STUDIES ON THE PREPARATION OF NUCLEOPROTEINS AND RIBONUCLEIC ACID FROM WHEAT GERM

A Thesis

by

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TABLE OF CONTENTS

Page

INTRODUCTION

I. GENERAL..... 1

II. HISTORICAL..... 3

III. PROBLEMS INVOLVED IN THE ISOLATION OF NUCLEIC ACID..... 7

(a) General..... 7

(b) Isolation of nucleoproteins..... 9

(c) Separation of nucleic acid..... 12

(d) Purification of nucleic acid..... 18

MATERIAL AND METHODS

I. WHEAT GERM..... 23

II. NITROGEN DETERMINATION..... 24

(a) General..... 24

(b) Digestion..... 24

(c) Distillation..... 25

(d) Titration..... 26

III. PENTOSE DETERMINATION..... 28

(a) General..... 28

(b) Procedure..... 30

(c) Experimental..... 31

IV. DETERMINATION OF HYDROGEN ION CONCENTRATION... 37

V. OTHER DETERMINATIONS..... 39

(a) Moisture..... 39

(b) Phosphorus..... 40

(c) Sodium..... 41

page

EXPERIMENTAL

I. WATER EXTRACTION OF A PROTEIN FRACTION FROM WHEAT GERM..... 42

(a) Non-protein nitrogen fraction..... 42

(b) Extraction of protein nitrogen with water.. 49

(c) Repeated extractions with water..... 57

II. ISOELECTRIC PRECIPITATION OF NUCLEOPROTEIN FRACTIONS..... 59

(a) Optimum hydrogen-ion concentration for precipitation of nucleoprotein..... 59

(b) Reprecipitation of nucleoprotein..... 64

(c) Variability of the composition of nucleoproteins..... 71

III. PREPARATION OF CRUDE NUCLEIC ACID SOLUTIONS.... 76

(a) Using barium acetate..... 76

(b) Denaturation and isoelectric precipitation of the protein..... 86

(c) Using ethyl alcohol..... 90

(d) Method of preparing a crude nucleic acid solution..... 99

IV. PREPARATION OF NUCLEIC ACID FROM SOLUTIONS OF CRUDE NUCLEIC ACID.....103

(a) Precipitation of nucleic acid.....103

(b) Purification of nucleic acid.....104

(c) Analyses of nucleic acids.....106
(1) Chemical analyses.....106
(2) Spectrophotometric analyses.....107

page

DISCUSSION

I. ANALYTICAL RESULTS.....113

II. NUCLEOPROTEINS FROM WHEAT GERM.....118

III. RECOVERY AND PURITY OF RIBONUCLEIC ACID FROM WHEAT GERM AT DIFFERENT STAGES IN THE ISOLATION PROCEDURE.....120

SUMMARY 125

CLAIMS TO ORIGINAL RESEARCH 128

BIBLIOGRAPHY 130

LIST OF TABLES

Table No.		Page
I.	Absorption data of the test and blank solutions in the pentose estimation with orcinol..	33
II.	Effect of volume and concentration of hydrochloric acid on colour development.....	35
III.	Effect of time of heating on colour development.....	36
IV.	Reliability of the estimation of hydrogen ion concentration between pH 4.0 and 7.0.....	39
V.	Non-protein nitrogen in wheat germ extracts...	43
VI.	Effect of trichloroacetic acid concentration on the non-protein nitrogen determination....	46
VII.	Effect of extraction time on total nitrogen extracted.....	50
VIII.	Effect of extraction time on nitrogen extracted.....	52
IX.	Effect of wheat germ:water ratio on extracted nitrogen.....	55
X.	Repeated extraction of wheat germ with water (Ratio 1:15).....	58
XI.	Repeated extraction of high concentrations of wheat germ with water.....	59
XII.	Effect of hydrochloric acid on nitrogen precipitation and hydrogen ion concentration of an extract.....	61
XIII.	Nitrogen precipitation as influenced by time of standing.....	64
XIV.	Successive precipitation of the nucleoprotein fraction.....	65
XV.	Effect of successive precipitations on the nucleoprotein fraction.....	67
XVI.	Effect of hydrogen ion concentration on solubility of a nucleoprotein reprecipitated three times.....	68

Table No.		Page
XVII.	Effect of hydrogen ion concentration on solubility of a twice precipitated nucleoprotein...	70
XVIII.	Separation of various nucleoprotein fractions from a nucleoprotein solution.....	73
XIX.	Effect of hydrogen ion concentration and amount of barium acetate on the precipitation and purification of nucleic acid.....	78
XX.	Effect of storage at 5°C. on precipitation of nucleic acid by barium acetate.....	83
XXI.	Effect of storage at room temperature on precipitation of nucleic acid by barium acetate...	85
XXII.	Solubility of denatured nucleoprotein at various hydrogen ion concentrations.....	87
XXIII.	Isoelectric and ethyl alcohol precipitation of a nucleoprotein solution prepared by the "mild" procedure.....	92
XXIV.	Isoelectric and ethyl alcohol precipitation of denatured nucleoproteins.....	93
XXV.	Effect of time of heating on nucleoprotein precipitation.....	94
XXVI.	Effect of order of addition of ethyl alcohol on nucleoprotein precipitation.....	96
XXVII.	Optimum time of heating and hydrogen ion concentration for precipitation.....	97
XXVIII.	Effect of hydrogen ion concentration on the precipitation of a denatured nucleoprotein.....	98
XXIX.	Chemical analyses of some nucleic acid preparations.....	108
XXX.	Light absorption data of some nucleic acid preparations.....	110

Table No. Page

XXXI. Theoretical sodium content of the possible "statistical" tetranucleotide salts.....114

XXXII. Recovery and purity of ribonucleic acid from wheat germ at different stages in the isolation procedure.....121

LIST OF FIGURES

Figure No. Page

1. Absorption spectra of the test and blank solutions in the colorimetric estimation of pentose. 34
2. Non-protein nitrogen in wheat germ extracts..... 44
3. Effect of trichloroacetic acid concentration on the non-protein nitrogen determination..... 47
4. Effect of extraction time on the total nitrogen extracted from wheat germ..... 51
5. Effect of extraction time on nitrogen extracted. 53
6. Effect of the wheat germ:water ratio on the extracted nitrogen..... 56
7. Effect of hydrogen ion concentration on nitrogen precipitated..... 62
8. Effect of hydrochloric acid on the hydrogen ion concentration of an extract..... 63
9. Effect of hydrogen ion concentration on the solubility of a nucleoprotein..... 69
10. Effect of hydrogen ion concentration and amount of barium acetate on nitrogen and pentose content of the precipitate..... 79
11. Effect of hydrogen ion concentration and amount of barium acetate on the precipitation of nucleic acid.....80
12. Influence of hydrogen ion concentration on solubility of denatured nucleoprotein..... 88
13. Purity of nucleic acid in denatured and undenatured nucleoprotein solutions at various hydrogen ion concentrations..... 89
14. Light absorption spectra of some nucleic acid preparations.....111

INTRODUCTION

I. GENERAL.

The annual production of wheat germ, as such, in the United States is estimated to be 30 to 50 million pounds. However, the present milling yield of about 0.5 per cent indicates a potential production of about 150 million pounds of germ (1). The wheat kernel is reported (2) to contain 2.2 - 3.0 per cent of germ, and, therefore, it is possible that, in the future, the milling yield and hence the potential production of wheat germ may be greatly increased. For Canada, assuming an annual production of 350 million bushels of wheat and a yield of only 0.5 per cent of germ, the potential production of wheat germ is about 100 million pounds per annum. Wheat germ, as a by-product of the milling industry, is used as animal feed, generally as component of the bran, and in limited amounts as a source of oil. Lusena and McFarlane (3) have sought to make wheat germ acceptable as a human food and thereby to make available a rich source of the B-vitamins (4, 5, 6) and a large amount of highest quality protein (7), in addition to fat and vitamin E (8). In spite of efforts from many sources the human consumption of wheat germ represents only a small fraction of the output and more industrial uses

have to be found for this valuable by-product of the milling industry.

Wheat germ could become the source of many needed biological compounds. Grewe and Le Clerc (9) have reviewed some of the important components found in wheat embryo. At the Chemistry Department, Macdonald College, McGill University, studies are being made on the isolation of tocopherol concentrates, lecithins, sterols, purified oils, proteins, starch and magnesium phosphate (one per cent of the germ). Many other products could be isolated on a large scale. For example, Channon and Foster (10) found that 7.8 per cent of the wheat germ oil consisted of phosphatides. Sullivan et al. (11) isolated 0.1 - 0.2 per cent glutathione from wheat germ, although they found (12) 0.46 per cent in a very fresh and specially purified sample. Schopfer et al. (13) believe that there are at least six growth factors present in wheat germ other than the known vitamins. Osborne and Harris (14) report 3.56 per cent nucleic acid in wheat germ and they isolated more than one per cent of a product containing 80 per cent nucleic acid. Therefore, wheat germ could be a rich source of ribonucleic acid and its preparation, if included in a larger scheme for the isolation of various biologically important components, might be economically feasible. A better utilization of wheat germ could be achieved and

wheat germ production could be expanded. The laboratory conditions for the large scale isolation of ribonucleic acid from wheat germ have not been studied in detail. Ribonucleic acid of a high degree of purity has never been isolated in good yield from wheat germ. Therefore, it is proposed in this investigation to determine the optimum conditions for the extraction and isoelectric precipitation of a definite nucleoprotein fraction; to investigate the required degree of denaturation of nucleoprotein for the separation of nucleic acid; and to develop a satisfactory method for the purification of ribonucleic acid.

II. HISTORICAL.

In 1899 Osborne and Campbell (15) isolated for the first time a ribonucleic acid from wheat germ which they called "tritico" nucleic acid. They extracted a large quantity of wheat germ meal with water, saturated the extract with sodium chloride and subjected the resulting precipitate to a vigorous pepsin digestion. They obtained a considerable quantity of a substance which they called "nuclein". The "nuclein", after washing with water, was dissolved in dilute potassium hydroxide and the solution

was faintly alkaline to phenolphthalein. The solution was treated cautiously with dilute hydrochloric acid until a precipitate formed. The precipitate was redissolved in potassium hydroxide solution and reprecipitated three times with dilute hydrochloric acid and ethyl alcohol. From the analytical data reported it seems that the preparation could have been at the best 80 - 85 per cent ribonucleic acid, assuming the tetranucleotide structure of Levene (16) at least as an average structure (i.e. assuming a "statistical" tetranucleotide structure).

In the same paper Osborne and Campbell reported the isolation of a nucleoprotein "leucosin" containing 20 - 30 per cent nucleic acid and representing 10 per cent of the embryo. A globulin was also isolated in amount equivalent to 5 per cent of the embryo and it contained 12 - 15 per cent nucleic acid. One third of the total nitrogen of the embryo was not extracted by water and salt solutions. This nitrogen was accompanied by phosphorus corresponding to about 6.75 per cent of nucleic acid which would account for two thirds of this insoluble nitrogen. In 1902, "tritico" nucleic acid was isolated again on a larger scale from wheat embryo by Osborne and Harris (14), employing minor changes in procedure. The composition of the various samples varied considerably

according to the analyses reported, the purest being at best 85 per cent nucleic acid. Employing 750 g. of the prepared nucleic acid, they made a thorough study of its properties and of some of its hydrolytic products. Among the latter they found adenine, guanine, a pentose and uracil and suggested the identity of "tritico"nucleic acid with yeast nucleic acid.

In 1910, Levene and La Forge (17) slightly improved the method of preparing "tritico" nucleic acid and found that on hydrolysis with ammonia in an autoclave at 145°C. it was decomposed into nucleosides and phosphoric acid, but they isolated only adenosine, guanosine and cytidine. They also showed that the pentose is identical with the one obtainable from yeast nucleic acid. They pointed out that tritico nucleic acid has "all the properties of nucleic acid from yeast" and that the two are "probably identical". In 1917, Read and Tottingham (18) prepared a guanine nucleotide from wheat germ, which was identical with the guanine nucleotide prepared from yeast. From the adenine-uracil dinucleotide prepared by them they isolated uridine nucleoside which was identical to the known uridine nucleoside from yeast.

In 1927, Calvery and Remsen (19), profiting from the work of Jones and Perkins (20), isolated and identified all four nucleotides of "tritico" nucleic acid and identified

them with the nucleotides of yeast nucleic acid. Levene and Bass (21) have stated that "tritico"nucleic acid is identical with yeast nucleic acid.

The foregoing research workers studied the composition of wheat germ ribonucleic acid, without being concerned with its isolation. They used Levene and La Forge's (17) modification of Osborne and Campbell's (15) method of preparation. Clarke and Schryver (22) in 1917, proposed a different procedure which involved extracting wheat germ, previously boiled with ethyl alcohol, with 10 per cent sodium chloride solution. A very crude product was obtained.

In 1946, Mirsky and Pollister (23), employing a new mild treatment, isolated a desoxyribose nucleoprotein complex from wheat germ, which they called "chromosin". Because of the precautions taken, this "chromosin" did not contain detectable quantities of ribose nucleoprotein, but, incidentally, they also achieved the extraction of ribose nucleoprotein, free of desoxyribose compounds. Belozerskii and Bazhilina (24) studied the interrelation between protein and nucleic acid in nucleoprotein by mixing weakly alkaline solutions of a non-basic protein and nucleic acid from wheat germ. On addition of acetic acid, nucleoproteins were precipitated. The upper limit of the

phosphorus content of the nucleoproteins was 2.08 to 2.20 per cent, corresponding to 25 - 27 per cent nucleic acid. They also submitted some evidence that the ability of these components to bind was partially due to free amino groups. These data are of limited value in connection with the study of the isolation of nucleoprotein and nucleic acid from wheat germ because the experimental procedures are very artificial. It seemed timely, therefore, to reinvestigate the details of the procedures for the isolation of ribonucleic acid from wheat germ.

