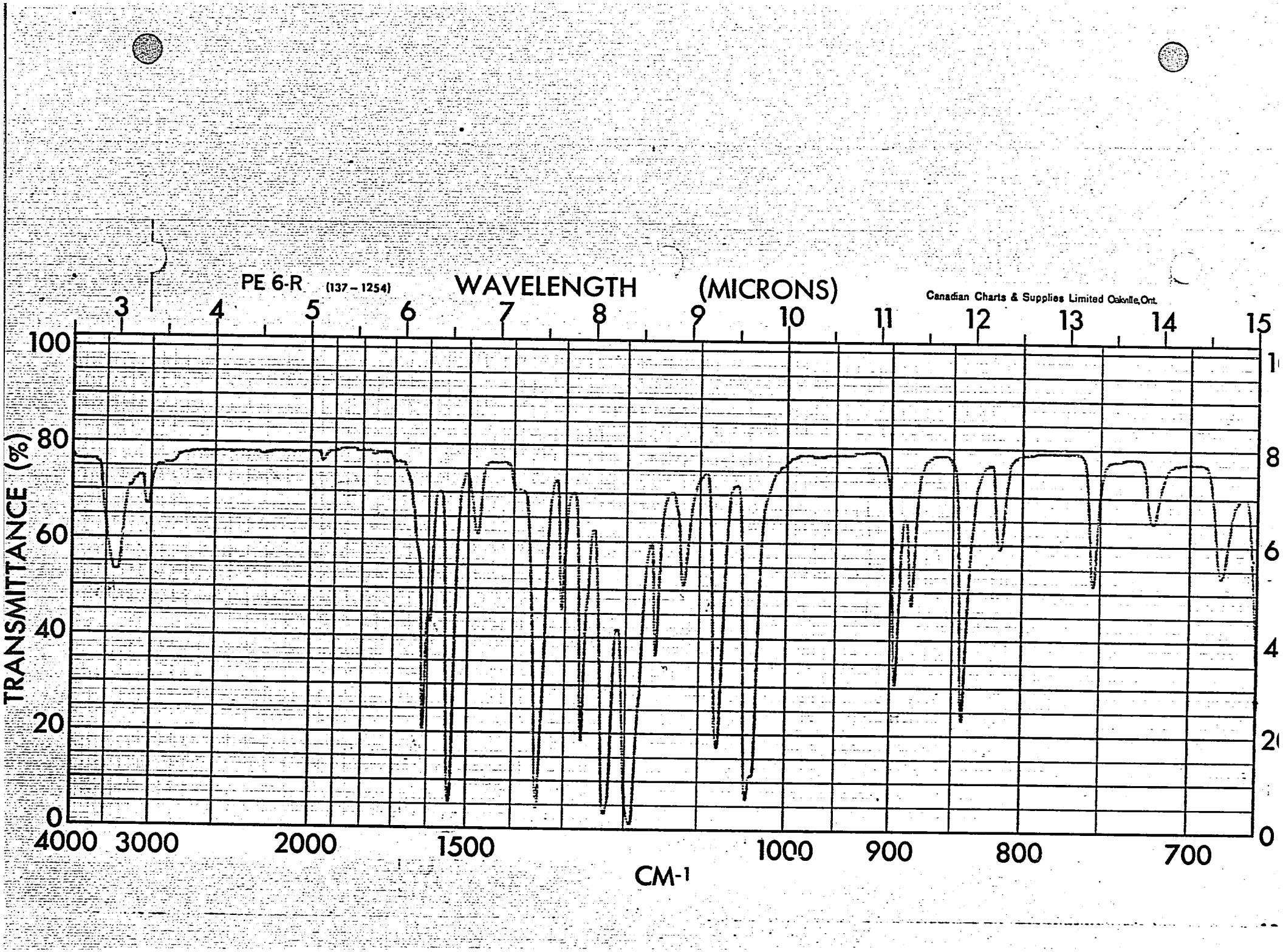

NO2
R1NH2
+
F
SO3Na
Protein
Sodium 4-fluoro-3-nitrobenzene sulphonate
(FNBSNa)
4 % Na2CO3
NO2
R1NH
SO3Na
Protein-NBSNa conjugate

was heated on a steam bath for 4 hours; it was then maintained at room temperature overnight and finally 30 ml of chilled water was added to it. The sulphoxide, obtained as a side product, precipitated out at this time. For oxidation of the sulphoxide to the corresponding sulphonic acid, a stream of air was bubbled through the mixture until most of the precipitate dissolved. The small amount of precipitate which persisted was removed by filtration through a sintered glass funnel. The product crystallized out on cooling and was recrystallized from warm HCl. The highly acidic product was then converted to the corresponding sodium salt by recrystallization from a saturated sodium chloride solution.

The evidence for the introduction of NO_2 and SO_3 groups into the fluorobenzene molecule was obtained by infrared studies (figure 19). The spectrum showed two extremely strong absorption bands at 1540 cm^{-1} and 1350 cm^{-1}, corresponding to the asymmetric and symmetric stretching vibrations of NO_2 group. The presence of the SO_3 group was identified as the intense band at 1200 cm^{-1} and 1040 cm^{-1} while the C-S stretching vibration appeared as a weak absorption at 670 cm^{-1}. In addition, the characteristic out-of-plane CH stretching vibration for each of the two adjacent ring H atoms and one isolated ring hydrogen atom at 850 cm^{-1} and 920 cm^{-1}, respectively, suggested a 1,3,4-trisubstituted aromatic ring. Thus, the product obtained was considered to be the desired compound.

Figure 19

INFRARED ABSORPTION SPECTRUM OF FNBS
OBTAINED IN KBr PELLET


PE 6-R (137 – 1254)
WAVELENGTH (MICRONS)
Canadian Charts & Supplies Limited Oakville, Ont.
3
4
5
6
7
8
9
10
11
12
13
14
15
100
80
60
40
20
0
TRANSMITTANCE (%)
4000
3000
2000
1500
1000
900
800
700
CM-1

The product was also subjected to qualitative analysis. Fifty-five ml of water containing 1.3 g of FNBS, 1.3 g of K_2CO_3 and 0.8 g of glycine, was warmed in a steam bath to 60°C. An orange-red colour appeared, indicating that coupling between the NH_2 group of the glycine and the product had occurred.

Preparation of insulin-nitrobenzene-sulphonic acid conjugate (IN-NBSNa)

All operations were carried out at 0 - 4°C. To 40 ml of 4% Na_2CO_3 containing 200 mg of the sodium salt of 4-fluoro-3-nitrobenzene sulphonic acid (FNBSNa) was added 200 mg of insulin. The mixture was stirred until all insulin dissolved and the resulting solution was incubated at 4°C for 24 hours. The reaction was terminated by adjusting to pH 7.5, and the clear yellow solution was then dialysed against frequent changes of 2 liter portions of buffered-saline (pH 7.5) for three days and then against distilled water for 24 hours. The small amount of precipitate, formed at the end of the dialysis, was removed by centrifugation. Finally, the solution was lyophilized to a bright yellow fluffy product. Further purification was achieved by chromatography on a Sephadex G-75 column (2.5 cm x 35 cm), which resulted in the complete separation of IN-NBSNa from FNBSNa and the sodium 4-hydroxy-3-nitrobenzene sulphonate (HO-NBSNa), which was obtained during the conjugation process as a result of the decomposition of

FNBSNa under alkaline conditions (figure 20). The latter procedure was found unnecessary after prolonged dialysis.

The product was subjected to the test given below in order to establish that conjugation had actually taken place. All analyses were performed in the same manner as described earlier for the characterization of IN-TDIC-BSA conjugate.

From chemical analyses (figures 21 and 22) it was deduced that under the experimental conditions used, all free NH_2 groups of insulin had been coupled to FNBSNa. Thus, the reaction of insulin with FNBSNa was similar to that with TDIC, inasmuch as both of these reagents reacted with all free amino groups of insulin; nevertheless, FNBSNa was far less reactive than TDIC. In the study of the coupling of insulin with TDIC, it was observed that the ε-amino group of the lysine residue could be reacted at pH 9.5. However, attempts to couple FNBSNa to insulin at a pH lower than 11.0 were unsuccessful. Thus, it appears that the reaction was catalysed in alkaline medium. However, it ought to be stressed that the phenolic groups of insulin had not reacted under the conditions used; this result confirms the earlier finding in the study for the conditions for coupling TDIC with insulin and that the OH groups of insulin were less reactive than the free NH_2 groups.

From the Schlieren records of the ultracentrifugal analyses shown in figure 23, it can be seen that the conjugate consisted of a single component having a sedimentation

Figure 20

SEPARATION OF IN-NBSNa CONJUGATE FROM FNBSNa AND HO-NBSNa ON SEPHADEX G-75 COLUMN

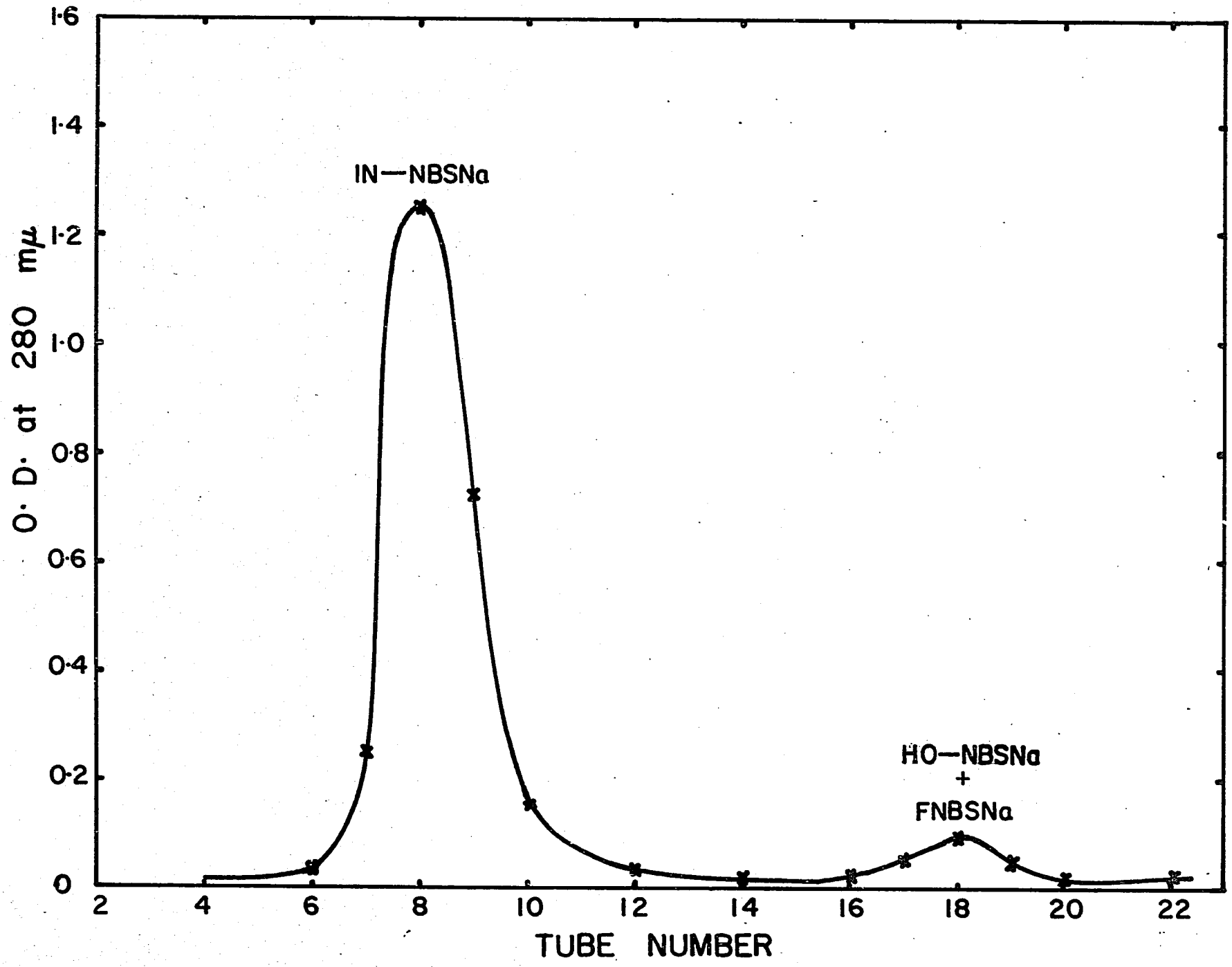

1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
O·D· at 280 mμ
IN—NBSNa
HO—NBSNa
+
FNBSNa
2
4
6
8
10
12
14
16
18
20
22
TUBE NUMBER

Figure 21

STANDARD CURVES FOR IN AND IN-NBSNa CONJUGATES OBTAINED WITH NINHYDRIN

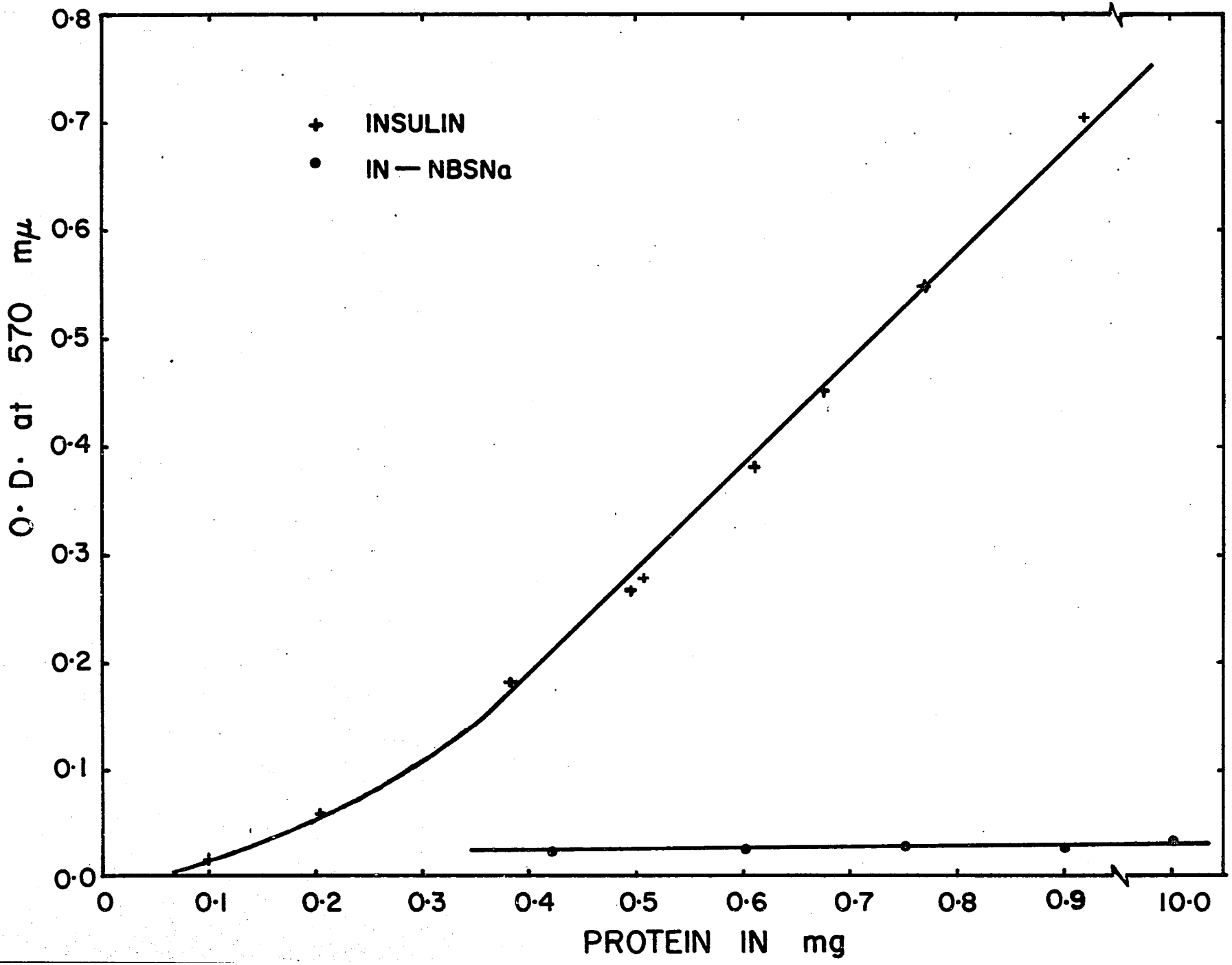

0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·0
O·D· at 570 mμ
INSULIN
IN — NBSNa
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
10·0
PROTEIN IN mg

Figure 22

STANDARD CURVES FOR IN AND IN-NBSNa CONJUGATES
OBTAINED WITH THE 'PHENOL REAGENT'

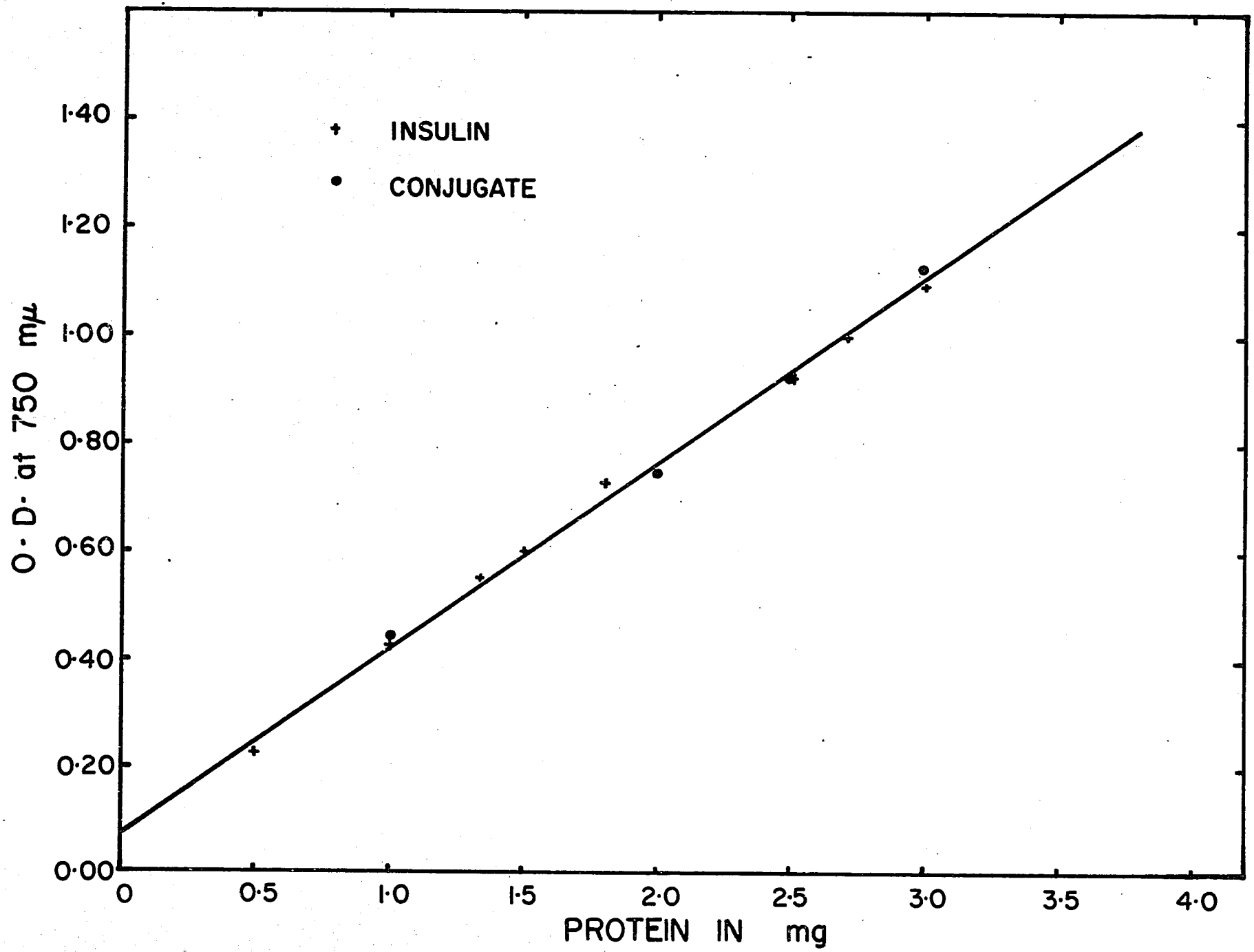
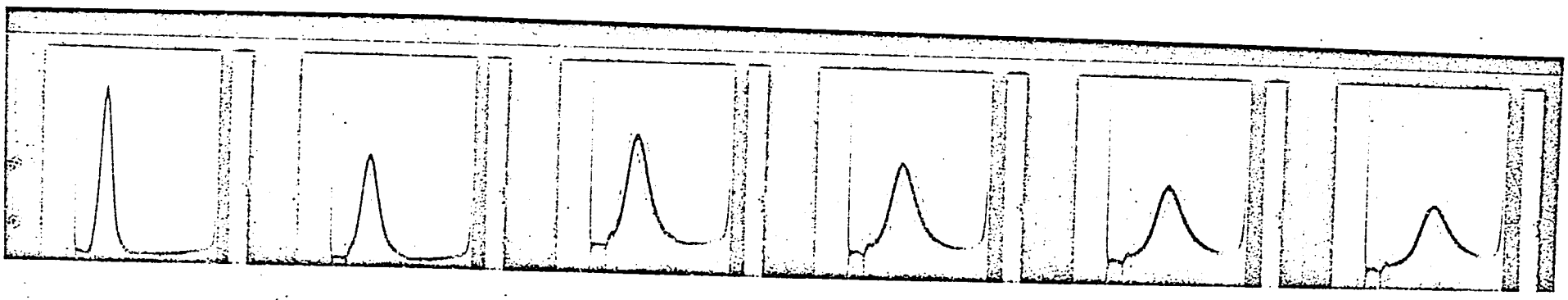

INSULIN
CONJUGATE
O·D· at 750 mμ
1·40
1·20
1·00
0·80
0·60
0·40
0·20
0·00
0
0·5
1·0
1·5
2·0
2·5
3·0
3·5
4·0
PROTEIN IN mg

Figure 23

ULTRACENTRIFUGAL PATTERNS OF IN-NBSNa CONJUGATE



coefficient of 4.2S. Since the conjugation reaction was carried out in 4% Na_2CO_3 (pH = 11.3), there is a possibility that the small amount of zinc, which is non=covalently bonded to insulin could have been dissociated from the crystalline insulin. Thus, the insulin, after the conjugation reaction, could have become zinc free. As already mentioned in Chapter II, zinc-free insulin is not homogeneous with respect to molecular weight, which depends on pH and insulin concentration. Previously, Marcker (114) reported that at a concentration up to 0.2% and a concentration up to 0.5%, the molecular weights of zinc-free insulin measured by osmometry at pH 9.10 were 12,000 and 24,000, respectively. On the other hand, by sedimentation experiment at pH 9 and 10, Fredericq (109) observed the sedimentation coefficient of 0.25% zinc-free insulin in potassium chloride-glycine solution (ionic strength 0.1) to be approximately 1.3. In addition, he demonstrated the presence of the monomer of insulin with molecular weight of 6,000 in dilute aqueous solution. In the present study, ultracentrifugal analysis of IN-NBSNa was carried out in borate buffer, pH 9.5, ionic strength 0.1. Under these experimental conditions, it is anticipated that a sedimentation coefficient much smaller than 3.3 should have been observed if the zinc ions had been dissociated from the crystalline insulin. The increase in sedimentation coefficient thus indicated that the zinc ions were not dissociated from the cystalline insulin. Moreover, this

result suggests a change in insulin structure brought about through the coupling between insulin and FNBSNa.

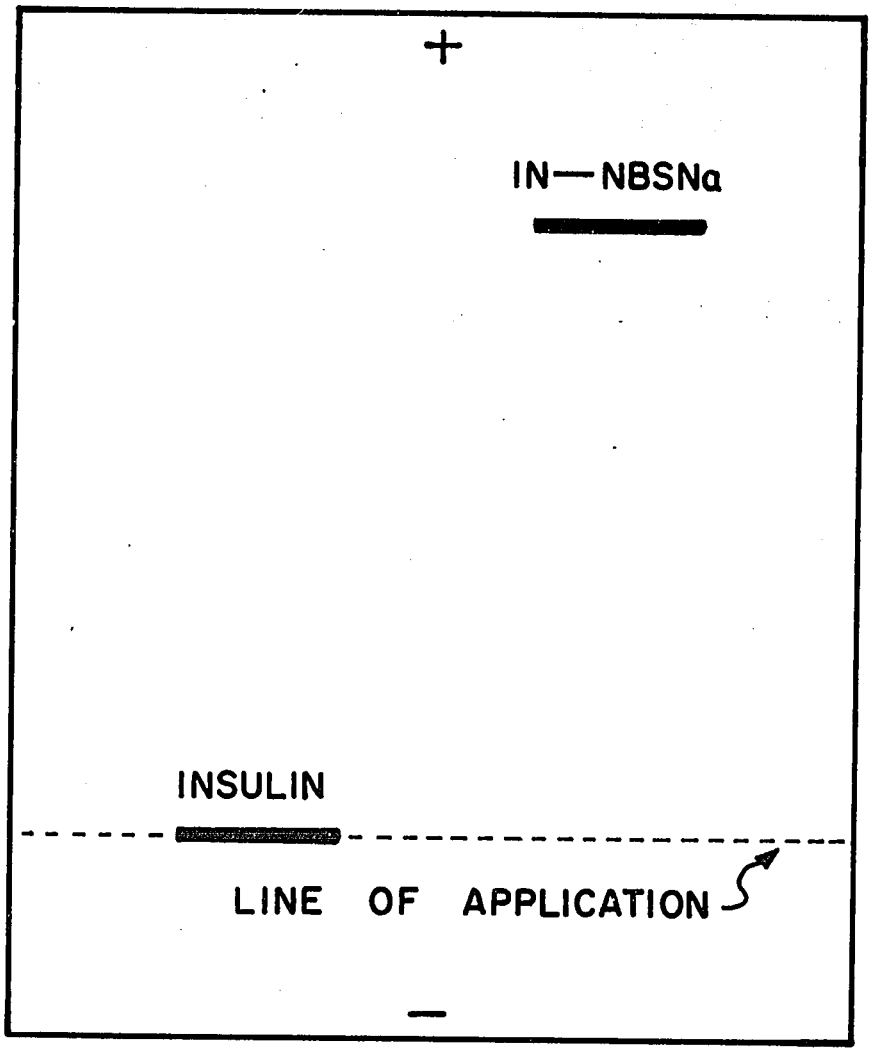
The blocking of the free NH_2 groups by FNBSNa was further demonstrated by the significant increase in the anodic mobility of the insulin derivative on paper electrophoresis (figure 24). Under the experimental conditions of the electrophoresis, i.e. at pH 6.0, the SO_3Na groups are fully ionized, i.e. they assume a negative charge; thus, the change in mobility is not due only to the elimination of the potentially positive NH_2 groups, but more importantly to the increase of the anionic groups.

Insulin is fairly soluble in a few organic solvents (183); however, its solubility in neutral aqueous solution is low. Previously, it was reported by Cohn et al. (184-185) that the solubility of insulin was about 10μg/liter in neutral aqueous medium and that with crystalline zinc insulin, containing 0.33 - 0.35% zinc, the solubility was 0.003 - 0.13 g/liter in the pH range 5.8 - 6.1 at 5°C. Usually high concentration of insulin solution can be obtained by dissolving it in a small amount of acid (pH = 1.0) or alkaline (pH = 12.0) and subsequently diluting it to the required pH with the appropriate buffer. In the present study, insulin was coupled to FNBSNa. Because of the incorporation of the NBSNa residues into the insulin molecule, the conjugate dissolved readily in neutral buffer up to a concentration of 2%.

Figure 24

Paper electrophoretic patterns of IN and IN-NBSNa conjugates in phosphate buffer pH 6.0, ionic strength 0.025

Voltage	:	110 V
Current	:	7.5 mA
Time	:	24 hours
Temperature	:	$4^{o}C$


+
IN—NBSNa
INSULIN
LINE OF APPLICATION
—

Solubility of I^{125}-IN-NBSNa in half-saturated ammonium sulphate

Method and Result

Diluent - The borate buffer used in these experiments had a pH of 8.3, ionic strength of 0.1*.

The saturated ammonium sulphate (SAS) was prepared at 4°C because all centrifugation was carried out at this temperature. Care was taken to prevent supersaturation by maintaining a small amount of undissolved ammonium sulphate in the 0°C stock SAS.

The normal rabbit serum (NRS) used in these experiments was separated from the clot within 24 hours of the time it was drawn. It was either used in this state or subsequently frozen and stored at -20°C until ready for use.

Antigen was diluted with 1:100 normal rabbit serum in borate buffer (1:100 NRS-borate). The 1:100 NRS-borate was necessary to prevent absorption of antigen on the surface of glassware and to prevent denaturation of I^{125}-IN-NBSNa by the effect of dilution.

I^{125}-IN-NBSNa was iodinated by Charles E. Frosst & Co., Montreal. This batch of I^{125}-IN-NBSNa, with specific

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*The borate buffer was prepared by dissolving 6.184 g boric acid, 9.356 g sodium tetraborate and 4.384 g sodium chloride in one liter to give pH 8.3 - 8.4.

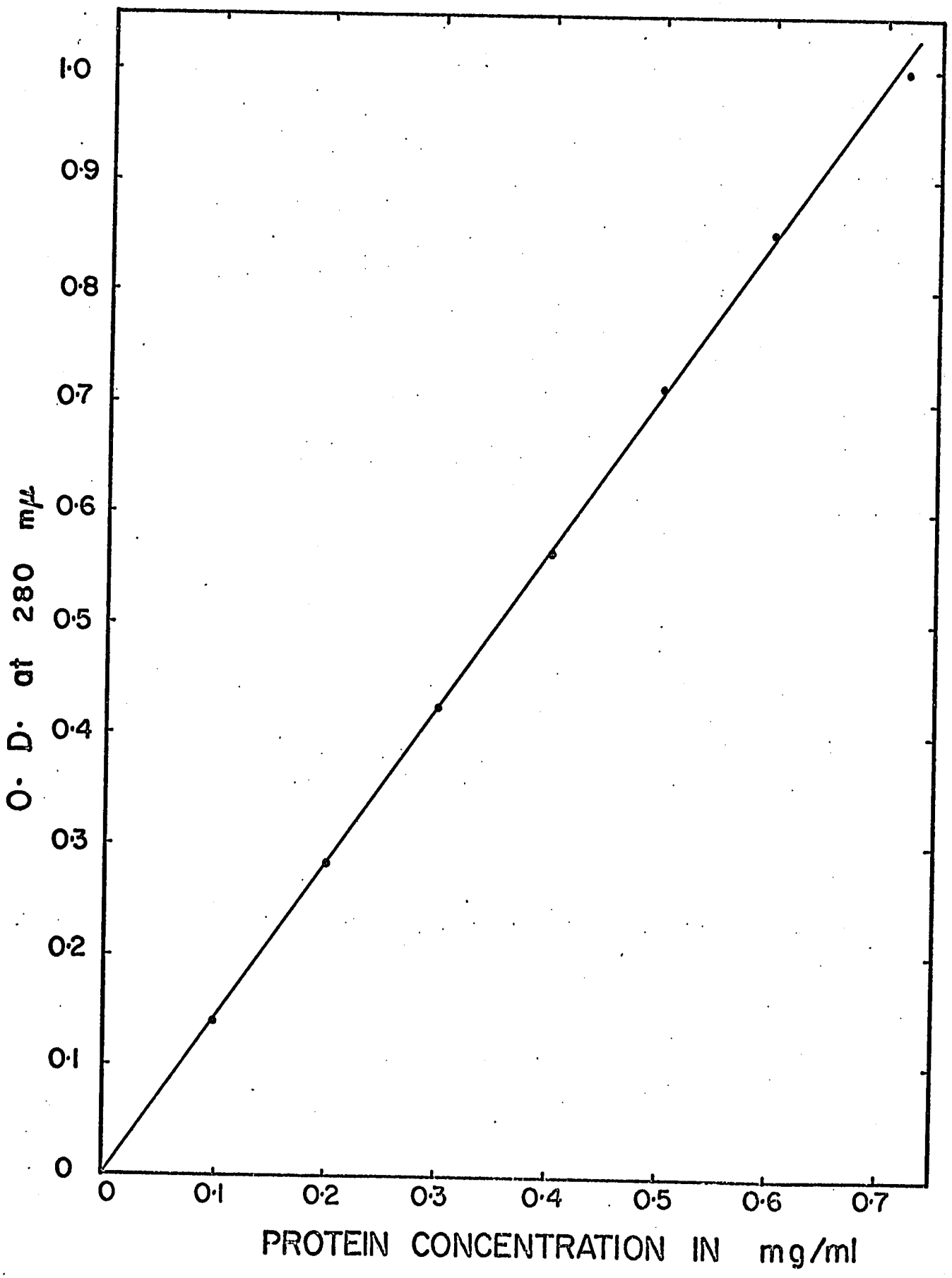
activity of approximately 2 mc/mg, was purified by passing through a column of Sephadex G-75 (1 cm x 40 cm). The I^{125}-IN-NBSNa thus purified contained less than 1% free I^{125} as measured by precipitation of protein with 10% trichloroacetic acid. For the analysis of the exact concentration of I^{125}-IN-NBSNa the optical method was employed. For this purpose, the optical densities of various concentrations of non-labelled IN-NBSNa in borate buffer, pH 8.3, ionic strength 0.1, were measured with a Beckman spectrophotometer (Model DU) at 280 mμ, and a standard curve was obtained by plotting the optical density vs. the protein concentration (figure 25). Then, the optical density of a stock solution in the borate buffer, containing relatively high concentration I^{125}-IN-NBSNa was similarly determined and the exact protein concentration could be read from the standard curve. The stock solution was further diluted with 1:100 NRS-borate to give a solution containing I^{125}-IN-NBSNa of the concentration in the order of 10 μg.