III. PROBLEMS INVOLVED IN THE ISOLATION OF NUCLEIC ACIDS.

(a) General:

A critical review of the main steps involved in the isolation of nucleic acids from various tissues other than wheat germ should clarify the problem. The terms, thymus nucleic acid or animal nucleic acid and yeast nucleic acid or plant nucleic acid, are now obsolete. Pollister and Mirsky (25, 26) proposed that the name "chromonucleic acid" should be used instead of thymus nucleic acid as the biological term for the substance described chemically as desoxyribose nucleic acid, and that"plasmonucleic acid" should be substituted for yeast nucleic acid as the biological equivalent of the chemical term ribose nucleic acid.

Gulland et al. (27, 28) stressed the necessity, in the light of present information, of defining a nucleic acid by referring both to its origin and its type. This second alternative should be used to avoid confusion, even though a recent paper by Mirsky and Pollister (23) gives very strong evidence in favour of their own nomenclature. Cohen (29) has also suggested, as a result of electrophoretic studies, that in the characterization of different nucleic acids the treatment in the course of isolation, such as degree of tissue autolysis, reagent used and other conditions should be described.

Many authors have attempted the direct extraction of nucleic acid (30, 31), but have found the methods unsatisfactory. Most of these methods involve the use of alkali to isolate nucleic acid from tissues such as yeast (30,31), pancreas (32), various animal tissues (33, 34) and bacteria (35, 36). Extraction with sodium chloride solutions, after denaturation with ethyl alcohol, yields ribonucleic acid from yeast and wheat germ (22) and liver (37); in the latter case a nucleoprotein is probably isolated and subsequently converted into the nucleic acid during purification. In general, it seems to be desirable to prepare nucleic acids by the isolation and subsequent decomposition of the nucleoprotein.

(b) Isolation of nucleoproteins:

Many of the methods for isolating nucleoproteins involve a stage, either during extraction or more generally in the precipitation process, which is relatively drastic and may produce changes in the chemical and physical properties of the nucleoprotein. The extraction processes vary considerably but generally employ water (38 to 45), dilute alkaline solution (14, 15, 17, 46), sodium chloride solution (36, 47), or buffer solutions of pH values ranging from 4 to 11 (48, 49, 50) followed in each case by precipitation with acids, usually glacial acetic or hydrochloric acid. These methods are now considered unsatisfactory (51) because of the possible rupture of the nucleic acid-protein bond and the variable composition of the product; the precipitated nucleic acid carrying with it varying quantities of loosely bound protein. Greenstein and Jenrette (52, 53) made a more controlled extraction of liver nucleoprotein. They treated the tissue with a solution containing 0.03 M sodium bicarbonate and 0.5 M potassium chloride and the nucleoprotein was precipitated by adjusting the solution to pH 4.2. According to Mirsky and Pollister (23) this precipitate was a mixture of desoxyribose and ribose nucleoprotein (27 per cent desoxyribose).

Carter and Hall (54) stressed the importance of a mild procedure and they extracted fresh pulped calf

thymus with 2 - 3 volumes of water at 5°C. for twenty-four to thirty-six hours. After clarifying the extract, they precipitated the nucleoprotein either by the addition of an equal volume of 2 per cent sodium chloride solution or of 0.2 per cent calcium chloride solution. The sodium salt of the nucleo-protein was soluble in water or 5 per cent sodium chloride solution, while the calcium salt dissolved either in 2 per cent calcium chloride solution or 5 per cent sodium chloride solution.

Mirsky and Pollister (23) developed a satisfactory mild procedure, apparently of general application for the isolation of desoxypentose nucleoproteins. This procedure depends on the rather remarkable changes in solubility of nucleoproteins in sodium chloride solutions of different strengths. The nucleoproteins dissolve in molar sodium chloride, forming viscous opalescent solutions, but are insoluble in 0.14 M sodium chloride solution, although soluble in 0.02 M or less or in pure water. The minced tissues, after being washed with 0.14 M sodium chloride solution to remove cytoplasmatic material, were extracted with molar sodium chloride (2 M in certain cases) and after clarification of the extract the nucleoprotein was precipitated in distinctly fibrous form by dilution with sufficient water to bring the sodium

chloride concentration to 0.14 M.

The nucleoprotein isolated by these mild methods (23, 54) from calf thymus has a molecular weight of the order of 2×10^6 (63) and its solution in molar sodium chloride shows a high viscosity and marked streaming birefringency generally associated with highly asymmetric macromolecules. The nucleoprotein may also be precipitated from the concentrated salt solution by the addition of alcohol (45, 55). Davidson and Waymouth (37) also used alcohol to precipitate the crude ribonucleic acid from liver extract (probably a denatured nucleoprotein).

Brues et al. (56) have isolated, from the same tissue sample, desoxyribose nucleoprotein and ribose nucleoprotein by precipitating the aqueous extract of minced rat liver with 0.4 per cent calcium chloride solution, and extracting the desoxypentose nucleoprotein from the precipitate with 10 per cent or molar sodium chloride solution. The pentose nucleic acid was extracted from the residue with boiling 10 per cent sodium chloride solution.

The isolation of nucleoproteins from cells possessing resisting walls has been satisfactorily accomplished (43, 51) by disintegrating the cells by means of intense audible sonic vibrations, the nucleoproteins being then extracted from the cellular debris with water and precipi-

tated with 0.1 N hydrochloric acid (43) or ammonium sulphate (51). Development of the technique of differential centrifugation has provided an important method whereby the macromolecular nucleoproteins, generally in association with lipoid material, may be isolated from various tissues with a minimum of chemical action. This has permitted the preparation of the active nucleoprotein fraction of the chick tumour I (57, 58) and of various fractions from mammalian cells and tissues (59, 60). The method is also used extensively for the isolation of the virus nucleoproteins (61, 62).

(c) Separation of nucleic acid:

The simplest and best available definition of a nucleoprotein would be to designate as such any protein with which nucleic acid is associated. The manner of association may refer either to primary (non polar) or to salt-like (polar) linkages between protein and nucleic acid. When the protein component is of a relatively simple protamine or histone nature, the nucleic acid can be separated by the addition of a neutral salt such as sodium chloride and it is difficult to see how the linkage between the nucleic acid and protein can be other than salt-like. The naturally-occurring defatted complex of protamine and desoxyribonucleic acid in the sperm heads

when treated with sodium chloride (64) swells and goes into solution and on diluting with water most of the protamine is precipitated, while the desoxyribose nucleic acid remains in solution to some extent. The nucleohistones, salts of histone and desoxyribonucleic acid dissolve in moderately concentrated salt solutions (39, 54, 56, 65) and, upon saturation with sodium chloride, the histone separates and is precipitated. Mirsky and Pollister (23) achieved the separation of the nucleohistones dissolved in molar sodium chloride solution by dialysis against molar sodium chloride solution, which results in the diffusion of the protein. When dissolved in molar sodium chloride solution, the desoxyribose nucleic acid and the histone components of a chromosin are to a considerable extent dissociated and the nucleic acid is highly polymerized (i.e. the solution is very viscous). They are not dissociated when the chromosin is dissolved in 0.02 M sodium chloride, but in this medium a partial depolymerization of the desoxyribose nucleic acid occurs.

When the protein component is of a more complex nature, separation of the two components by metathesis is not so readily achieved. Here a profound intramolecular rearrangement and disorientation of the protein, and possibly of the nucleic acid as well, by denaturing

processes is a necessary condition for such a separation. This is the case with most pentose-containing nucleoproteins. The virus nucleoproteins have been most widely studied from this standpoint. Bawden and Pirie (66) have paid considerable attention to the effect of various salts and neutral organic compounds which effect denaturation of various virus nucleoproteins with separation of protein and ribonucleic acid and also have demonstrated the range of susceptibility of the viruses to these reagents. Certain nucleoproteins require stronger denaturing salts than others, but in every case tested at least one neutral substance could be found which would effect the separation of protein and nucleic acid. This treatment frequently results in a disaggregation of the nucleoproteins into smaller particles accompanied by a loss in anisotropic properties (67, 68) and the liberation of sulphydryl groups (67, 69) and presumably other groups (70). As this denaturation is effected by the addition of neutral salts such as guanidine hydrochloride or of neutral organic compounds such as urea, it is difficult to avoid the impression that here too the binding forces between protein and nucleic acid are of a salt-like character. However, it should be realized that the stability of such polar linkages is dependent upon the

native configuration of both protein and nucleic acid and upon their mutual orientation. Other methods of removing the nucleic acid from the protein component of the virus nucleoproteins include heating (71, 72), high pressure (73), and treatment with acids and bases (72). The use of more selective agents such as the nucleases, which are specific for nucleic acid, gives results which are inconclusive (74, 75). In the case of the tobacco mosaic virus nucleoprotein, only an indefinite complex between enzyme and nucleoprotein is formed in which the virus is reversibly inactivated without loss of nucleic acid.

The decomposition of a ribose nucleoprotein, not isolated from viruses, into its constituent nucleic acid and protein is most easily brought about by alkaline hydrolysis (21), followed by the removal of protein with colloidal iron and precipitation of the nucleic acid in acid solution. An obvious objection, however, is that the alkalinity also causes some degradation of the nucleic acid. This degradation has no doubt often been considerable (76), but in some cases this is apparently the only method available; an alternative successful procedure is hydrolysis of the crude nucleoprotein with pepsin (14, 15, 17). According to Sevag et al. (43), complete separation of the protein and nucleic acid of nucleo-

proteins may be effected by decomposing the nucleoprotein at 50°C. in 0.5 per cent sodium carbonate solution for one to two hours, neutralizing to pH 7 and shaking the solution with chloroform containing a small amount of a foam-preventing agent such as amyl alcohol. The protein concentrates at the interface forming a chloroform-protein gel, easily separated by centrifugation. The same authors (51) prepared two pentose nucleoproteins from aqueous extracts of streptococci, one by acid precipitation and the other by ammonium sulphate precipitation. From a direct comparison of the two methods of precipitation (43) they suggested that electrovalent bonds are formed in the acid precipitation. The ammonium sulphate-precipitated nucleoproteins, unlike their acid-precipitated congeners, were completely soluble on the acid side of their isoelectric point. The acid-treated nucleoproteins formed an immediate precipitate with neutral calcium chloride solution, whereas no precipitation occurred with the natural nucleoprotein. These results imply either that the phosphoric acid groups of the natural nucleoprotein are bound in such a manner as not to be available for reaction with calcium ions, or alternatively that the calcium salt of the natural nucleoprotein is soluble. Whatever may be the true explanation, it is evident that acid precipitation changes

the properties of pentose nucleoproteins from streptococci.

The generalization has been made by Mirsky (77) that the bonds between pentose nucleic acid and protein are covalent, whereas those between desoxypentose nucleic acid and protein are electrovalent, but the evidence obtained with carefully prepared nucleoproteins is insufficient to warrant this generalization, which is certainly not in agreement with the results obtained with virus nucleoproteins. The separation of the nucleoprotein components with alkali has been considered to indicate the presence of a non-polar bond between them. Greenstein (78) has suggested that it is difficult to distinguish between the following possibilities: (a) that alkali actually splits an electrovalent bond, presumably an ester linkage, by hydrolysis; (b) that alkali induces denaturation and the consequent internal rearrangement and disorientation of protein and nucleic acid lead to the separation of the salt in a manner essentially similar to that induced by neutral substances; (c) that, conversion of the protein to an anion by alkali, brings about separation of the two components through electrostatic repulsion of the similarly-charged nucleic acid anion.

(d) Purification of nucleic acid:

Most of the methods of purifying crude nucleic acid involve dissolving in alkali (sodium hydroxide or ammonia) and precipitation by addition of acid (acetic or hydrochloric acid) amd/or ethyl alcohol (21). Conditions are not generally well defined and they involve very drastic treatment with alkali and acid. It is true that the nucleic acid, after separation from the bulk of the protein, is not in the native state, but it does not seem advisable to carry this breakdown any further. Jorpes (55) precipitated barium salts of protein-free pentose nucleic acid from pancreas by dissolving the crude nucleic acid in distilled water and adding 0.25 volume of 20 per cent barium acetate solution, adjusted to pH 6.8. The main part of the desoxyribonucleic acid of the pancreas is removed with the mother liquor. When the mother liquor is made distinctly alkaline with barium hydroxide, a pentose nucleic acid precipitates, but it is greatly contaminated with desoxypentose nucleic acid.

Levene and Jorpes (79) treat the barium salts of nucleic acid with hydrochloric acid and dissolve them in water with the aid of strong sodium hydroxide or sodium carbonate solutions, avoiding an alkaline reaction. The nucleic acid is precipitated on the addition of ten volumes of glacial acetic acid. If the barium

salt is not protein-free, a precipitate appears on the addition of the first traces of acetic acid. The last traces of protein are removed by separating this precipitate before adding more glacial acetic acid. The pentose nucleic acid is precipitated with glacial acetic acid and the thymus nucleic acid remains in the mother-liquor. Jorpes (55) reports that the pentose nucleic acid isolated by their procedure gives analytical data that agree quite closely with the theoretical values for a pentose nucleotide.

The separation of the nucleic acid from a nucleoprotein by the formation of a chloroform-protein gel, is described by Sevag et al. (43), as a method of purification. Khouvine and Grégoire (82) purified crude ribonucleic acid from the larvae of Calliphora erythrocephala by the method of Sevag et al. (43) and by electrophoresis. The per cent nitrogen and phosphorus in the two products obtained are given in the following table as a basis for comparing the two methods of purification.

Nucleic Acid Sample	Nitrogen (per cent)	Phosphorus (per cent)	Nitrogen/Phosphorus ratio
Crude	15.83	8.75	1.81
Purified by Sevag's method	15.60	9.00	1.73
Purified by electrophoresis	15.90	9.30	1.71
Theoretical	16.12	9.52	1.69

Vendrely, Sarciron and Doucet (83) improved the classical methods of purification of nucleic acid which are based on acidification. They studied the conditions in more detail using a commercial preparation of yeast ribonucleic acid as the starting material. The best results are obtained when the crude nucleic acid is dissolved in a minimum amount of dilute sodium hydroxide solution (no other details are given). This solution, containing about 5 per cent nucleic acid, is poured into 5 volumes of 0.5 N hydrochloric acid. After centrifuging to remove a precipitate, containing proteins and carbohydrates impurities, the nucleic acid is precipitated from the supernatant solution with alcohol (the concentration is not given). This procedure is repeated until the desired purity is obtained. The crude commercial preparation contains 85 per cent ribonucleic acid and the first precipitation recovers 65 per cent of the nucleic acid of a purity of 93 per cent. Repeating the precipitation twice gives practically pure nucleic acid with a recovery of 55 per cent. The impurities consist of a polysaccharide and a basic protein, the latter being very difficult to remove.

The purity of the samples can be estimated from the nitrogen and phosphorus content. However, other procedures are now available for confirmation. The

desoxyribonucleic acid can be analyzed for desoxyribose by the Feulgen (84, 85) or the Dische (86, 87) reactions and the pentose content of ribonucleic acid can be determined by the orcinol test (88, 89, 90). However, if contamination with desoxyribonucleic acid is expected, the use of the more recent method of Hahn and v. Euler (91) is advisable. A micromethod for the estimation of purine nitrogen involving a copper precipitation has been developed (92) and also a method for nucleotide, nucleoside and free purine nitrogen (93, 94). The pyrimidine ring structure, which is common to purine and pyrimidine bases, strongly absorbs ultraviolet radiation with a sharp maximum in the region of 260 millimicrons (95, 96). Frequent use has been made of this fact to measure nucleic acid even in nucleoproteins "in vivo" or in isolated cells (97, 98, 99). The absorption curves of ribonucleic and desoxyribonucleic acid are practically identical. The extinction coefficient at this maximum is proportional to the nucleic acid concentration.

Most of the above mentioned tests can be applied to determine the content of nucleic acids in nucleoproteins and cells. Such tests, however, only reveal the presence of the components, sugar, nitrogenous bases or phosphorus, but they are not specific for nucleic acid as such. The actual isolation of nucleic acid is the only absolute

measure. However, these tests are useful when their limitations are recognized and when due precautions are taken to remove interfering substances. The application of some of these tests is thoroughly discussed by Davidson and Waymouth (100).

MATERIAL AND METHODS

I. WHEAT GERM.

Pure wheat germ should have been used in this study, but it was impossible to secure large enough quantities, however, the best commercial wheat germ with a guaranteed purity of 95 per cent, was employed. The first ten batches received were tested for purity. Two samples of exactly 1.0 g. each were sorted by hand to remove impurities. The pure wheat germ and the impure fractions were weighed and the purity of the original wheat germ was calculated. The average purity for ten samples was 95.7 per cent with a range of 95.5 to 95.8. The per cent recovery (i.e. the sum of the two fractions divided by the original sample weight and multiplied by 100) averaged 99.1 with a range of 97.9 to 99.5. Naturally the error in estimating the purity would be much greater if the low recovery were due to impurities lost rather than wheat germ. The wheat germ, as received from the flour mill, was ground to pass the finest screen of the Wiley Mill. Some fat was lost in the process and the residual material left in the mill contained a larger percentage of bran as indicated by the colour. The amount of bran could not be determined because the particles of wheat germ and bran were partially milled. Samples of the finely milled wheat germ were taken for

nitrogen analysis and the flour was stored in tightly stoppered glass jars. The nitrogen content on an air dry basis varied from 5.48 to 5.27 per cent with an average of 5.35 per cent. Fresh samples were received monthly and no sample was stored longer than two months at a temperature of -10°C. unless otherwise stated.

II. NITROGEN DETERMINATION.

(a) General:

A micro-procedure was essential in most cases because the work was carried out on small samples or on very dilute solutions. The micro-Kjeldahl procedure described below is the official method of the Association of Official Agricultural Chemists (101), as modified by Chibnall et al. (102) and Miller and Houghton (103). A slightly modified Parnas-Wagner micro-Kjeldahl distilling apparatus was used because of its extreme reliability and ease and speed of operation (up to 10 distillations including the titrations can be performed in one hour).

(b) Digestion:

The size of the sample was so adjusted as to require a titration of 3 to 5 ml. of 0.02 N hydrochloric acid (i.e. the sample contained between .84 and 1.4 mg. of nitrogen). If a powder was analyzed, 10 - 15 mg. were weighed to the

fifth decimal place on a micro-balance. The weighing was carried out on a cigarette paper, which was folded to contain the sample and then dropped into a 30 ml. micro-Kjeldahl flask. To avoid sampling error, the sample was allowed to come into equilibrium with atmospheric moisture. If a solution was analyzed, a 1 or 2 ml. aliquot, properly diluted, was transferred from a pipette to the bottom of the flask. If the solution contained ethyl alcohol, the aliquot was evaporated by placing the digestion flask in a boiling water bath, thus preventing excessive foaming at the beginning of digestion. The catalyst, a mixture of 62.5 g. of finely ground potassium sulphate and 5 g. of mercuric oxide, was measured by volume to give approximately 0.5 g. (from 0.45 to 0.55 g.). After adding 1.5 ml. of concentrated sulphuric acid, the digestion flasks were placed on a sand bath and gently heated until frothing ceased. The temperature was then increased until the mixture boiled vigorously and the vapours of the acid rose to within 5 cm. of the mouth of the flask. Heating was continued for one hour after clearing.

(c) Distillation:

The digest, after cooling and diluting to about 8 ml., was transferred quantitatively to the distillation chamber of the Parnas-Wagner apparatus. To facilitate the transfer

the lip of the digestion flask was smeared with a thin film of vaseline, thus preventing any liquid from running down the outside of the flask. The digestion flask was rinsed four times with small volumes (about 3 ml.) of water. A small excess of alkali was added, the solution being prepared by mixing 40 g. of sodium hydroxide and 5 g. of sodium thiosulphate with 55 ml. of freshly boiled water. After filtering through asbestos, the solution was stored in a bottle fitted with a carbon dioxide trap. Distillation was carried out quite briskly and a large flow of water was kept running through the silver condenser. In less than 5 minutes 15 ml. of distillate were collected in the receiver, which contained 5 ml. of a 2 per cent boric acid solution. The indicator (0.1 per cent methyl red in ethyl alcohol) was added at the rate of 10 ml. per liter of boric acid solution. The receiver was lowered and, after allowing a further one ml. to distil, the tip of the condenser was rinsed with distilled water and distillation discontinued.

(d) Titration:

A colour standard was prepared by diluting 5 ml. of the boric acid solution to about 80 ml. in a container similar to the receiver flask. With these concentrations a very distinct titration end-point was obtained. The distillate-boric acid mixture was titrated almost to the

end point, then diluted to about 80 ml. rinsing the side of the flask and titration was completed to the end point. Blank determinations were carried out with all the reagents. If cigarette paper was used the blank correction was equivalent to 0.1 ml. of 0.02 N hydrochloric acid and to 0.05 ml. without cigarette paper. The microburette (5 ml. volume) was graduated in 0.01 ml. and allowed readings to 0.005 of ml. The end point was clearly exceeded by 0.02 ml. of 0.02 N hydrochloric acid, but the titre was easily estimated to 0.01 ml.

The standard 0.02 N hydrochloric acid was prepared from constant boiling hydrochloric acid by weighing an exact amount and transferring it to a 2 l. retested volumetric flask (104). No correction was made for temperature variations which ranged from 22° to 24°C. In one instance the standard solution was checked by the Official Borax Method (105) and the normalities agreed within 1 in 2000. If duplicate determinations showed an error of more than 0.5 per cent, the analyses were repeated. The average error was between 0.3 and 0.2 per cent. The greatest difficulties were encountered in the sampling. When enough sample was available, solutions were prepared and aliquots analyzed. The same solution was diluted and used for the pentose estimation. The reliability of the nitrogen determination was also confirmed by the results obtained

on the purest samples of nucleic acid which agreed very closely with the theoretical.

III. PENTOSE DETERMINATION.

(a) General:

The quantitative Bial's test as developed by Mejbaum (88, 89) served as a quick analytical tool to determine the relative ribonucleic acid content of various solutions. The literature contains many discussions (90, 100, 106, 107, 108) on the question of converting these relative values into absolute values, since it is not definitely known what proportion of the pentose in nucleic acid will react. Such ambiguity does not inspire confidence in the method. It was later found that the method gives theoretical values if certain assumptions and minor changes are made in the procedure. The colour reaction is given by pentoses and polyuronides. Hahn and v. Euler (91) report that desoxy-ribose reacts as though it contained about 10 per cent ribose. Glucose, certain polysaccharides, lead and nitrates interfere by developing extraneous colours. Therefore, the test becomes a measure of ribonucleic acid content only after repeated precipitations of the nucleic acid as a nucleoprotein. A phosphorus estimation would not be more specific as wheat germ contains inorganic phosphorus and phosphorus containing substances including desoxyribonucleic

acid (23).

To utilize the orcinol test for pentose as the basis of a quantitative estimation of nucleic acid, it was necessary to make certain initial assumptions, e.g.:

(1) It must be assumed that the isolated nucleic acid of wheat germ is ribonucleic acid or that desoxyribonucleic acid is present in traces only.

(2) It is also assumed that the fundamental structure of this ribonucleic acid is a tetranucleotide, according to Levene (16) and is composed of four phosphoric acid molecules, four ribose molecules, two purine bases (adenine and guanine) and two pyrimidine bases (uracil and cytosine). It is appreciated that this is an over-simplification and that complex polymers of the tetranucleotide are also present.

(3) Under the conditions of the orcinol reaction, only the ribose linked to purine bases gives the blue colour and hence it is assumed that only 50 per cent of the ribose molecules present give the colour reaction (89).

At the conclusion of this investigation, when purified wheat germ ribonucleic acid had been prepared, it was possible to show that these assumptions were justified as pentose determinations on the product gave values equivalent to half the theoretical value for a tetranucleotide.

(b) Procedure:

A 2 ml. aliquot of a solution containing 5 to 25 µg. of free pentose or an equivalent weight of a substance containing bound reacting pentose is placed in a pyrex 10 ml. graduated glass-stoppered cylinder. A 2 ml. aliquot of orcinol reagent is added. The reagent contains 1.0 per cent orcinol and 0.1 per cent ferric chloride in concentrated hydrochloric acid and is prepared immediately before use. A blank is also prepared by adding to a 2 ml. aliquot of the solution to be analyzed, 2 ml. of 0.1 per cent ferric chloride in concentrated hydrochloric acid. Thus the blank reagent does not contain orcinol. The cylinders are shaken and then placed in a briskly boiling water bath for 60 minutes, then the tubes are cooled, the solutions made up to volume, mixed and the colour intensities read in an Evelyn photoelectric colorimeter using the 660 mµ. filter and the galvanometer adjusted to 100 per cent transmission with the blank solution. The colour reaction obeys Beer's Law over a range of concentrations of 0.5 and 2.5 µg. per ml. which under the conditions outlined above corresponds to a range of 65 to 20 per cent transmission. However, the Beer's Law constant varied from.0263 to .0281 during the two years of this investigation and each constant had an average variation of ± 0.0004 for a range of concentrations of 5 to 25 µg. If precise work is required a

determination on a standard solution should be carried out simultaneously with the analysis of the unknown. The value $\frac{2-\log G}{K}$ (where G is the corrected galvanometer reading and K is the Beer's Law constant) gives the micrograms of reacting pentose in the two ml. aliquot of the test solution. Using any of the constants found during the last two years, the error could be as great as 10 per cent, whereas by establishing the constant for each determination the error does not exceed 2 per cent. It is suggested here that this variation might be due to the difference in the strength of the concentrated hydrochloric acid.

The quality of the orcinol is very important; a very old sample (Schuchardt) was used and the reagent was mixed just before use, because it quickly deteriorates and develops a pinkish-brown colour overnight. Another worker in this laboratory, using a new sample of orcinol (Eastman) and another colorimeter found the reaction to be at least 10 per cent more sensitive and that Beer's Law was not strictly obeyed. The setting of the colorimeter to 100 per cent transmission was constant over the two year period when readings were made on the blank solution or various solutions of pentose sugars, nucleic acids and nucleoproteins.

(c) Experimental:

It was found that ribose, arabinose and equivalent

amounts of pure yeast nucleic acid (assuming that 50 per cent of the pentose reacts) develop the same colour intensity if the test is conducted under the conditions just described. This is contrary to what Cori and Cori (108) and Davidson and Waymouth (100, 107) found. However, they did not report the purity of the nucleic acid used and their procedure was slightly different from the one described here. Using a Coleman spectrophotometer with cuvettes 1 cm. in diameter, readings for absorption spectra were taken on a blank and a test solution containing 5 µg. per ml., heated 20 minutes in a boiling bath. Table I and Figure 1 show the data expressed as optical densities at the specific wave length ($E = (\log I_o/I)_\lambda$, for 1 cm. thickness and a concentration of 5 µg. per ml. The maximum absorption for the green-blue colour of the pentose orcinol complex is at 660 mµ. and at that wave length the yellow colour of the ferric chloride solution, which is mainly responsible for the colour of the blank solution, does not absorb much light.