The measurement of radioactivity from I^{125} was performed in the automatic liquid scintillation spectrometer (Ansitron, Wallingford, Conn.). Bray's solution (186) was chosen as liquid scintillator because of its high efficiency and high water holding capacity (187). The scintillation solution consisted of 4 g of 2,5-diphenyloxazole (PPO), 0.2 g of 1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene (DM-POPOP), 60 g of naphthalene, 20 ml of ethylene glycol, and 100 ml of

Figure 25

STANDARD CURVE FOR DETERMINATION OF I^{125}-IN-NBSNa

BY OPTICAL DENSITY METHOD AT 280 mμ


1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
O· D· at 280 mμ
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
PROTEIN CONCENTRATION IN mg/ml

methanol dissolved in p-dioxane to make up a total volume of 1 liter.

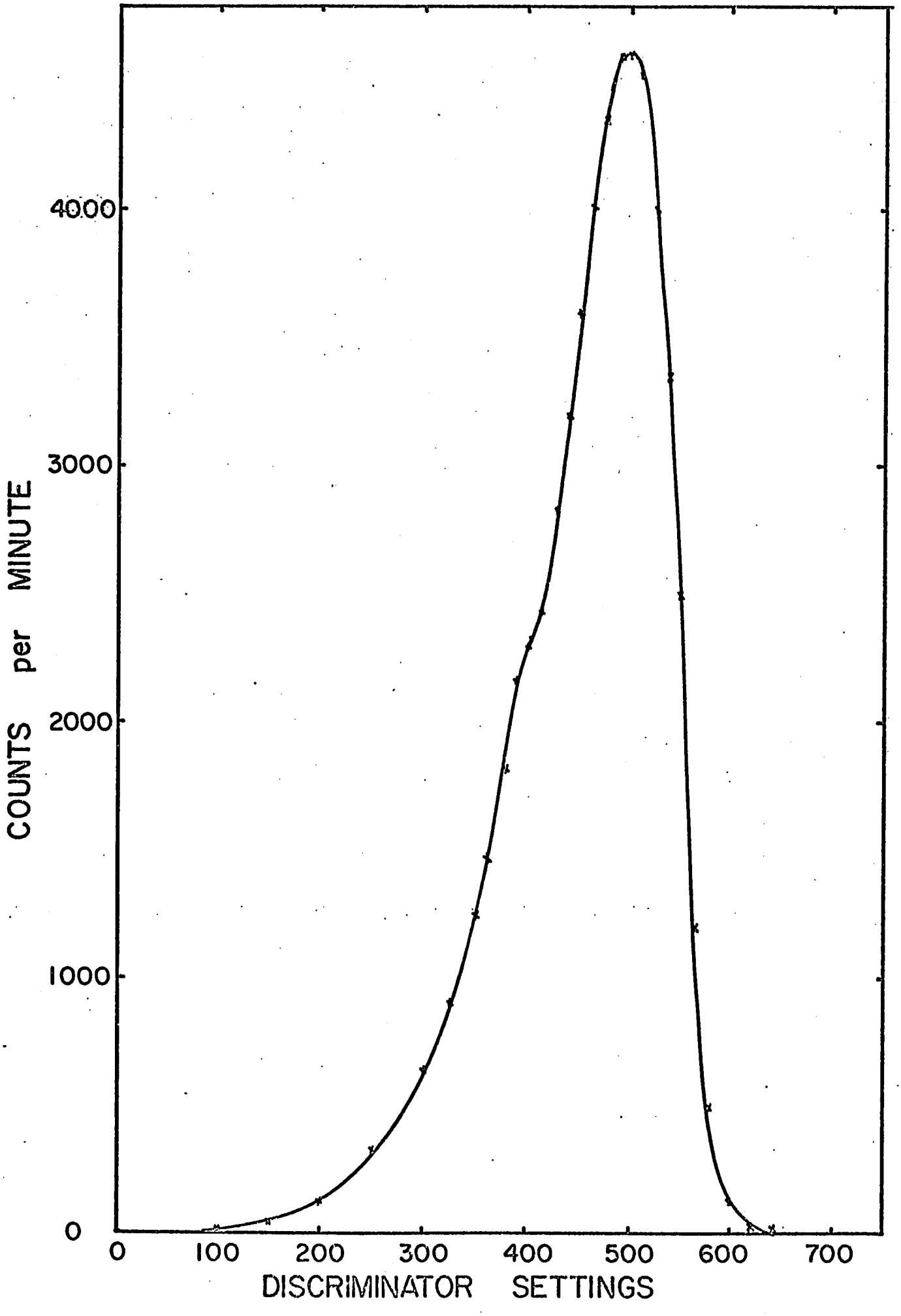
To determine the energy spectrum of I^{125} activity, a small volume ($\sim$0.1 ml) of I^{125}-IN-NBSNa stock solution was added to 15 ml of the scintillation solution in a vial, which was then placed in the spectrometer. The single channel analyser in the spectrometer with a window opening of 5 units (0.5% of the maximum lower discrimination level scale) was used to scan through the entire spectrum. The counting rate obtained at each lower discrimination level setting is given in figure 26. The peak is due to the gamma rays of 35.5 keV emitted during the decay of I^{125} by electron capture. The lower side of the peak exhibits a shoulder, which is due to the K X-rays of 31.8 keV emitted as a result of electron capture in the decay of I^{125}. After the spectrum had been determined, the single channel analyser of the spectrometer was set to include counts in the peak only for all subsequent countings of the protein samples. This was done to minimize background counts. All measurements were carried out at a temperature of 4^{o}C for sufficient time (5 minutes) to obtain a statistical error of less than 1%.

For the determination of the solubility of I^{125}-IN-NBSNa in half-saturated ammonium sulphate solution, 1 ml of 1:100 NRS-borate containing I^{125}-IN-NBSNa at 4^{o}C was added to an equal volume of the saturated ammonium sulphate in a 15-ml centrifuge tube. The mixtures were shaken vigorously to

Figure 26

LIQUID SCINTILLATION SPECTRUM OF I^{125}

IN BRAY'S SOLUTION


4000
3000
2000
1000
0
COUNTS per MINUTE
0
100
200
300
400
500
600
700
DISCRIMINATOR SETTINGS

ensure thorough mixing, incubated at 4^{o}C for 30 minutes and centrifuged at a speed of 2,000 r.p.m. for 30 minutes in an International centrifuge. The supernatant was separated by decantation and 0.4 ml of the supernatant was added to 15 ml of the scintillation liquid for counting. All experiments were set up in triplicate. Results of the present investigation (table VII) indicate that at half-saturation with ammonium sulphate solution, at which 98% of antibody globulins and the corresponding antibody-antigen complexes are precipitated, 65 - 70% of the I^{125}-IN-NBSNa was also precipitated. Since for kinetic studies it is essential to establish unequivocally the concentrations of the free and bound form of the antigen, it became obvious that this approach was not going to be fruitful, and it was decided to abandon the study of kinetics of this reaction. Nevertheless, the greatly increased solubility of IN-NBSNa with respect to that of insulin was taken advantage of in the study of the specificity of the antibodies to insulin.

Table VII

SOLUBILITY OF I^{125}-IN-NBSNa AT HALF-SATURATION WITH AMMONIUM SULPHATE

Concentration in µg/ml	Original activity in total No. of counts	Activity in supernatant in total No. of counts	% in supernatant
0.5	20,118	6,679	33.2
	"	7,163	35.6
	"	6,820	33.9
1.5	58,257	19,190	33.0
	"	19,426	33.4
	"	20,552	35.4
3.1	122,900	39,920	33.3
	"	39,168	31.8
	"	42,104	34.2
5.0	19,754	60,447	30.6
	"	65,978	33.4
	"	69,336	35.1

CHAPTER 4

STUDIES ON PRODUCTION AND SPECIFICITY OF ANTIBODIES TO INSULIN

Introduction

A procedure for coupling insulin to proteins by stable covalent bonds was described in the previous chapter. The possibility that the immunogenicity of insulin might be enhanced by coupling it to a carrier protein was investigated by immunizing rabbits with IN-TDIC-BSA conjugates. The antibodies produced in this manner were characterized by immunochemical techniques and the results are described in this chapter.

Method

Preparation of Antisera

Solutions containing IN-TDIC-BSA conjugates were used for immunization of albino rabbits weighing 3 to 4 kilograms. These animals received a series of intravenous injections of 1 ml of a 1% sterile saline solution of the immunizing antigens, three times a week for five weeks*. The immunizing antigens

*The author is indebted to Mr. Rabasse for his help in immunizing the rabbits.

consisted of BSA and the conjugates of IN-TDIC-BSA and IN-TDIC, which had been described in Chapter III. Blood samples were collected periodically starting six days after the last injection and the presence of antibody was detected by ring test. The animals whose sera did not contain antibodies by this test were re-immunized according to the same schedule after a rest period of two weeks. The rabbits which produced antibodies were bled once a week (50 to 70 ml) and re-injected with 1 ml of the immunizing antigens immediately after bleeding. This schedule was followed for about 6 months. The blood samples were allowed to clot at room temperature and were then placed in a cold room to allow the clot to shrink. The serum was then separated by centrifugation, Seitz filtered into sterile vials, and stored at 4^{o}C until required.

Demonstration of precipitating antibodies

Ring test

Ring tests were performed in small test tubes (4.5 cm x 0.5 cm) by layering carefully 0.5 ml of different concentration of insulin in veronal buffered saline, pH 7.5, ionic strength 0.1, over 0.5 ml of the antiserum. The tubes were kept at room temperature for 1 to 2 hours. The presence of antibody was manifested by the appearance of a precipitate in the form of a ring at the interface. The sera giving a ring within 2 - 5 minutes were graded as (3) +, (2) +, and (+)

respectively. The specificity of the reaction was demonstrated by the absence of a ring when the same solution of the antigen was layered over a normal rabbit serum.

Micro-Ouchterlony technique

Standard microscope slides (17.5 cm x 6 cm) were first coated with a thin layer of agar by applying 0.5 ml of a hot solution of a 1% agar in distilled water and evaporating off the water in a stream of hot air. A warm solution of 1% agar in 0.9% saline, containing one part of merthiolate in 5,000, was then layered over the agar film to give a thickness of 2 mm. When the gel had solidified, the desired pattern of wells for the antibody and antigen solutions was prepared by removing the agar with a dull syringe needle (gauge #13) attached to a water aspirator. The distance between the wells varied between 4 and 8 mm. The wells were filled only once and the slides were placed in an air-tight container saturated with water vapour.

Precipitin reaction

In all experiments 1.0 ml portions of the antiserum were added to a series of tubes each containing decreasing amounts of the antigen solution in 1 ml of veronal buffer (pH 8.6, ionic strength 0.1). After mixing the two reactants, the solutions were incubated for 4 hours at 37°C and then for 48 hours at 4°C. The precipitates were packed to the bottom

of the tubes by gentle centrifugation and washed three times with 2 ml volumes of chilled saline. The amount of precipitated protein in each tube was determined by the micro-Kjeldahl procedure of McKenzie and Wallace (188). For this purpose, the precipitated proteins were digested with 0.8 ml of concentrated H_2SO_4 containing about 100 mg of K_2SO_4 plus 0.2 ml $HgSO_4$*; during this process, the protein nitrogen was converted to $(NH_4)_2SO_4$. To each tube was then added 5 ml of 40% NaOH and the ammonia liberated was steam distilled into a solution of boric acid** containing methyl red and methylene blue as an indicator. Titration was performed with 0.001 M $KH(IO_3)_2$*** solution.

Micro-hemagglutination

Rabbit erythrocytes coated with the antigen, i.e. 'sensitized' erythrocytes, were used in the passive hemagglutination technique. Blood was collected from the marginal ear vein into an equal volume of Alsever's solution (189) from rabbits which had not been previously bled or which had been allowed to rest for at least six weeks after an

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*20 g of HgO dissolved in 200 ml of 4N H_2SO_4.

**15 g of boric acid + 10 ml of 0.2% methyl red + 5 ml of 0.2% methylene blue per 1.5 liter.

***$KH(IO_3)_2$ of analytical grade was purchased from Fisher Scientific Co., Montreal.

earlier bleeding*. The blood in Alsever's solution was not used for more than a week and was stored at 4°C. The serum used as diluent was isolated from the blood of the same rabbit which supplied the erythrocytes. The complement in this serum was inactivated by heating at 56°C for 30 minutes, and the serum was then diluted 100-fold with veronal-buffered saline (pH 7.5, ionic strength 0.1).

Bis-diazotized-benzidine (BDB) was prepared according to the method previously used in this laboratory (77); it was essential to maintain the temperature of the reaction mixture at 0°C. The reaction was allowed to proceed for 30 minutes with intermittent stirring. The final product was a pale yellow solution; small volumes of $\sim$1.5 ml of this solution were then placed into 4 ml vials, frozen at -80°C (in dry ice-acetone) and maintained at -20°C until required. Before coupling the antigen to erythrocytes, the frozen BDB solution was melted quickly by rotating the vial in the palm, and 1 ml of it was added to 14 ml of isotonic phosphate buffer**, pH 7.3, ionic strength 0.15, which had been pre-cooled to 4°C (this solution will be referred to hereafter as BDB-phosphate

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*If the erythrocytes were obtained from rabbits bled within a shorter interval, false positive hemagglutination occurred, probably due to the fragility of the red blood cells formed by the animal under this stress.

**The phosphate buffer was prepared by mixing 215 ml of 0.15 M Na_2HPO_4 with 49 ml of 0.15 M KH_2PO_4.

solution). This solution must be used as soon as possible so as to minimize its deterioration, which is detected by the formation of coloured products.

Sensitization of the erythrocytes

The packed cells were washed three times by resuspending them in cold physiological saline and recentrifugation and were then suspended in an equal volume of saline /50% (v/v) red cell suspension/. In all experiments, 3 ml of isotonic phosphate buffer solution containing the optimal quantity of insulin was mixed with 0.1 ml of this cell suspension. The optimal amount of BDB-phosphate was then added and the suspension gently mixed and allowed to stand at room temperature for 15 minutes with occasional stirring. The 'sensitized cells' were separated by centrifugation and the supernatant was discarded. These cells were then washed with 3.5 ml of diluent and finally were suspended in 5 ml of diluent.

Standardization of the method

It was necessary to determine the optimal ratio of BDB to insulin for the sensitization of erythrocytes. When excess insulin was used, the cells were not agglutinated by the insulin antisera. If too little insulin was used, agglutination and lysis occurred after the addition of BDB-phosphate to the cell suspension. To establish the optimal amounts of each batch of BDB and antigen for a constant number

of erythrocytes, the following procedure was used: (i) the volume of BDB-phosphate was varied keeping the antigen concentration constant, and (ii) the antigen concentration was varied and the volume of BDB-phosphate kept constant. The proportion of the BDB-phosphate solution to antigen was considered optimal when the highest hemagglutination titers were obtained with a given antiserum and when no false positive patterns were observed in controls. For optimal sensitization, 0.1 ml of a 50% (v/v) red cell suspension mixed with 3 ml of insulin solution (containing 1.0 mg) required 0.25 ml of BDB-phosphate buffer.

Performance of the hemagglutination test

The micro-hemagglutination test was performed with the Microtit kit, purchased from Metrimpex, Budapest, Hungary. The method was essentially similar to that developed by Takatsy (190)*. The plexiglass plates had 6 rows of 12 wells; the wells were conical at the bottom.

To remove non-specific agglutinins, all sera were treated (i.e. adsorbed) with an equal volume of packed red blood cells for one hour at room temperature. The cells used for absorption and sensitization were obtained from the same rabbit. A volume of 0.05 ml of diluent was placed into each

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*The calibration of the droppers and loops had been done in this laboratory by Mrs. A. Reiner.

well with a calibrated dropper (2 drops). Two series of two-fold serial dilutions of the absorbed antisera were prepared in plexiglass plates using stainless steel loops of known volume (0.05 ml). To each well was then added one drop (0.025 ml) of the sensitized cell suspension. The plates were gently shaken and the reaction was allowed to proceed for 3 hours at room temperature. Results of the reaction were read after 4 to 6 hours. The titer of each antiserum was expressed as the reciprocal of the highest dilution of the antiserum which still gave a positive test.

For the demonstration of the specificity of immunochemical reaction by the hemagglutination inhibition procedure, 10 μg of insulin in 0.025 ml was added to each well; for additional controls, 0.025 ml of the diluent was added to each well in another serial dilution of the same antiserum.

Immunoelectrophoresis

Immunoelectrophoresis was carried out with the LKB immunoelectrophoresis apparatus (Model 6800 A). The method of preparing the agar slide was essentially the same as that for the Ouchterlony test except that a different buffer was used for preparing the agar gel. Six standard microscope slides were placed on a rectangular slide frame, three on each side. It was important to place the slides closely together so as to have a clearance of about 2 cm between the ends of the outer slides and the frame (figure 27). The frame was then placed

Figure 27

THIS DIAGRAM SHOWS SUCCESSIVE STEPS FOR THE IMMUNOELECTROPHORESIS RUN

(1) Slide frame with six microscope slides in place

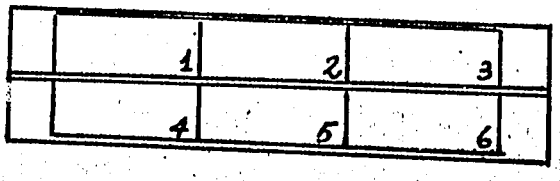
(2) Application of agar solution

(3) Punch of a pattern consisting of two troughs and a well in the gel

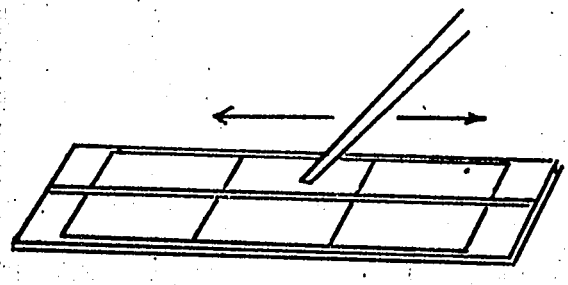
(4) Application of rabbit antiserum

(5) Electrophoresis run

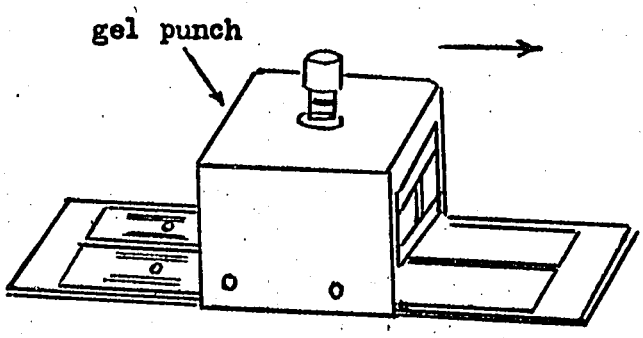
(6) Removing agar bits from trough with a gel knife



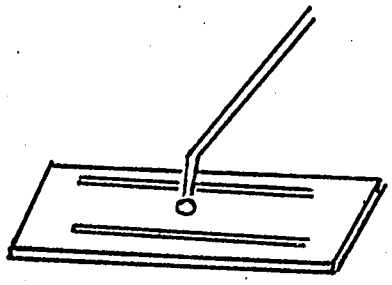
(1)



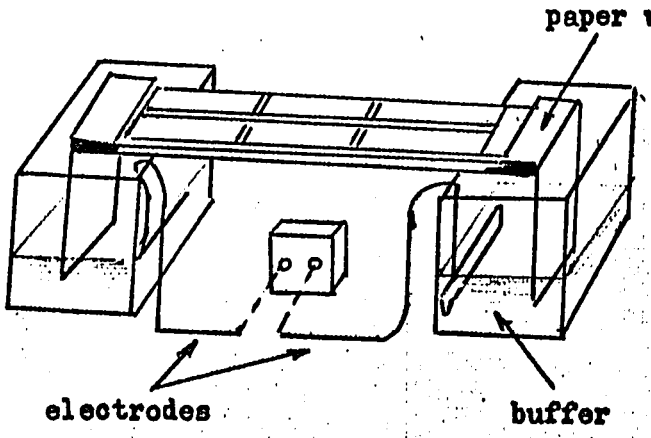
(2)



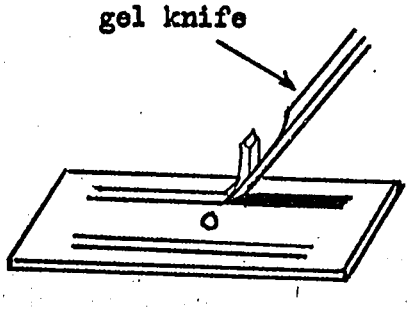
(3)



(4)



(5)



(6)

on a levelling table, which was adjusted to horizontal. A film of hot agar solution in water was applied to the glass slides and then air-dried for at least 15 minutes. This film sealed the joints between adjacent slides and ensured adhesion of the agar gel layer to the glass slides; the latter was formed by pouring 10 ml of warm agar solution in veronal buffer* (pH 8.6, ionic strength 0.025) uniformly over the microscope slides. The entire frame section, including the part outside the microscope slides near the edge, was thus covered with agar. The standard pattern consisting of a hole (2 mm in diameter) at the centre of the slide and of two longitudinal troughs on both sides of and 10 mm from the hole was cut with a gel punch. The agar in the central hole was carefully removed by aspiration with a suction needle, while the agar in the troughs was left in place until after electrophoresis. In most experiments, 5 μl of the rabbit antiserum was applied to the hole. The frame was placed in a casette and was used as a bridge between the electrode vessels containing veronal buffer (pH 8.6, ionic strength 0.1). The electrode consisted of an anode made of a platinum wire and a loop of stainless steel for the cathode. The potential applied during electrophoresis, which lasted usually 50 minutes,

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*The buffer consisted of 3.68 g of diethyl barbituric acid, 20.62 g of sodium barbiturate and 8.2 g of sodium acetate dissolved in 8 liters of distilled water.

was 250 V and the current was 8 mA. The frame was taken out from the casette; the agar in the troughs was removed with the knife provided with the LKB set, and the two troughs were filled with a goat antiserum to rabbit serum proteins* and with a solution of insulin, respectively. The slide was then put into a humid chamber at room temperature overnight for the development of the precipitin bands.

Results

1. Ring test

The majority of rabbits produced precipitating antibodies to insulin approximately five weeks after the first injection of antigens. Since the solution used for immunization contained IN-TDIC-BSA conjugate, IN-TDIC and BSA, as would be expected, ring tests were obtained with each of the components, i.e. with insulin, BSA, IN-TDIC and IN-NBSNa but not with glucagon, which had been present in the insulin preparation as a contaminant (table VIII). Eight of the 13 antisera had a sufficiently high antibody content to give well-defined precipitin rings; two antisera gave ambiguous reactions and one antiserum had no detectable antibodies (table IX, p.129). Only two of the 13 rabbits, which had been immunized, died before the end of the immunization programme. It appears, therefore,

*The author is indebted to Mr. Fred Kisil for the preparation of this goat antiserum to rabbit serum proteins.

Table VIII

RING TESTS OBTAINED WITH ANTISERUM FROM No. 2 RABBIT

Test antigen	Reaction
BSA	+++
IN-TDIC	++
IN	+
IN-NBSNa	+
Glucagon*	-

*Crystalline glucagon was purchased from Connaught Medical Research Laboratory, Toronto, Ontario.

that repeated intravenous injections of adequate amounts of IN-TDIC-BSA conjugates over a sufficiently long period of time consistently elicited antibodies to insulin in rabbits.

2. Micro-Ouchterlony test

As can be seen from figure 28, precipitin bands were formed between rabbit antiserum and the conjugate, insulin, IN-TDIC and BSA. Moreover, a reaction of partial identity was observed between IN-TDIC and IN (figure 29A). Pre-incubation of the antiserum with IN-TDIC resulted in the inhibition of the precipitin bands formed with both IN-TDIC and IN, whereas absorption of the antiserum with IN solution caused the disappearance only of the arc with IN (figure 29B). The presence of antibodies specific to IN, but not to glucagon, is illustrated in figure 29C; this demonstrates that glucagon was neither immunogenic nor did it inhibit the reaction with IN. Furthermore, a band of identity was obtained between the antiserum and IN and IN-NBSNa (figure 30); this band was completely inhibited when the antiserum was pre-incubated with either of these antigens in excess.

3. Precipitin reaction

The absolute amounts of antibodies to insulin present in the different antisera were determined by the quantitative precipitin reaction. A typical precipitin curve is shown in figure 31. The antibody content of this serum was calculated

Figure 28

PRECIPITIN BANDS IN AGAR-GEL OBTAINED WITH RABBIT ANTISERUM (No. 6) TO IN-TDIC-BSA CONJUGATES

Central well - Antiserum (No. 6)

Wells No. 1,5 - BSA (0.5 mg/ml solution)

3,7 - IN (1.0 mg/ml solution)

2,4,6,8 - immunizing antigens (IN-TDIC-BSA, IN-TDIC and BSA) (1.0 mg/ml solution)

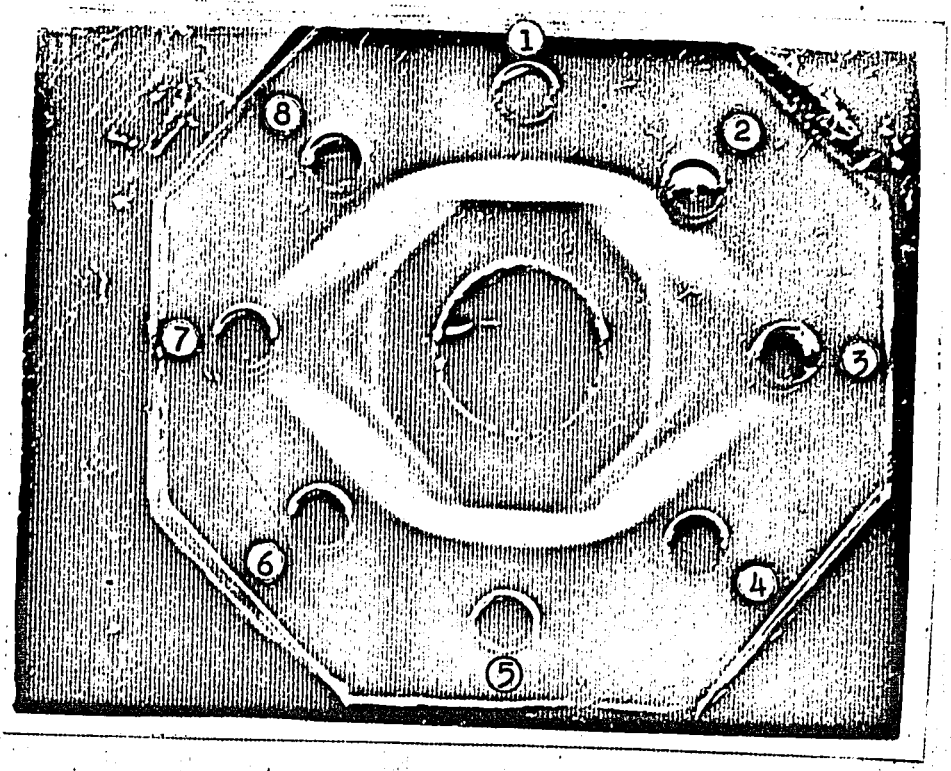

1
2
3
4
5
6
7
8

Figure 29

INHIBITION OF PRECIPITIN BANDS IN AGAR-GEL DIFFUSION

(A) Well No. 1,3 - antiserum

2 - IN (1 mg/ml solution)

4 - IN-TDIC (25 μg/ml solution)

(B) Well No. 1 - antiserum pre-incubated with insulin (0.5 ml of antiserum + 0.5 ml of 0.2% solution)

2 - IN (1 mg/ml solution)

3 - antiserum pre-incubated with IN-TDIC (0.5 ml of antiserum + 0.5 ml of 0.1% IN-TDIC solution)

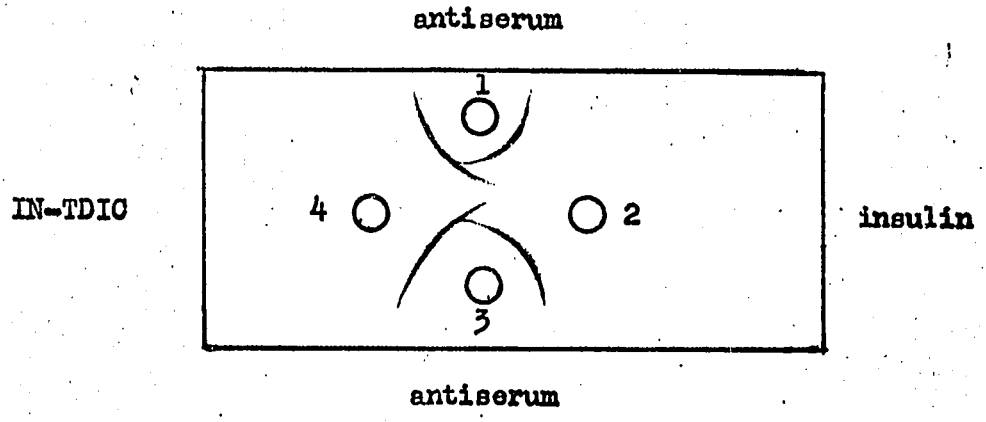
4 - IN-TDIC (25 μg/ml solution)

(C) Well No. 1 - antiserum

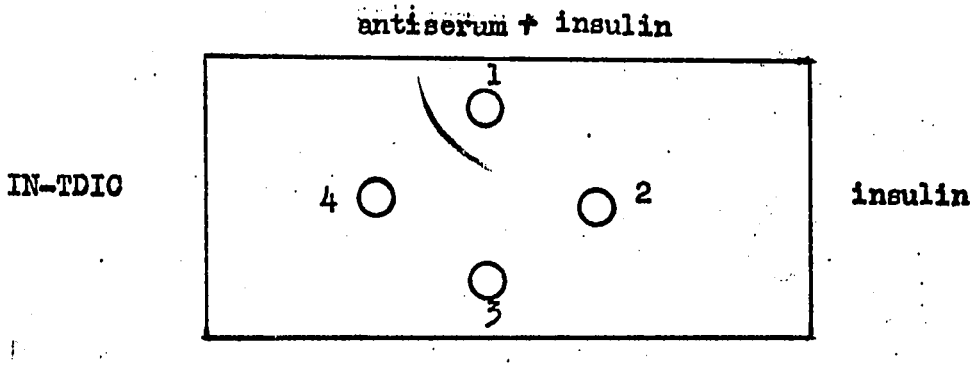
2 - IN (1 mg/ml solution)

3 - antiserum pre-incubated with glucagon (0.5 ml of antiserum + 0.5 ml of 0.1% glucagon solution)

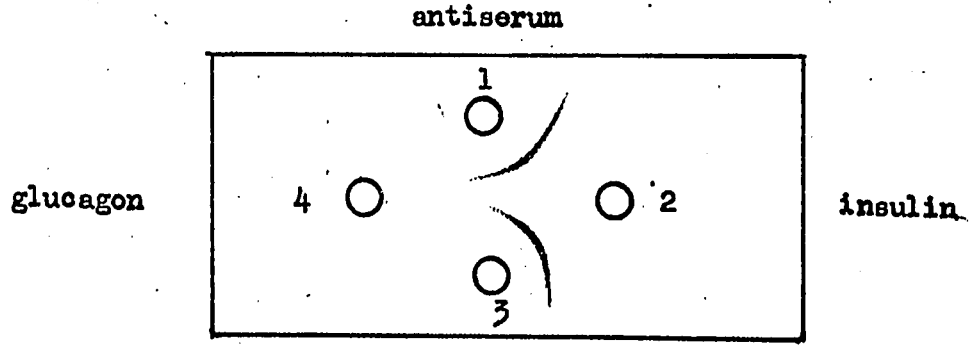
4 - glucagon (1 mg/ml solution)


antiserum
1
IN-TDIC
4
2
insulin
3
antiserum

(A)


antiserum + insulin
1
IN-TDIC
4
2
insulin
3
antiserum + IN-TDIC

(B)


antiserum
1
glucagon
4
2
insulin
3
antiserum + glucagon

(C)

Figure 30

PRECIPITIN BANDS BETWEEN RABBIT ANTISERUM TO IN-TDIC-BSA CONJUGATES AND IN AND IN-NBSNa

Well No. 1 - IN (1 mg/ml solution)

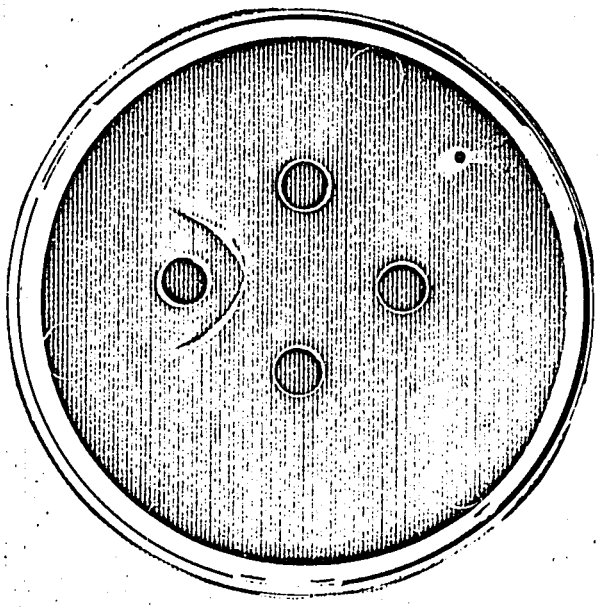
2 - normal rabbit serum

3 - IN-NBSNa (1 mg/ml solution)

4 - antiserum (No. 6)

Insulin

Antiserum to the insulin conjugate preparation



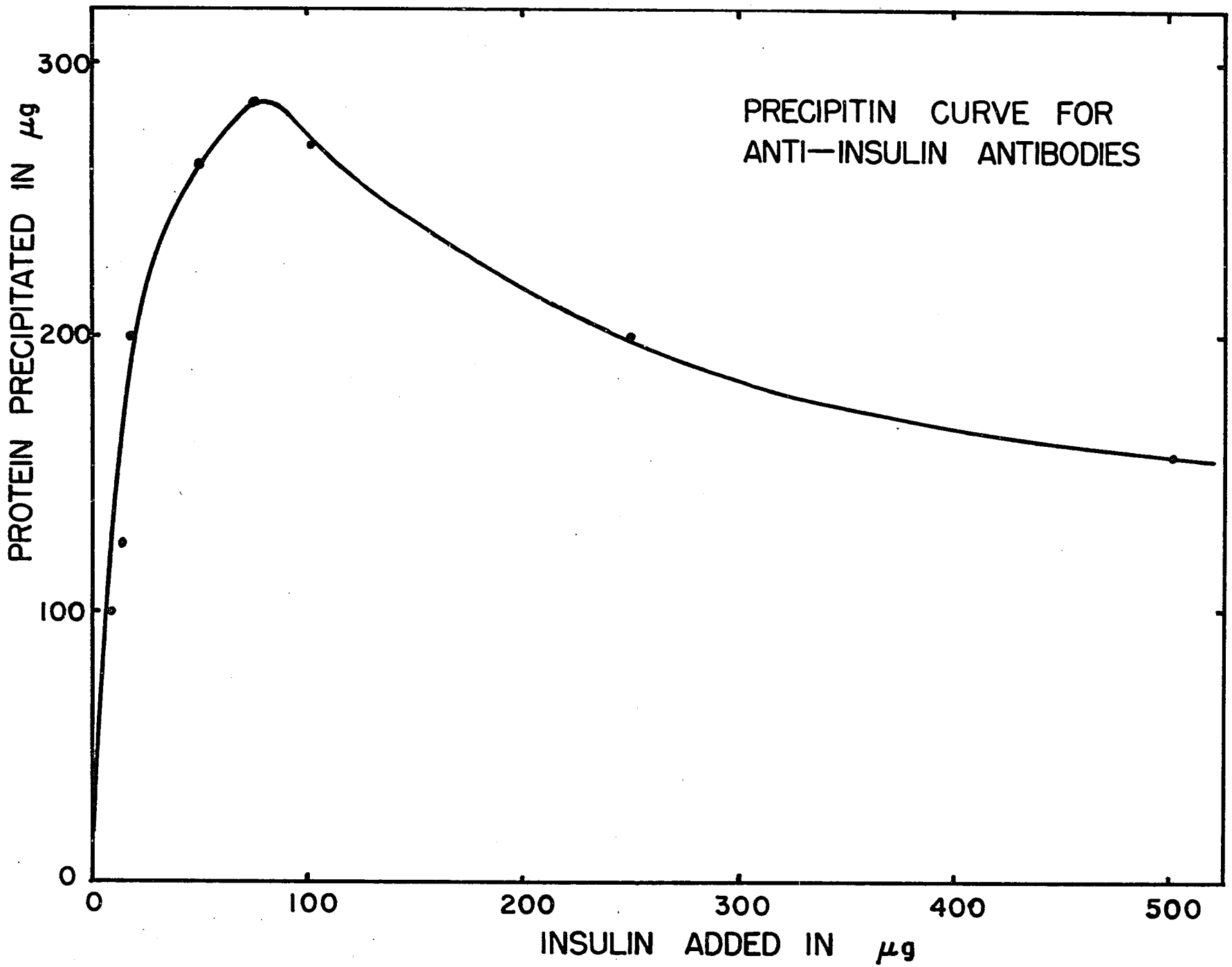
Normal rabbit serum

IN-NBSNa

Figure 31

PRECIPITIN CURVE FOR INSULIN ANTIBODIES

(antiserum No. 10)


PRECIPITIN CURVE FOR
ANTI—INSULIN ANTIBODIES
PROTEIN PRECIPITATED IN μg
INSULIN ADDED IN μg
0
100
200
300
400
500

from the difference between the amounts of Ag-Ab complexes precipitated per ml of antiserum in the optimum zone and the amount of antigen added. For different antisera which gave positive ring tests, the antibody content varied from 200 μg to 1 mg per ml (table IX). It can be seen from the precipitin curves with IN and with IN-NBSNa that practically the same amounts of antibodies were precipitated at the optimum zone by these two different compounds and that the curve obtained with IN-NBSNa was almost identical to that obtained with IN as antigen (figure 32). The specificity of the precipitin reaction was also demonstrated by the lack of any precipitation with these two antigens when a normal rabbit serum was incubated with the antigen.

4. Titration of anti-insulin antibodies in rabbit sera by hemagglutination

Hemagglutination was obtained at serum dilutions up to 2048 (table X). The specificity of hemagglutination reaction was demonstrated by inhibition of hemagglutination with free insulin. In these experiments, 10 μg of insulin was added to each dilution of the antiserum prior to the addition of the sensitized erythrocytes. In the case of one serum with an initial hemagglutination titer of 256, complete inhibition of hemagglutination was achieved. However, in the case of the other ten, the hemagglutination titers were considerably diminished to values of the order of 2 - 32. In view of the

Table IX

COMPARISON OF THE INTENSITY OF RING TESTS WITH THE ANTIBODY CONTENTS DETERMINED BY THE PRECIPITIN REACTION

Antigen used - insulin

Antiserum	Ring test	µg/ml
1	+	210
2	+	310
3	++	380
4	±	?
5	+++	530
6	++	480
7	++	370
8	+++	980
9	±	?
10	+	280
11	-	-

Figure 32

PRECIPITIN CURVES FOR INSULIN ANTIBODIES
OBTAINED WITH IN AND IN-NBSNa

(antiserum No. 6)

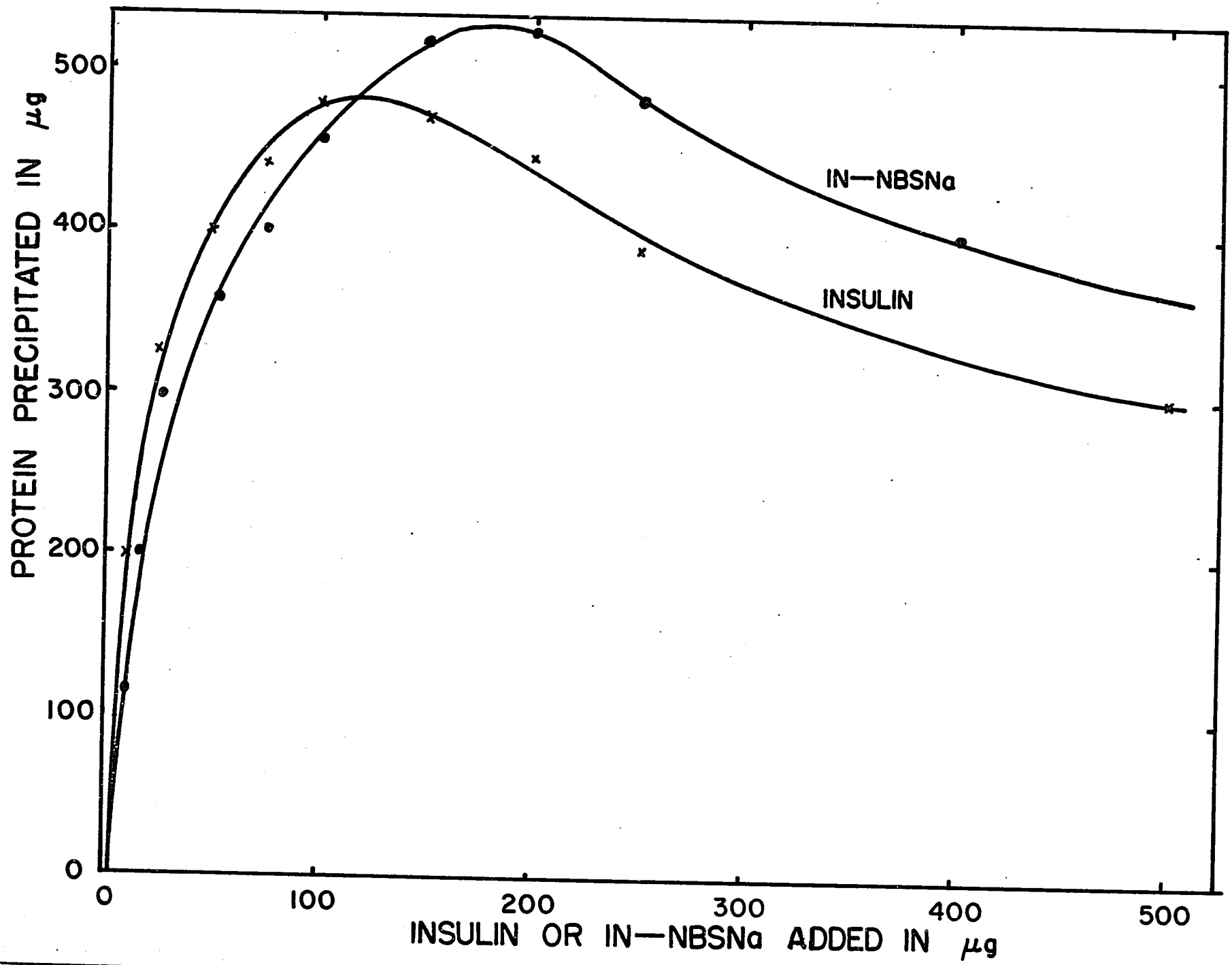

PROTEIN PRECIPITATED IN μg
0
100
200
300
400
500
IN—NBSNa
INSULIN
0
100
200
300
400
500
INSULIN OR IN—NBSNa ADDED IN μg

Table X

DEMONSTRATION OF ANTIBODIES TO INSULIN BY THE INHIBITION OF HEMAGGLUTINATION WITH INSULIN

Antiserum	Hemagglutination titer prior to inhibition	Titer after inhibition with 10 μg insulin
1	256	complete inhibition
2	512	2
3	2048	4
4	128	2
5	2048	4
6	512	2
7	1024	8
8	2048	32
9	64	4
10	512	2
11	64	2

significant decrease of the hemagglutination titer in the presence of 10 μg of insulin with all sera, this phenomenon was considered to reflect the specificity of the inhibition reaction for antibody directed against insulin. Higher concentrations of insulin were not used in this inhibition test, since in inhibition experiments with the separated insulin chains comparable concentrations had to be used because of solubility considerations and the limited amount of materials available. (See chapter V.)

A comparison of the hemagglutination titers with the antibody contents, determined by the precipitin test (tables IX and X), revealed a very good agreement between the results of the two tests. Thus, the hemagglutination titer appears to be a reflection of the relative amount of the antibody in the antiserum specific to insulin. However, the hemagglutination titers determined are relatively low when compared with values obtained with other precipitating antibodies. Previously, Gyenes and Sehon (79), using BDB and TDIC as coupling agents in the passive hemagglutination reaction, observed that the hemagglutination titers of the anti-BSA sera were practically identical with the two techniques, whereas the TDIC-hemagglutination titers for six out of seven rabbit anti-WSR sera were considerably lower than the corresponding BDB-titers. It was suggested by these authors that, for the WSR-anti-WSR system, distinct antibodies were produced against determinants containing free amino groups and that on coupling of the

antigen to erythrocytes with TDIC some of these groups were blocked; consequently, the sensitivity of the hemagglutination reaction for this antigen was reduced when TDIC was used for coupling it to erythrocytes. By the same token, in the present study, the observed low hemagglutination titers with BDB as a coupling agent might be attributed to the fact that the determinant groups of insulin contain tyrosyl or histidyl residues, or are situated in the vicinity of these groups. This conclusion is further supported by the finding of Izzo et al. (191) that incorporation of more than six iodine atoms per molecule of insulin (M.W. 6,000) lowered the capacity of iodoinsulin to bind with specific antibodies to insulin.

5. Immunoelectrophoresis

A distinct precipitin line was obtained with antisera containing high amounts of antibodies (i.e. 400 μg/ml). The line had a typical single arc in the gamma-globulin region (figure 33).

Discussion

As already noted in Chapter II, the properties of antibodies to insulin are governed by many factors, such as the species from which the insulin is derived and the recipient. Thus, the majority of diabetic patients treated with insulin have been found to produce 'non-precipitating' antibodies to

Figure 33

IMMUNOELECTROPHORETIC ANALYSIS OF THE RABBIT ANTISERUM (No. 6) DEVELOPED WITH INSULIN 0.025 mg/ml

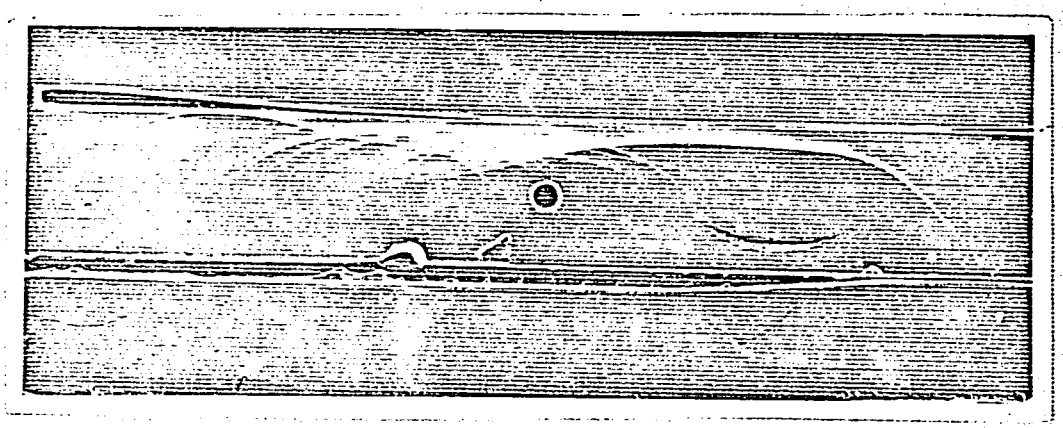
Upper trough - goat antiserum to rabbit serum proteins

Lower trough - insulin (0.025 mg/ml solution)

Central well - rabbit antiserum to IN-TDIC-BSA conjugates (No. 6)

Goat antiserum to rabbit serum proteins

+



-

Insulin

insulin, i.e. antibodies which did not lead to the formation of a precipitate when reacted with the homologous antigen (129, 138,192). Similarly, in the earlier studies, attempts to produce precipitating antibodies to insulin by immunization of various animals were also not successful (133,135,193). Moloney and Aprile (139) were the first to obtain precipitating antibodies to insulin in a horse immunized with crystalline ox insulin with Freund's adjuvant. More recently, several investigators have reported the production of precipitating antibodies in laboratory animals, when immunization was carried out in the presence of adjuvants (140-144).

In the present study, rabbits were immunized with insulin-BSA conjugate, an artificial antigen. Results of the ring tests and micro-Ouchterlony agar-gel experiments demonstrated readily the appearance of the precipitating antibodies against insulin itself. Furthermore, with these antisera a single line of precipitation was obtained at all times by double diffusion in agar against insulin.

The specificity of antibodies for insulin produced in this study was clearly demonstrated by inhibition experiments. Thus, in agar-gel diffusion experiments, pre-incubation of the antisera with IN-TDIC inhibited the formation of the arcs with IN-TDIC as well as with insulin, whereas absorption of antisera with insulin only inhibited the arc with insulin but not with IN-TDIC. These results would suggest that injection of IN-TDIC-BSA conjugate into rabbits elicited the formation of

antibodies against the determinants of the insulin as well as against the haptenic TDIC group. A similar pattern of inhibition was observed in the hemagglutination reaction; pre-incubation of the antisera with 10 μg of insulin diminished the hemagglutinating titer dramatically. However, glucagon did not give a precipitate with anti-insulin antibodies, neither did it inhibit the reaction between these antibodies and insulin. These findings thus eliminate the possibility that the antisera contained antibodies specific to glucagon which is the most likely contaminant present in the insulin sample.

Although it had been possible to produce precipitating antibodies to bovine insulin in the guinea pig (140-144), this was achieved with difficulty in other animals such as the rabbit, and then primarily with the help of adjuvants. Thus, although Lowell and Franklin (194) used insulin emulsified with complete Freund's adjuvant and Arquilla and Stavitsky (133) immunized rabbits with alum-precipitated crystalline insulin, these investigators were not able to induce the production of precipitating antibodies. On the other hand, Steigerwald and associates (195), using aluminum hydroxide with insulin (as much as 1.5 g of insulin), obtained appreciable amounts of precipitating antibodies. Similarly, Hirata and Blumenthal (141-142), using complete adjuvant containing paraffin oil, mannide monodate and M. butyricum and only 19 mg of insulin, obtained precipitating antibodies in four out of six rabbits,

as demonstrated by paper electrophoresis with insulin-I^{131}. However, in this study, the antibody content of only one pool of rabbit antiserum was determined by standard precipitin reaction and amounted to 400 μg/ml.

In the present investigation immunization of rabbits by intravenous injections with insulin, which had been coupled to BSA, i.e. with IN-TDIC-BSA, elicited readily and consistently the formation of appreciable amounts of precipitating antibodies to insulin without causing increased mortality among the immunized animals. Thus, it appears that the attachment of insulin to BSA enhanced the immunogenicity of the former.

Insulin, by itself, is weakly antigenic, the induction of antibodies against it requiring large amounts of antigen, in the absence of adjuvants, which must be administered over a long period of time (129). There is no clear cut evidence explaining the cause for the weak antigenicity of insulin. The small size of the insulin molecule is probably one factor responsible for its weak antigenic properties. Consistent with this is the ease with which Steigerwald and Spielmann (196) were able to induce insulin antibodies in rabbits by immunization with a mixture of insulin-resistant serum and insulin. It is likely that, in this instance, the insulin molecule was combined with the larger antibody molecule, and that the immunogenic form was the corresponding antibody-antigen complex. Further support for this suggestion comes from the result of the present

investigation. The coupling of insulin covalently to a carrier protein - BSA did increase the immunogenicity of insulin. Previously, it was suggested that a significant fraction of plasma insulin is bound to a normal plasma protein (197) or exists in the form of a high molecular weight compound (198). Thus, the possibility that insulin, upon intravenous injection, becomes associated into complexes with proteins in tissue fluids and plasma cannot be ruled out. On the other hand, Berson and Yalow (199) demonstrated that on addition of I^{131}-labelled insulin to plasma or to buffer solutions containing only serum albumin, 60 - 80 per cent of the insulin was not bound. However, it is conceivable that under abnormal metabolic circumstances insulin might become coupled to serum or tissue proteins under the action of an enzyme. In fact, Ensinck et al. (200) demonstrated that reduced I^{131}-labelled B chain, derived enzymically from I^{131}-labelled insulin, was bound to albumin. Therefore, it would seem that the antigenicity of insulin might depend upon its combination with body proteins, which would imply that insulin does not act as a true antigen, but as a hapten.

Another important factor responsible for the weak antigenicity of insulin might be attributed to its close resemblance with the host's insulin. It is noted that beef insulin differs from rabbit insulin only in the C-terminal amino acid (B30) of B chain and the amino acids in positions 8 and 10 of A chain (table II). Unfortunately the three

dimensional conformation of different insulin molecules is not known. If the antigenic properties of the protein molecule are determined by the primary structure, only these three amino acids should represent sites of antigenicity. The small difference between the beef insulin and the insulin of the host renders it difficult for the antibody forming cells to recognize the beef insulin as 'non-self'. In this connection, it might be mentioned that in earlier studies in this laboratory, it had been demonstrated that low molecular weight metabolites, such as estrone (201), 5-OH indole acetic acid (202), and serotonin (203), which are normal constituents of the animal body, did not induce the formation of specific antibodies in the host; however, upon coupling them to appropriate carrier proteins, they became immunogenic. By analogy, in the present study, it was anticipated that beef insulin, after being covalently coupled to BSA, would become immunogenic. The result presented substantiates this original expectation.

Reaction of all three amino groups in the insulin molecule with FNBSNa did not block the antigenic determinants of the molecule as demonstrated by micro-Ouchterlony and precipitin tests. These results would suggest that the N-terminal amino group of both A and B chains, as well as of the B29 lysyl residue, may not contribute markedly to the structure of the determinant groups of insulin.

CHAPTER 5

ELECTROLYTIC REDUCTION OF INSULIN

Introduction

Antigen-antibody reactions are exquisitely specific, inasmuch as antibodies will react only with the antigen used for immunization or with molecules possessing groups which are sterically closely related to the determinant groups of the original antigen (15). As already discussed in Chapter 1, the forces participating in the reaction between an antibody and an antigenic group are short-range forces and could not be responsible for the specificity of the reaction unless these interactions are accompanied by the complementary juxtaposition of the surfaces of the determinant group of the antigen and of the corresponding combining site of the antibody.

The importance of the three-dimensional conformation of the determinant group was demonstrated in the classical studies of Landsteiner (15) and Pressman and his colleagues (204). Thus, it was shown that a slight modification in the structure of the hapten resulted in complete loss of binding. By analogy, it would also be expected that deformation of the structure of a protein antigen would lead to loss of antigenic properties of the molecule. Among the interchain interactions which are known to stabilize protein conformations, disulphide

linkages are undoubtedly the most stable and important. Therefore, in the present study the role of disulphide bonds in maintaining the structure of the insulin molecule responsible for its antigenicity was considered.

Various methods have been employed for the rupture of the disulphide bonds in insulin. Oxidation with peracids requires the presence of 98% formic acid and was shown to lead to partial oxidation of insulin (205). Chemical reducing agents, in the absence of denaturing agents, cleave only the intrachain disulphide bond of the A chain and render the molecule insoluble (206); reduction in the presence of high concentrations of urea, however, results in complete cleavage. Cecil and Wake (207) have shown that sodium sulphite cleaves the interchain bonds, but cleavage of the intrachain bond was accomplished only in the presence of high concentration of guanidine-HCl and $HgCl_2$ or of phenylmercuric hydroxide (208). Reduction with mercurial has the disadvantage that it leads to an asymmetrical fission, the products being $-SSO_3$ and -SHgR groups. An alternative procedure is oxidative sulfitolysis (209-212), which leads to symmetrical fission with only $-SSO_3$ groups as products, but unless denaturing agents are present alkaline solutions are used. Consequently, it was deemed desirable to seek for another reduction method in which all three disulphide bonds of insulin could be reduced under the same experimental condition. In the present study the electrolytic reduction method under controlled potential was

chosen for some of its immediately apparent advantages.

With the exception of cystine, amino acids do not produce a reduction wave at the dropping mercury electrode unless a reducible group is present, e.g. iodine in thyroxine. Disulphides and aromatic thiocyanates are the only types of sulphur groupings which have been reported to be reducible at the dropping mercury electrode. Electrolytic reduction of disulphide bonds in proteins was first realized in 1930 when Heyrovsky and Babicka (213) discovered a polarographic effect of sulphur containing proteins, which consisted of a characteristic wave at a potential of -1.6 V on the current-voltage curve of a solution of 0.1 N ammonia and 0.1 N ammonium chloride containing traces of proteins. The wave, however, was not due to the reduction of the protein present; rather, it was due to the evolution of hydrogen, which was catalysed by the presence of the protein at the cathode. In a subsequent study, Brdicka (214) revealed that the height of this catalytic wave was affected not only by pH, but also by the kind of buffer and the concentration of the buffer constituents. On the basis of these observations, Brdicka (214) suggested that this catalytic* wave was attributable to a direct reduction of the SH group:

$$- SH + e \longrightarrow - S^{-} + H$$

---

*This is an example of acid-base catalysis in the Brönsted sense.

and that the S^- ions formed would react with any acid, A, present to reform the sulfhydryl group:

$$- S^- + A \longrightarrow - SH + B$$

where B is the conjugate base of A. Thus, the overall reaction occurring at the cathode would be the discharge of hydrogen from the acid A. A similar polarographic wave was also reported for the cystine-cysteine system. However, this wave occurred at a potential more negative than the reduction potential of cystine to cysteine. Hence, it would seem that the evolution of hydrogen was catalysed by the SH group rather than the S-S group. Furthermore, for the cystine to produce a catalytic wave, the disulphide bonds must have been electro-reduced at the mercury cathode. In fact, Brdicka (215) observed that waves given by a solution of cystine were identical with those obtained with solutions of cysteine having twice the concentration of the cystine solution. Moreover, Mulli and Werner (216) reported that solutions containing trace amounts of insulin produced the same polarographic effect as those containing other sulphur-containing proteins. Thus, it would appear that the electrolytic reduction at the mercury cathode is specific for the disulphide bond in protein. The advantages of the electrolytic reduction under controlled potential are: (i) complete reduction is achieved in neutral conditions and without the use of reducing or denaturing agents; (ii) the reduction is highly specific; (iii) since no

reducing agent is added, analysis for the formed sulphydryl groups can be carried out during the course of reduction; (iv) partial or total fission of the disulphide bonds can be accomplished as desired.

Materials and Method

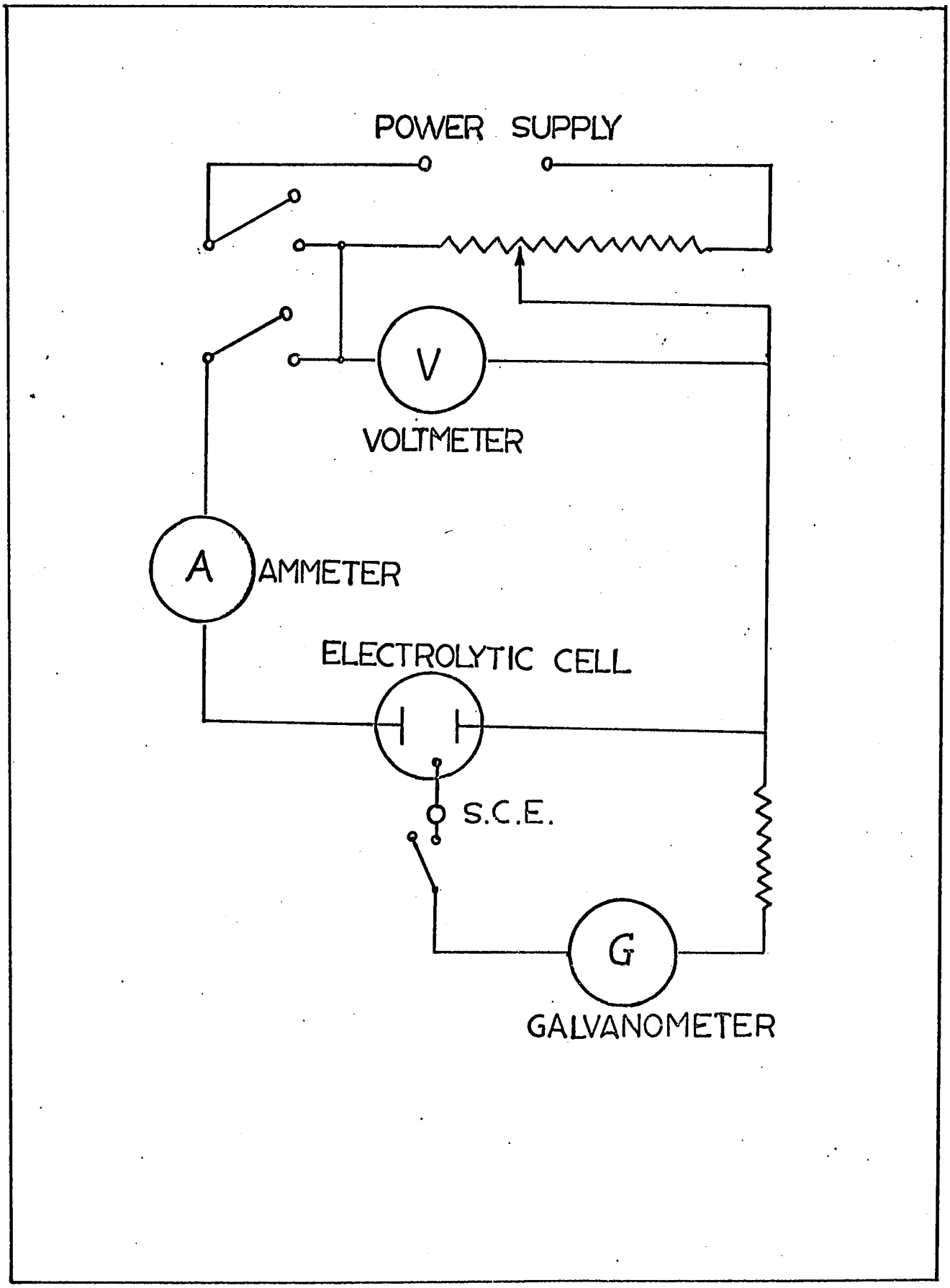
All chemicals, unless otherwise stated, were of analytical reagent grade and were purchased from Fisher Scientific Co., Montreal. Silver nitrate was a Fisher primary standard product; a solution of 2×10^{-3} M of $AgNO_3$ was made up and stored in the dark. Urea was a Fisher analytical reagent, which was further purified by re-crystallization from the filtrate of a concentrated solution at $50^{\circ}C$. This purification, as well as the use of de-ionized water throughout the whole procedure, was found to be absolutely necessary. Unflavoured No. 1 gelatine, purchased from Knox Gelatine (Canada) Limited, Montreal, was used in all titrations. Triple distilled mercury was obtained from Mallinckrodt Chemical Works, Montreal.

Apparatus and general technique

The method used for the reduction of insulin was similar to that described by Lingane (217). Figure 34 shows a simple circuit for the manually controlled potential electrolysis. The total e.m.f. applied to the cell was regulated by a voltage divider (a variable 2 K resistor of 2 W)

Figure 34

SCHEMATIC DIAGRAM OF THE CIRCUIT FOR THE MANUALLY CONTROLLED POTENTIAL ELECTROLYTIC REDUCTION


POWER SUPPLY
V
VOLTMETER
A
AMMETER
ELECTROLYTIC CELL
S.C.E.
G
GALVANOMETER

with direct current from a rectifier. A potential of 100 V was applied across this voltage divider, and the corresponding fraction applied to the cell was read with a multi-range voltmeter, while the microammeter (full range 10 μamps) indicated the total current passing through the electrolytic cell. The potential of the cathode against the saturated calomel electrode was measured in the ordinary manner with a galvanometer in series with a 9.65 megohm resistance. Using this circuit, the applied voltage against the saturated calomel electrode was maintained at a constant value by frequent adjustments of the voltage divider during the course of the electrolysis.

Galvanometer

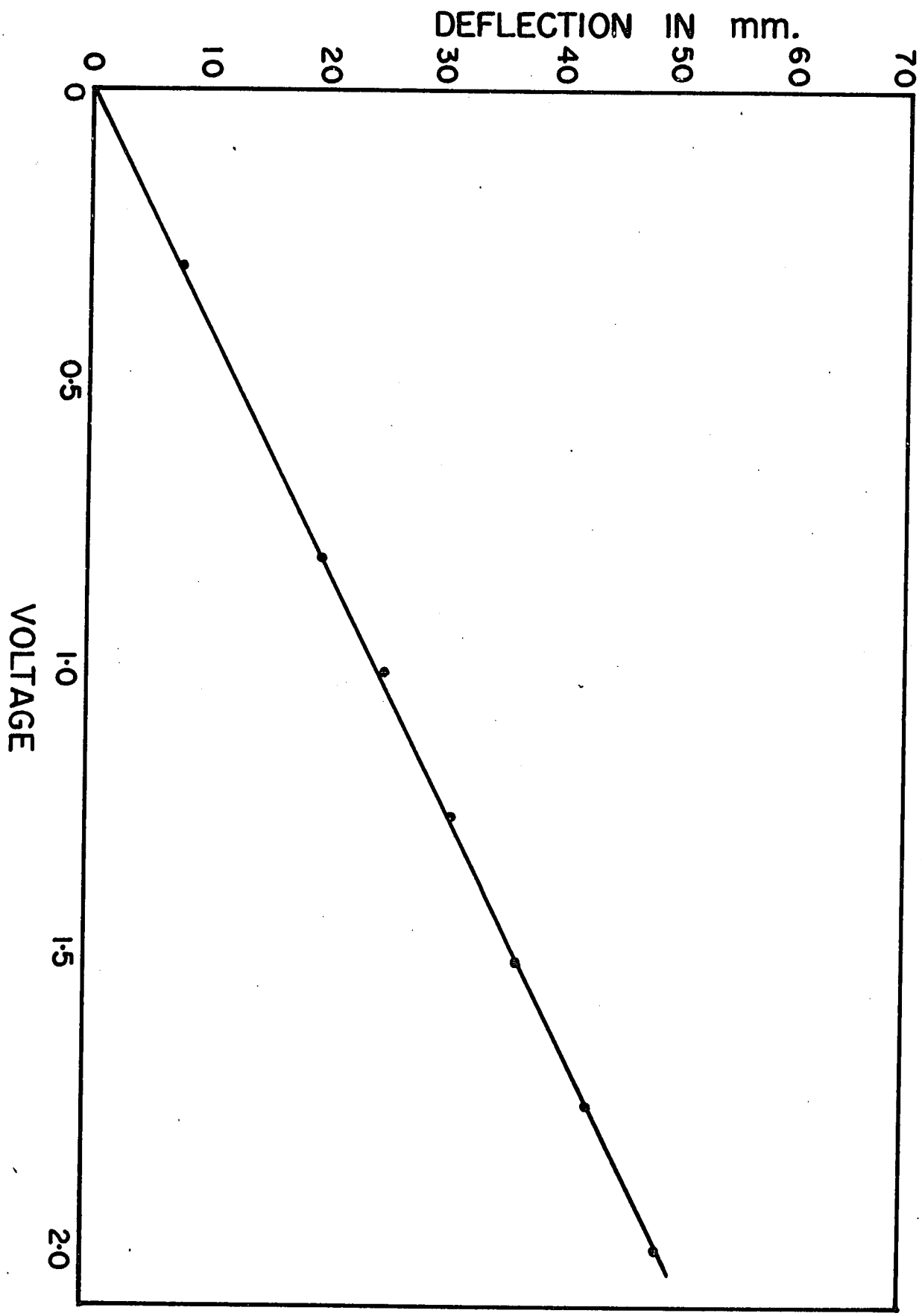
The galvanometer used to measure the cathode voltage was calibrated as follows. Various voltages were applied across the galvanometer in series with a resistance of 9.65 megohms and the corresponding deflections measured in millimeters were recorded. The calibration curve obtained is shown in figure 35. Thus, the galvanometer had a sensitivity of 0.003 μamp per mm deflection when it was adjusted to produce a full scale deflection with an applied e.m.f. of 2V.