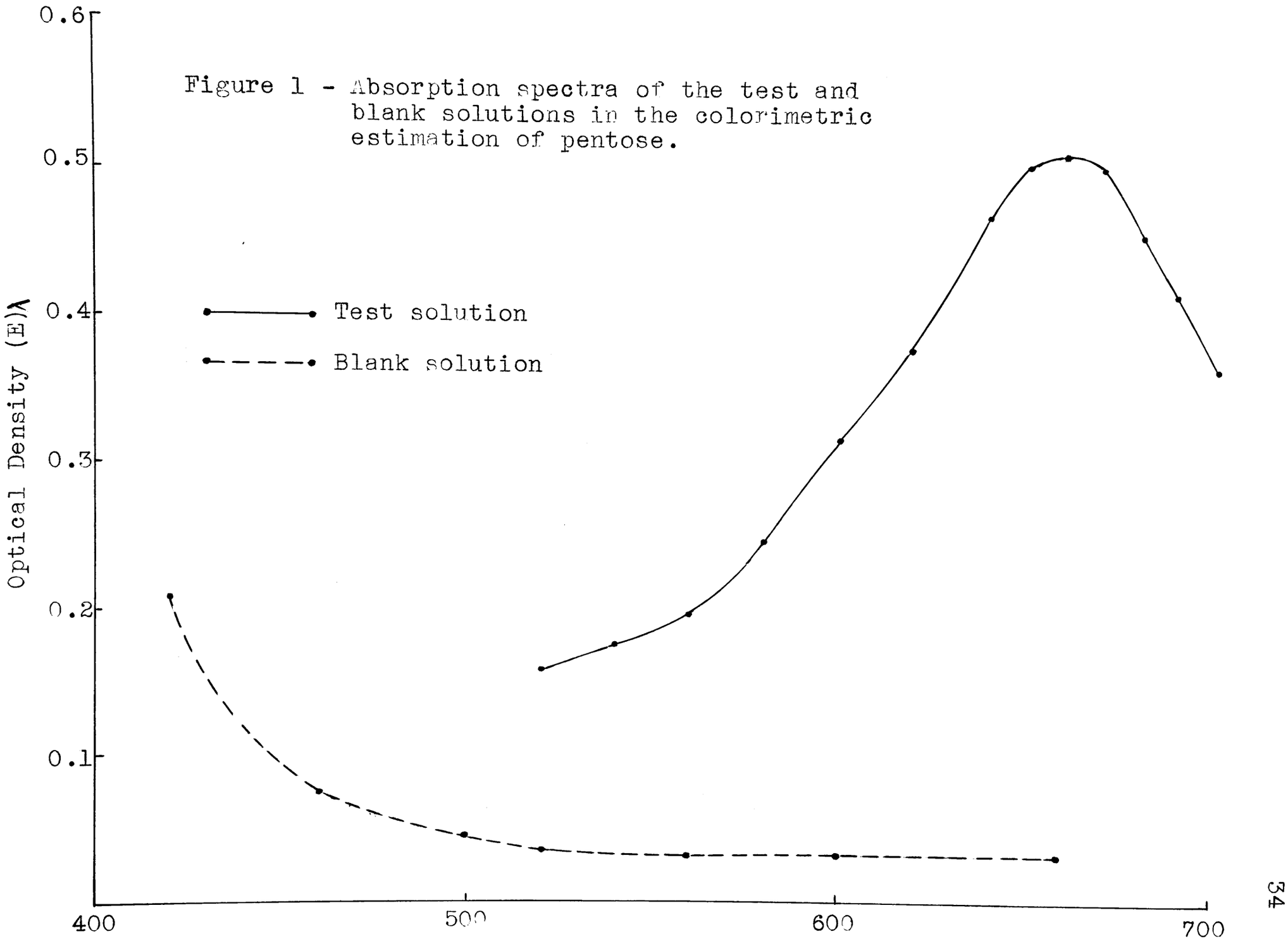
The reaction between orcinol and pentose sugars is very critical to temperature variations; unreliable results are obtained unless the water bath, in which the reaction vessels are placed, is kept boiling vigorously; a device to keep the water level constant is essential to avoid sudden changes in temperature when water is added to the

TABLE I - Absorption data of the test and blank solutions in the pentose estimation with orcinol.

Wave Length (mμ.)	Optical Density (E_λ)*	
	Test Solution	Blank Solution
700	.364	----
690	.417	----
680	.453	----
670	.502	----
660	.505	.031
650	.502	----
640	.469	----
620	.377	----
600	.319	.031
580	.248	----
560	.197	.031
540	.177	----
520	.161	.036
500	----	.046
460	----	.076
420	----	.207

* $E_\lambda = (\log I_o/I)_\lambda$

Figure 1 - Absorption spectra of the test and blank solutions in the colorimetric estimation of pentose.



bath. The reagent was the same as described by Mejbaum (88). Equal volumes of the reagent and test solution are used and, after the development of the colour, 3 volumes of distilled water are added to 2 volumes of the mixture of test solution and reagent.

A 1 ml. aliquot of a solution containing 25 µg. of arabinose and 2 ml. of the orcinol reagent were added to several cylinders containing varying amounts of water and concentrated hydrochloric acid. After heating, all the solutions were diluted to 10 ml. and read on the colorimeter. Table II shows the results, which indicate that the conditions as described by Mejbaum are not necessarily optimal, but they are probably the most convenient because a ratio of 1:1 hydrochloric acid to water corresponds approximately to constant boiling hydrochloric acid. After the reaction mixture is diluted from 4 ml. (2 ml. of reagent and 2 ml. of test solution) to 10 ml., further dilution may be made with a solution containing 1 part of 0.1 per cent ferric chloride solution in concentrated hydrochloric acid to 4 parts of water, obtaining proportionate

TABLE II - Effect of volume and concentration of hydrochloric acid on colour development.

Water added (ml.)	Hydrochloric acid added (ml.)	Total volume (ml.)	Water hydrochloric acid ratio	Per cent light transmission
0	1	4	1 : 3	25.5
0	0	3	1 : 2	17.25
1	0	4	1 : 1	20.0
2	0	5	1 : 0.66	23.0

intensities of colour. However, the proportions of pentose sugar to reagent are critical for quantitative determination, therefore, solutions of the proper dilution must be prepared before developing the colour. Varying the time of standing at different stages of the procedure did not effect the results and the colour was stable overnight. With arabinose, yeast nucleic acid and wheat germ nucleoprotein the duration of heating is quite important as shown in Table III. All samples contained approximately the equivalent of 15 µg. of reacting pentose in the 2 ml. aliquot tested.

TABLE III - Effect of time of heating on colour development.

Time (minutes)	Per cent light transmission		
	Arabinose	Nucleic Acid	Nucleoprotein
20	48.0	46.75	42.75
30	42.5	43.0	40.0
40	41.0	42.25	38.75
50	41.25	41.75	38.0
60	40.5	41.25	37.0
70	40.75	40.75	37.5
100	40.5	41.0	37.25
120	40.75	41.0	36.75

Ribose gave maximum colour development in 20 minutes with very slight fading. Mejbaum (88) originally advocated

heating for 20 minutes but Schlenk (109) extended the time to 30 minutes and the method was so used by Davidson and Waymouth (100, 107) and Cori and Cori (108). The data in Table III might explain the inconsistencies in their results, e.g. heating for 60 minutes seems to be necessary for optimum colour development with arabinose, nucleic acid and wheat germ nucleoprotein. Some confirmation of these findings is contained in a paper by Albaum and Umbreit (110).

The protein present in nucleoproteins does not seem to interfere with the colour development. When the nucleic acid content of a nucleoprotein solution is determined by the orcinol method and by absorption in the ultraviolet, theoretical comparison is obtained. After removal of the protein from a nucleoprotein solution, the pentose is accounted for quantitatively.

IV. DETERMINATION OF HYDROGEN ION CONCENTRATION.

The accurate measurement of hydrogen ion concentration was a most important factor in this investigation. Bates et al. (111) have reviewed the major practical problems to be encountered. A Beckman pH meter, Model G, was used. Hydrogen ion concentration measurements on standard buffer solutions of the same pH (i.e. pH 7.0) as procured from various companies, showed a variation of as much as 0.5

of a pH unit. Therefore, a set of crystalline standards was purchased from the National Bureau of Standards, Washington, D. C. A 0.025 M phosphate buffer solution pH 6.87 at 22°C. and a 0.05 M acid potassium phthalate buffer solution pH 4.00 at 22°C. were prepared each month and used to check the electrodes. Two similar buffer solutions, prepared with ordinary chemicals, were standardized to be used daily in the checking of the instrument. New electrodes were frequently found to deviate as much as 0.5 of a pH unit at pH 4 when the instrument was standardized at pH 7. Accurate readings can be obtained between pH 7 and 4 only when the performance of the instrument is checked with reliable standards of hydrogen ion concentrations in the range of pH 4 - 7. An overall check on the accuracy with which these two buffer solutions were prepared, and on the reliability of the instrument and electrodes, and on the ability of the operator to measure small differences was desirable. Therefore, readings were made on a buffer solution of pH 4.00, while the instrument was standardized at pH 6.87, using all combinations of three calomel electrodes and four glass electrodes. The results are given in Table IV. The alkaline range was occasionally checked with 0.01 M sodium tetraborate (borax) buffer solution pH 9.20 at 22°C. prepared according to the National Bureau of Standards' instructions. The reading of the

TABLE IV - Reliability of the estimation of hydrogen ion concentration between pH 4.0 and 7.0.

Glass electrodes (No.)	Calomel electrode (No.)		
	I	II	III
1	4.01	4.01	4.00
2	4.01	3.99	4.00
3	4.00	3.99	4.01
4	4.00	4.00	3.99

instrument, standardized at pH 7, was accurate within 0.05 of a pH unit. At higher alkalinities, the readings were probably much less accurate. The hydrogen ion concentrations of thick colloidal solutions and alcoholic solutions were measured with difficulty as the instrument took a long time to reach a steady reading.

V. OTHER DETERMINATIONS.

(a) Moisture:

Before sampling, the preparation to be analyzed was allowed to come to equilibrium with atmospheric moisture. Small glass weighing bottles were dried to constant weight and weighed. They were then allowed to stand exposed to the atmosphere for one hour and reweighed. About 0.2 g. of the sample was then added and weighed. The samples were dried for six hours in a vacuum oven at about a pressure of 2 mm. mercury and a temperature of 70 - 75°C. At the

end of this period air was admitted into the oven through concentrated sulphuric acid. The samples were placed in a dessicator for 20 minutes and then weighed. Drying was repeated for two hour periods until constant weight was reached. The weight of the weighing bottle plus sample minus the weight of the bottle after standing one hour exposed to the atmosphere is the weight of the sample. The constant weight reached after drying minus the weight of the dried weighing bottle is the weight of the dry matter in the sample. A micro-balance weighing to the fifth decimal place was used, but, in spite of all precautions, an error greater than 2 per cent was found between triplicate determinations. Therefore, the values for moisture are not significant beyond the first decimal place.

(b) Phosphorus:

Total phosphorus was determined gravimetrically as described in the "Book of Methods of the Association of Official Agricultural Chemists" (112). A 0.3 g. portion of nucleic acid (equivalent to about .03 g. of phosphorus) was digested and put into solution as described in Section 2.8 (c) page 22. The determination was carried out on the whole sample. Phosphorus was weighed as anhydrous magnesium pyrophosphate, but the results were calculated as per cent phosphorus in the dry sample. The values reported are

averages of three determinations which checked within 0.5 per cent.

(c) Sodium:

Sodium was determined gravimetrically by precipitation as sodium magnesium uranyl acetate as described in the "Book of Methods of the Association of Official Agricultural Chemists" (113). A 0.2 g. sample of nucleic acid was used which gave a precipitate weighing between 0.1 and 0.6 g. Triplicate determinations showed an error of about 4 per cent. Results expressed as per cent sodium in sample at equilibrium with atmospheric moisture are reported only to the first decimal place.

EXPERIMENTAL

I. WATER EXTRACTION OF A PROTEIN FRACTION FROM WHEAT GERM.

(a) Non-protein nitrogen fraction:

The nitrogen-containing compounds which are extracted by water and are soluble in trichloroacetic acid are referred to here as non-protein nitrogen. The value representing the difference between the total nitrogen extracted from wheat germ and the non-protein nitrogen is taken as a measure of the protein nitrogen extracted, this value being utilized as a criterion of the most effective and least denaturing extraction procedure. The results of non-protein nitrogen estimations on aqueous extracts of wheat germ were quite variable and apparently depended on details in the procedure; therefore, it was found necessary to study the factors responsible for these variations.

Two similar extraction procedures were compared with one which was quite different, i.e. procedure I involved shaking wheat germ with an appropriate aliquot of a trichloroacetic solution for 20 minutes and then filtering through a Whatman 42 filter paper. Procedures II and III involved shaking the germ with an appropriate aliquot of distilled water for 20 minutes, then adding trichloroacetic acid to give the desired concentration and filtering through Whatman 42 filter paper either after standing for 10 minutes or immediately. As these determinations were done on small

aliquots (5 ml.) of the water extracts, filtering did not take more than 30 seconds. The nitrogen determinations were carried out on the filtrates, and the non-protein nitrogen was expressed as per cent of the nitrogen content of the wheat germ. These procedures were compared with various concentrations of wheat germ and with different strengths of trichloroacetic acid. The results are given in Table V and Fig. 2.

TABLE V - Non-protein nitrogen in wheat germ extracts.

Wheat germ : water ratio.	Pro-cedure *	N.P.N. as per cent of total nitrogen in the wheat germ extracted Conc. of trichloroacetic acid in per cent 1	2	5	10
1:80	1	13.1	12.9	13.1	17.0
	2	18.3	14.7	12.7	14.7
	3	19.0	16.5	11.9	12.1
1:40	1	12.1	11.7	12.3	15.2
	2	16.2	13.8	12.3	13.9
	3	18.1	16.0	11.8	11.7
1:10	1	11.4	11.4	11.9	16.0
	2	15.9	13.1	12.0	14.5
	3	16.2	11.9	10.9	10.6

* 1) Extracted 20 minutes with trichloroacetic acid and immediately filtered.

2) Extracted 20 minutes with water, trichloroacetic acid then added and filtered after 10 minutes.

3) Same as 2) but filtered immediately.

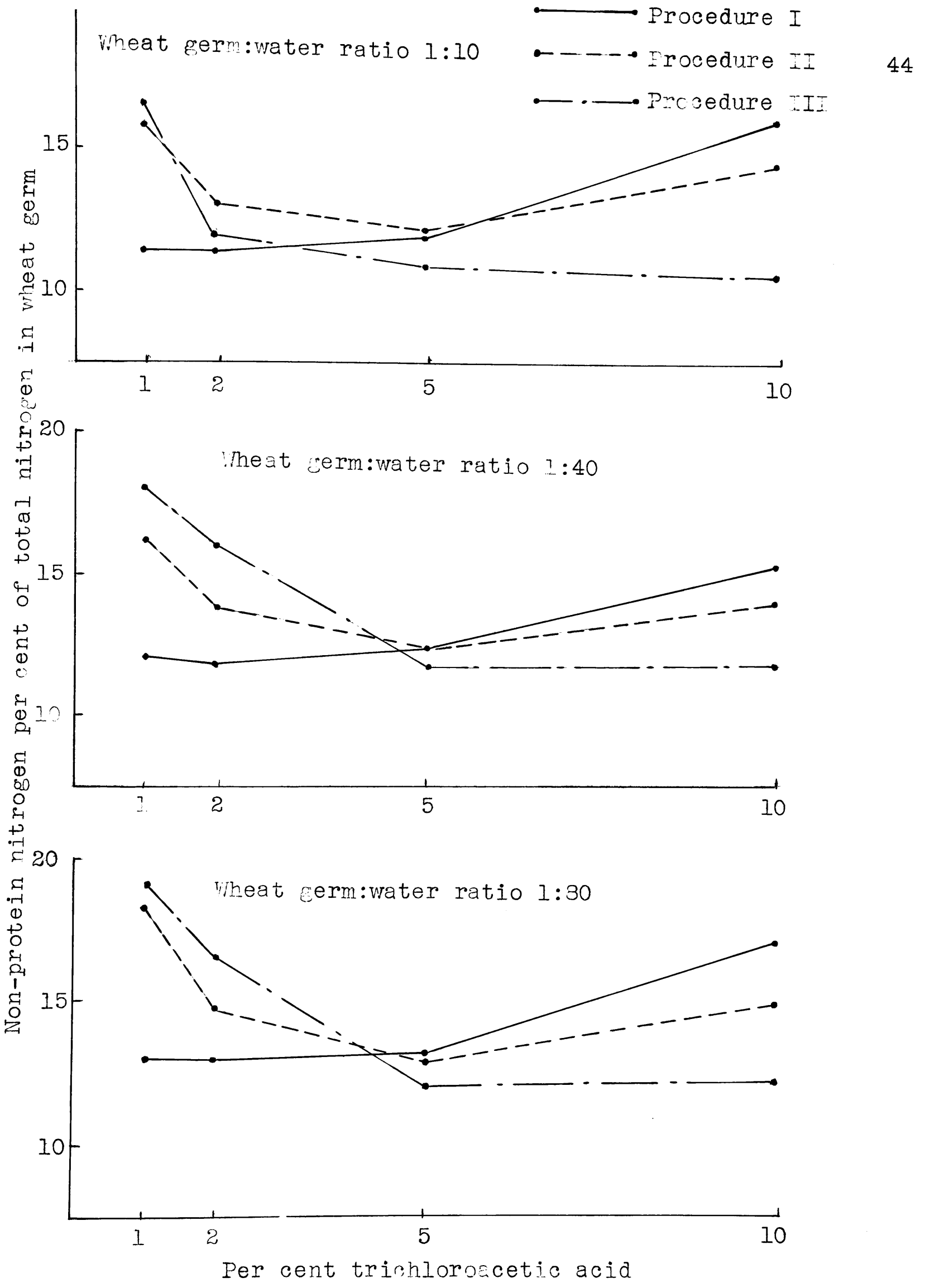


Figure 2 - Non-protein nitrogen in wheat germ extracts.

Incomplete precipitation and hydrolysis by the trichloroacetic acid are the two major sources of error and they both tend to give high results. Therefore, the lowest values are more likely to be an indication of the non-protein nitrogen content of the extracts. Extracting wheat germ with trichloroacetic acid solution (Procedure I) requires a lower trichloroacetic acid concentration to obtain minimal values; higher concentrations of trichloroacetic acid apparently hydrolyze the proteins. The precipitation of protein nitrogen from a water extract of wheat germ by trichloroacetic acid (Procedures II and III) seems to depend largely on adherence to the details of the procedure. Allowing to stand for ten minutes after the addition of trichloroacetic acid and before filtering resulted in more efficient precipitation at lower concentration, but more marked hydrolysis at higher concentration. The results also indicate that in the case of the less concentrated extracts hydrolysis occurs more easily, whereas with highly concentrated extracts more trichloroacetic acid is needed to give complete precipitation of protein nitrogen.

Trichloroacetic acid extraction (Procedure I) was therefore discarded, because we are interested here in non-protein nitrogen only as a means of deducing what fraction of the total nitrogen extracted with water from wheat germ is protein in nature. Of the two procedures

involving the precipitation of the protein nitrogen from a water extract, that employing immediate filtration after the addition of trichloroacetic acid was chosen for further use as it gave lower results, probably because there was less protein hydrolysis, as evidenced by the fact that when the concentration of trichloroacetic acid was increased to 10 per cent the same results, or lower results in the case of the more concentrated extracts, were obtained.

TABLE VI - Effect of trichloroacetic acid concentration on the non-protein nitrogen determination.

Trichloroacetic acid concentration (per cent)	Non-protein nitrogen as per cent of total nitrogen in wheat germ		
	Wheat germ : Water ratio		
	1:80	1:40	1:10
1	19.0	18.1	17.6
2	16.5	16.0	14.6
3	13.0	13.3	12.7
4	12.7	12.5	11.9
5	11.9	11.8	11.3
6	11.7	11.8	11.2
7	12.0	11.7	11.0
8	12.1	11.7	10.5
10	12.1	11.7	10.0

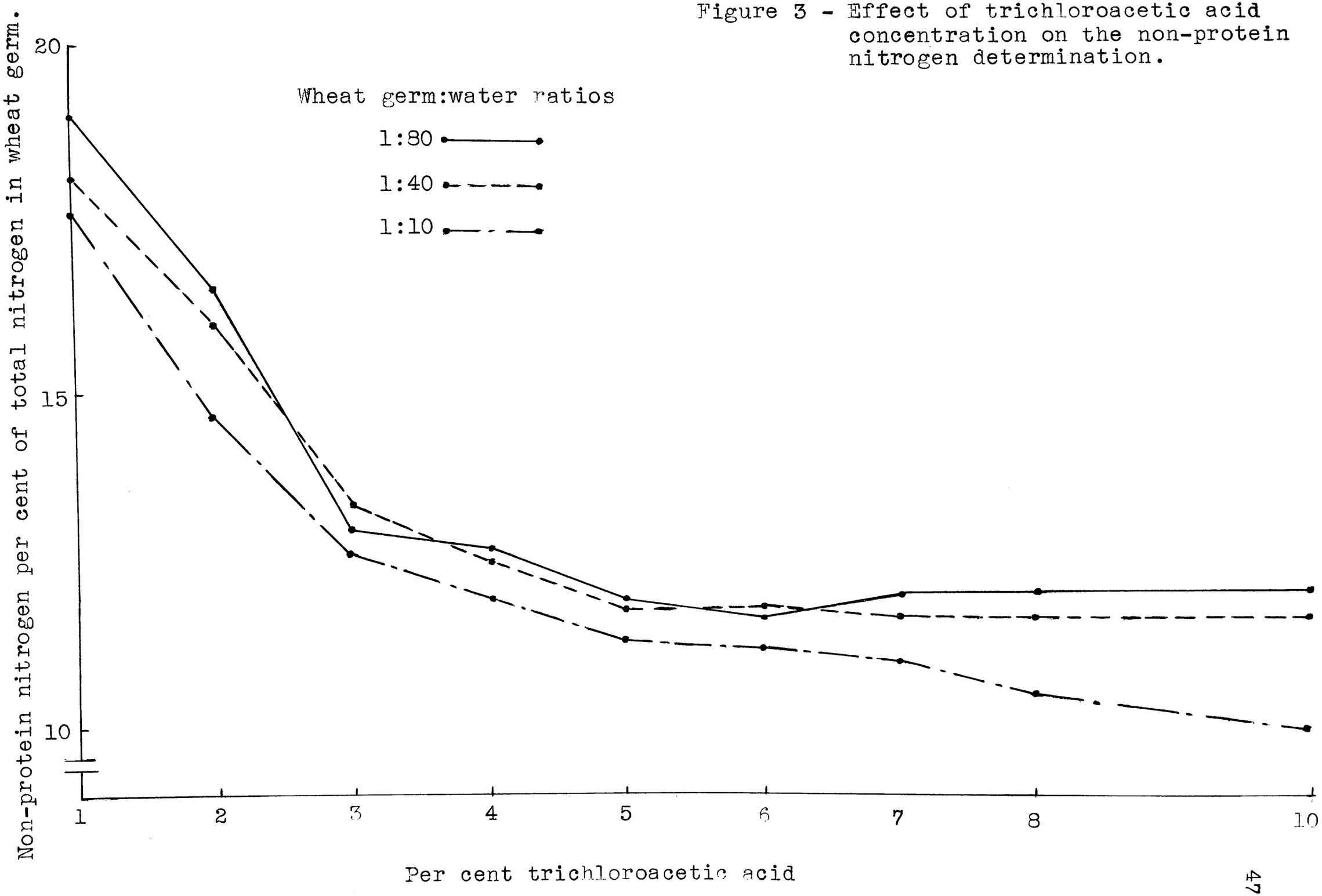


Figure 3 - Effect of trichloroacetic acid concentration on the non-protein nitrogen determination.

The results presented in Table VI and illustrated in Figure 3 show that when wheat germ is extracted for 20 minutes with water and the extract immediately filtered after the addition of the acid, a concentration of 10 per cent trichloroacetic acid is required to obtain minimal non-protein nitrogen values in the case of extracts obtained from one part of wheat germ to 10 parts of water, while extracts with wheat germ : water ratio of 1:40 and 1:80 need only 5 per cent trichloroacetic acid. More non-protein nitrogen is present in the less concentrated extracts. This might indicate that with the more dilute extracts (1) more breakdown occurs during extraction or (2) the hydrolysis is affected more by trichloroacetic acid or (3) all the nitrogenous compounds are not precipitated. The second possibility seems most plausible because the non-protein nitrogen content is almost constant with increasing concentrations of trichloroacetic acid from 5 to 10 per cent. However, it may be that the hydrolysis is counter-balanced by a more complete precipitation. Suppositions (1) and (3) mean essentially the same thing, except (1) involves an artifact while (3) is based on the solubility of the nitrogenous compounds in wheat germ. It must be pointed out here that care was taken to adjust all the solutions to the same final volume regardless of the ratio of wheat germ to water in

extraction or the concentration of trichloroacetic acid in precipitation. Concentrations of trichloroacetic acid exceeding 10 per cent were not tested because of the impracticability.

Since the lowest non-protein nitrogen content was chosen at the outset as a criterion of the best procedure, it was decided to precipitate the protein nitrogen from an aqueous extract of wheat germ using 10 per cent trichloroacetic acid and filtering immediately through a Whatman 42 filter paper.

(b) Extraction of protein nitrogen with water:

The next objective was to obtain maximum extraction of protein nitrogen from wheat germ under the mildest conditions and keeping the volumes to a workable level. In the following experiments certain experimental details were kept constant so that the results could be compared. In the extraction the volume of water was always 50 ml. and a 125 ml. Erlenmeyer flask was used. Different amounts of wheat germ were added and the flask was shaken on a mechanical shaker at room temperatures of 22 - 24°C. The duration of shaking is referred to as the extraction time. As soon as the flask was removed from the shaker the solution was filtered through ordinary filter paper and aliquots were taken for total nitrogen and non-protein nitrogen determinations as soon as enough filtrate was

obtained (about 10 ml.). To 5 ml. of filtrate a 5 ml. aliquot of 20 per cent trichloroacetic acid solution was added, the solution immediately filtered through a Whatman 42 filter paper and an aliquot taken for nitrogen analysis. The results are expressed as per cent of the nitrogen in wheat germ to indicate how the nitrogenous compounds are fractionated.

TABLE VII - Effect of extraction time on total nitrogen extracted.