Saturated-Calomel Electrode (S.C.E.)

In making calomel electrodes every care must be taken that the solid phase, i.e. the calomel, is in complete

Figure 35

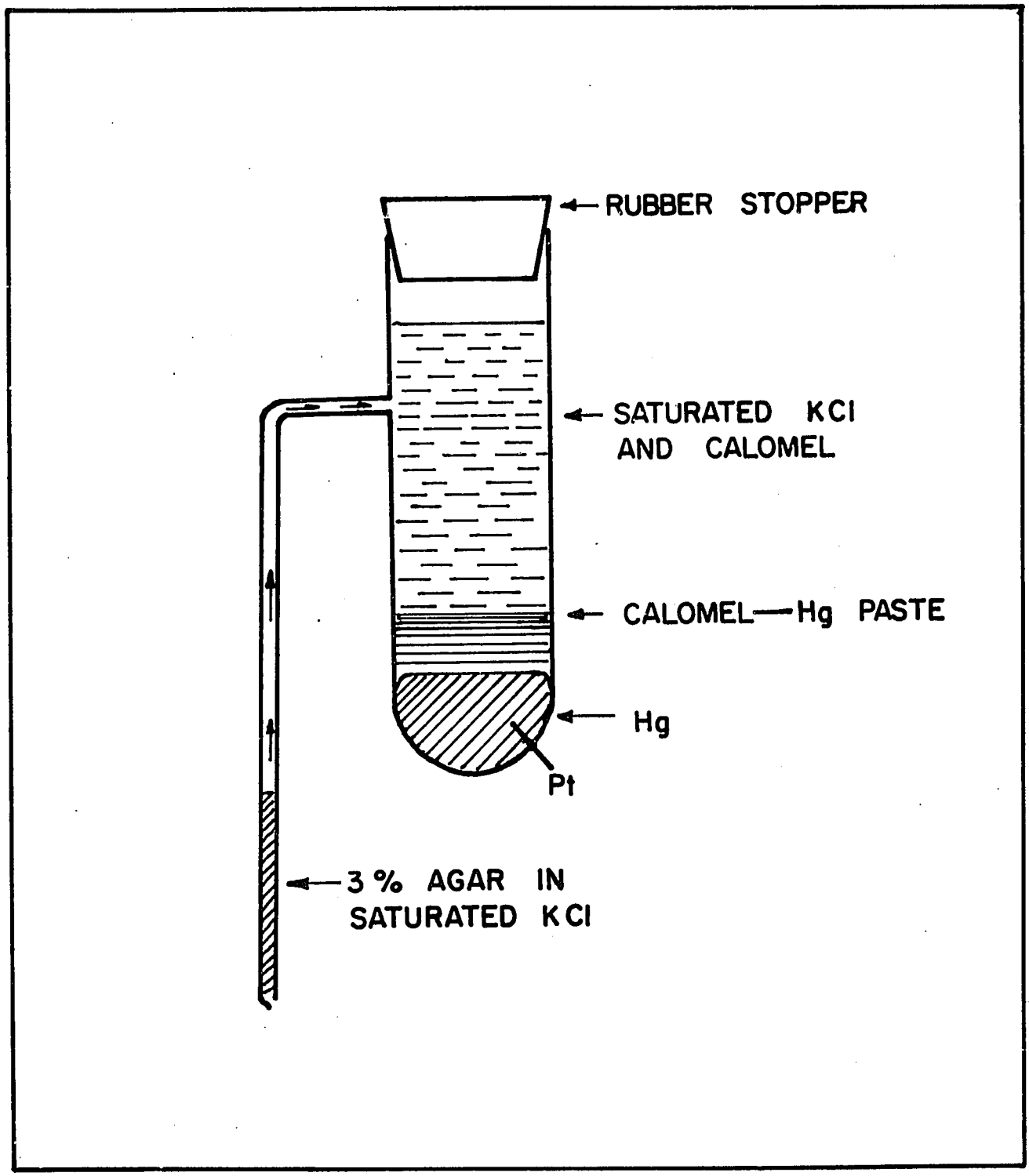
THE CALIBRATION CURVE OF THE GALVANOMETER


DEFLECTION IN mm.
0
10
20
30
40
50
60
70
VOLTAGE
0
0·5
1·0
1·5
2·0

equilibrium with the potassium chloride solution. The solution must be saturated with calomel, and an electrode should not be used until it has acquired a steady and correct potential. Various forms of vessels are used for calomel electrodes; however, the accuracy and efficiency of calomel electrodes are not influenced by the shape of the vessel. The calomel electrode convenient for this study is shown in figure 36. Into the closed end of a glass tubing (2 cm internal diameter) was sealed a platinum wire, which was covered by mercury. Above the mercury was a paste of calomel and mercury, and above this a layer of a saturated solution of potassium chloride, which was also saturated with calomel. The calomel-mercury paste was made by grinding calomel with mercury in a mortar using a small volume ($\sim$ 5 ml) of the potassium chloride solution; after washing several times with potassium chloride, the paste was thoroughly shaken with more KCl solution and then poured into the electrode vessel. The tube was closed with a rubber stopper. The bridge consisted of a small glass tubing (6 mm internal diameter) bent at right angle and joined to the electrode. To minimize changes in the concentration of the solution in the electrode vessel when the side arm was dipped into the cathode compartment of the electrolysis cell, the end section of this side arm was filled with a 3% agar gel in saturated potassium chloride. The saturated calomel electrode so prepared was allowed to stand at room temperature for a few days in order to attain equilibrium.

Figure 36

DIAGRAM OF THE SATURATED CALOMEL ELECTRODE


RUBBER STOPPER
SATURATED KCl
AND CALOMEL
CALOMEL—Hg PASTE
Hg
Pt
3 % AGAR IN
SATURATED KCl

No correction was made for changes in temperature.

The saturated calomel reference electrode must be placed as closely as possible to the outside surface of the cathode. The observed cathode potential is related to the true potential by the equation

$$E_{observed} = E_{true} - iR$$

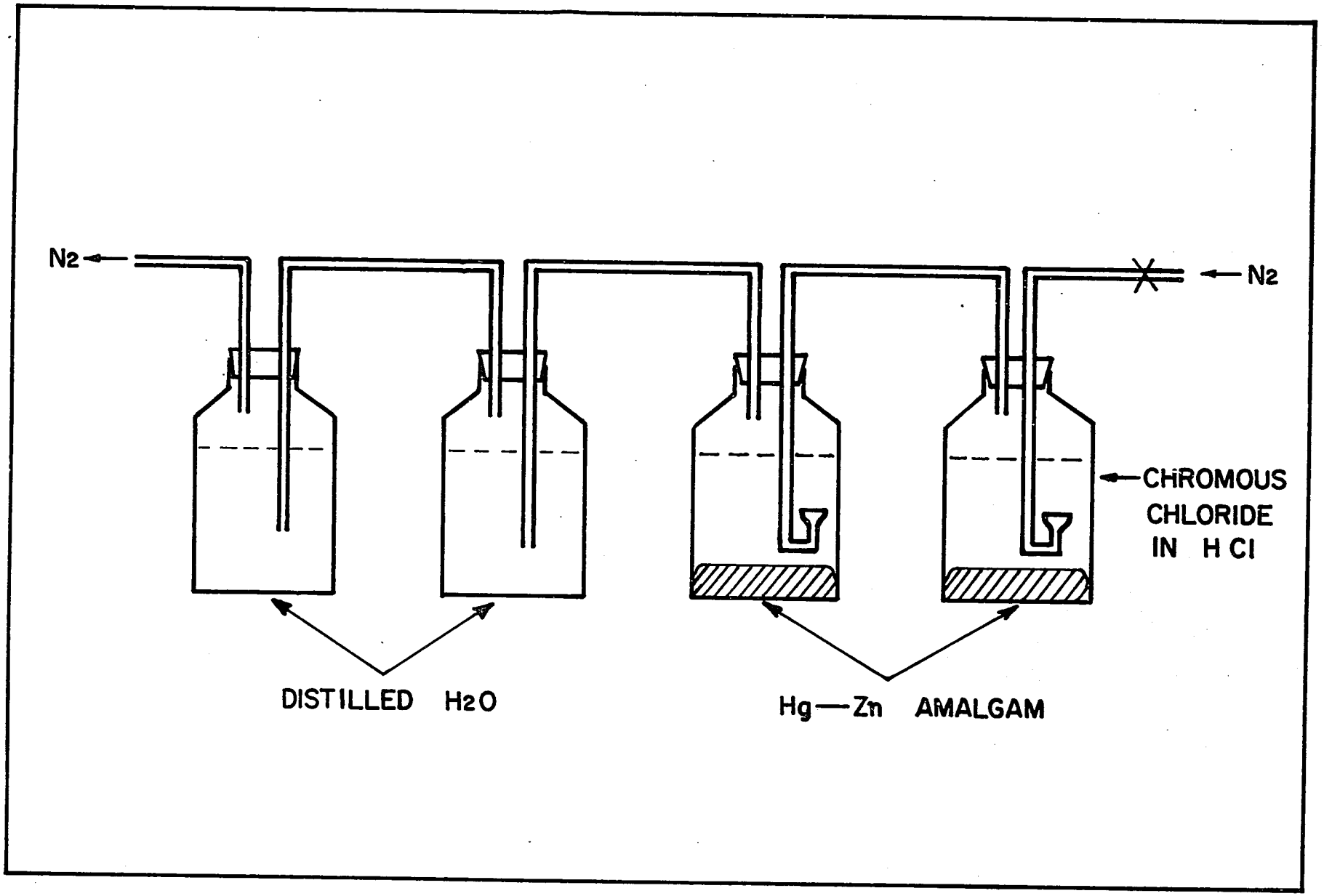
where iR is the ohmic potential drop in the solution between the cathode surface and the tip of the salt bridge from the reference electrode. By placing the tip of the salt bridge very close to the cathode, and on the outside of it, iR is kept negligibly small.

Removal of dissolved air

Since oxygen is easily reduced at the mercury electrode, it is usually necessary to remove dissolved air from the solution to prevent any change of pH of the medium. Most commonly, air is removed by bubbling nitrogen through the solution. The commercial nitrogen from high pressure cylinders contains traces of oxygen, which can be removed by passing the gas through two wash bottles containing a solution of chromous chloride in 1 N HCl, as shown in figure 37. The small amount of acid which was carried over with the nitrogen was removed by passing through two wash bottles containing distilled water. To prevent blockage of the delivery tube by

Figure 37

NITROGEN PURIFICATION TRAIN


N2
N2
CHROMOUS
CHLORIDE
IN H Cl
DISTILLED H2O
Hg—Zn AMALGAM

zinc amalgam, it was fitted with a coarse sintered glass funnel in an upward position.

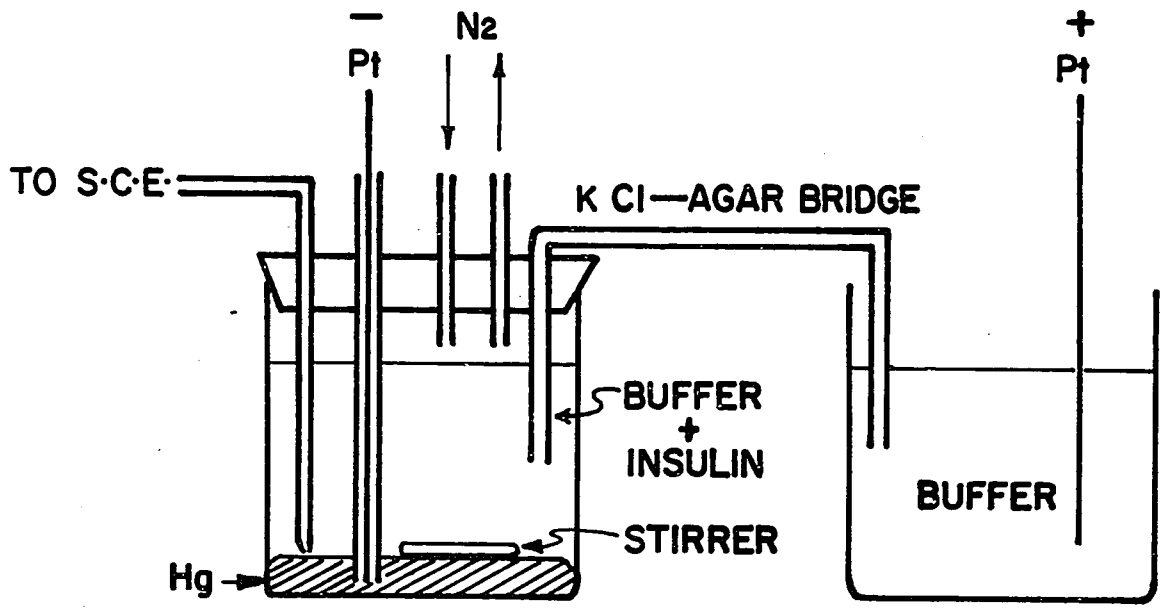
The solution of chromous chloride in 1 N HCl was prepared according to the method of Kolthoff and Sandell(218). Thus, to 600 ml of 2% mercuric nitrate solution and 2 to 4 ml of concentrated nitric acid was added 600 g of pure 20 to 30 mesh zinc in a wash bottle. The mixture was stirred thoroughly for five to ten minutes; the supernatant was decanted from the zinc and was then further washed with the same mercury nitrate solution, two or three times by decantation. The amalgamated zinc should have a bright silvery lustre. Enough solution of 0.1 M chromic sulphate in 1 N HCl was added to the amalgamated zinc to fill two-thirds of the wash bottle, which was then stoppered and connected to the purification train.

Procedure for electrolytic reduction

The cell for electrolytic reduction (figure 38) was similar to that described by Dohan and Woodward (219). The cathode vessel was a 200 ml-beaker containing approximately 20 ml of triply-distilled mercury and the area of the mercury pool cathode was about 20 cm^2. The mercury-solution interface was kept in rapid agitation by a magnetic stirring bar. The mercury pool was negatively charged by a platinum electrode sealed into a mercury-filled glass tube, and care was taken that the platinum wire was covered with mercury in the cathode

Figure 38

THE CELL FOR CONTROLLED POTENTIAL ELECTROLYTIC REDUCTION WITH MERCURY CATHODE



CELL FOR CONTROLLED POTENTIAL
ELECTROLYTIC REDUCTION WITH Hg CATHODE

compartment, which was stoppered with a cork. The tip of the salt bridge from the S.C.E. was placed within about 1 mm from the mercury cathode when the mercury was at rest, so that the ohmic drop, included in the observed cathode potential, would be negligible. The most satisfactory results were obtained when the tip of the salt bridge was positioned just at the mercury-solution interface when the stirrer was in motion. The potential of the mercury cathode was measured continuously against the S.C.E. during electrolysis and controlled by changing the total e.m.f. applied to the cell.

Into this cathode compartment was pipetted 140 ml of phosphate buffer, pH 7.2, ionic strength 0.02. Purified nitrogen was bubbled through the phosphate buffer for 15 minutes and flushed over the surface of the buffer solution throughout the reduction process. At the same time, 150 mg of insulin was dissolved in 10 ml of de-oxygenated 0.04 N NaOH with the nitrogen gas flushing over the surface of the solution and was then added to the buffer solution in the cathode compartment. The anode compartment consisted of a 200 ml-beaker containing phosphate buffer and a Pt wire as an electrode. The anode compartment was connected to the cathode compartment by a bridge filled with 1% agar in saturated KCl. After the rectifier was switched on, the voltage applied across the cell was increased gradually to the desired cathode voltage and adjusted manually from time to time. The extent of the reduction was followed by

amperometric titration with silver nitrate. The reduced insulin was alkylated with 20% excess of iodoacetic acid at room temperature for one hour. The solution was then dialysed exhaustively against distilled water at 4°C and freeze-dried.

Amperometric titration of SH and S-S groups with silver nitrate

In amperometric titrations, the current passing through the titration cell between an indicator electrode and an appropriate depolarized reference electrode, at a suitable applied e.m.f., is measured as a function of the volume of a suitable titrating solution. Generally, the end-point of an amperometric titration is obtained by back extrapolation on a plot of current increments against the volume of the titrating reagent added. The performance of an amperometric titration becomes particularly simple when the potential difference between the indicator and reference electrodes is sufficient to discharge the titrating ion so that no e.m.f. needs to be applied to the cell. The rotating electrode is placed in the solution to be titrated; electrolytic contact is made with the reference electrode, and the current which flows through the cell during the titration is read on a microammeter. The diffusion current of the substance titrated is measured at the potential of the rotating electrode.

The method used in the present study to follow the

extent of the electrolytic reduction of insulin was of the latter type as described by Benesh et al. (220). In this method, which is both sensitive and specific, protein SH groups are titrated with silver ions in the form of a complex with tris(hydroxymethyl)-aminomethane (Tris) (221), in neutral, buffered solutions. The reference electrode was the Hg-HgO-saturated $Ba(OH)_2$ electrode of Samuelson and Brown (222), which had a potential of -0.01 V versus the saturated calomel electrode. The reference electrode was made by placing a layer of mercury at the bottom of the flask described by Kolthoff and Harris (223); this was then covered with a slurry of red mercuric oxide and solid barium hydroxide, and the flask was then filled with water saturated with the two reagents. The electrode thus prepared was stable for several months when protected from light with a black cover. The reference electrode was connected to the rotating platinum electrode by a bridge which consisted of a coarse sintered glass funnel filled with a 3% agar gel in saturated potassium chloride as shown in figure 39. This funnel was attached to a 60 cm long piece of Tygon tubing which led to a No. 2 three-way stopcock, which, in turn, was connected to the reference electrode solution as far as the three-way stopcock and with saturated KCl from the stopcock to the funnel. It is important that no air bubble should be trapped in the stopcock. The arrangement permitted daily flushing of the saturated KCl part of the bridge. The platinum electrode was made

Figure 39

APPARATUS FOR AMPEROMETRIC TITRATION

A - reference electrode

B - tygon tubing

C - three-way stopcock

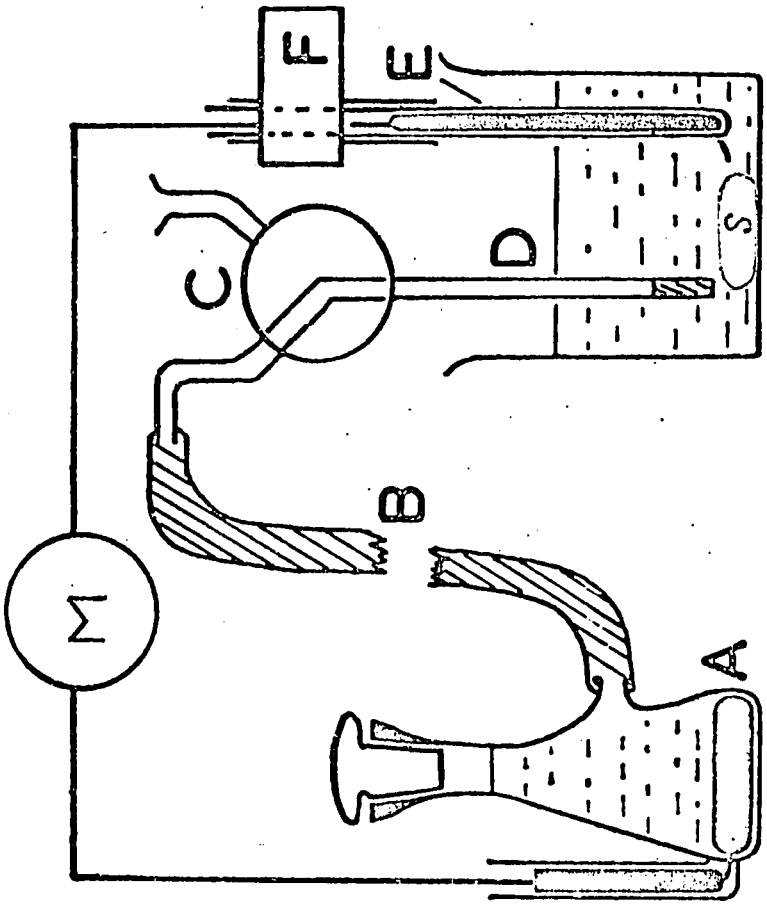
D - saturated KCl bridge ending with sintered glass funnel filled with 3% agar in saturated potassium chloride

E - rotating platinum electrode

F - electrode motor

M - microammeter (full range 25 microamps)

S - magnetic stirrer


F
E
S
D
C
B
Σ
A

according to the procedure of Kolthoff and Harris (223). It consisted of a soft glass tubing with a platinum wire sealed at the end; the glass tubing was then filled with mercury. The platinum electrode thus prepared was fitted into the hollow shaft of a pulley, which was driven by a rotator. Electrical contact was maintained by another platinum wire dipping into the mercury well at the top of the electrode. The current was observed on a microammeter (full range 25 μamps). If the electrode became sluggish, it was dipped into concentrated HNO_3 for thorough cleaning.

Titrations of SH groups were carried out in 100 ml beakers containing a total of 30 ml of solution. The titration mixture, the final pH of which was 7.4 at room temperature, was prepared by mixing 4.0 ml of 1.0 M Tris, 3.4 ml of 1.0 M HNO_3, 0.3 ml of 1.0 M KCl, 1.0 ml of 1.8×10^{-3} M ethylenediaminetetraacetate (EDTA), 0.1 ml of 3% gelatine, and 14.4 g of re-crystallized urea. After addition of glutathione or reduced insulin, the solution was made up to 30 ml and titrated against $AgNO_3$ (2×10^{-3} M). To check the precision of this apparatus, ten samples of glutathione (GSH) of known concentrations (ranging from 1 μmole to 10 μmoles) were titrated against $AgNO_3$; the maximum error was found to be 3%. No difference was observed when these titrations were performed in the absence of 8 M urea. For the titration of the reduced insulin, 5 ml of the cathode solution was pipetted into the titration mixture and the solution was titrated

immediately. The typical titration obtained is shown in figure 40.

For the determination of S-S bonds in insulin, the method of Carter was employed (224). The titration mixture was prepared by mixing 0.5 ml of the insulin solution in 0.04 N NaOH, 1.0 ml of EDTA in a concentration of 1.8×10^{-3} M, 14.4 g of re-crystallized urea, and 0.1 ml of saturated Na_2SO_3 at 27°C. The resulting mixture was diluted to 30 ml with de-ionized water and was incubated at 27°C for thirty minutes. It was then titrated against $AgNO_3$ of 2×10^{-3} M. The titration curves of the cystine residues of insulin and insulin fragments are presented in figure 41.

Amino acid analysis

Method

(A) Introduction

All amino acid analyses were carried out in the Beckman Amino Acid Analyser (Model 120B), shown in figure 42. The equipment includes storage facilities for all reagents used during the analytical run, complete apparatus for the automatic separation and for the quantitative analysis, a multipoint recorder, the pumping system, and controls for selecting and establishing the operating and timing parameters for a complete variety of different analyses. The separation of the amino acids in a sample is effected by chromatography

Figure 40

AMPEROMETRIC TITRATION OF GLUTATHIONE

AND REDUCED INSULIN

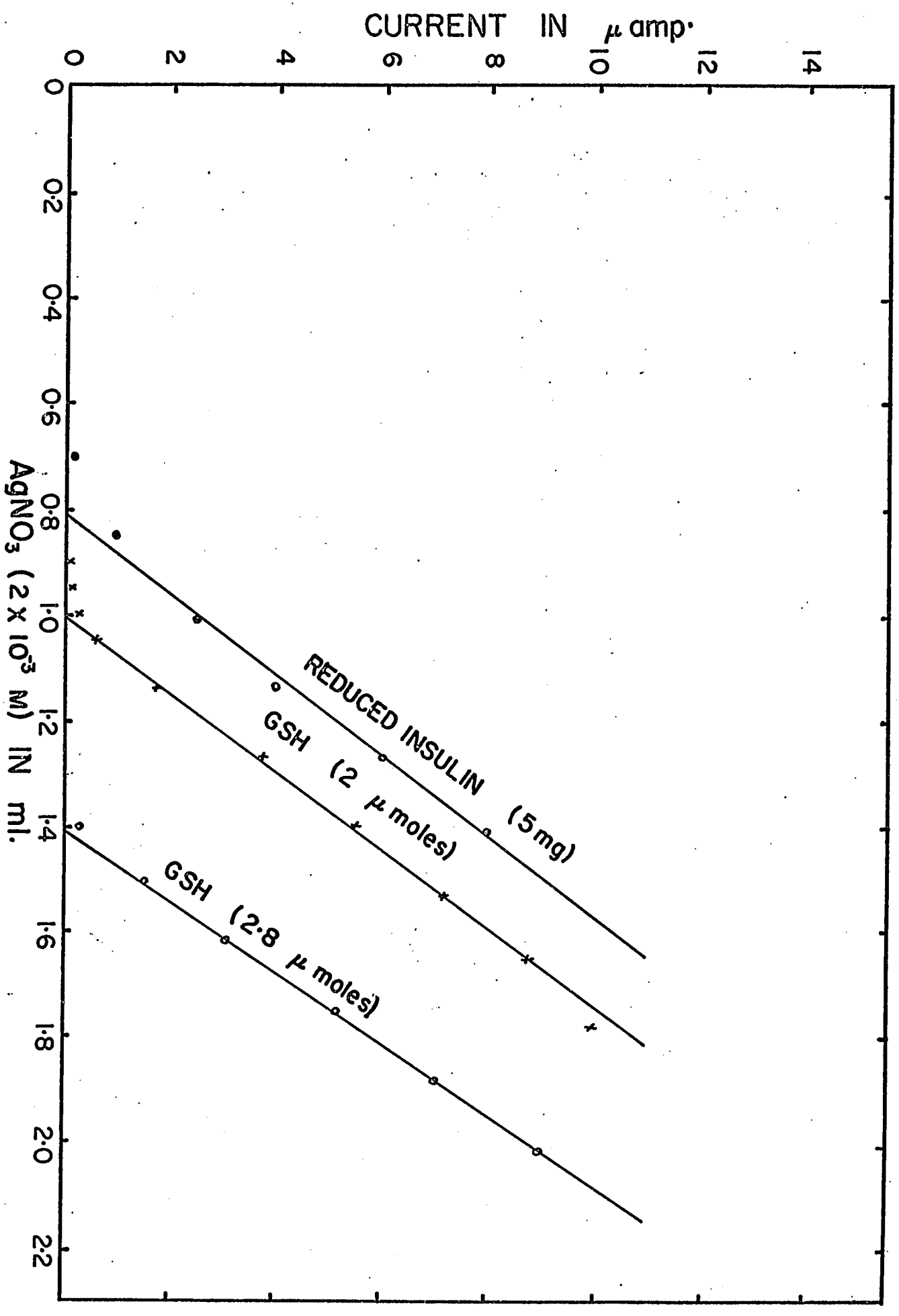

CURRENT IN μ amp.
0
2
4
6
8
10
12
14
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0
2·2
AgNO3 (2 x 10-3 M) IN ml.
REDUCED INSULIN (5mg)
GSH (2 μ moles)
GSH (2·8 μ moles)

Figure 41

AMPEROMETRIC TITRATION OF S-S BONDS IN INSULIN AND INSULIN FRAGMENTS

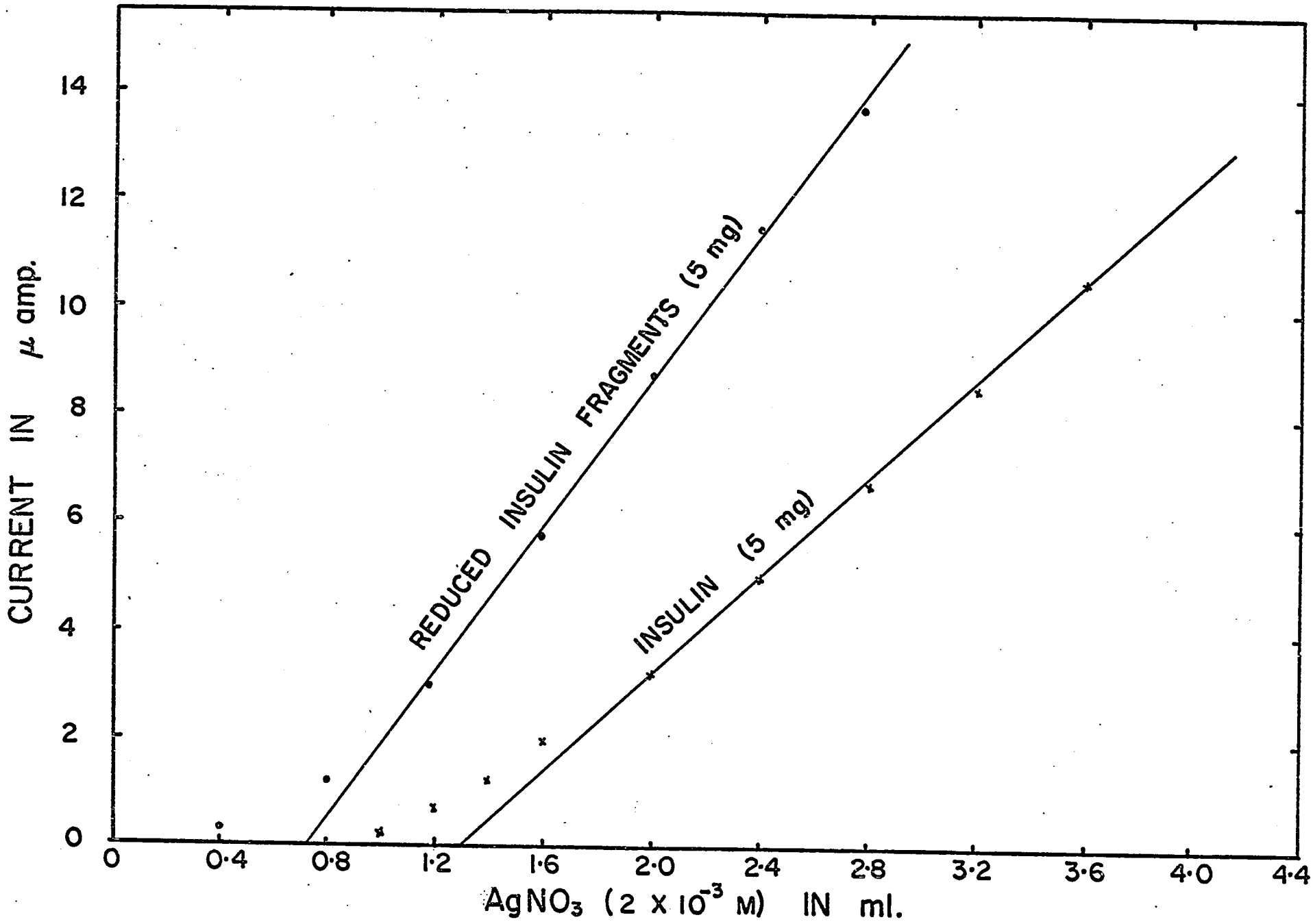
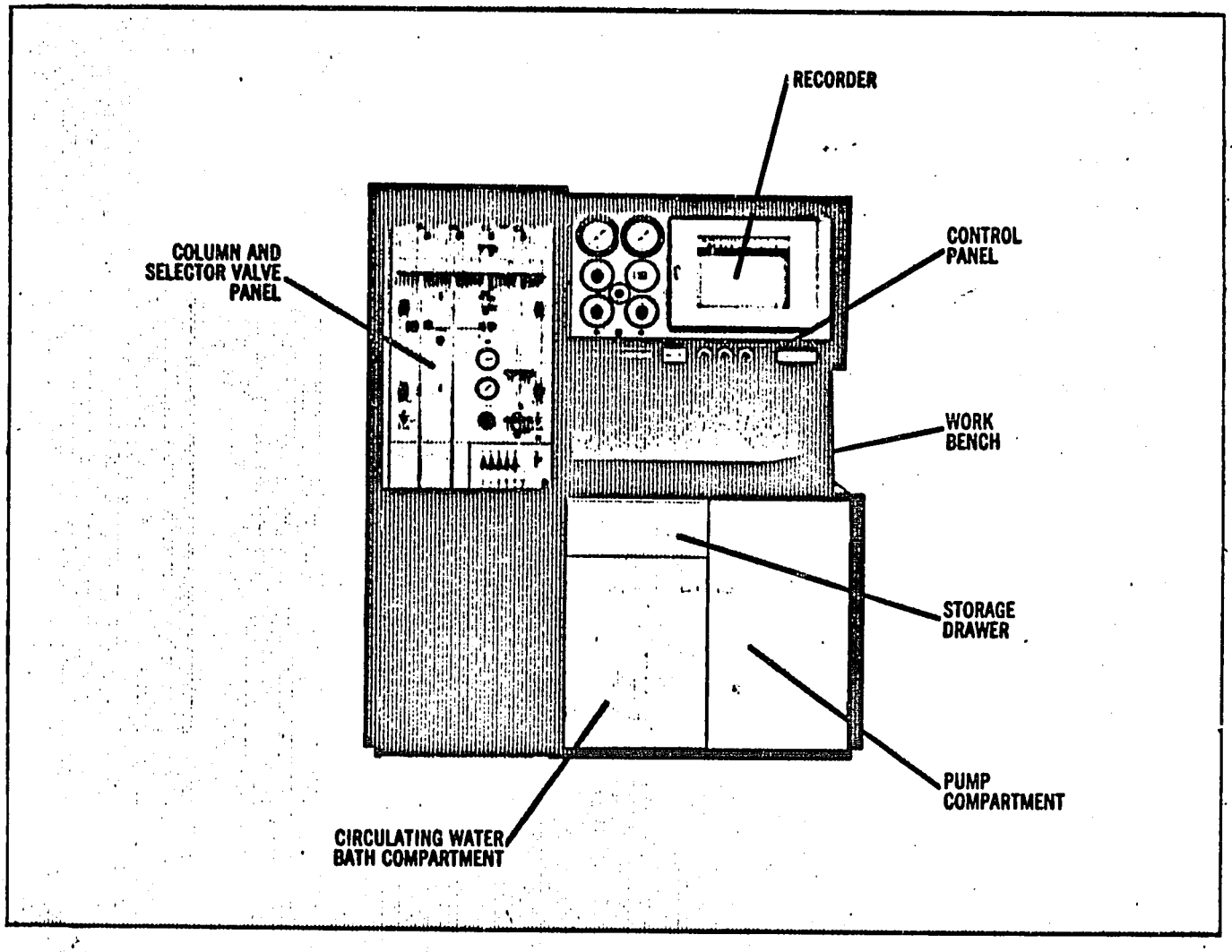

CURRENT IN μ amp.
0
2
4
6
8
10
12
14
0
0·4
0·8
1·2
1·6
2·0
2·4
2·8
3·2
3·6
4·0
4·4
AgNO3 (2 X 10-3 M) IN ml.
REDUCED INSULIN FRAGMENTS (5 mg)
INSULIN (5 mg)

Figure 42

BECKMAN AUTOMATIC AMINO ACID ANALYSER

(Model 120B)


RECORDER
CONTROL PANEL
COLUMN AND SELECTOR VALVE PANEL
WORK BENCH
STORAGE DRAWER
PUMP COMPARTMENT
CIRCULATING WATER BATH COMPARTMENT

on a column of the sodium salt of a polysulphonic acid resin, the cation exchanger. When an amino acid is placed on top of the column, ion exchange takes place between the Na ion of the resin and the positively charged amino group of the amino acid. This is a reversible reaction and equilibrium takes place. Under a given set of conditions (such as chemical composition, resin particle size and resin pore size, diameter and length of the packed column, pH and ionic strength and rate of flow of the eluting buffer, and the temperature), the amount of a given quantity of an amino acid, which is bound to the ion-exchange resin relative to that remaining in solution at equilibrium, is usually expressed as a distribution coefficient, K. The magnitude of this coefficient depends on the structure of the individual amino acid. If the capacity of the resin is not exceeded under an appropriate and reproducible set of conditions, the amino acids in a sample are separated from each other by the time they emerge from the column, each amino acid emerging in a specified displacement (elution) volume.