Extraction time (hours)	Total nitrogen extracted as per cent of nitrogen in wheat germ.	
	Wheat germ : water ratio	
	1:20	1:40
0.25	60.2	61.2
0.50	60.5	61.8
1.0	59.0	61.0
2.0	57.0	62.0
4.0	56.7	58.2
6.0	57.5	60.0

Table VII and Figure 4 show the effect of extraction time on the total nitrogen extracted employing two concentrations of wheat germ. Maximum extraction is definitely achieved in less than an hour and prolonged extraction reduces the solubility of the nitrogen compounds. The decrease in solubility with increasing extraction time

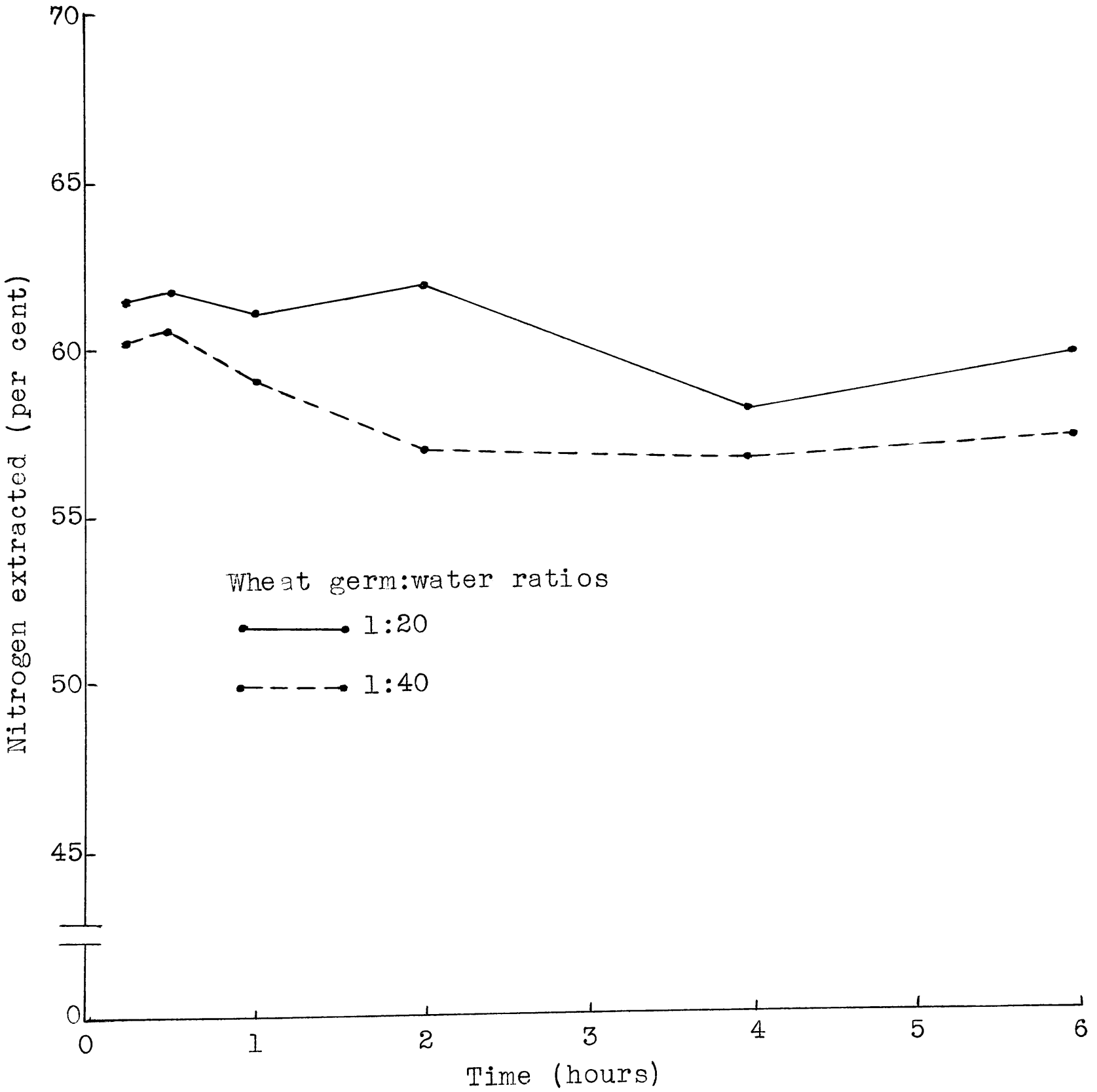


Figure 4 - Effect of extraction time on the total nitrogen extracted from wheat germ.

might be explained as being due to the denaturation of the proteins and it was noted that foaming occurred. After six hours the solubility starts to increase again which might be due to more advanced denaturation and/or bacterial decomposition. The results comparing different concentrations of wheat germ are hard to explain but the more dilute extracts show more complete extraction.

The effect of extraction time was next studied in more detail and estimations of non-protein nitrogen were also carried out. The results are shown in Table VIII and Figure 5.

TABLE VIII - Effect of extraction time on nitrogen extracted.

Wheat germ : water ratio.	Extraction time (minutes)	Total N. as per cent of N. in wheat germ.	N.P.N. as per cent of N. in wheat germ.	Calculated protein N. as per cent of N. in wheat germ.
1:15	0	41.7	8.4	33.3
	5	57.0	10.2	46.8
	10	60.5	10.6	49.9
	20	59.5	10.8	48.7
	30	59.2	11.0	48.2
	45	59.8	11.2	48.6
1:20	0	39.6	8.2	31.4
	5	55.0	9.8	45.2
	10	58.3	10.0	48.3
	20	57.7	10.6	47.1
	30	57.8	10.5	47.3
	45	58.4	10.7	47.7

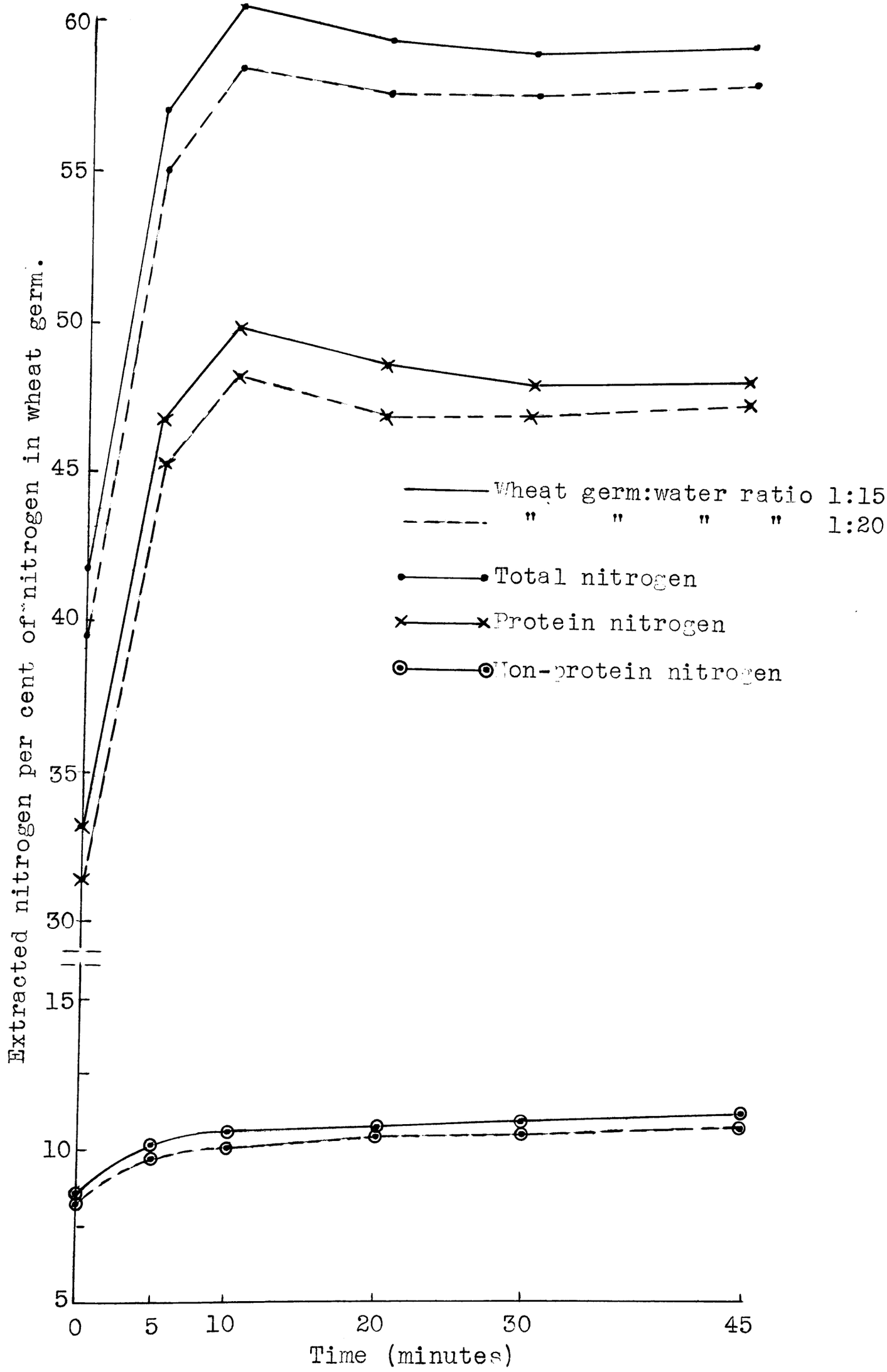


Figure 5 - Effect of extraction time on nitrogen extracted.

The results show no appreciable difference between extraction times of 10 and 45 minutes and, therefore, it is preferable to extract for the shortest time to avoid denaturation of the protein. The two ratios of wheat germ to water chosen are too close to show any marked difference, but the two experiments are useful in showing that the results can be duplicated. Almost 50 per cent of the nitrogen is extracted as protein nitrogen by shaking for 10 minutes with water at room temperature and this extraction procedure was adopted in future work.

In the next experiment the effect on nitrogen extraction of varying the wheat germ : water ratio was determined. Two samples of wheat germ were used, one was freshly milled and the other had been stored for two months at 5°C. The results are shown in Table IX and Figure 6. These results show that protein nitrogen is more readily extracted from fresh wheat germ than from stale wheat germ and this difference is more marked with the more concentrated suspensions of wheat germ. Extraction with higher concentrations of fresh wheat germ seems to be most satisfactory, because on dilution changes apparently occur which reduce the solubility of the protein. This is also shown by the fact that the value for non-protein nitrogen is almost constant in the case of the stale wheat germ, but increases with dilution in the case of the fresh sample. A ratio of

TABLE IX - Effect of wheat germ : water ratio on extracted nitrogen.

Wheat germ sample	Wheat germ : water ratio.	Total N. as per cent of N. in sample of wheat germ	N.P.N. as per cent of N. in sample of wheat germ	Calculated protein N. as per cent of N. in wheat germ.
Freshly Milled	1 : 10	61.2	10.6	50.6
	1 : 15	61.0	12.0	49.0
	1 : 20	60.5	11.8	48.7
	1 : 40	60.0	13.7	46.3
	1 : 80	62.5	15.7	46.8
Two months old	1 : 10	56.1	10.4	45.7
	1 : 15	55.2	10.0	45.2
	1 : 20	55.4	10.3	45.1
	1 : 40	56.4	10.5	45.9
	1 : 80	56.7	11.4	45.3

one part of wheat germ to 5 parts of water was also tried, but it was difficult to handle because the extract was too viscous. Ratios of 1 : 10 and 1 : 20 with a 10 minutes extraction time are acceptable.

Assuming that the volume of the extract is equal to the amount of water added, although naturally some is absorbed by the residue, 45 to 50 per cent of the nitrogen in wheat germ is extracted as protein nitrogen, and 55 to

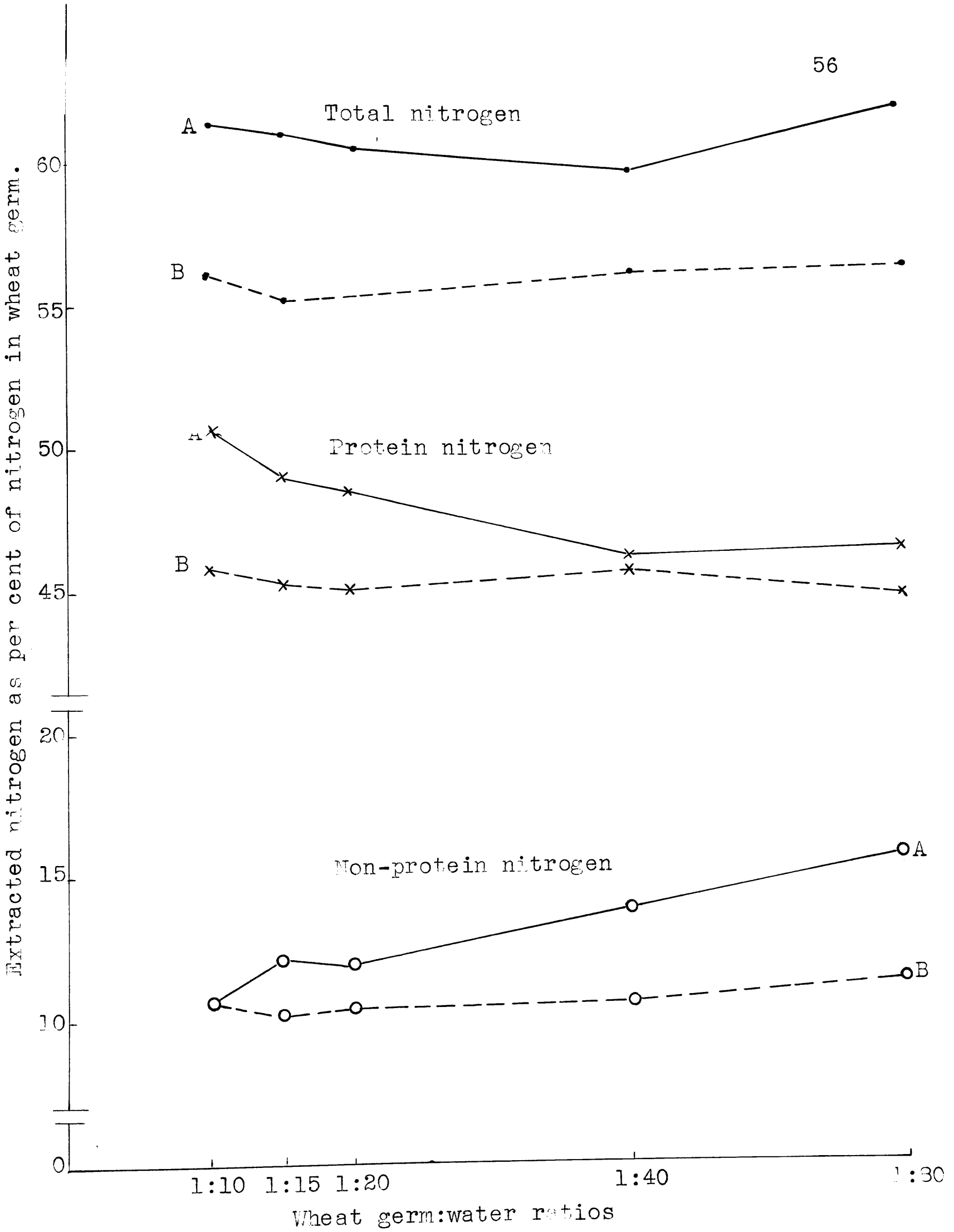


Figure 6 - Effect of the wheat germ:water ratio on the extracted nitrogen.

(A - fresh wheat germ
B - stale wheat germ)

61 per cent as total nitrogen, the values depending on the freshness of the wheat germ and the extent of denaturation during extraction.

(c) Repeated extractions with water:

In an attempt to ascertain if more protein is recovered by successive extractions and to determine if other less soluble protein fractions are extracted, the following experiments were carried out: To 2 g. of wheat germ was added 30 ml. of water (ratio 1 : 15), the suspension shaken for 10 minutes and centrifuged for 10 minutes. The volume of the decanted supernatant was measured and the solution analyzed for nitrogen. From these figures the amounts of decanted nitrogen and the "left-over" extracted nitrogen were calculated, assuming that the liquid wetting the wheat germ residue had the same nitrogen content as the solution decanted. The sum of these two values represents the nitrogen extracted in the first extraction. The residue, after being shaken for 10 minutes with 30 ml. of distilled water was again centrifuged. From the analysis of the supernatant the decanted nitrogen and the "left-over" nitrogen were again calculated. Subtracting the "left-over" nitrogen of the previous extraction from the sum of these two values, the nitrogen extracted in the second extraction is calculated. The

results for subsequent extractions were calculated in a similar manner. The complete data are given in Table X.

TABLE X - Repeated extraction of wheat germ with water (Ratio 1 : 15).

Extraction	N. conc. in extract mg./ml.	Decanted N. in per cent	Left-over N. in per cent (calculated)	Extracted N. in per cent (calculated)
1st	2.3	60.8	4.8	65.6
2nd	0.3	8.7	0.4	4.3
3rd	0.1	2.7	0.3	2.6
4th	0.05	1.3	0.1	1.1
5th	0.03	0.8	0.0	0.8

The data are only relative but it shows that wheat germ contains an easily soluble group of nitrogenous compounds, and that the nitrogen extracted in successive extractions is much less soluble and insignificant in amount.

Table XI gives the results of a similar experiment but with a wheat germ : water ratio of 1 : 3 in the first extraction and a 1 : 2 ratio in the succeeding six extractions. The first extract contained almost one per cent of nitrogen, which indicates the high solubility of this nitrogen fraction. In the second extraction the "left-over" nitrogen of the first extraction depresses the solubility of the less soluble nitrogen, which is again more soluble in the third extraction.

TABLE XI - Repeated extraction of high concentrations of wheat germ with water.

Extraction	N. conc. in extract mg./ml.	Decanted N. in per cent	"left-over"N. in per cent (calculated)	Extracted N. in per cent (calculated)
1st	9.5	36.0	18.0	54.0
2nd	3.2	13.1	6.1	1.2
3rd	1.9	8.1	3.3	5.3
4th	1.1	4.6	1.9	3.2
5th	0.7	2.9	1.3	2.3
6th	0.4	1.8	0.8	1.3
7th	0.3	1.2	0.5	0.9

From these observations it is concluded that to obtain a uniform extraction it is advisable to extract once only and with no more than 15 volumes of water. Perhaps 10 volumes of water would be better and so obtain a nitrogen content above 3.2 mg. per ml. of extract, which is the value obtained in the second extraction (Table XI) and where apparently the solubility of the least soluble nitrogenous compounds is depressed.

II. THE ISOELECTRIC PRECIPITATION OF NUCLEOPROTEIN FRACTIONS.

(a) Optimum hydrogen ion concentration for precipitation of nucleoprotein.

Twenty ml. aliquots of a wheat germ extract, containing 60.5 per cent of the nitrogen of the sample in a

concentration of 2.2 mg. per ml., were placed in 50 ml. centrifuge tubes; to each was added different volumes of distilled water and 0.05 N hydrochloric acid to give a final volume of 10 ml. After standing one hour, the tubes were centrifuged for 10 minutes at 3000 r.p.m. and the hydrogen ion concentration and nitrogen content of the supernatant were determined. The results are shown in Table XII and Figures 7 and 8. Maximum precipitation of the nitrogenous compounds is obtained at pH 4, but the slope of the curve in the region of the maximum is not very steep and there are only slight differences between pH 3.8 and 4.2. About 68 per cent of the nitrogen is precipitated; that is, 32 per cent remains in solution. From the data in Table IX it is observed that about 19 per cent of the extracted nitrogen is non-protein nitrogen, therefore, about 13 per cent of the nitrogen remaining in solution is protein nitrogen. The graph obtained by plotting the amount of hydrochloric acid added against the hydrogen ion concentration (Fig. 8) is similar to a protein titration curve with the onset of buffering at $>$ pH 6 and a strong buffer region from $<$ pH 4.

Table XIII shows the effect of time of standing after adding hydrochloric acid to an extract, exactly the same amount of acid being added in all cases. One hour standing gives maximum precipitation and longer periods are

TABLE XII - Effect of hydrochloric acid on nitrogen precipitation and hydrogen ion concentration of an extract.

Hydrochloric acid (ml. of 0.05 N)	pH	Nitrogen precipitated (per cent)
0	6.75	0
1	6.3	11.5
2	5.9	37.8
3	5.35	52.7
4	4.8	62.3
4.5	4.65	64.0
5	4.3	66.9
5.5	4.2	67.5
6	4.0	68.1
6.5	3.8	67.5
7	3.6	66.2
8	3.3	64.5
9	3.1	63.8
10	2.9	57.1

not so effective, probably because protein breakdown increases the solubility of the nitrogen.

The proteins precipitated had a high content of phosphorus and pentose. It was assumed that these components were contributed by ribonucleic acid, and that the wheat germ ribonucleic acid is identical with yeast nucleic

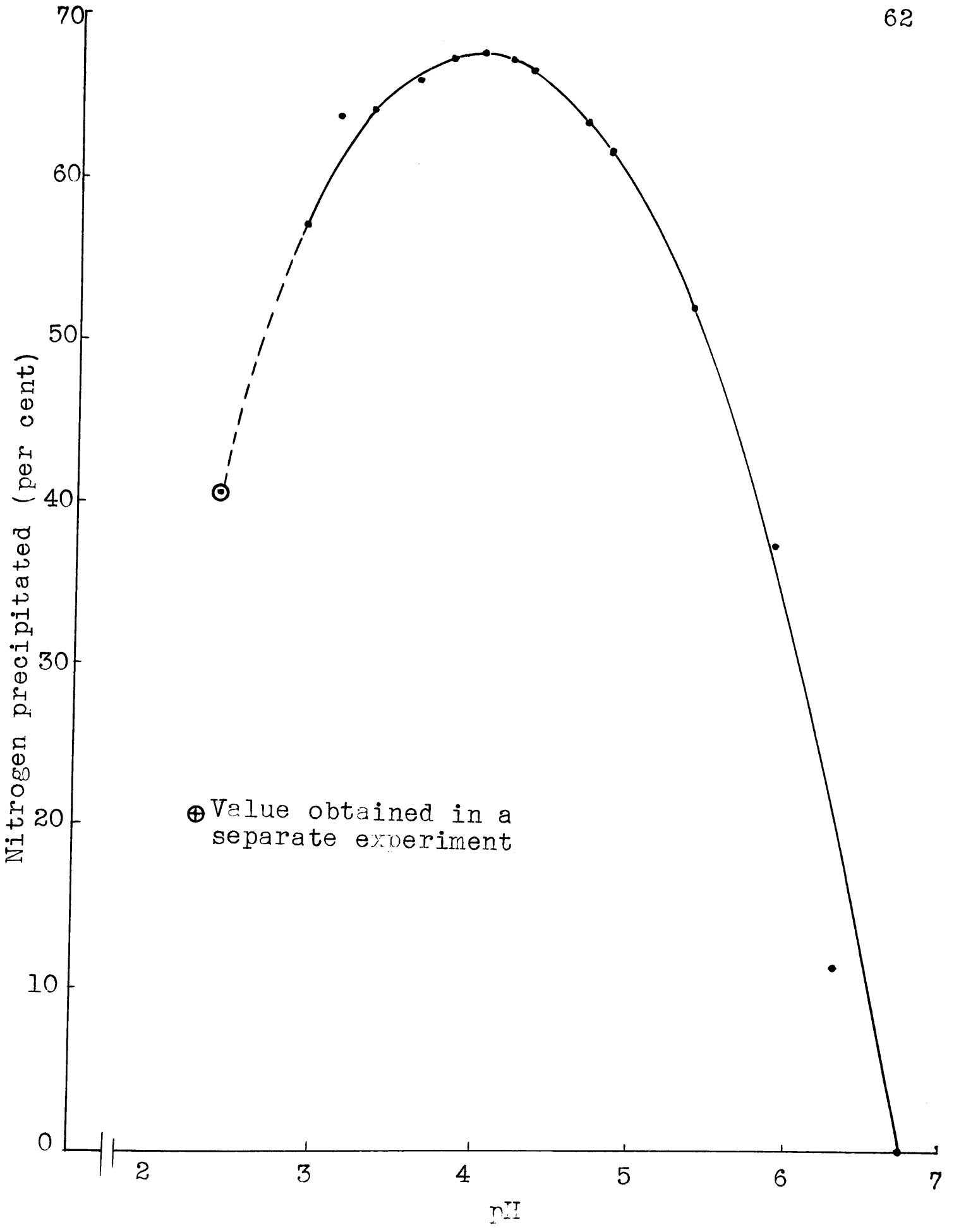


Figure 7 - Effect of hydrogen-ion concentration on nitrogen precipitated.

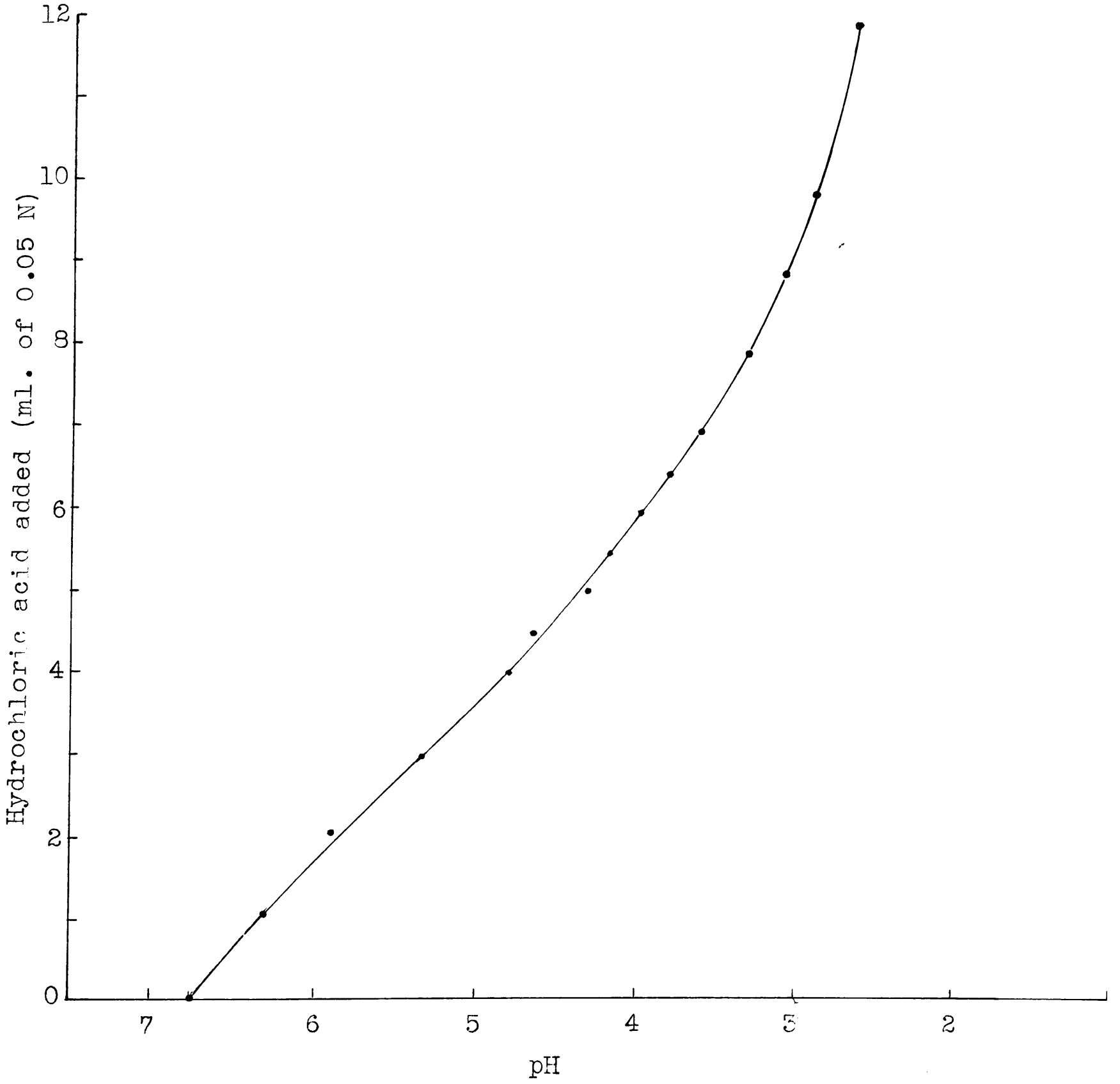


Figure 8 - Effect of hydrochloric acid on the hydrogen-ion concentration of an extract.