Two columns (a long column and a short column) are used for protein analysis. For complete protein analysis to be carried out in five hours in the Model 120B, buffers are forced through the column by positive displacement pumps working at several atmospheric pressures. For the analysis of a protein hydrolysate, two buffers are used with automatic change-over from the first at pH 3.28 to the second at pH 4.25,

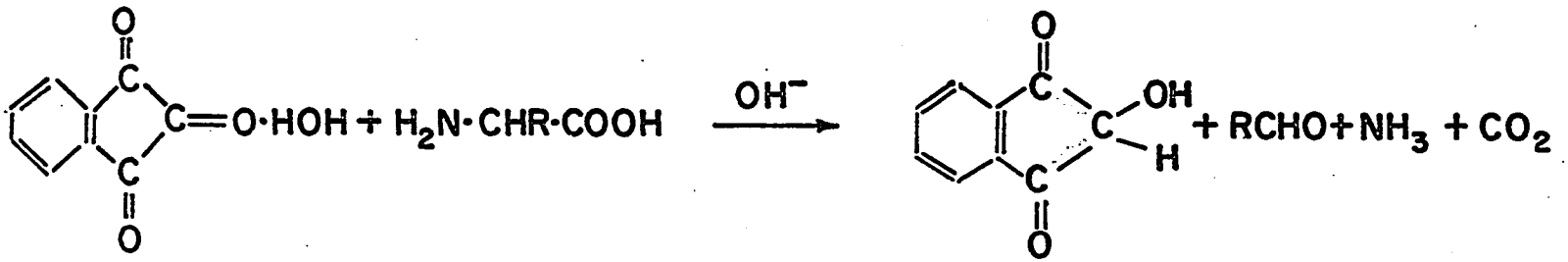
at 90 minutes, after the long column has been started for the analysis of the neutral and acidic amino acids. A third buffer of pH 5.28 and higher ionic strength is used with a second column for the analysis of the basic components in the sample.

After separation, each amino acid in the effluent is made to mix with ninhydrin and the mixture then flows through the reaction bath system, maintained at the boiling point of water in order to assure constant environmental factors. With an α-amino acid, ninhydrin participates in a deaminative oxidative decarboxylation and then condenses further to give a blue compound; the colour formed from the reaction with the imino acids proline and hydroxyproline is yellow (figure 43). By using a colorimeter in conjunction with a photovoltaic cell, the colour developed is proportionally converted into electric current. This electric current is then used to drive a conventional multipoint recorder which plots the results of the analysis as absorbance versus time. To account for two different colours which may develop, two separate colorimeter readings are recorded simultaneously in different colours on the chart. One photometer unit of the colorimeter measures absorbance of the imino acids proline and hydroxyproline at a wave-length of 440 mμ and the second photometer unit measures at 570 mμ for the rest of the amino acids. Amino acids are then identified by the order and position of the corresponding peaks on the chart, and the concentration of each amino acid

Figure 43

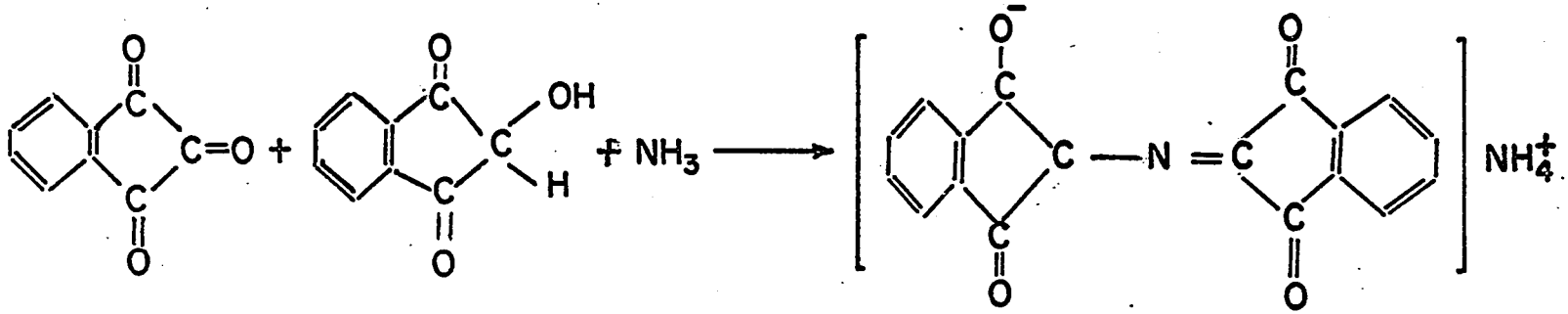
NINHYDRIN REACTION WITH α-AMINO ACID

(a)



NINHYDRIN AMINO ACID

(b)



RESONANCE HYBRID
(COLOURED)

is determined by measuring the area under its peak.

(B) Buffers

All buffers were prepared in de-ionized water according to the recipes recommended by Beckman Instruments, Inc. (table XI) with analytical grade reagents purchased from the companies specified by Beckman. These sodium citrate buffers were used as eluents. Prior to use, portions of the appropriate buffers were filtered and then placed in the reservoirs of the Model 120B Analyser. With the use of octanoic acid as preservative, buffers could be made on a large scale and stored at room temperature rather than at $4^{o}C$. The high concentration of thiodiglycol in the pH 2.2 buffer and the filling buffer helps to minimize the conversion of small amounts of methionine to the methionine sulphoxides during the addition of the sample to the column. For uniform drop size polyoxyethylene lauryl alcohol (BRIJ-35) was added to the buffers. The BRIJ-35 solution was prepared by dissolving 50 g of the solid detergent in 100 ml of hot water.

(C) Preparation of the buffered solution of ninhydrin reagent

The 4 N sodium acetate buffer was prepared by dissolving 1,008 g of $NaOAc.3H_2O$ in one liter of de-ionized water in a 2-liter volumetric flask by stirring overnight. Then 200 ml of glacial acidic acid was added and the flask was

Table XI

SODIUM CITRATE BUFFERS

pH	2.2 ± 0.03 sample dilutor	3.28 ± 0.01 long column	4.25 ± 0.02 long column	5.28 ± 0.02 short column	3.28 ± 0.01 filling buffer
Sodium concentration	0.20 N	0.20 N	0.20 N	0.35 N	0.20 N
Sodium citrate.$2H_2O$	19.6 g	784.3 g	784.3 g	1372.6 g	19.6 g
Concentrated HCl	16.5 ml	493 ml	335 ml	260 ml	12.3 ml
Thiodiglycol (TG)	20 ml	200 ml	200 ml	-	20 ml
BRIJ-35 solution	2 ml	80 ml	80 ml	80 ml	2 ml
Octanoic acid	0.1 ml	4 ml	4 ml	4 ml	0.1 ml
Final volume	1 liter	40 liters	40 liters	40 liters	1 liter

filled with water almost to the mark. The pH of the solution was expected to be at 5.5 ± 0.03. For final adjustment of the pH, pellets of NaOH were added (5 g of NaOH was added, which corresponded to about 0.04 pH unit), and the solution was made up to 2 liters.

Because peroxides quantitatively destroy the reducing power of stannous chloride, a small amount of peroxides which might be present in methyl cellosolve becomes intolerable for this analysis. The presence of peroxides in methyl cellosolve was checked by mixing 3 ml of this solvent with 3 ml of a 4% aqueous solution of potassium iodide. In the absence of peroxides, the resulting solution should be colourless. If there was a slight yellow colour observed, the methyl cellosolve was discarded.

The buffered solution of ninhydrin reagent must be prepared in the absence of oxygen. To this end, the whole preparation was carried out under nitrogen atmosphere. Then 3 liters of filtered peroxide-free methyl cellosolve was added to one liter of filtered 4 N sodium acetate buffer. The resulting solution was stirred magnetically while N_2 was made to bubble through for fifteen minutes. Eighty g of ninhydrin and 1.600 g of $SnCl_2.2H_2O$ were added; stirring was continued until completely dissolved. The solution of ninhydrin reagent thus prepared was transferred to the reservoir, a 4-liter bottle, which was painted black to eliminate photochemically induced reactions.

(D) Preparation of the sample for amino acid analysis

Approximately 5 mg of protein was weighed in a standard 16 x 150 mm Pyrex test tube, which had been previously cleaned with chromic acid and rinsed thoroughly with de-ionized water and dried. The sample was then dissolved in 0.5 ml of reagent grade concentrated HCl and diluted with 0.5 ml of de-ionized water. To remove any dissolved air, the tube was sealed onto the manifold of a vacuum system provided with a mechanical pump. The solution was then frozen by immersion in a Dewar flask containing a mixture of dry ice-alcohol and the tube evacuated to a pressure less than 50 micron Hg.* This de-aeration procedure has proved essential for the accurate determination of carboxy methylcysteine and advantageous for determining serine, threonine, tyrosine and cystine. The sample tube was then sealed under reduced pressure and placed in an oven regulated at 110 $\pm$ 2^{o}C for 22 hours. Then the tube was chilled and opened. Removal of HCl was achieved by freeze-drying. To ensure that all HCl was completely removed, the residue was redissolved twice in one ml of distilled water and subjected again to freeze-drying. Finally, the residue was dissolved in 5 ml of pH 2.2 buffer ready for analysis.

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* The author is indebted to Mr. R. Patel for his help in this part of the work.

(E) Preparation of columns

Two grades of sulphonated styrene-8% divinyl benzene copolymer resins were used for the chromatographic analysis of protein hydrolysates. They differed with respect to the size of the particles and were used in two columns, a long one (65 cm) and a short one (25 cm). The resin used in the long column for the separation of the neutral and acidic amino acids contained particles ranging from 31 to 41 microns in size. For the analysis of basic amino acids, the short column filled with resin particles ranging from 19 to 25 microns was used.

Because of the grinding operation in the process of manufacture of the resin, many particles may be partially fractured, so that on subsequent handling small fines of 3 to 10 microns in size may break off. These would easily clog the column and were, therefore, removed by free sedimentation in the buffer subsequently to be used for elution, except that the BRIJ-35 and thiodiglycol were left out.

After removal of the fines, a glass column of 0.9 - 1.0 cm diameter was packed in sections; the slurry of the resin was stirred rapidly in a beaker with a glass rod and then poured into the column tube fitted with a sintered disc and stoppered at the bottom. The first portion was allowed to settle under gravity for a few minutes and then under air pressure of 15 psi until no further drop in height of the

surface of the resin occurred. The buffer in the tube was allowed to drain; when the level of the buffer had fallen almost to the surface of the resin, a second portion of slurry was added. Care was taken not to disturb the surface of the resin already in the column. The other portions were added similarly to bring the height of the resin to about 55 cm. To avoid disturbance of the resin surface during the application of the sample, it was covered with a sintered disc. Then the appropriate buffer was pumped through the column, which connected to the reaction bath system under a back pressure of about 170 - 180 psi. The flow rate was adjusted to 68 ml/hour. Before use, the column was washed with 0.2 N NaOH and then equilibrated with the pH 3.28 buffer.

The short column was prepared in a similar manner with a slurry of resin in 0.35 N buffer, pH 5.28. When the buffer was passed through the column, the back pressure was 20 - 30 psi.

(F) Operation of columns

To save operational time, the analysis was first carried out in a short column. The buffer above the disc was removed and one ml of the sample solution was introduced into the column from a bent tip pipette; at the end of the delivery the tip was slightly touched against the wall of the column immediately above the solution surface. The sample was forced into the column under an air pressure of 15 psi.

Application of air pressure was discontinued as soon as the solution disappeared from the surface of the disc. At no time was the air bubble forced into the resin. Then it was washed in with three aliquots of 0.2 ml buffer, pH 2.2. The column was filled with the pH 5.28 buffer and was then connected to the reaction bath system and the buffer was passed through the column. Under these conditions, all acidic and neutral amino acids were eluted as one peak. Prior to the elution of the basic amino acids, the base lines of the multipoint recorder were adjusted. The total operational time was approximately 60 minutes. As no amino acids were irreversibly retained by the column, regeneration could be easily achieved by passing the buffer through for an additional half-an-hour. Usually, good resolution was obtained.

For analysis of the neutral and acidic amino acids, 1 ml of the sample was applied to the long column with care and, after entering the column material, the surface of the column was gently washed with three 0.2 ml volumes of the buffer at pH 2.2. The automatic timer was set to 90 minutes for change of buffers from the first at pH 3.28 to the second at pH 4.25 and the column was ready for analysis. The protein hydrolysate containing a mixture of amino acids was resolved into their acidic and neutral components while the basic amino acids were retained by the column. For regeneration, the long column was washed with 0.2 N NaOH and

was then re-equilibrated with the pH 3.28 buffer.

(G) Estimation of amino acids

As mentioned before, each amino acid has its own characteristic elution time under constant environmental factors. Thus, by comparing an unknown chromatogram with that previously obtained with a synthetic mixture of known composition, every individual amino acid present in the sample can be identified. In addition, the elution rate of the column is kept at a constant value of 68 ml per hour, while the chart travels at a speed of 6 inches per hour. Furthermore, to facilitate the measurement along the time scale, the recorder prints a dot every 6 seconds for each curve on the chart, which is calibrated across in absorbance units on a log scale from zero to infinity. Therefore, the area, which is proportional to the amount of a given amino acid, can be readily integrated on the absorbance-time graph by the height-width method. The area under the peak was determined as recommended in the Beckman manual by multiplying the height (H) of the peak by the width (W) which is measured at the mid-point of the height. The peak height can be read from the chart directly, while the width is measured by counting the number of dots above the width at half of the height (i.e. the total time for the chart to travel along the half-width). The concentration of each amino acid is calculated by the following equation:

$$C_u = \frac{H_u \times W_u}{H_c \times W_c} C_c$$

where H_c, W_c and C_c are the height (absorbance), width (dots = time) and concentration (micromoles) of the calibration mixture, and H_u, W_u and C_u the corresponding terms for the sample of known composition.

The chromatogram of the synthetic mixture, containing eighteen amino acids, each 0.5 μmoles, is shown in figure 44. As can be seen, the basic amino acids and ammonia, recorded in the shorter curve at the far left, were separated on the short column (25 cm) operated at 56°C and with the pH 5.28, 0.35 N sodium citrate buffer. The acidic and neutral amino acids, shown in the longer second curve, were separated on the long column (65 cm), which was also operated at 56°C. The elution was started with the pH 3.28, 0.2 N sodium citrate buffer and changed to the pH 4.25 buffer at 90 minutes. The half-cystine peak is well separated from alanine and the valine peak is sharpened by the pH 4.25 buffer front. Figure 45 illustrates the amino acid composition of insulin, and each individual amino acid present was identified. It is noted that methionine is absent from this chromatogram, as insulin contains no methionine. With the aid of the above equation, the concentration of each amino acid in the insulin sample could be determined. These values, calculated on a molar

Figure 44

THE CHROMATOGRAM OF THE SYNTHETIC MIXTURE OF 18 AMINO ACIDS OBTAINED FROM BECKMAN MODEL 120B AMINO ACID ANALYSER

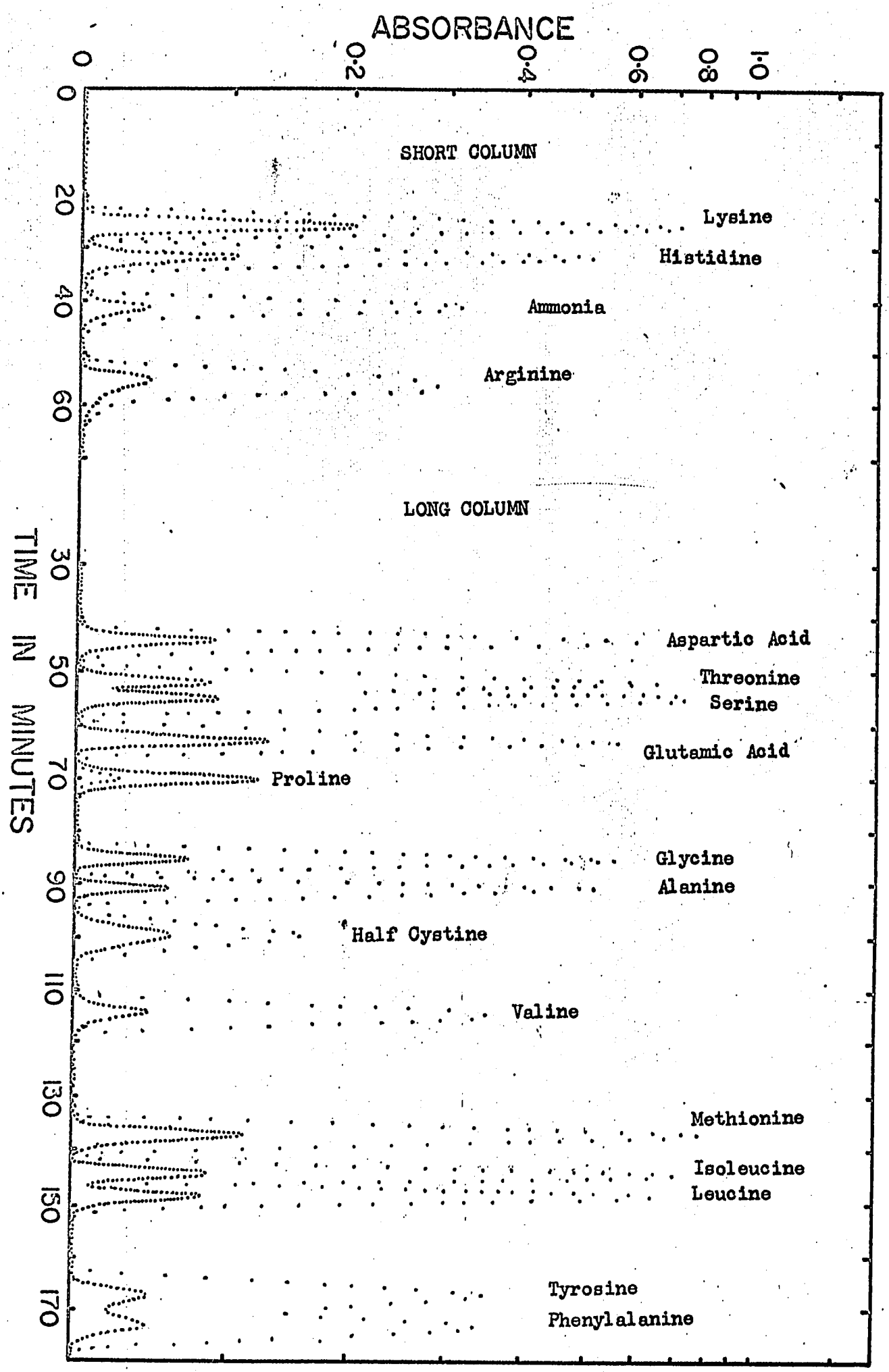
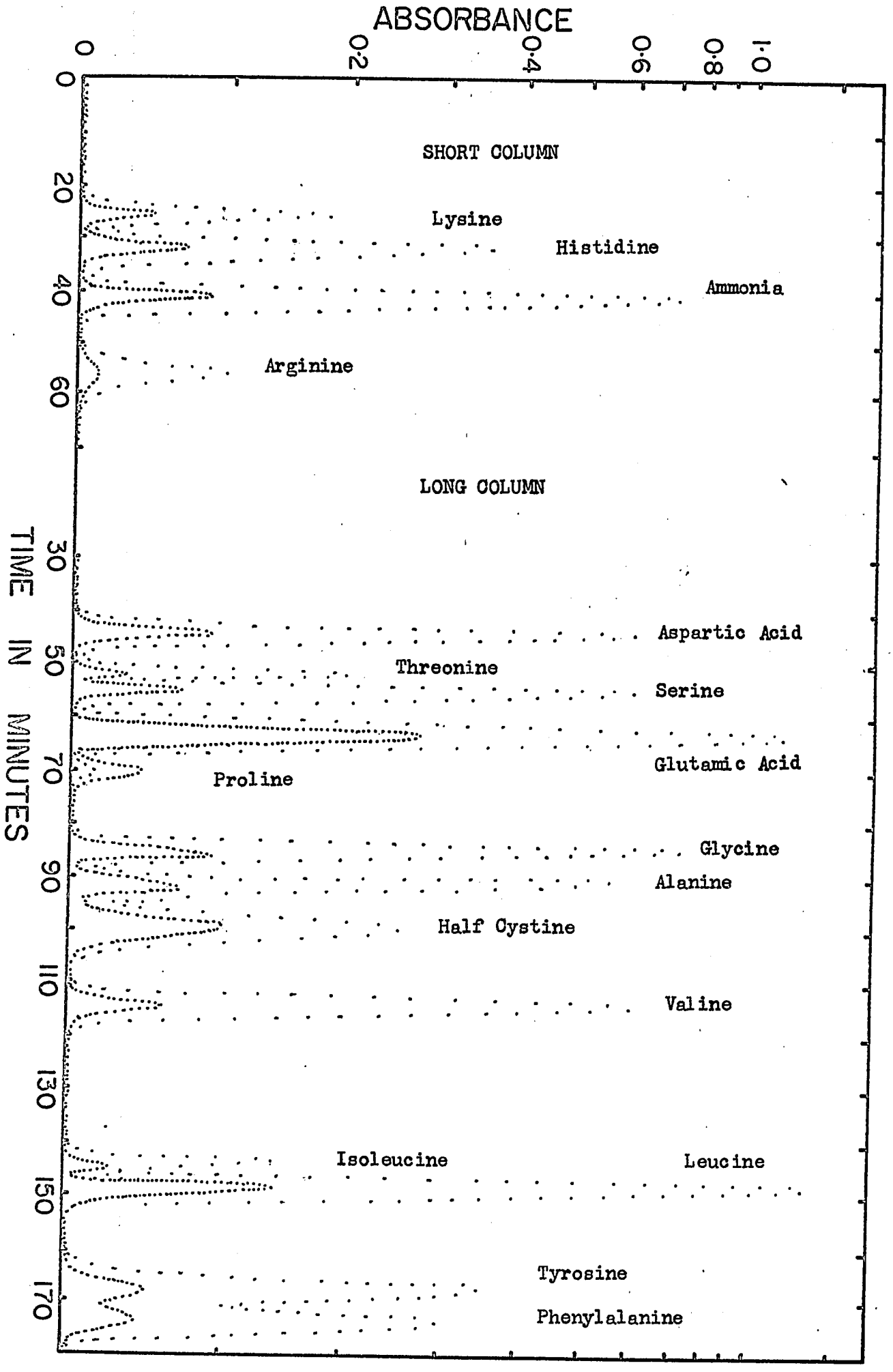

ABSORBANCE
0
0·2
0·4
0·6
0·8
1·0
TIME IN MINUTES
0
20
40
60
30
50
70
90
110
130
150
170
SHORT COLUMN
Lysine
Histidine
Ammonia
Arginine
LONG COLUMN
Aspartic Acid
Threonine
Serine
Glutamic Acid
Proline
Glycine
Alanine
Half Cystine
Valine
Methionine
Isoleucine
Leucine
Tyrosine
Phenylalanine

Figure 45

THE CHROMATOGRAM OF THE HYDROLYSATE OF INSULIN OBTAINED FROM BECKMAN MODEL 120B AMINO ACID ANALYSER


ABSORBANCE
0
0·2
0·4
0·6
0·8
1·0
SHORT COLUMN
Lysine
Histidine
Ammonia
Arginine
LONG COLUMN
Aspartic Acid
Threonine
Serine
Glutamic Acid
Proline
Glycine
Alanine
Half Cystine
Valine
Isoleucine
Leucine
Tyrosine
Phenylalanine
TIME IN MINUTES
0
20
40
60
30
50
70
90
110
130
150
170

basis relative to threonine, are listed in table XII and are compared with the theoretical values. The values obtained in five separate experiments were fairly reproducible. The lower values observed for serine, isoleucine and tyrosine, which were consistent in these experiments, might be attributed to the conditions of hydrolysis of the sample. Therefore, in the analysis of insulin fragments, hydrolysis was carried out under the same conditions as used for calibration of the instrument with the hydrolysate of insulin itself, and the results obtained were compared with the experimental standard.

Result and Discussion

When the e.m.f. was applied to the electrolysis cell and was gradually increased, there was no current flowing through the cell until a threshold voltage was reached. On further increase of the applied e.m.f., the current rose rapidly as demonstrated in figure 46. The threshold voltage, referred to as the decomposition voltage of the cathode solution, was obtained by extrapolation of the line to zero current. Frequently, a small current (the residual current) was observed even before the decomposition potential was reached and was attributed to the reduction of the trace amount of oxygen remaining in the cathode solution. As expected, the current-cathode potential curves, obtained with

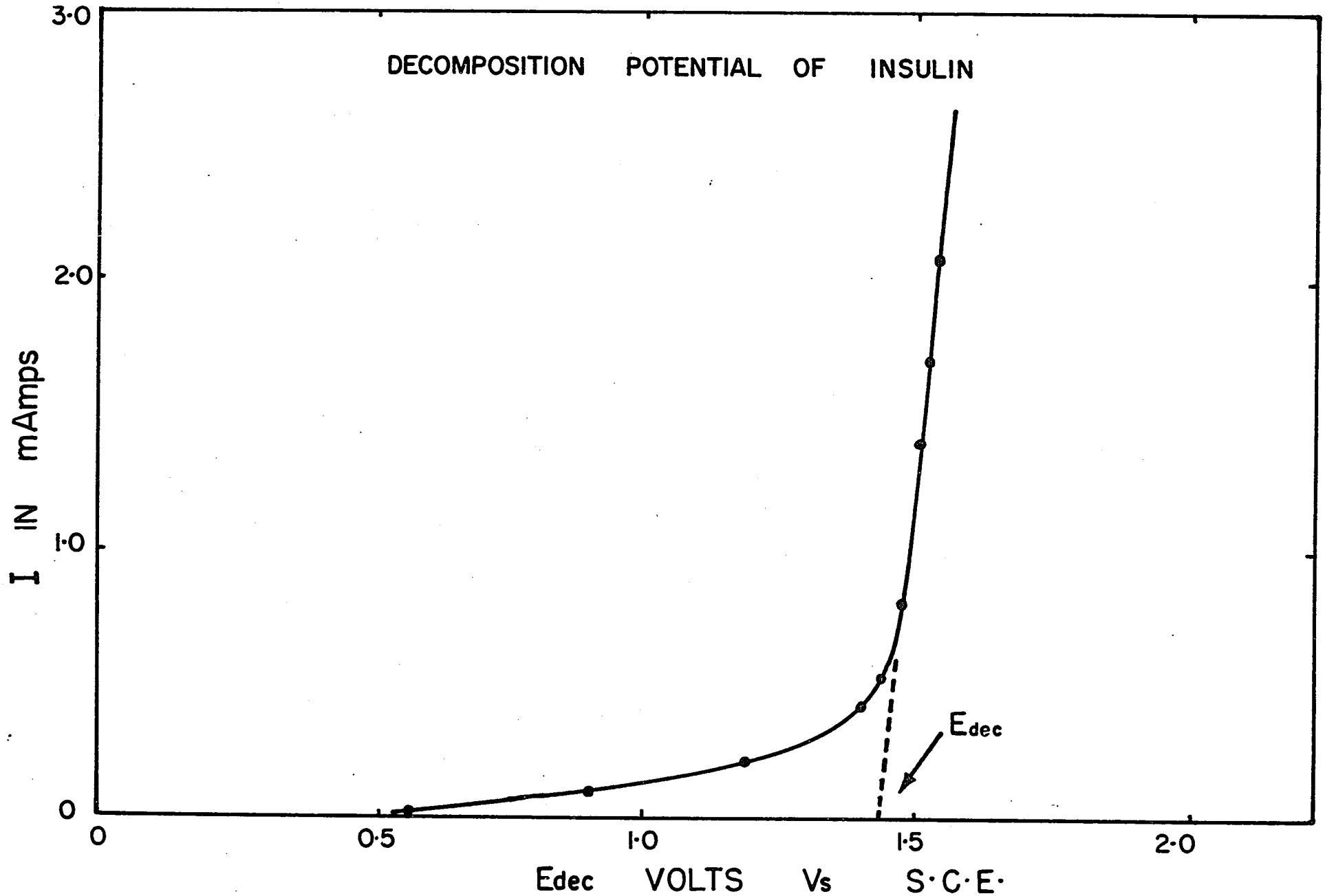
Table XII

AMINO ACID ANALYSIS OF INSULIN

Amino acid	Expected	Observed
Lysine	1	0.9
Histidine	2	2.0
Arginine	1	1.1
Aspartic acid	3	3.1
Threonine	1	1.0
Serine	3	2.5
Glutamic acid	7	7.5
Proline	1	1.3
Glycine	4	4.3
Alanine	3	3.2
Valine	5	4.9
Half-cystine	6	5.8
Isoleucine	1	0.7
Leucine	6	6.2
Tyrosine	4	3.4
Phenylalanine	3	2.9

Figure 46

CURRENT-VOLTAGE CURVE OF 5 μMOLES OF INSULIN IN PHOSPHATE BUFFER, pH = 7.2, IONIC STRENGTH 0.02


DECOMPOSITION POTENTIAL OF INSULIN
3·0
2·0
1·0
0
I IN mAmps
0
0·5
1·0
1·5
2·0
Edec
Edec VOLTS Vs S·C·E·

the large mercury cathode and with a well-stirred solution, did not show the limiting current (i.e. did not reach a plateau value) that is characteristic of the dropping mercury electrode polarograms. The occurrence of a limiting diffusion-controlled current depends on the attainment of an extreme state of concentration polarization at the electrode surface, whereas the use of a large mercury electrode reduces concentration polarization to a minimum. Hence no limiting current was observed. Therefore, by using the present method, it was difficult to determine whether there were three distinct decomposition potentials for the three disulphide bonds present in the insulin molecule.

The decomposition potential measured was -1.46 ± 0.05 V vs S.C.E.. Previously Kolthoff and Barnum (225), in their study on the cathodic waves of cystine, found that the depolarization potential of cystine solution was dependent on the pH. At pH values between 4 and 9.2, the depolarization potentials of the buffered solutions saturated with cystine varied from -0.2 V to -0.75 V vs S.C.E. and further increase in applied voltage resulted in a slow rise in current with a flat slope. However, when the solutions were saturated with a capillary-active substance, such as camphor, at pH values between 4 and 9.2, the waves shifted to a more negative potential and the current-voltage plot had a steep slope, all the waves starting at about -1.35 V (vs S.C.E.). In order to explain the shift of the cystine waves to a more negative

potential, Kolthoff and Barnum (225) postulated that the cystine had to be oriented in a favourable position at the surface of the dropping mercury electrode to be reduced. Apparently capillary-active substances counteract or prevent the orientation of cystine at the interface and thus displace the waves to more negative potentials. In the present study, the reduction potential measured at the mercury cathode for the disulphide bonds of insulin also occurred at a more negative value than that of the simple cystine. Thus, it would appear that for the reduction of S-S bonds in the insulin molecule, which itself is surface active and complicated in structure, it had to be in an appropriate orientation itself, so that the cystine residues were in a favourable position at the mercury-solution interface.

As mentioned in the introduction of this chapter, with chemical reducing agents in the absence of denaturing agent cleavage of either intrachain or interchain disulphide bonds were accomplished. The differential reduction of these two types of disulphide bonds in insulin indicates that probably there might be two different environments which embed these bonds. Thus, in the present study, for reduction of the interchain disulphide bonds, a voltage of -1.6 V vs. S.C.E. was applied to the cathode in order to attain a decent current (2 to 3 mamps) while a cathode voltage of slightly below -2.0 V was applied for total reduction.

Aside from the cystine residues in insulin, the

reducible substances present in the cathode compartment were Na^+, K^+ ions and a trace amount of Zn^{++}, which was present in the crystalline beef insulin. All these ions could be electro-separated in the mercury cathode and subsequently amalgamated with the mercury when the cathode voltage reached their corresponding decomposition potentials. The half-wave potentials of Na^+, K^+ and Zn^{++} are -2.11 V, -2.14 V and -0.993 V (vs S.C.E.), respectively (226). Thus, with the cathode voltage kept at -1.6 V or slightly less than -2.0 V (vs S.C.E.), only the cystine and Zn^{++} were reduced. However, there was no significant rise in current at -0.993 V vs S.C.E. probably due to a very low concentration of Zn^{++} ions.

Figure 47 shows the change of current with time. As the cathode voltage was adjusted manually from time to time, the current decreased in a stepwise fashion. The histograms were similar for both reductions when the cathode voltage was kept either at -1.6 V or slightly less than -2.0 V. The formation of SH groups was followed by amperometric titration, as this method gave direct and quick results.

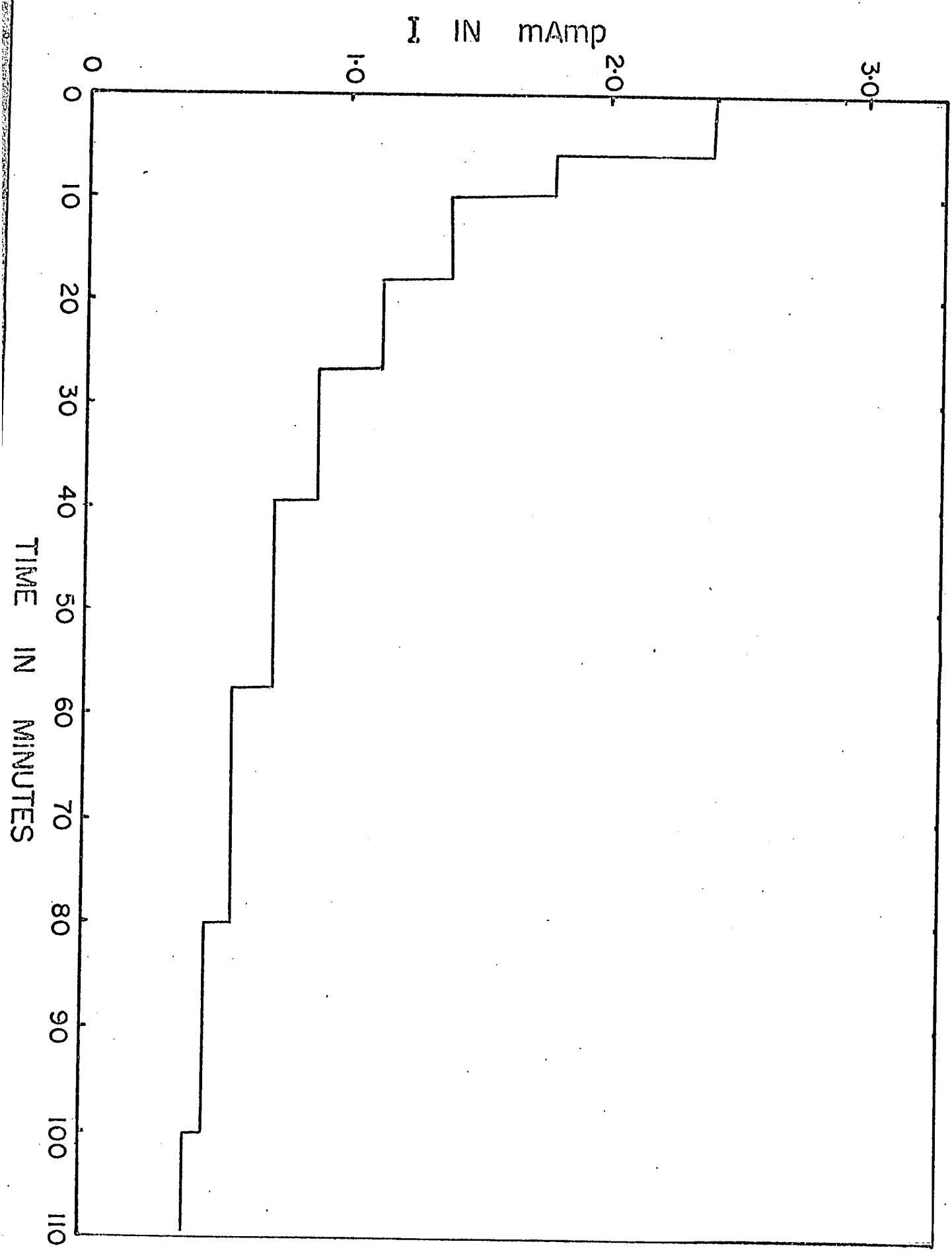
Methods of Analysis

(A) Paper electrophoresis

Since the products of reduction were poorly soluble under mild pH range (pH 4.0 - 9.0) i.e. solubility was less than 1 mg/ml, paper electrophoresis was carried out in 20%

Figure 47

CURRENT VS TIME DURING THE COURSE OF ELECTROLYTIC REDUCTION OF INSULIN AT -1.6 V VS S.C.E.


I IN mAmp
0
1·0
2·0
3·0
TIME IN MINUTES
0
10
20
30
40
50
60
70
80
90
100
110

formic acid (pH = 1.2) according to the method described in Chapter 3. Optimal separations were generally achieved by passing a current of 10 mA (at 110 V) for 24 hours at $4^{\circ}C$. The identity of the bands was deduced from the electrophoretic properties at pH = 1.2.

The A chain after complete reduction and alkylation of the 4 SH groups contains (see page 21) one terminal amino group (charge + 1 unit), two glutamic acid residues of pK = 4.7, one terminal carboxyl group of pK = 3.6 (113) and four S-carboxymethylcysteine of pK $\sim$ 3. The last three types of amino acids have probably a negligible charge at the pH of of 1.2 and, therefore, the overall charge of the A chain at this pH was expected to be + 1 unit. As the charge contribution from S-carboxymethylcysteine groups would be at best negligible, the net charge of the A chain, with the intrachain disulphide bond intact, would be expected to be the same as that of the totally reduced A chain. The A chain with the intrachain disulphide bond intact will be referred to hereafter as A(S-S) chain, whereas the totally reduced A chain, i.e. with the reduced intrachain S-S bond, will be referred to in future simply as the A chain.

The B chain contains one terminal amino group, one lysine residue, one arginine residue, two histidine residues (i.e. overall charge of + 5 units) and for similar reasons, as stated before, the charge contribution from the two glutamic acid residues, the terminal carboxyl group, and the two

S-carboxymethylcysteine would be negligible. The overall charge of the B chain, therefore, would be + 5 units. On the same grounds, the net charge of the intact insulin under these conditions would be expected to be + 6 units.

When an average of 2.9 S-S bonds was reduced at a cathode voltage of slightly less than -2.0 V S.C.E., the products separated into two distinct bands on paper electrophoresis at pH 1.2: one remaining at the line of application and the other with its cationic mobility slightly slower than that of the intact insulin (figure 48). These two bands are identified with their corresponding electrophoretic mobilities as A and B chains, respectively. The present results indicate that even under mild experimental conditions all three disulphide bonds of insulin were electrolytically reduced and that no air oxidation or re-combination occurred to form insulin after the reduction. Furthermore, at pH 1.2, the two chains were separated from each other without any apparent aggregation.

Figure 49 shows the products of the reduced insulin when an average of 2.0 S-S bonds was reduced at a cathode voltage of -1.6 V vs S.C.E. Only two bands were observed: one corresponding to either a totally reduced or A(S-S) chain and the other corresponding to B chain. If the three disulphide bonds of insulin had been reduced at random, the products ranging from intact insulin, insulin with one or two disulphide bonds reduced and insulin fragments with all

Figure 48

Paper electrophoretic patterns of insulin and of the products of insulin reduced at a cathode voltage of slightly less than -2.0 V vs S.C.E.

Solution	-	20% formic acid
Voltage	-	110 V
Current	-	10 mA
Time	-	24 hours
Temperature	-	4 ^{o}C

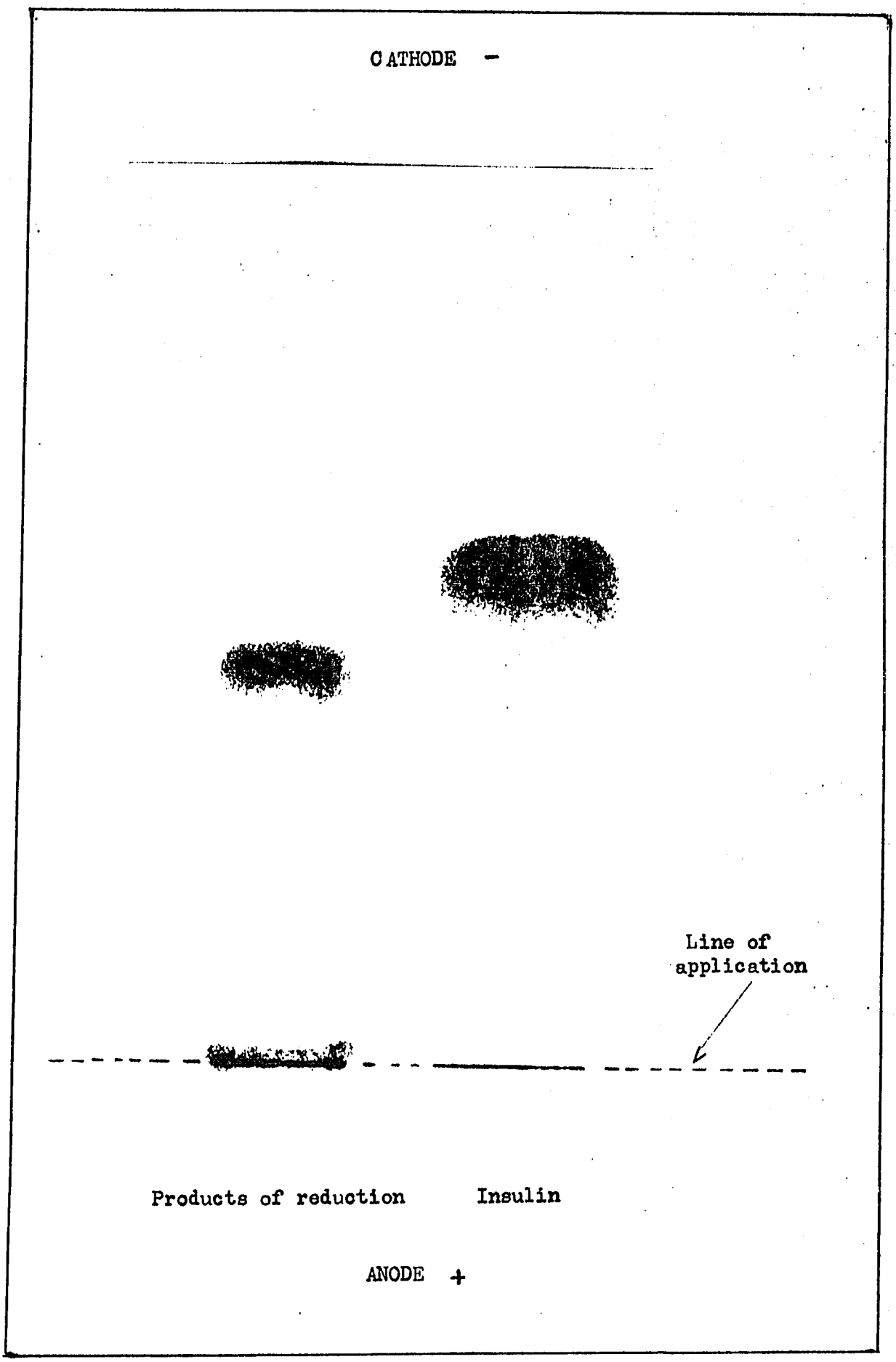
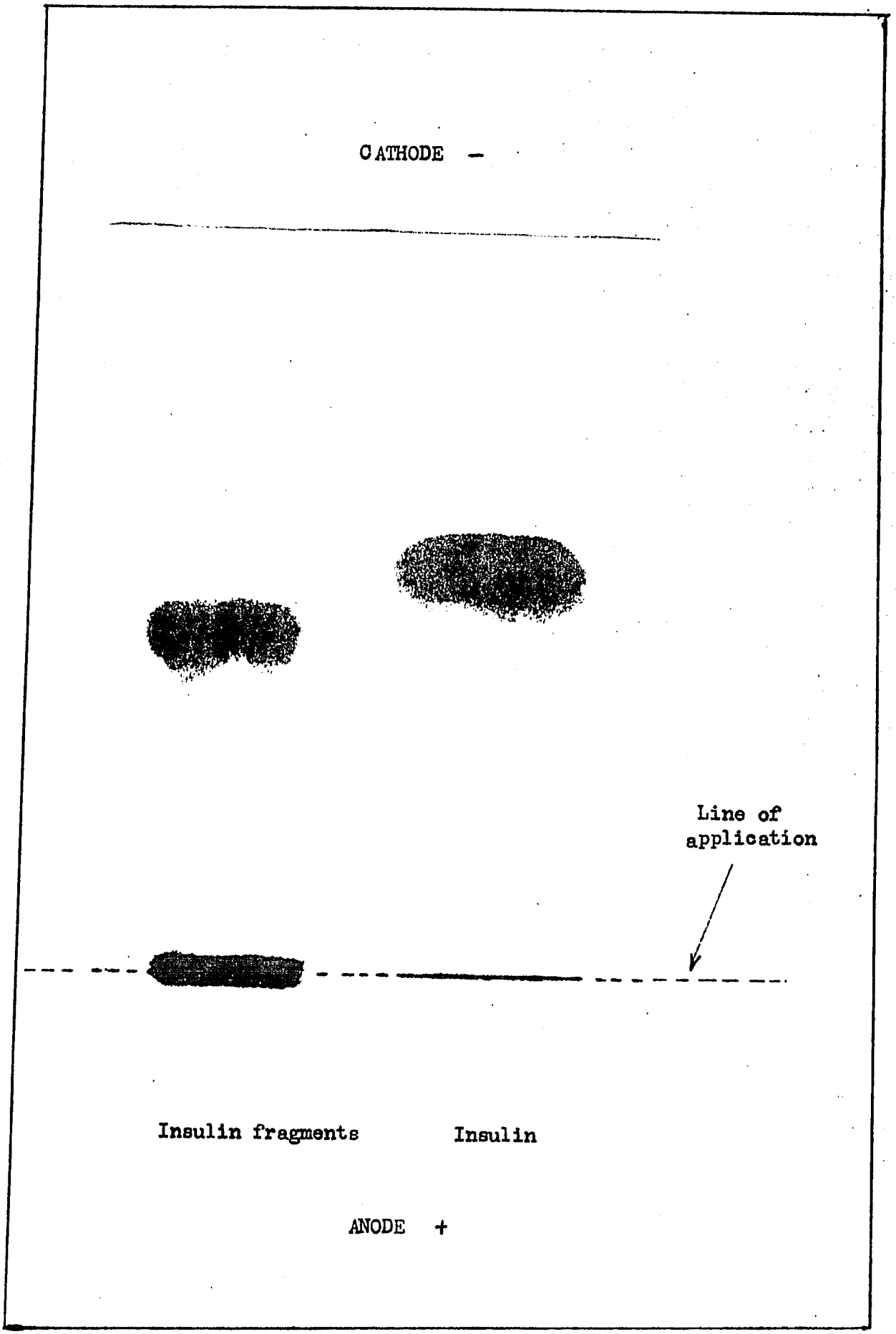

CATHODE -
Line of application
Products of reduction
Insulin
ANODE +

Figure 49

Paper electrophoretic patterns of insulin and of insulin fragments obtained when an average of 2.0 S-S bonds was reduced at a cathode voltage of -1.6 V vs S.C.E.

Solution	-	20% formic acid
Voltage	-	110 V
Current	-	10 mA
Time	-	24 hours
Temperature	-	$4^{\circ}C$


CATHODE –
Line of application
Insulin fragments
Insulin
ANODE +

disulphide bonds reduced would have been obtained in different quantities. For a random process, the distribution of the products can be readily calculated according to the law of probability. Thus, in a population of insulin molecules, each molecule has three disulphide bonds. On the average, two disulphide bonds are broken after the electrolytic reduction. Hence, the probability that a given disulphide bond in a molecule is broken is

$$p = \frac{2}{3}$$

and the probability that a given disulphide bond in a molecule remains intact is

$$q = \frac{1}{3}$$

The probability to find an insulin molecule with x disulphide bonds broken can be expressed as

$$P_x = \frac{n!}{(n - x)!\, n!}\, p^x\, q^{n-x}$$

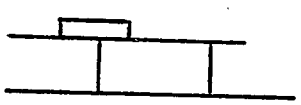
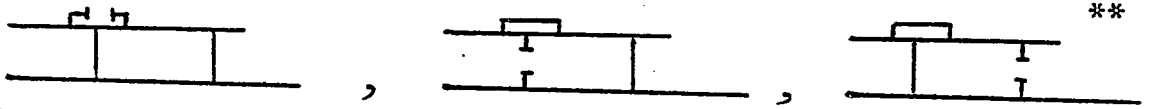
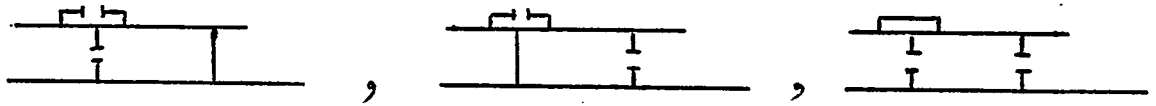
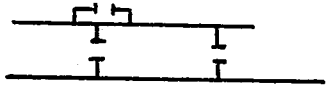
where n = total disulphide bonds in insulin = 3

x = number of disulphide bonds broken.

From this equation, the probabilities to find an insulin molecule with 0, 1, 2, 3 disulphide bonds broken are evaluated (table XIII). As can be seen from this table, the most probable products after electrolytic reduction would have been: (i) B chain, (ii) totally reduced A chain, (iii) A chain

Table XIII

EXPECTATION VALUES OF DIFFERENT TYPES OF PRODUCTS WHEN AN AVERAGE OF TWO S-S BONDS OF INSULIN IS REDUCED AT RANDOM

Probability	Types of Products
$P_o = \frac{1}{27}$	 *
$P_1 = \frac{6}{27}$	 **
$P_2 = \frac{12}{27}$	
$P_3 = \frac{8}{27}$	

*The diagram represents an insulin molecule with A and B chains joined together by two interchain disulphide bonds at B7 - A7 and at B19 - A20. A third intrachain disulphide bond occurs at A6 - A11.

**The broken line refers to the reduced disulphide bond.

with intrachain S-S bond intact, (iv) insulin with the B7 - A7 interchain disulphide bond intact and the intrachain disulphide bond and the B19 - A20 interchain disulphide bond reduced, and (v) the B19 - A20 interchain disulphide bond intact and the intrachain S-S bond and the B7 - A7 interchain disulphide bond reduced. The products of the (iv) and (v) types would assume an overall charge which would be different from those of A and B chains, but equal to those of the intact insulin and of insulin with one of the three disulphide bonds reduced at pH 1.2. Thus, it is anticipated that upon electrophoresis at pH 1.2, at least three bands should have been observed. The absence of the band or bands corresponding to those of (iv) and (v) suggests that the three disulphide bonds of insulin had not been ruptured at random, but that the two interchain disulphide bonds were preferentially reduced at the cathode voltage of -1.6 V vs S.C.E. From this evidence one would deduce that the intrachain disulphide bond would be reduced at a higher cathode voltage, which is in agreement with the observation of Cecil and Wake (207) that the interchain disulphide bonds are in general more labile than the intrachain disulphide bonds in protein. Moreover, the band remaining at the line of application would thus represent the A(S-S) chain, i.e. with the intrachain disulphide bond intact.

When, on the average, 1.0 S-S bond was reduced at a cathode voltage of -1.6 V vs S.C.E., the electrophoretic pattern of the products of reduction revealed three bands:

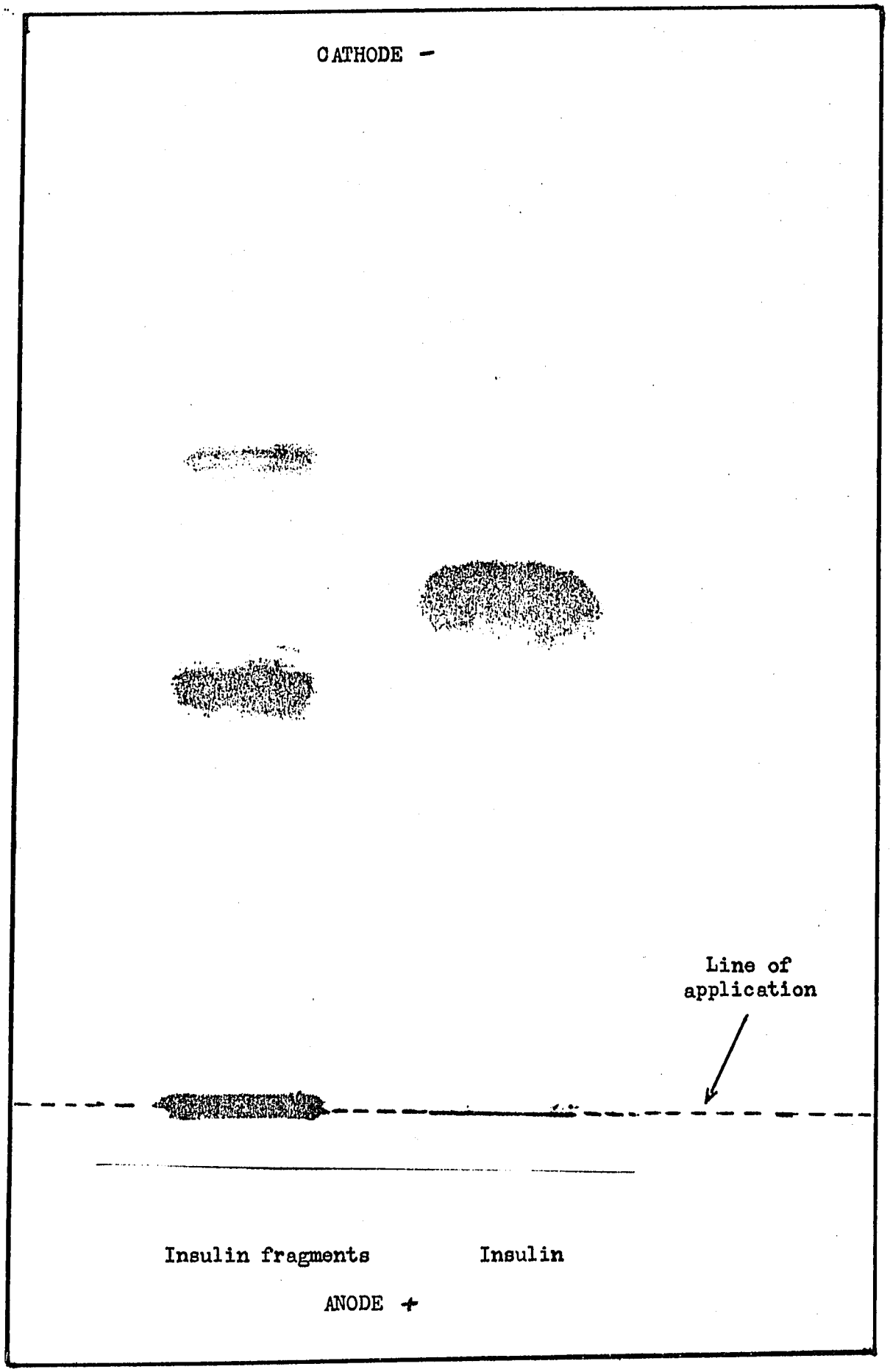
one remaining at the line of application with mobility corresponding to that of either the totally reduced A chain or the A(S-S) chain, one with a cationic mobility slightly slower than that of intact insulin and corresponding to B chain, and a third one with a cationic mobility slightly faster than that of crystalline zinc insulin (figure 50). As already discussed above, at this cathode voltage, rupture of the three disulphide bonds of insulin molecule does not occur at random; therefore, one can deduce that the two interchain disulphide bonds are preferentially reduced. Thus, it is anticipated that when, on the average, 1.0 S-S bond was reduced at a cathode voltage of -1.6 V vs S.C.E., the main products would be the A(S-S) chain and the B chain, and there would be a substantial amount of intact insulin remaining in solution. Therefore, it would seem reasonable to identify the band remaining at the line of application in figure 50 as the A(S-S) chain.

The insulin used in this investigation was crystalline zinc insulin with a molecular weight of 36,000. It has been suggested that the site of binding of the zinc is through the co-ordination of histidine residue with the Zn^{++} ion (112,113). As noted before, the half-wave potential of Zn^{++} ions is -0.993 V vs S.C.E. Hence, with the cathode voltage kept at -1.6 V vs S.C.E., the Zn^{++} ions would be electro-reduced and subsequently amalgamated with the mercury in the cathode compartment. Therefore, any crystalline insulin remaining in

Figure 50

Paper electrophoretic patterns of insulin and of insulin fragments obtained when an average of 1.0 S-S bonds was reduced at a cathode voltage of -1.6 V vs S.C.E.

Solution	-	20% formic acid
Voltage	-	110 V
Current	-	10 mA
Time	-	24 hours
Temperature	-	$4^{\circ}C$


CATHODE -
Line of application
Insulin fragments
Insulin
ANODE +

solution would have become zinc-free insulin. The molecular weight of zinc-free insulin in aqueous solution is dependent on pH and on the insulin concentration, and at the pH used for paper electrophoresis (pH = 1.2) insulin exists as a dimer (114). Thus, the third band in figure 50 with a cationic mobility slightly faster than that of crystalline zinc insulin (a hexamer) might represent zinc-free insulin (a dimer); the observed change in mobility is considered to be brought about through the alteration in electrokinetic properties of the insulin molecule as it dissociates from a hexamer into a dimer.

(B) Ion-exchange chromatography

As insulin and its separate A and B chains have different isoelectric points, it would be reasonable to expect that these can be separated by chromatography on an ion-exchange resin. Therefore, the products of reduction, obtained when an average of 1.0 S-S bond was ruptured at a cathode voltage of -1.6 V vs SCE., were separated in a column (1.0 cm x 20 cm) of Dowex 50-X2 according to the method of Bailey and Cole (227). The column was equilibrated with 6 M urea buffered at pH 2.2 with 0.2 M sodium citrate overnight in a cold room. A sample of 20 mg in 1.0 ml of the same buffer was applied to the column. The glycyl chain passed through the column unretarded and the phenylalanyl chain was eluted with 6 M urea buffered at pH 7.6 with 0.2 M sodium phosphate. The third component was eluted with 6 M urea buffered at pH 8.5 with 0.2 M

Tris-phosphate. After dialysis against distilled water to remove urea, the fractions obtained were concentrated by lyophilization. These fractions will be referred to hereafter as fraction I, II and III, respectively.

Figure 51 represents the chromatogram of the insulin fragments. As can be seen, three peaks were observed, fraction I corresponding to the A(S-S) chain, fraction II representing the B chain, and fraction III being the component which had the fastest cationic mobility on paper electrophoresis at pH 1.2. Each fraction was further identified by paper electrophoresis in 20% formic acid (pH = 1.2). The electrophoretogram (figure 52) reveals that fraction I remained at the line of application and that there was no contamination with either B chain or with insulin. Fraction II migrated with a mobility corresponding to that of B chain; however, tailing was observed. Thus, this result suggests that the latter fraction was probably contaminated with a small amount of A chain. Fraction III corresponded to the third component on paper electrophoresis. These fractions were subjected to amino acid analysis.

(C) Amino acid analysis

As the totally reduced A chain, the A(S-S) chain, the B chain and intact insulin differ from each other in their primary structure, analysis of their compositions was expected to lead to their unequivocal identification. Thus, the

Figure 51

SEPARATION OF INSULIN FRAGMENTS IN A COLUMN (1.0 x 20 cm) OF DOWEX 50-X2

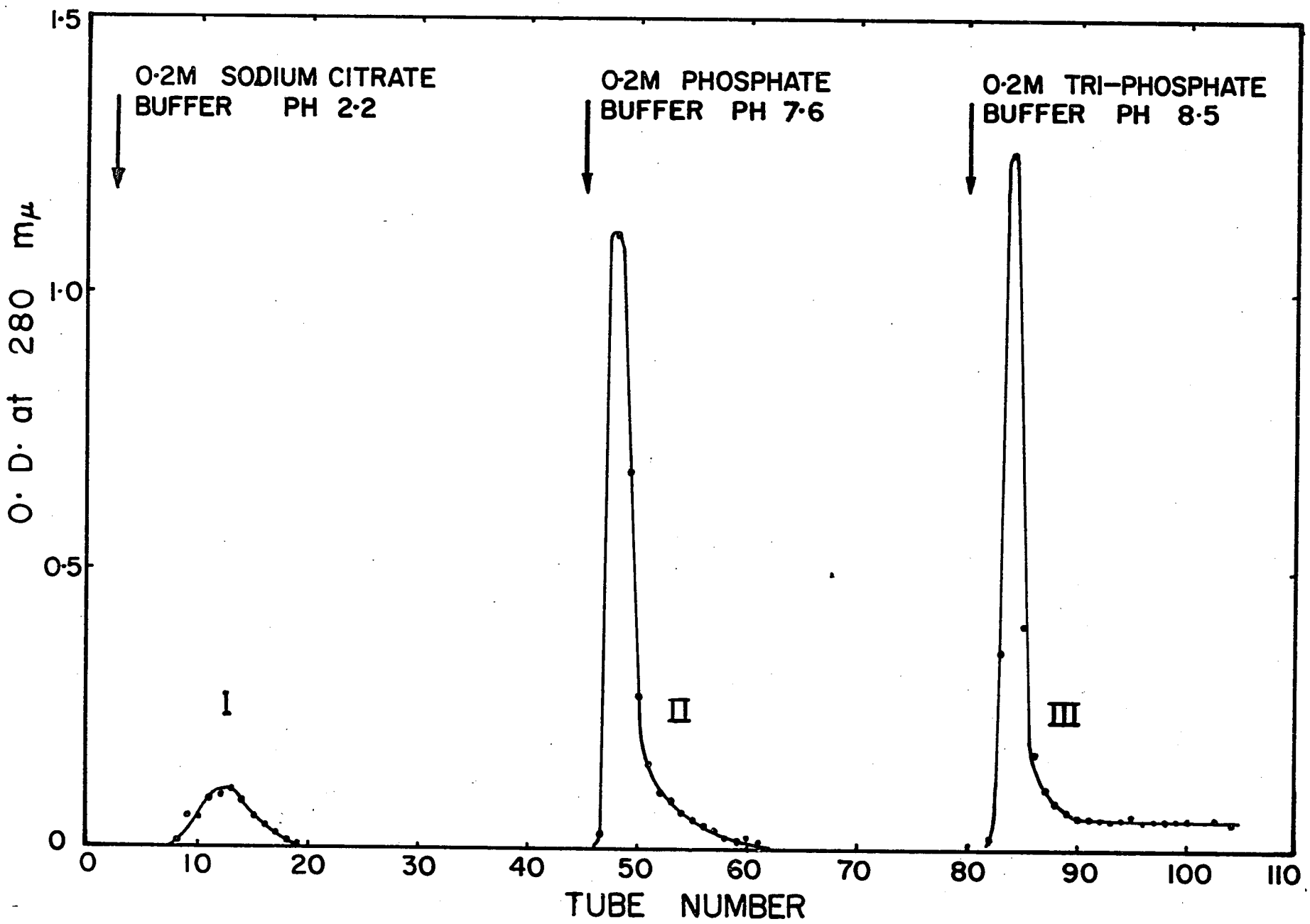
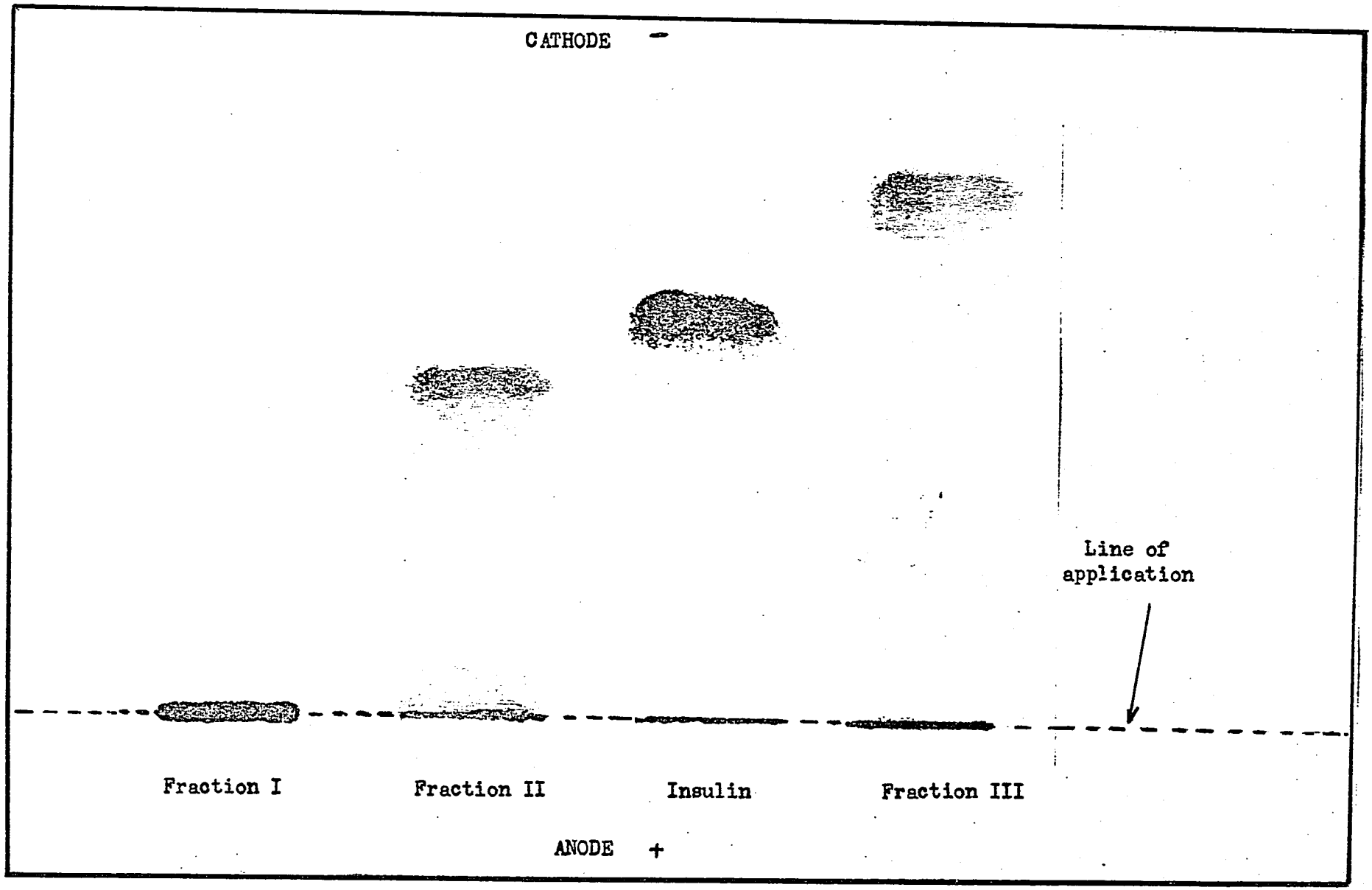

0·2M SODIUM CITRATE
BUFFER PH 2·2
0·2M PHOSPHATE
BUFFER PH 7·6
0·2M TRI-PHOSPHATE
BUFFER PH 8·5
O·D· at 280 mμ
1·5
1·0
0·5
0
I
II
III
0
10
20
30
40
50
60
70
80
90
100
110
TUBE NUMBER

Figure 52

ELECTROPHORETIC PATTERNS OF FRACTIONS I, II AND III IN 20% FORMIC ACID

Voltage	-	110 V
Current	-	10 mA
Time	-	24 hours
Temperature	-	$4^{o}C$


CATHODE −
Line of application
Fraction I
Fraction II
Insulin
Fraction III
ANODE +

fractions separated from a column of Dowex 50-X2 were subjected to duplicate amino acid analysis and, from the chromatograms obtained (figures 53, 54, 55), the relative molar concentration of each amino acid present in each fraction could be evaluated.

The chromatogram of fraction I (figure 53) demonstrated that there were only trace amounts of lysine, histidine, and threonine and that the peaks corresponding to arginine, proline and phenylalanine were not observed. As these amino acids are absent from the A chain of insulin, the result of this analysis indicates that fraction I contained A chain, which was contaminated with trace amounts of B chain. Upon comparing the relative concentration of each amino acid present in this fraction with that of A chain, it can be seen from table XIV that there is a very good agreement. Thus, this fraction undoubtedly contained A chain. In addition, in figure 53 there is a new peak appearing two minutes before the elution time of aspartic acid; this new acidic amino acid, which is not present in the original insulin, was therefore identified as the S-carboxymethylcysteine. This evidence, together with the presence of two half-cystine residues, suggests that fraction I consisted of A(S-S) chain, i.e. A chain with the intrachain disulphide bond intact. The present result thus confirms the earlier conclusion derived from the analysis of paper electrophoresis that reduction of the insulin molecule did not occur at random and that the cleavage of one out of

Figure 53

THE CHROMATOGRAM OF THE HYDROLYSATE OF FRACTION I OBTAINED FROM BECKMAN MODEL 120B AMINO ACID ANALYSER

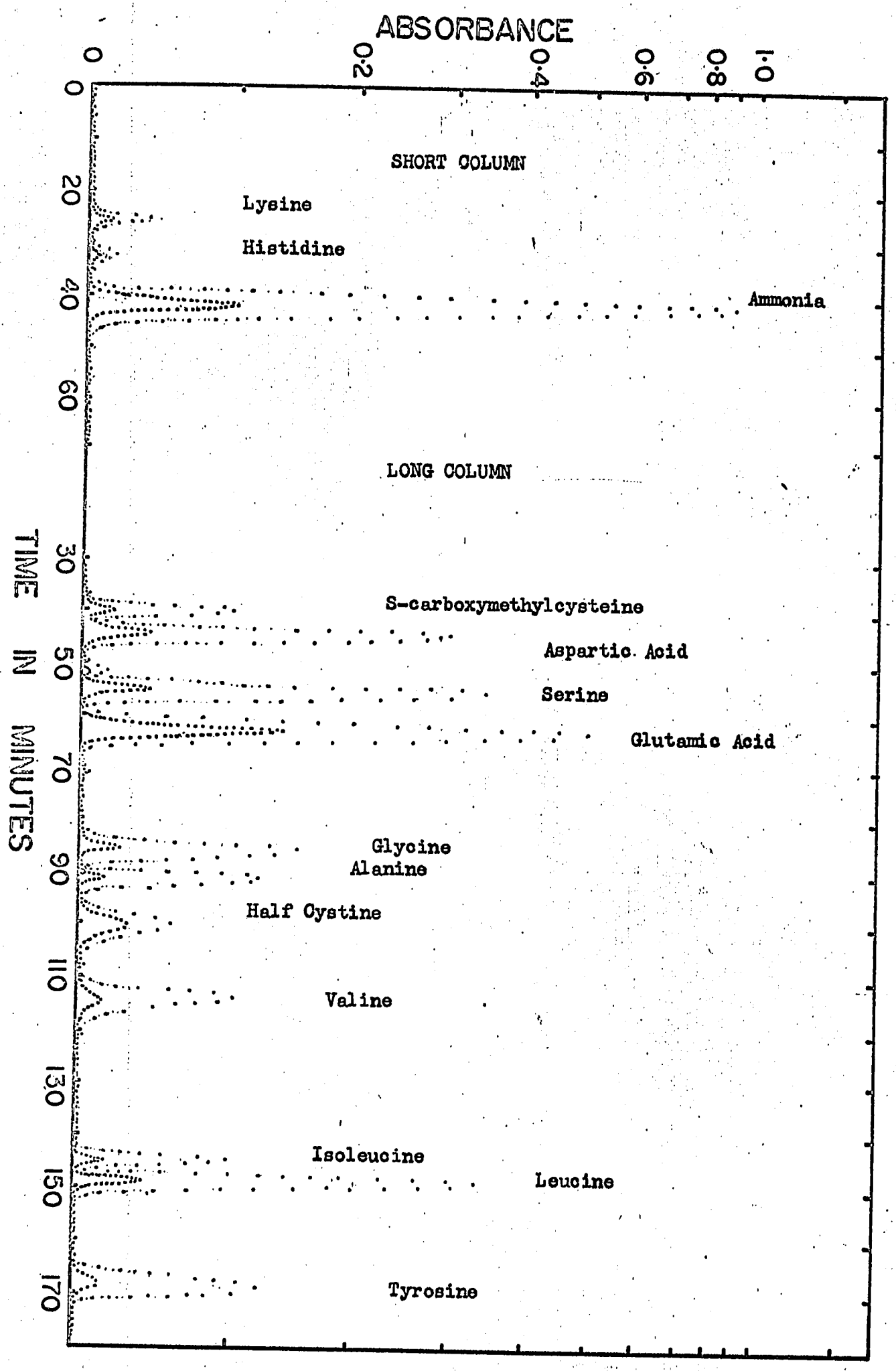

ABSORBANCE
0
0·2
0·4
0·6
0·8
1·0
TIME IN MINUTES
0
20
40
60
30
50
70
90
110
130
150
170
SHORT COLUMN
Lysine
Histidine
Ammonia
LONG COLUMN
S-carboxymethylcysteine
Aspartic Acid
Serine
Glutamic Acid
Glycine
Alanine
Half Cystine
Valine
Isoleucine
Leucine
Tyrosine

Figure 54

THE CHROMATOGRAM OF THE HYDROLYSATE OF FRACTION II OBTAINED FROM BECKMAN MODEL 120B AMINO ACID ANALYSER

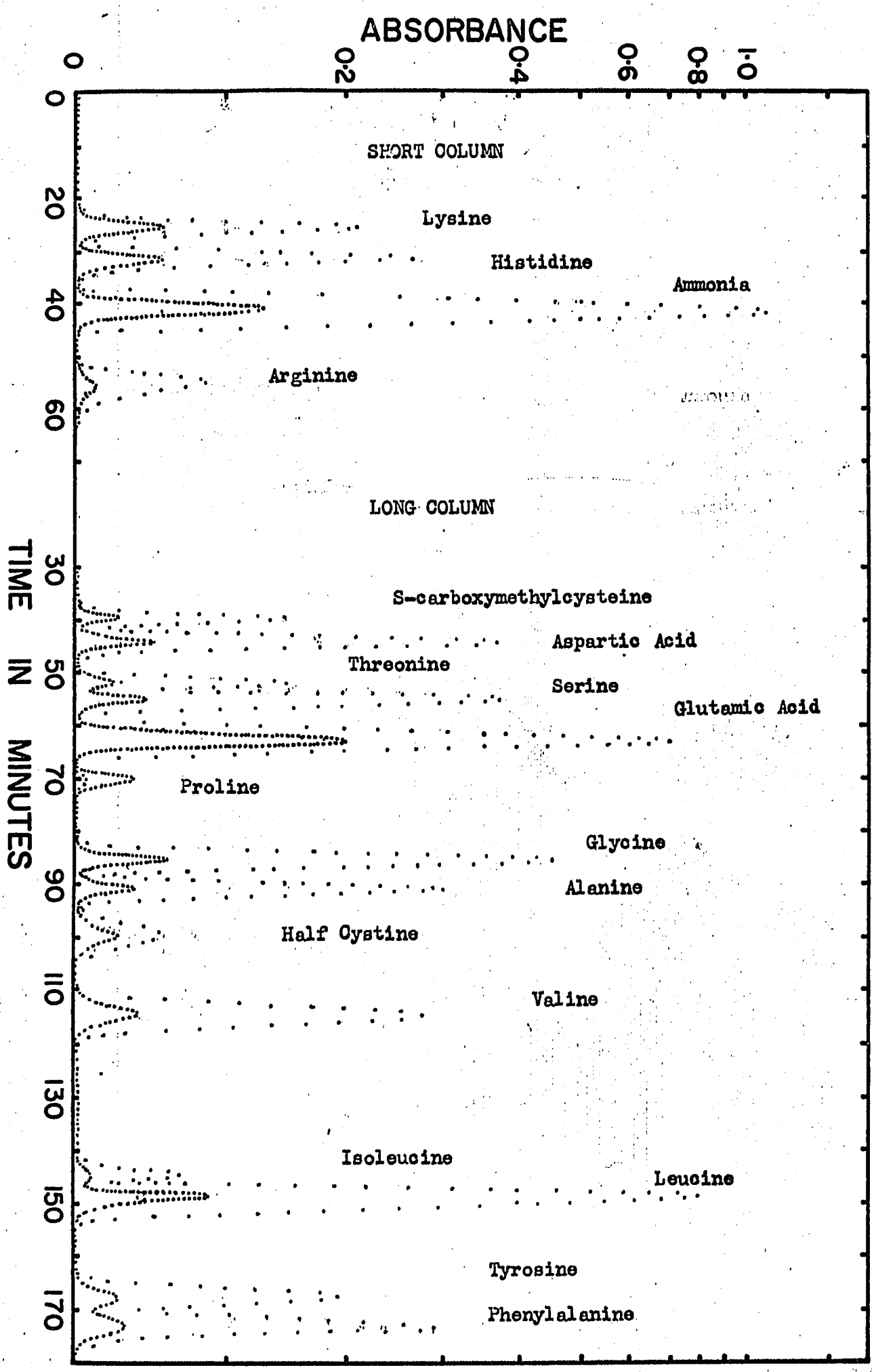

ABSORBANCE
0
0·2
0·4
0·6
0·8
1·0
TIME IN MINUTES
0
20
40
60
30
50
70
90
110
130
150
170
SHORT COLUMN
Lysine
Histidine
Ammonia
Arginine
LONG COLUMN
S-carboxymethylcysteine
Aspartic Acid
Threonine
Serine
Glutamic Acid
Proline
Glycine
Alanine
Half Cystine
Valine
Isoleucine
Leucine
Tyrosine
Phenylalanine

Figure 55

THE CHROMATOGRAM OF THE HYDROLYSATE OF FRACTION III OBTAINED FROM BECKMAN MODEL 120B AMINO ACID ANALYSER

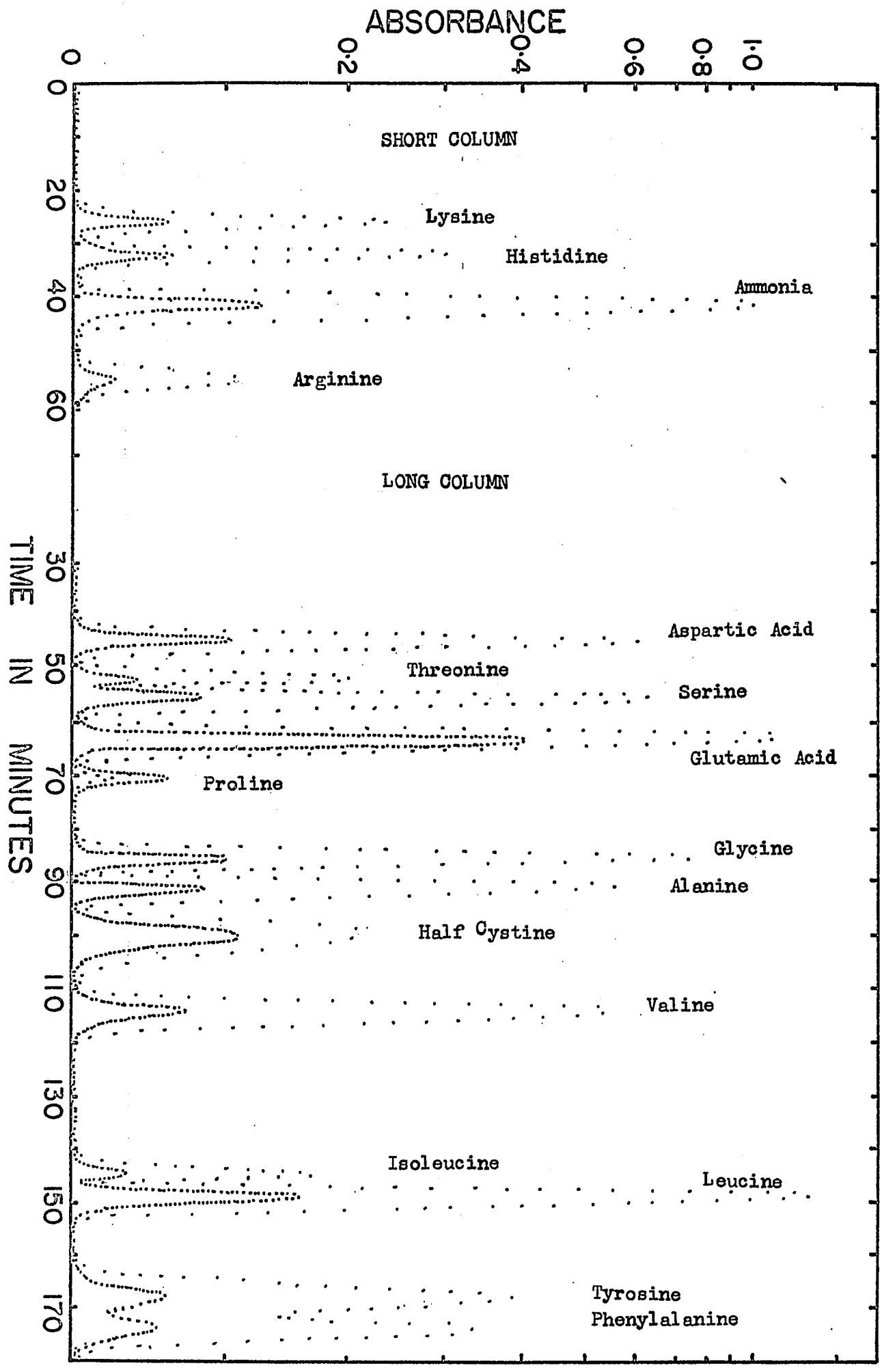

ABSORBANCE
0
0.2
0.4
0.6
0.8
1.0
TIME IN MINUTES
0
20
40
60
30
50
70
90
110
130
150
170
SHORT COLUMN
Lysine
Histidine
Ammonia
Arginine
LONG COLUMN
Aspartic Acid
Threonine
Serine
Glutamic Acid
Proline
Glycine
Alanine
Half Cystine
Valine
Isoleucine
Leucine
Tyrosine
Phenylalanine

Table XIV

AMINO ACID COMPOSITION OF FRACTION I

(The data presented below are the average values obtained in two analyses)

Amino Acid	Alkylated A chain expected	Fraction I observed
S-Carboxymethylcysteine	4	-*
Lysine	0	0.1
Histidine	0	-
Arginine	0	-
Aspartic acid	2	1.8
Threonine	0	-
Serine	2	1.9
Glutamic acid	4	4.0
Proline	0	-
Glycine	1	1.2
Alanine	1	1.0
Half-cystine	0	2.0
Valine	2	1.5
Methionine	0	-
Isoleucine	1	0.6
Leucine	2	2.1
Tyrosine	2	1.5
Phenylalanine	0	-

*The relative molar concentration of S-carboxymethylcysteine was not evaluated as the standard for this amino acid was not available at the time of analysis.

the three disulphide bonds was most likely achieved at a higher cathode voltage.

All the B chains produced by reduction were expected to be eluted in fraction II. From the determination of the relative molar concentrations of the amino acid residues present in this fraction (table XV), it is seen that there is a discrepancy. The presence of a small amount of isoleucine and half-cystine residues, which are not the constituents of B chain, suggests that this fraction was contaminated with either A chain or insulin. Nevertheless, amino acid analysis alone could not establish the type of contamination. However, as revealed by paper electrophoresis (figure 52), there was some tailing behind the band of the B chain, which is interpreted to indicate that some of the A chain was eluted together with B chain.

Figure 55 shows the chromatogram of the amino acid analysis of fraction III. It is noted that the S-carboxymethylcysteine was absent from this fraction, and the relative molar concentration of each residue agrees well with that of the insulin molecule (table XVI). Hence this fraction contained pure insulin molecules. This result suggests that the two interchain disulphide bonds behaved indistinguishably at the applied potential. Furthermore, as observed from the electrophoretic pattern of fraction III (figure 52), this fraction, which contained pure and zinc-free insulin, had a mobility slightly faster than that of the crystalline zinc insulin. This finding supports the earlier conclusion drawn

Table XV

AMINO ACID COMPOSITION OF FRACTION II

(The data presented below are the average values obtained in two analyses)

Amino Acid	Alkylated B chain expected	Fraction II observed
S-Carboxymethylcysteine	2	-*
Lysine	1	1.1
Histidine	2	1.8
Arginine	1	1.0
Aspartic acid	1	1.1
Threonine	1	0.7
Serine	1	1.1
Glutamic acid	3	3.8
Proline	1	1.0
Glycine	3	2.5
Alanine	2	1.9
Half-cystine	0	0.8
Valine	3	2.6
Methionine	0	-
Isoleucine	0	0.3
Leucine	4	3.7
Tyrosine	2	1.6
Phenylalanine	3	2.8

*The relative molar concentration of S-carboxymethylcysteine was not evaluated as the standard for this amino acid was not available at the time of analysis.

Table XVI

AMINO ACID COMPOSITION OF FRACTION III

(The data presented below are the average values obtained in two analyses)

Amino acid	Insulin expected	Fraction III observed
Lysine	1	0.7
Histidine	2	1.8
Arginine	1	1.0
Aspartic acid	3	3.2
Threonine	1	1.0
Serine	3	2.8
Glutamic acid	7	7.6
Proline	1	1.3
Glycine	4	4.3
Alanine	3	3.3
Half-cystine	6	5.8
Valine	5	4.8
Methionine	0	0
Isoleucine	1	0.8
Leucine	6	6.1
Tyrosine	4	3.5
Phenylalanine	3	2.9

from the analysis of paper electrophoresis (section A, this chapter) that the observed difference in mobility is attributed to changes associated with the dissociation of insulin from a hexamer to a dimer.

(D) Molecular sieve chromatography

The A(S-S) chain and B chain, after passage through a column of Dowex 50-X2, had a much lower solubility (< 0.1 mg/ml in pH 6.0 - 12.0) than those before the chromatography. This change in solubility may be attributed to some structural change in the free A and B chains with respect to their structure in the intact insulin molecule or to some possible denaturation caused during ion-exchange chromatography at low pH and in the presence of a high concentration of the denaturing agent used. Because of possible denaturation and low solubility, the separated fragments were not considered to be suitable materials for testing their antigenicity. Hence, an attempt was made to separate the products of the reduction under milder conditions provided by molecular sieve chromatography at slightly alkaline pH. For this purpose, a sufficient amount of Sephadex G-75 of medium size was equilibrated with borate buffer, pH 8.5, ionic strength 0.1, overnight in a cold room. After the fines had been removed by free sedimentation, a column (2 cm x 40 cm) was packed in several sections. The buffer was passed through at a flow rate of 5 ml/h with a constant pressure head of 1.5 psi until

there was no further drop in the height of the gel in the column. As the particles of Sephadex G-75 were quite light, care was taken not to disturb the surface during the introduction of the sample. In general, a sample of 20 mg in two ml of the same buffer gave a good separation in this column. For a good separation it was important to pass the buffer through the column at a relatively slow flow rate, since fast flow rates led to poor separation of the insulin and insulin chains. The fractions obtained were dialysed exhaustively against distilled water* and then lyophilized.

Each fraction obtained was further studied by analytical ultracentrifugation and paper electrophoresis. In addition, to determine the distribution of both the A and B chains in the second fraction, the eluates of the corresponding tubes in ten separate experiments were pooled, dialysed exhaustively against distilled water and freeze-dried. The cystine residues per mole of A chain (M.W. 2,500) were then determined amperometrically in duplicate.

Results and Discussion

After passing through the column of Sephadex G-75, the products of the reduction (with an average of 1.0 S-S bond reduced) were separated into two fractions: SI and SII (fig.56).

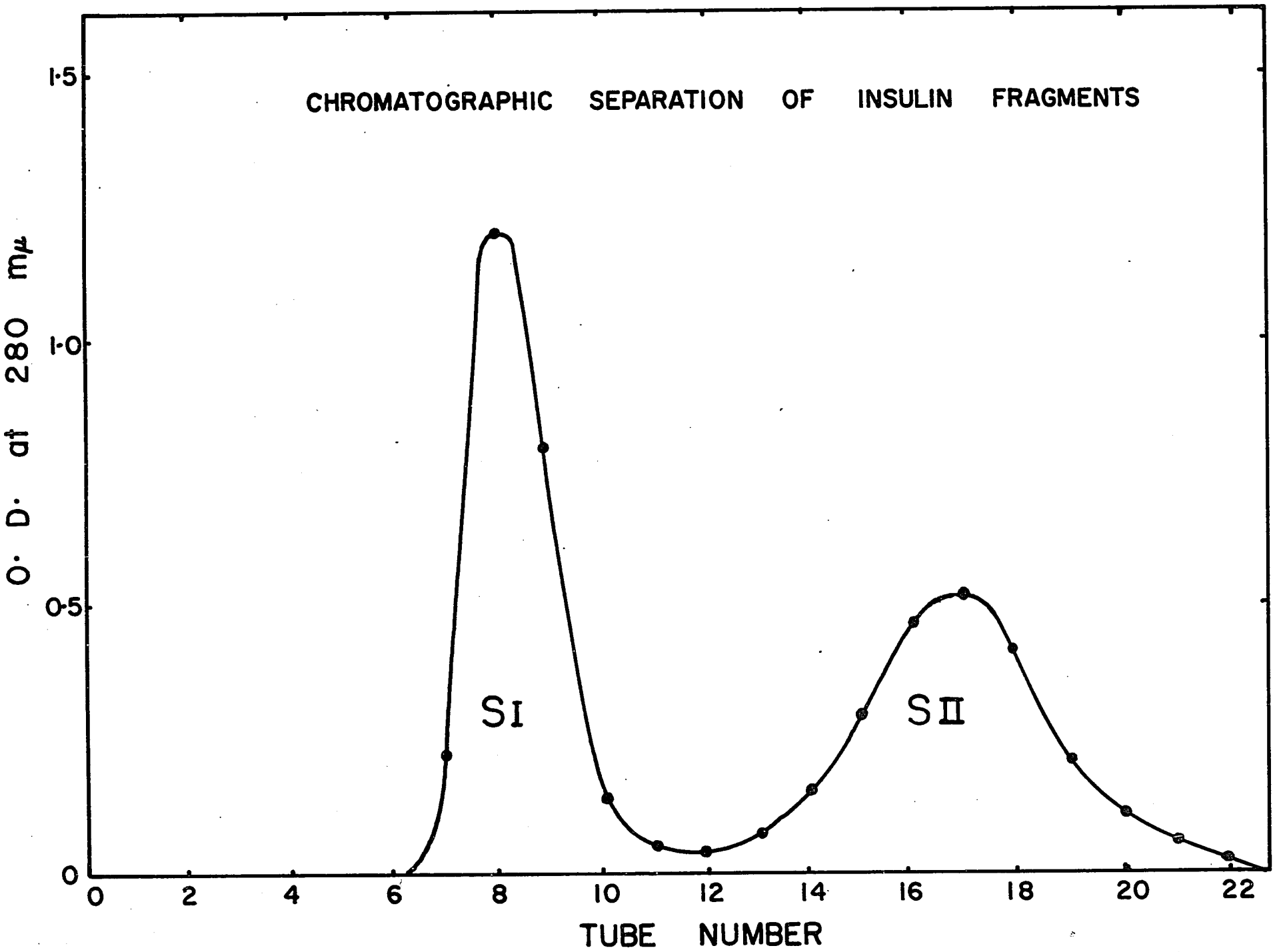
---

*For dialysüs, the 18/32 Visking tubing was used. If any losses occurred during dialyses, they were negligible.

Figure 56

SEPARATION OF INSULIN FRAGMENTS IN

A COLUMN OF SEPHADEX G-75


CHROMATOGRAPHIC SEPARATION OF INSULIN FRAGMENTS
O. D. at 280 mμ
1·5
1·0
0·5
0
0
2
4
6
8
10
12
14
16
18
20
22
TUBE NUMBER
SI
SII

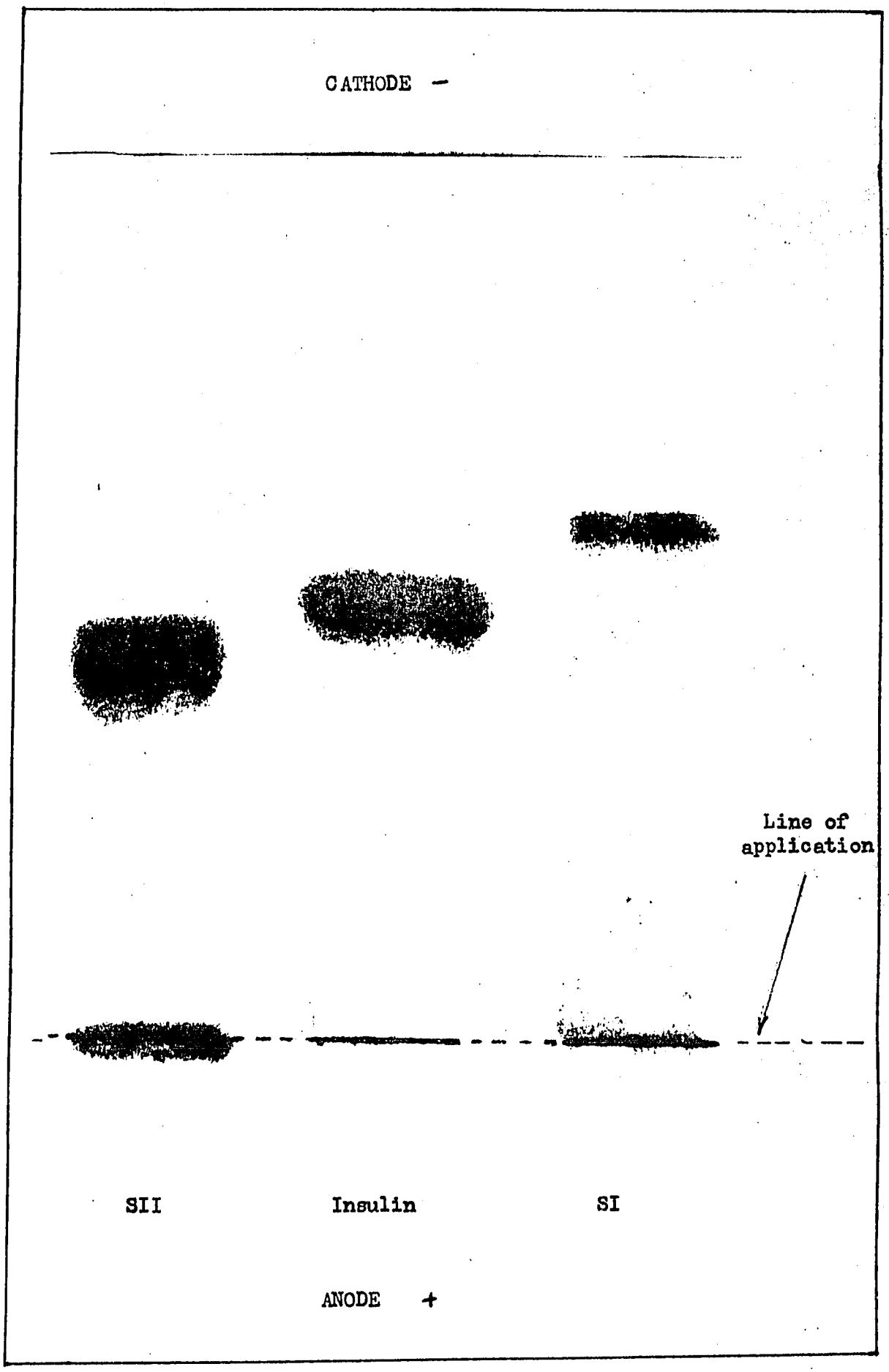
As already discussed in the section dealing with the analysis of ion-exchange chromatography and amino acid analysis, the products of the partial reduction consisted of zinc-free insulin, B chain and A(S-S) chain. Therefore, on a molecular weight basis, it was anticipated that the zinc-free insulin would be eluted in fraction SI immediately after the elution volume of the column. Since the molecular weights of A and B chains are approximately 2,500 and 3,000 respectively, these two peptides were eluted together in fraction SII. This was confirmed by paper electrophoresis at 20% formic acid (pH = 1.2). As illustrated in figure 57, fraction SI was composed of protein, having a cationic mobility higher than that of the crystalline zinc insulin and corresponding to that of zinc-free insulin. In addition, it was observed that a small amount of the peptide chains was also eluted in this fraction. Fraction SII, on electrophoresis, could be separated into two distinct bands, with mobilities corresponding to those of the B and A chains; this fraction was free of insulin.

As already noted, for partial reduction of the insulin sample, the cathode voltage was kept at -1.6 V vs S.C.E., which was higher than the decomposition voltage of Zn^{++} ion. Therefore, the Zn^{++} ion was electro-separated and amalgamated with Hg, and the insulin remaining at the end of this reduction was free of zinc. This conclusion was supported also by ultracentrifugal study of fraction SI. The molecular

Figure 57

ELECTROPHORETOGRAM OF FRACTION SII, FRACTION SI AND INSULIN AT 20% FORMIC ACID

Voltage	-	110 V
Current	-	10 mA
Time	-	24 hours
Temperature	-	$4^{o}C$


CATHODE −
Line of application
SII
Insulin
SI
ANODE +

weight of cystalline zinc insulin is constant within mild pH range (∿pH 4 - 10) while that of the zinc-free insulin depends on the protein concentration, pH and ionic strength of the buffer used. Previously, Marcker (114) reported that at a concentration up to 0.2% and a concentration up to 0.5%, the molecular weights of zinc-free insulin measured by osmometry at pH 9.10 were 12,000 and 24,000, respectively. On the other hand, by sedimentation experiments at pH 9 and 10, Fredericq (109) observed the sedimentation coefficient of 0.25% zinc-free insulin in potassium chloride-glycine solution (ionic strength 0.1) to be approximately 1.3. In addition, he demonstrated the presence of the monomer with molecular weight of 6,000 in dilute aqueous solution. In the present study, from the results recorded in table XVII, it is clearly seen that fraction SI had a sedimentation coefficient of 1.41S, which was much smaller than that of the crystalline zinc insulin (3.3S) under the same experimental conditions. Thus, these results indicate that insulin in fraction SI was free of zinc, and that consequently the polymeric insulin dissociated in slight alkaline pH. On the other hand, fraction SII consisted purely of A and B chains. The sedimentation coefficient observed for this fraction was 0.74, which was more or less the same as that obtained for the reduction product when all disulphide bonds of the insulin molecule were ruptured (0.73S). Therefore, the present findings suggest that fraction SII was composed of two chains. In addition,

Table XVII

Sedimentation coefficients of the reduced insulin and insulin fragments in borate buffer pH = 9.5, ionic strength 0.1

Item	S*
Crystalline zinc insulin	3.30
Fraction SI	1.41
Fraction SII	0.74
Product with 2.9 S-S bond reduced	0.73
Product with 2.1 S-S bond reduced	0.72

*S in Svedberg unit = 10^{-13} sec^{-1}

these chains were separated from each other in solution, i.e. did not aggregate to form an insulin molecule. Had the chains polymerized in solution, a higher sedimentation coefficient should have been observed for this fraction. A similar sedimentation coefficient was calculated for the reduction product when an average of 2.1 S-S bond was reduced. This observation implies that the reduction product contained mostly two chains of insulin, hence confirming the earlier conclusion reached from the results of paper electrophoresis that reduction of the disulphide bonds was not at random, but that the two interchain disulphide bonds in the insulin molecule were reduced preferentially, i.e. prior to the intrachain S-S bond, at a cathode voltage of -1.6 V vs S.C.E.

The homogeneous distribution of the two chains in fraction SII was investigated. As seen from table XVIII, the cystine residue, i.e. A(S-S) chain, was concentrated in the rear portion of fraction SII. Thus, the relatively pure A(S-S) chain could be obtained.

Antigenic activities of the A and B chains of insulin

Materials and Method

Diluent - The veronal buffered saline* used in these experiments had a pH 7.4, ionic strength 0.15, and

*This buffer consisted of 85 g of NaCl, 5.75 g of 5,5-diethyl barbituric acid and 3.75 g of sodium 5,5-diethyl barbiturate dissolved in 10 liters to give pH 7.3 - 7.4.

Table XVIII

DETERMINATION OF CYSTINE RESIDUES IN FRACTION SII

Tube No. in fraction SII	S-S residue per chain
14	0.10
15	0.25
16	0.47
17	0.70
18	0.88
19	0.92
20	0.95

contained 1% normal rabbit serum.

The B chain and the totally reduced A chain were purified by gel filtration procedure described in section D of this chapter. For this purpose, a sample of the reduced insulin with all three disulphide bonds ruptured was passed through a column (2 cm x 40 cm) of Sephadex G-75 in borate buffer (pH 8.5, ionic strength 0.1). The B chain was recovered from the eluate, collected from tubes 12 to 15, while the totally reduced A chain was recovered from the eluate obtained from tubes 19 to 21.

For the purification of the A chain with the intrachain disulphide bond intact, a sample of reduced insulin with an average of 1.0 S-S bond ruptured was passed through a column (2 cm x 40 cm) of Sephadex G-75 in borate buffer (pH 8.5, ionic strength 0.1). The A(S-S) chain was recovered from the rear portion of the second fraction (from tube 19 onwards). The partially reduced A chain purified by this procedure contained 0.90 - 0.95 S-S bond per mole of A chain (M.W. 2,500).

The rabbit antiserum to insulin was obtained by the immunization of rabbits with IN-TDIC-BSA conjugates, as described in Chapter IV.

The precipitin reaction in agar-gel and the inhibition of the precipitin reaction in agar-gel, as well as the inhibition in hemagglutination, were carried out as described in Chapter IV.

To determine the inhibitory activity of the A(S-S)

chain relative to that of the original insulin, the hemagglutination-inhibition test was first calibrated. For this purpose, various amounts of insulin (ranging from 10 μg to 0.50 μg) were added as inhibitor to the hemagglutination reaction. The hemagglutination titers in the presence of various amounts of insulin were diminished to different extents, decreasing with the increase of insulin added. In the test, 10 μg of A(S-S) chain were added as an inhibitor instead; the hemagglutination titer was observed and the percentage inhibitory activity of the A(S-S) chain, relative to that of insulin, can be evaluated by comparing 10 μg of A(S-S) chain to the amount of insulin, which inhibited the hemagglutination reaction to the same extent.

To study the effect of B chain on the inhibitory activity of the A(S-S) chain, 0.05 ml of diluent containing various amounts of B chain and a constant amount of A(S-S) chain were added to the hemagglutination reaction as inhibitors. The corresponding titers obtained were recorded.

Results and Discussion

As can be seen from table XIX, both the B chain and the totally reduced A chain were immunologically inactive, as demonstrated by the direct agar-gel diffusion and by the inhibition tests in agar-gel diffusion and hemagglutination reaction. As the insulin molecule itself is antigenic, the

Table XIX

ANTIGENIC ACTIVITIES OF THE A AND B CHAINS OF INSULIN

Antiserum: rabbit antiserum to insulin

	Direct agar-gel diffusion	Inhibition	
		agar-gel	hemagg.
A chain with intrachain disulphide bond intact	-	+	+
A chain (totally reduced)	-	-	-
B chain	-	-	-
A chain + B chain (both totally reduced)	-	-	-

non-reactivity of the totally reduced A chain or of the B chain might be attributed to the conformational changes resulting from the rupture of the disulphide bond. Fission of the disulphide bridges of the insulin molecule might alter the original secondary and tertiary structures of the peptide chain, which might lead to the masking of the original determinant groups present on the surface of the molecule, at the same time exposing new antigenic groups on the surface of the molecule. In fact, rotatory dispersion measurements on the separated, reduced and alkylated A and B chains indicated that both chains were present in solution as random coils (154). This finding is in agreement with the observation of Berson and Yalow (152), who reported that neither A nor B chain alone would inhibit insulin binding.

As expected, the A(S-S) chain, i.e. A chain with intrachain disulphide bond intact, did not give a direct agar-gel reaction. It, however, inhibited both the agar-gel diffusion and hemagglutination reaction between insulin and its homologous rabbit antibodies. The A(S-S) chain used for the inhibition study contained 0.90 - 0.95 S-S bond per mole of A chain. This might be interpreted as indicating that the A(S-S) sample might have been contaminated with a small amount of B chain and/or a small amount of the totally reduced A chain. It is conceivable that the intrachain disulphide bond of insulin, which was not reduced until a higher cathode voltage had been reached, might undergo interchange reaction

with the interchain SH groups formed during the electrolytic reduction. Previously, Wardlaw and Dixon (228) demonstrated that 0.7 - 1.5% of A and B chains present together in solution may recombine to form insulin. The possibility that the observed inhibitory activity might be attributed to the small amount of the recombined insulin may be refuted from the result that the mixture containing an equal amount of A and B chains did not have any inhibitory activity. Further support for this conclusion comes from the finding that the inhibitory activity of the A(S-S) chain was independent of the presence of B chain (table XX). In addition, a decrease in the amount of A(S-S) added as inhibitor resulted in an increase in the hemagglutination titer. This observation indicates that the antigenic activity of the A(S-S) chain is entirely due to the A chain with the intrachain disulphide bond intact.

The inhibitory capacity of the A(S-S) chain was compared with that of the insulin for four separate antisera. As can be seen from table XXI, in all antisera tested, both insulin and the A(S-S) chain invariably inhibited the hemagglutination reaction between insulin and its homologous rabbit antibodies, but to a different degree. There is no correlation between the titers obtained in the presence of insulin and those obtained in the presence of the same amount of A(S-S) chain. These results would suggest that antibodies elicited in different rabbits possess different affinities towards the A(S-S) chain, or it might indicate that the

Table XX

EFFECT OF B CHAIN ON THE INHIBITORY ACTIVITY OF THE A(S-S) CHAIN

Antigen: insulin
Antiserum: rabbit antiserum to insulin

No.	µg of B chain added as inhibitor	µg of A(S-S) chain added as inhibitor	Hemagg. titer
1	0	0	128
2	0	10.0	8
3	2.5	10.0	8
4	5.0	10.0	8
5	7.5	10.0	8
6	10.0	10.0	8
7	5.0	5.0	32

Table XXI

Comparison of the inhibitory activity of insulin with that of the A(S-S) chain for four rabbit antisera to insulin

(All experiments were set up in triplicate)

Antigen: insulin
Antiserum: rabbit antiserum to insulin

No.	Original titer	Titer after inhibition with	
		10 μg of insulin	10 μg with A(S-S) chain
1	128	complete	4
		"	8
		"	8
2	526	complete	64
		"	64
		"	64
3	1024	2	32
		2	32
		4	32
4	2048	4	128
		4	128
		4	64

relative amount of antibodies directed against the determinant in A(S-S) chain varies from rabbit to rabbit.

Table XXII shows the extent of inhibition in hemagglutination for antiserum No. 1 when different amounts of insulin were added as inhibitor. The hemagglutination titer of the antiserum No. 1 was 128. In the presence of 10 μg of the A(S-S) chain, the titer decreases to 8 whereas only 0.75 - 1.0 μg of insulin was required to inhibit the hemagglutination reaction to the same extent. Thus, the inhibitory capacity of the A(S-S) chain was 7.5 - 10% of the original insulin. However, it must be stressed that this inhibitory capacity of A(S-S) varied from serum to serum.

The A(S-S) chain itself did not give direct precipitin reaction. However, it did inhibit the reaction between insulin and its homologous rabbit antibodies. These results suggest that the A(S-S) chain is univalent. Further reduction of the intrachain disulphide bond resulted in complete loss of its inhibitory activity. Thus, it would appear that the intrachain disulphide bond retained the complementary configuration within the loop A6 - A11, which enables its reaction with the corresponding antibody combining site. Implicit in this interpretation is that one of the antigenic determinants of insulin is located in A6 - A11, including the intrachain disulphide bond. As already discussed in Chapter IV, beef insulin differs from rabbit insulin only in the C-terminal amino acid (B30) of B chain and the amino

Table XXII

CALIBRATION FOR THE HEMAGGLUTINATION-INHIBITION TECHNIQUE

Antigen: insulin
Antiserum: rabbit antiserum to insulin

No.	μg of insulin added as inhibitor	Hemagglutination titer
1	-	128
2	10.0	complete
3	7.5	2
4	5.0	2 - 4
5	2.5	4
6	1.0	8
7	0.75	8 - 16
8	0.50	16

acids in positions 8 and 10 of A chain (table II). If the antigenic properties of the protein molecule are determined by the primary structure, it is anticipated that only these three amino-acid acids should represent the site of antigenicity. The results of the present investigation indicate that amino acids A8 and A10, situated within the loop of the intrachain disulphide bond, indeed constitute a site of antigenicity.

More recently, it was demonstrated by Yagi et al. (229) that the S-sulphonated B chain could be degraded by different enzymes into smaller peptides, which were then purified by gel filtration and high voltage electrophoresis. The chemically characterized peptides were then labelled with I^{125}. The antigenic activity of each peptide with respect to the guinea pig anti-B chain antiserum was determined by equilibrium dialysis and co-precipitation. These authors concluded that the B_{1-9} peptide was active with respect to all antisera, whereas the B_{23-30} peptide was capable of interacting with a few antisera only. The association constants measured for interaction of these two peptides with the anti-B antisera were in the order of 10^7 - $10^6 M^{-1}$. Removal of the phenylalanine resulted in serious loss of combining capacity; similarly, regions in the middle portions of the B chain, such as B_{11-16}, B_{17-25}, and B_{2-10}, were active. Thus, these results indicated that there were two independent antigenic regions residing in the B chain. As already noted, bovine B

chain differs from guinea pig B chain in 10 of the 30 amino acids (B3, B4, B10, B14, B17, B20, B21, B22, B27, and B30). To this marked difference in composition of the two B chains, one may attribute the immunogenicity of the beef B chain to the recognition of the antigenic determinants of beef B chain by the antibody-forming cells of the guinea pig. On the other hand, bovine B chain differs from rabbit B chain in only one amino acid (B30). In the present study, it was demonstrated that bovine B chain did not interact with rabbit antibodies specific to insulin. If the antigenic determinant is controlled by the primary structure of the protein molecule, the only region that could have been the antigenic site is the B30. However, in view of the fact that A chain with the intrachain disulphide bond intact was active, whereas the totally reduced A chain was not active, the non-reactivity of the B chain might be attributed also to the possible loss of its rigid three-dimensional configuration associated with the original B chain in the insulin molecule. Thus, the present result did not eliminate the possibility that the B chain in the intact bovine insulin might be also antigenic.

In contrast, a striking observation was reported by Wilson and Aprile (230); they showed that sera from human diabetics resistant to bovine insulin provoked passive cutaneous anaphylaxis (PCA) in the guinea pig challenged with synthetic B chain double peptide $(B_{1-8})_2$. This result was unexpected as the only difference between the amino acid

sequences of human and bovine insulins is at position 8 - 10 of the A chain and position 30 of the B chain. To account for this immunological manifestation, a relatively rigid molecular model for insulin was proposed in which B_{1-6} region was considered to be situated closely to the main antigenic locus from A_{8-14}. Furthermore, the B_{9-18} and B_{20-30} regions form a twin alpha-helix, so that the two ends of the B chain lie on either side of the N-terminal glycyl residue of the A chain. Similarly, the guinea pig antiserum to ox insulin provoked PCA reaction when challenged with the synthetic peptide A_{10-21}. This PCA reaction was inhibited when the guinea pig antiserum had been pre-incubated with 0.5 μg of A_{10-21}, or 50 μg of $(B_{1-8})_2$ or 50 μg of $(B_{17-30})_2$. These results would indicate that the main antigenic determinant region is located in A_{10-21}, including a small portion of B_{1-8} and B_{17-30}. Implicit in this interpretation is that B_{1-8} and B_{17-30} are located closely to A_{10-21}. In the study of Wilson et al. (230) the beef A chain differs from guinea pig A chain in 8 amino acids (A4, A8, A9, A10, A12, A13, A14, and A18). The peptide A_{10-21} contains most of the amino acids that are different in these two types of A chains. Thus, it is anticipated that A_{10-21} is the locus of the antigenic determinant. On the other hand, results of the present study clearly indicated that an antigenic determinant of A chain with respect to its homologous rabbit antibodies specific to beef insulin is located in the A6 - A11 region, including the intrachain disulphide bond. This

portion of the beef A chain contained the two amino acids (A8 and A10) that are different from those of rabbit A chain. Thus, it appears that the antigenic region of the A chain varies, depending on the recipient, and that results obtained with sera of one species cannot be unreservedly considered to hold for another species.

GENERAL DISCUSSION

Ever since the classical studies of Landsteiner, the studies of the production of antibodies to low molecular weight and well-defined compounds and of their interactions with these haptens have provided the basis for the understanding of the specificity of the antigen-antibody reaction at the molecular level. From such studies it was deduced that the antibody combining site was configurationally complementary with respect to small chemical groups of the antigen molecule, and not to the antigen molecule as a whole, and that this site represented only a small portion of the antibody molecule. However, the nature of the determinant groups on the surface of a protein molecule is in general not fully understood. This is partly due to the limited knowledge of the primary, secondary and tertiary structures of the protein molecules and partly due to the dependence of the antigen-antibody reaction on the three-dimensional configuration of the protein molecule.

In the present study, insulin has been chosen as an antigen for the reason that it is a relatively small protein molecule (M.W. 6,000) consisting of two polypeptide chains (A chain of 21 amino acids and B chain of 30 amino acids), the primary structure of which is known for insulins from a variety of animals (84-98). Thus, it was considered to provide a good model for the study of the nature of the antigenic determinant groups occurring on the surface of the protein molecule.

As already noted, insulin itself is a weak antigen, the induction of antibodies against it requiring large amounts of antigen, in the absence of adjuvants, which must be administered over a long period of time (129). Immunization of laboratory animals with insulin in the presence of adjuvants has evoked the formation of precipitating antibodies in some instances. However, success in immunization was mainly confined to guinea pigs receiving doses of beef insulin in the presence of adjuvants (140-144). Among the factors responsible for the weak antigenicity of insulin one may cite (i) its low molecular weight, (ii) the injection of large doses of insulin causes often the death of the animals due to the hypoglycemic effect of insulin, and (iii) structural similarity to autologous insulin. To overcome these limitations, in the present study insulin was coupled to a carrier protein, BSA. It was anticipated that the insulin-protein conjugates would elicit in rabbits the formation of antibodies specific to insulin and that these specific antibodies could be used for the study of the antigenicity of the insulin molecule.

It has been shown in this investigation that insulin could be coupled to bovine serum albumin through the formation of ureido linkage (R-NH-CO-R'). To this end, the tolylene-2, 4-diisocyanate (TDIC) was used as a coupling agent because the isocyanate group in position 4 is more reactive than the one in position 2. Hence, stepwise reaction with each of the two proteins to be coupled could be controlled, i.e. it was possible

to conjugate the diisocyanate first with insulin in the first stage to form IN-TDIC adduct, which was subsequently reacted to BSA in the second stage to form IN-TDIC-BSA conjugates. Moreover, it has been demonstrated that, on the average, two IN-TDIC were coupled per BSA molecule and a higher molecular weight compound $(IN\text{-}TDIC)_3$-BSA was also formed, but to a lesser extent.

It is obvious from this outline that, in order to minimize destruction or blocking of the determinant groups of the protein molecule responsible for its antigenicity, it is advantageous to react the antigen molecule with the coupling agent in the second step. Unfortunately, attempts to produce first the BSA-TDIC adduct and subsequently to react it with insulin in the second stage were not successful. Similarly, no coupling had occurred when bovine gamma globulin was reacted either in the first stage or in the second stage in the conjugation reaction with insulin.

It has been shown in the present work that, during the conjugation reaction, all three amino groups of insulin were blocked by TDIC molecules. Previously, Halikis and Arquilla (231) coupled the amino groups of insulin with fluorescein isothiocyanate. It was demonstrated by these authors that fluorescein insulin-sensitized sheep erythrocytes were agglutinated at lower dilutions of antiserum than sheep erythrocytes sensitized with crystalline insulin, when tested with rabbit antibodies against crystalline insulin in the

hemagglutination reaction. However, no immunological difference was observed between the fluorescein-insulin conjugate and crystalline insulin in cross-inhibition experiment. It appears, therefore, that substitution of the amino groups with large haptenic groups decreased the immunological reaction presumably through steric hindrance. Thus, in the present study, blocking the amino groups of insulin with TDIC probably did not impair the immunogenic property of the molecule. On the other hand, coupling the amino groups of insulin is not without advantage, as it was demonstrated in this work that the hormonal activity of insulin was dramatically reduced. Therefore, the loss of biological activity of insulin in a way simplified the immunization process of rabbits, i.e. injection with large doses of insulin-protein conjugates did not cause death to the immunized animals.

The immunogenicity of insulin was greatly enhanced by its being covalently coupled to a large carrier protein - BSA. This was demonstrated by the ease and readiness in stimulating the formation of antibodies specific to insulin in rabbits. As already mentioned, low molecular weight metabolites, such as estrone (201), 5-OH indole acetic acid (202) and serotonin (203), which are normal constituents of the animal body, do not induce by themselves the formation of specific antibodies in the host; however, upon coupling with appropriate carrier protein, they became immunogenic. Thus, in the present study, aside from

the increase in molecular weight, the enhanced immunogenic activity of insulin might be attributed to the fact that, under these circumstances, it acted as if it were a hapten.

A highly solubilizing molecule, sodium 4-fluoro-3-nitrobenzene sulphonate, was coupled to all free amino groups of the insulin molecule. The antigenic activity in vitro of the resulting insulin conjugate was shown to be comparable with that of the native insulin. This may be attributed to the fact that the free amino groups of insulin, i.e. the N-terminal glycyl residue of A chain, the N-terminal phenylalanyl residue of B chain and the B29 lysyl residue of the B chain, do not contribute markedly to the antigenic determinants of the insulin molecule with respect to its homologous rabbit antibodies. This result is not unexpected as the insulin used for immunization was also blocked with TDIC.

It has been demonstrated in the present study that at half-saturation with ammonium sulphate solution, at which 98% of antibody globulins and the corresponding antibody-antigen complexes are precipitated, 65 - 70% of the I^{125}-IN-NBSNa was also precipitated. Since for kinetic studies it is essential to establish unequivocally the concentrations of the free and bound form of the antigen, it was thus decided to abandon the study of kinetics of the reaction between insulin and its homologous antibodies. Previously, it was shown that poly-D, L-alanyl derivatives of a protein, produced by reaction with D,L-alanine N-carboxy anhydride, had an increased solubility

without markedly impaired enzymic or antigenic properties (232-234). Thus, in the future, the solubility of insulin in half-saturated ammonium sulphate could be increased by coupling insulin to poly-D,L-alanine of various chain lengths and the study of kinetics of the reaction between insulin and its homologous antibodies might be possible.

The role of disulphide bridges in the insulin molecule in maintaining the structure responsible for the antigenicity of insulin was investigated. For this purpose, the disulphide bonds were reduced electrolytically at a constant cathode voltage. It was shown in this work that the decomposition potential of insulin in phosphate buffer, pH 7.2, ionic strength 0.02, was -1.46 ± 0.05 V vs S.C.E. However, at this voltage the reduction was extremely slow and the cathode potential was therefore increased to -1.6 V vs S.C.E., where the two interchain disulphide bonds behaved indistinguishably, and were reduced preferentially. On the other hand, the intrachain disulphide bond was ruptured only at -2.0 V. Obviously, from these data it can be concluded that the decomposition potential of one of the two interchain disulphide bonds was -1.46 ± 0.05 V vs S.C.E., but it is not possible to state which of the two, or if both interchain disulphide bonds were reducible at this voltage. As already noted, the current-cathode potential curves (figure 46) obtained with a large mercury cathode and with a well-stirred solution, did not show the limiting current that is characteristic of the

dropping mercury electrode polarograms. Therefore, by using the present method, it was difficult to determine whether there were three distinct decomposition potentials for the free disulphide bonds present in the insulin molecule. To this end, the dropping mercury electrode polarograms should be employed.

The extent of the reduction was followed by amperometric titration with $AgNO_3$ and the SH groups formed were immediately stabilized by alkylation under nitrogen atmosphere with 20% excess iodoacetic acid. Under these experimental conditions, the only unavoidable side reaction, which may occur during the electrolytic reduction and before the alkylation, is the interchange reaction between the SH groups already formed and the unreduced disulphide bonds of the insulin molecule.

When this work was being completed, Markus (154) reported the electrolytic reduction of insulin according to the method of Dohan and Woodward (219) with slight modification. The reduction was carried out in 0.1 M bicarbonate buffer pH 8.5 (with a current of 20 mA and at a potential of 10 V), and at $0^{\circ}C$ two reduction rates were observed. In addition, from the behaviour of optical rotation as a function of the state of reduction, it was concluded by these authors that reduction of the three disulphide bonds did not occur in a random sequence, but that cleavage of one out of the three bonds was relatively slow and perhaps contingent on the

previous reduction of the other two. With this method, they were able to obtain A chains with 0.6 intrachain S-S bond intact. Results of the present work confirm their finding and, at a cathode voltage sufficient to reduce the intrachain disulphide bond, it is anticipated that the rate of reduction of the two interchain S-S bonds should proceed faster as these two S-S bonds are reduced at a less negative potential. At approximately the same time, Leach et al. (235) reported the reduction of insulin in a H-type cell at a constant cathode voltage adjusted at pH 2.9 (glycine buffer, cathode potential -1.25 V vs S.C.E.), although slow and incomplete reduction occurred at lower pH values. More extensive reduction was reported to occur at pH 9.0 (Tris buffer, cathode potential -1.2 V vs S.C.E. for 1 - 2 hours and then -2.0 V vs S.C.E.) and after 18 hours 4 thiol groups were detectable per mole of insulin. However, these authors did not analyse the products. If the 4 thiol groups had been formed as a result of the preferential reduction of the two interchain disulphide bonds, their results would have indicated that a cathode voltage more negative than -2.0 V vs S.C.E. would be required for the reduction of the intrachain disulphide. On the other hand, results of the present study indicate that at about -2.0 V vs S.C.E. the intrachain disulphide bond was reduced in phosphate buffer pH 7.2, ionic strength 0.02. As already pointed out, the decomposition potential is pH dependent even for a simple disulphide compound such as cystine (see page 180) and the

decomposition potential becomes more negative as the pH increases. In addition to pH, the decomposition potential of disulphide bond would probably be dependent on the type of buffer used. Previously, Christian et al. (236) showed that insulin exhibited a polarographic wave at -0.643 V vs S.C.E. in orthophosphate buffer pH 7.4. The potential reported by these authors was more positive than that obtained with ordinary disulphide groups. It was thus suggested that the insulin wave arised from the reduction of the intrachain disulphide bond. However, it must be stressed that this argument should be regarded as purely speculative as Leach et al. and Christian et al. did not analyse their products of reduction.

It has been demonstrated in the present study that the A and B chains could be separated from each other by molecular sieve chromatography on a Sephadex G-75 column under mild conditions. The A(S-S) chain purified by this method contained 0.90 - 0.95 S-S bond per mole of A chain, which could be interpreted as indicating that this A(S-S) chain was contaminated with a small amount of B chain and/or a small amount of totally reduced A chain. The presence of a small amount of B chain might aggregate with A(S-S) chain to reform insulin, hence vitiating the results of the test on the antigenic activity of the A(S-S) chain. Previously, it was demonstrated by Ensinck et al. (200) that insulin could be reduced into its component A and B chains after incubation of insulin with glutathione and the enzyme glutathione-insulin

transhydrogenase isolated from a complex with albumin, which could be separated from the A chain by a simple dialysis. The dissociation of the B chain from albumin was effected by mere heat denaturation or by ion-exchange chromatography on Dowex 50. It was thus suggested by these authors that the interaction between polypeptide and protein provides a means of solubilizing the B chain under physiological conditions. In the present study, it has been demonstrated that a mixture containing equal amounts of A and B chains did not have any inhibitory activity in the hemagglutination reaction. This may be attributed to the fact that the B chains have formed complexes with rabbit serum albumin, which was present in the diluent used for the hemagglutination reaction. Hence, in the present study, to eliminate complexing of A(S-S) chain with any contaminating B chain, veronal buffered saline, pH 7.4, ionic strength 0.15, containing 1% normal rabbit serum was used for all experiments in determining the antigenic activity of the A(S-S) chain. The purification by molecular sieve chromatography was rather tedious and, in retrospect, one should have used isoelectric precipitation.

Earlier, Berson and Yalow (152) showed that insulin-binding antibodies in the sera of subjects immunized with beef and pork insulins reacted with insulins of four ungulate species to a different extent, the order of reactivity being beef $\simeq$ sheep $>$ pork $>$ horse. The insulins differing in only one of the A8 - A10 residues reacted more alike than insulins

differing in two or more residues. These results suggested that the antigenic site was located within the intrachain disulphide region. Unequivocal support for the conclusion that the A chain is the principal site of the antigenicity was provided by the studies of Wilson, Dixon, and Wardlaw (153). Cod and beef insulins were split into their respective A and B chains and the corresponding hybrid insulin, i.e. cod A chain-beef B chain and beef A chain-cod B chain were synthesized. Cod A chain-beef B chain, like cod insulin reacted weakly with guinea pig antibodies to beef insulin, whereas beef A chain-cod B chain reacted as strongly as beef insulin. This result indicated that the antigenicity is controlled by the A chain. Similarly, in the present investigation, it has been shown that an antigenic site is located in A6 - A11 region, encompassing the intrachain disulphide bond.

Further reduction of the intrachain disulphide bond of the A chain resulted in complete loss of activity. As already discussed, at least one antigenic site resides in the A chain within the A6 - A11 loop; the non-reactivity of the totally reduced A chain may be attributed to the conformational change resulting from the rupture of the disulphide bond. In fact, from optical rotatory dispersion measurements on the A(S-S) chain, Markus (154) concluded that at least some features of the original conformation of the loop between amino acids 6 and 11 had been preserved by the intrachain bond and

that the totally reduced alkylated A chains are present in solution as random coils. Changes in the secondary and tertiary structures of the polypeptide chains might lead to the masking of the original determinant groups present on the surface of the molecule, at the same time exposing new antigenic groups on the surface of the molecule. Thus, in the studies by Yagi et al. (155), antibodies were produced in guinea pigs against A and B chains of bovine insulin. It was observed that antibodies against A chain reacted with A chain, but not with B chain or native insulin. Antibodies against B chain reacted strongly with both B chain and insulin, but not with A chain. Antibodies against insulin reacted strongly with insulin, weakly with B chain, and not at all with A chain. From these findings it was concluded that the anti-insulin antibodies in antisera to the B chain and to insulin differed in their specificities inasmuch as they were directed against different individual antigenic regions of the insulin molecule. Hence, the non-reactivity of the totally reduced A chain, demonstrated in the present study, appears to be attributable to the change in its three-dimensional configuration as a result of the rupture of the intrachain disulphide bond.

SUMMARY

The summary is divided into two sections.

(A) Studies on rabbit antibodies to to beef insulin

(1) Beef crystalline zinc insulin (IN) was coupled to bovine serum albumin (BSA) through the formation of ureido bonds (R-NH-CO-R') involving the free amino groups of insulin and BSA. For this purpose, the tolylene-2,4-diisocyanate (TDIC) was coupled to insulin at 0°C in borate buffer, pH 9.5, ionic strength 0.1. At this temperature, the N=C=O group in the para position of the TDIC reacted with the free amino group of insulin. The IN-TDIC conjugate was subsequently reacted with the free amino group of BSA at 37°C; the higher temperature made possible the coupling between the N=C=O group in the ortho position of the TDIC and the free amino group of BSA.

(2) Chemical analyses (ninhydrin procedure and phenol reagent method) on the insulin-TDIC conjugate indicated that the three amino groups of insulin had reacted with TDIC and that the phenolic groups of insulin were not reacted. Furthermore, the hormonal activity of the IN-TDIC conjugate was greatly diminished.

(3) Evidence for the formation of insulin-BSA conjugate was obtained by paper electrophoresis and ultracentrifugal analyses. The number of insulin molecules coupled per molecule of BSA were determined semi-quantitatively by Tiselius electrophoresis analysis. It was deduced that, on the average, two molecules of insulin were coupled per molecule of BSA; a higher molecular weight compound, i.e. $(IN\text{-}TDIC)_3$-BSA, was also formed to a lesser extent.

(4) A highly soluble insulin derivative was prepared by coupling sodium 4-fluoro-3-nitrobenzene sulphonate (FNGSNa) to insulin in 4% Na_2CO_3 at $0^{\circ}C$. Chemical analyses (ninhydrin procedure and phenol reagent method) revealed that three NBSNa residues were coupled per insulin molecule through the reaction between FNBSNa and the free amino groups of insulin.

(5) The IN-TDIC-BSA conjugate was used for the immunization of rabbits and was shown to elicit the formation of antibodies specific to insulin. The specificity of these antibodies was demonstrated by the inhibition of this reaction in the agar-gel diffusion test and by the hemagglutination technique. In addition, glucagon, which was the most likely contaminant in the insulin sample, was shown to be neither reactive nor to have any inhitibory activity.

(B) Studies on antigenic activities of the A and B chains of insulins

(1) The disulphide bonds of insulin were reduced electrolytically by the application of controlled potential at a mercury cathode. The extent of the reduction was followed amperometrically with $AgNO_3$. The decomposition potential of insulin observed in phosphate buffer, pH 7.2, ionic strength 0.02, was -1.46 ± 0.05 V vs S.C.E. At a cathode potential of -1.6 V vs S.C.E., the two interchain disulphide bonds behaved indistinguishably and were preferentially reduced, and total reduction of the three disulphide bonds of the insulin molecule was achieved at a cathode voltage of slightly less than -2.0 V vs S.C.E.

(2) The SH groups formed during the reduction were stabilized by alkylation with iodoacetic acid. Evidence for the electrolytic reduction and for the preferential reduction was obtained from the results of paper electrophoresis, ion-exchange chromatography, amino acid analysis, and ultracentrifugal analysis.

(3) The B chain, totally reduced A chain, and the A chain with the intrachain disulphide bond intact were purified under mild conditions by molecular sieve chromatography. The A chain with the intrachain disulphide bond intact thus obtained contained 0.90 - 0.95 S-S bond per mole of A chain.

(4) It was demonstrated by standard immunological procedures that the totally reduced A chain and B chain were not active, i.e. they did not give a precipitation reaction nor did they inhibit the reaction between insulin and its homologous rabbit antibodies. On the other hand, the A chain with the intrachain disulphide bond intact was found to be a univalent fragment, which inhibited the reaction between insulin and its homologous rabbit antibodies, as demonstrated by the inhibition experiments in agar-gel diffusion and hemagglutination reaction. This inhibitory activity of the A chain with the intrachain disulphide bond intact was independent of the presence of the specific B chain.

(5) The inhibitory activity of the A(S-S) chain relative to that of the original insulin amounted to 7.5 - 10.0% in one antiserum.

CLAIMS TO ORIGINALITY

1. Insulin was coupled to bovine serum albumin through the formation of ureido linkage (R-NH-CO-R'), using tolylene-2,4-diisocyanate as the cross-linking agent.

2. The conjugate consistently and readily evoked the formation of antibodies specific to insulin without affecting the normal life span or state of health of the immunized animals.

3. A soluble insulin derivative was prepared by coupling the sodium 4-fluoro-5-nitrobenzene sulphonate (FNBSNa) to insulin. The conjugate obtained was reactive with respect to its homologous rabbit antibodies in vitro.

4. A simple apparatus was built for electrolytic reduction of insulin at a constant cathode voltage.

5. Reduction of all three disulphide bonds of insulin was accomplished at neutral pH and in the absence of denaturing agents.

6. The decomposition potential of insulin in phosphate buffer (pH 7.2, ionic strength 0.02) was -1.46 ± 0.05 V vs S.C.E. However, at this voltage the reaction was extremely slow and the reduction potential was, therefore, increased to -1.6 V vs S.C.E., where the two interchain disulphide bonds

were reduced indistinguishably. The intrachain disulphide bond was ruptured only at -2.0 V vs S.C.E.

7. The A chain-6,11-disulphide-7,20-bis-carboxymethyl-cysteine, prepared by electrolytic reduction, i.e. the A chain with intrachain disulphide bond intact, was isolated by molecular sieve chromatography and was shown to contain 0.90 - 0.95 S-S bond per mole of A chain.

8. Both B chain and the totally reduced A chain were not active with respect to rabbit anti-insulin antibodies.

9. The antigenic determinant of the A chain was shown to reside between amino acids 6 - 11, i.e. in a site encompassing the intrachain S-S bond.

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