TABLE XIII - Nitrogen precipitation as influenced by time of standing.

Time of Standing (hrs.)	pH	Nitrogen precipitated (per cent)
0.0	4.10	59.9
0.25	4.10	61.8
0.50	4.10	63.5
0.75	4.15	66.5
1.00	4.15	67.2
2.50	4.20	62.6
4.50	4.20	60.2

acid. It was also assumed that the fundamental unit of the complex nucleic acid was the tetranucleotide structure as postulated by Levene (16) and that exactly 50 per cent of the ribose reacted in the quantitative orcinol test. On the basis of these assumptions the proteins contained about 21 per cent nucleic acid and they were called nucleoproteins.

(b) Reprecipitation of nucleoprotein.

A 1:15 extract of 160 g. of fresh wheat germ was prepared and 60.9 per cent of the nitrogen was extracted. The hydrogen ion concentration was adjusted to pH 4 by careful addition of dilute hydrochloric acid and after one hour the precipitate was collected by centrifuging and the supernatant analyzed for nitrogen. The precipitate

was resuspended in a smaller volume of distilled water at a hydrogen ion concentration lower than pH 7 by adding a small volume of a dilute sodium hydroxide solution and the solution analyzed. After readjusting to pH 4, a second precipitate was collected and a third precipitation was carried out in a similar manner. The results are shown in Table XIV.

TABLE XIV - Successive precipitation of the nucleoprotein fraction.

Precipitation	pH	Nitrogen concentration in extract (mg./ml.)	Nitrogen concentration in supernatant (mg./ml.)	Nitrogen precipitated Per cent of extracted N. (calculated)	Nitrogen precipitated Per cent of wheat germ N. (calculated)
1st	4.05	2.4	0.86	63.7	38.7
2nd	4.10	2.5	0.21	90.0	34.8
3rd	4.05	4.0	0.31	93.3	32.5

The volumes involved in each extraction were carefully noted to permit calculation of the values shown. In the third precipitation 93 per cent of the nitrogen is recovered. Most of the non-protein nitrogen and the protein nitrogen not precipitated at pH 4 was discarded in the supernatants. The final precipitate weighed 19 g. (air dry) and contained 15.19 per cent nitrogen and 4.81 per cent pentose (reacting), which is equivalent to 20.8 per cent nucleic acid. Therefore, about 4 g. of nucleic acid

were recovered from 160 g. of wheat germ. Thus, wheat germ contains at least 2.5 per cent of ribonucleic acid as a nucleoprotein.

A similar experiment was carried out using a 1:15 extract of 100 g. of stale wheat germ containing only 52.2 per cent nitrogen (Table XV). Five precipitations were carried out and a portion of each precipitate was analyzed for nitrogen and pentose. The precipitate was dried with absolute ethyl alcohol and ether and allowed to come to moisture balance with the air. The recoveries were less than in the previous experiment, even slightly less than the lower extraction would account for. For example, the extraction obtained in the experiment recorded in Table XV is 85.6 per cent as efficient as the extraction obtained in the experiment recorded in Table XIV, but the nucleoprotein recovered on the third precipitation recorded in Table XV is only 79.9 per cent of the nucleoprotein recovered on the third precipitation recorded in Table XIV. The values for nitrogen and pentose show that the precipitates are fairly similar; part of the differences might be due to moisture which cannot be measured because the samples are too small. More than 97 per cent of the nitrogen is recovered in the fifth precipitation.

A study of the solubility of these nucleoproteins at

TABLE XV - Effect of successive precipitations on the nucleoprotein fraction.

Precipitation.	pH	Nitrogen concentration in extracts (mg./ml.)	Nitrogen concentration in supernatant (mg./ml.)	Nitrogen precipitated Per cent N. in extract (calculated)	Nitrogen precipitated Per cent N. in wheat germ (calculated)	Composition of precipitate (air dry basis) Nitrogen (per cent)	Composition of precipitate (air dry basis) Reacting pentose (per cent)	Composition of precipitate (air dry basis) Nucleic acid (per cent)
1st	4.20	2.0	0.78	60.4	31.4	14.8	4.5	19.5
2nd	4.17	2.1	0.23	89.5	28.1	15.3	4.8	20.8
3rd	4.09	2.2	0.14	92.4	25.9	15.4	5.1	21.8
4th	3.95	2.3	0.12	95.5	24.8	15.3	4.7	20.4
5th	4.05	3.4	0.10	97.5	24.2	15.1	4.6	20.0

various hydrogen ion concentrations and after two and three precipitations was carried out and the data are shown in Tables XVI and XVII and Figure 9.

TABLE XVI - Effect of hydrogen ion concentration on solubility of a nucleoprotein reprecipitated three times.

pH	Nitrogen in supernatant (mg./ml.)	Precipitated Nitrogen (per cent)
9.65	0.510	0.0
7.5	0.505	1.0
6.27	0.502	1.5
5.43	0.475	6.9
4.98	0.288	41.6
4.52	0.112	78.2
4.40	0.025	95.0
4.10	0.013	97.5
3.75	0.022	96.0
3.37	0.048	91.0
3.00	0.104	79.6
2.58	0.325	36.3

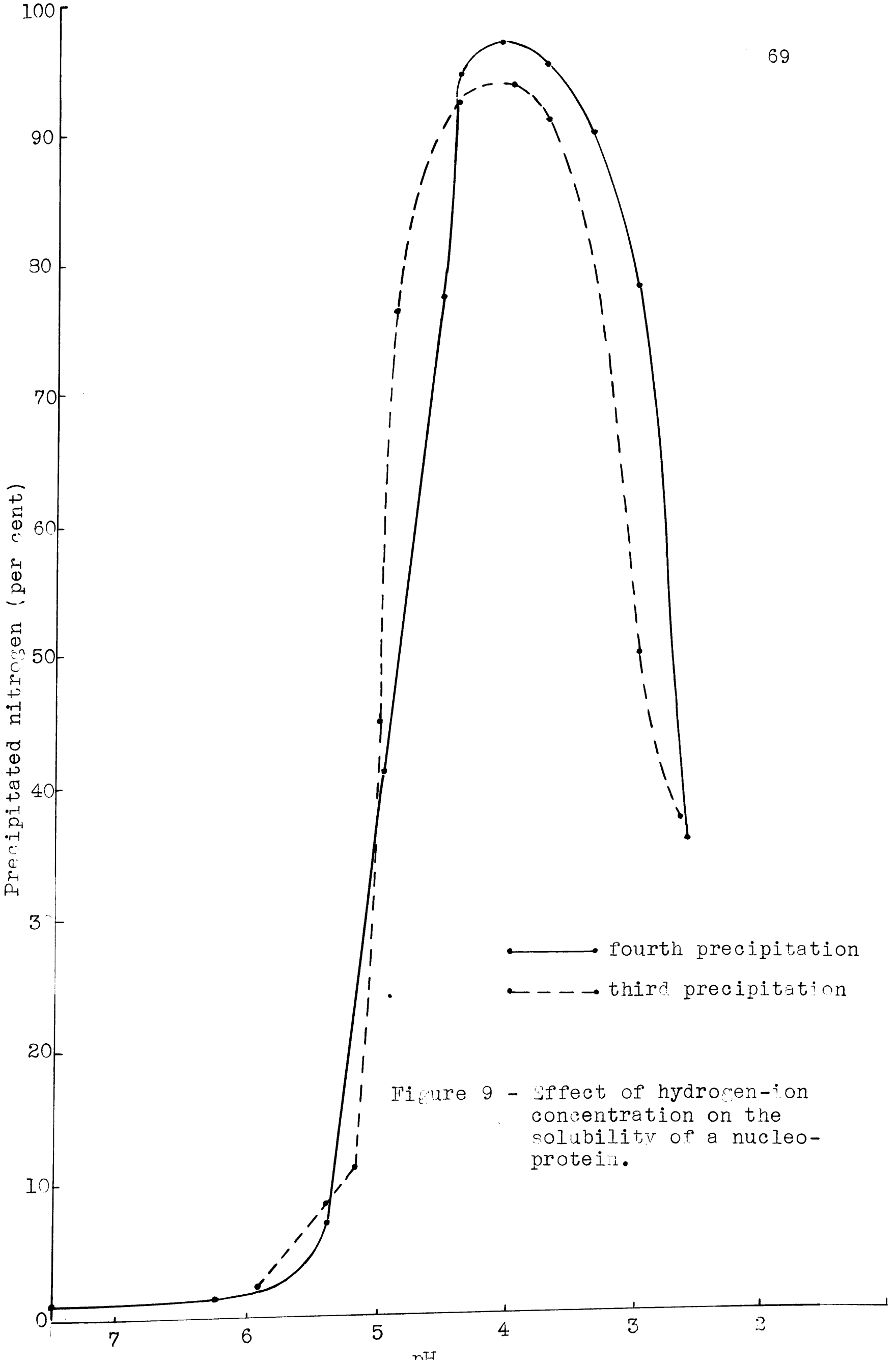


Figure 9 - Effect of hydrogen-ion concentration on the solubility of a nucleo-protein.

TABLE XVII - Effect of hydrogen ion concentration on solubility of a twice precipitated nucleoprotein.

pH	Nitrogen precipitated (per cent)	Pentose precipitated (per cent)	Calculated purity of nucleic acid remaining in solution.
5.95	2.1	1.2	19.8
5.65	5.4	1.8	20.3
5.40	8.5	4.8	20.4
5.20	11.3	5.0	21.1
5.00	45.2	34.6	21.5
4.90	76.9	73.6	22.3
4.45	92.9	91.2	24.7
4.00	94.5	98.1	18.9
3.70	92.0	94.4	15.0
3.00	50.5	61.9	15.2
2.60	37.4	48.7	16.0

Again the nucleoproteins are least soluble at pH 4. The two curves are very similar to that in Figure 7 except that repeated precipitation gives a greater recovery in the precipitate and the hydrogen ion concentration for precipitation is more critical. Table XVII shows that pH 4 is also maximal for precipitation of pentose (i.e. nucleic acid) and in the last column the per cent nucleic acid in the supernatant is given as calculated from the

nitrogen : pentose ratio (i.e. disregarding any contaminants not containing nitrogen). These figures show that there is considerable variability in the composition of the nucleoproteins precipitated at different hydrogen ion concentrations. At pH 4 the nucleoprotein precipitated may have a slightly higher nucleic acid content than the solution from which it is derived.

(c) Variability of the composition of nucleoproteins.

From a study of the data recorded in the last column of Table XVII, it was realized that nucleoproteins of different nucleic acid content could be isolated depending upon the hydrogen ion concentration for precipitation. This was confirmed in several experiments not recorded in this thesis. Quantitative results cannot be reported because substances are present in the aqueous extract of wheat germ which interfere with the pentose determination. Double precipitation of the nucleoproteins is recommended, otherwise the pentose test cannot be regarded as entirely reliable. This variability in the composition of nucleoproteins may be due either to the fact that in nature there exists a series of nucleoprotein complexes with different percentages of nucleic acid, or, that, during the isolation and precipitation some of the protein is denatured. Possibly both factors are involved. These

observations, however, do not alter the earlier conclusion that precipitation at pH 4 is best for a maximum recovery of nucleic acid in nucleoproteins containing about 21 per cent nucleic acid.

The following experiment indicates the possibility of isolating nucleoproteins of different nucleic acid content from the same solution. A 500 g. portion of wheat germ was extracted for 20 minutes with 5 liters of water. The nucleoproteins were twice precipitated at pH 4 and resuspended in water containing sufficient sodium hydroxide to give 1200 ml. of solution of pH 6.8 (Solution A). A 500 ml. aliquot of Solution A was adjusted to pH 6.0 with 0.25 normal hydrochloric acid, and centrifuged after standing 3 hours (Solution B). The residue from Solution B was precipitated three times at pH 6 and redissolved (Solution C). The supernatants from these three precipitations were combined (Solution D). Supernatant Solution B was adjusted to pH 5 and centrifuged (Solution E). The residue from Solution E was precipitated three times at pH 5 and redissolved (Solution F). The supernatants from these three precipitations were again combined (Solution G) The Solutions C, D, F, G, and E were analyzed for nitrogen and pentose and were of known volume so that the results could be calculated as per cent of the original amount in the 500 ml. aliquot of Solution A. The total

recovery was also calculated (H) as a percentage of the original. These data are presented in Table XVIII.

TABLE XVIII - Separation of various nucleoprotein fractions from a nucleoprotein solution.

Letter in text	Description of Material	Fresh Nucleoprotein Solution			Nucleoprotein Solution stored at pH 8.8		
		As per cent of total		Calculated per cent nucleic acid in fraction	As per cent of total		Calculated per cent nucleic acid in fraction
		Nitrogen	Pentose		Nitrogen	Pentose	
C	Precipitated 3 times at pH 6	27.5	19.1	21.2	23.8	16.6	16.8
D	Combined supernatants at pH 6	38.4	34.4	21.8	41.2	32.9	19.4
F	Precipitated 3 times at pH 5	17.2	16.6	23.3	29.7	28.1	23.2
G	Combined supernatants at pH 5	2.9	3.5	29.4	1.8	3.2	43.2
E	Left in solution after removal of the two precipitates	9.9	23.9	58.6	8.2	20.9	62.0
H	Total recovery	97.9	97.5	24.6	104.7	101.7	23.6

Another 500 ml. portion of Solution A was adjusted to pH 8.8, stored in the ice box for a week and then treated exactly as the first portion. This experiment involved an error of at least 4 per cent because the per cent recovery varied from 97.5 to 104.7 and the per cent nucleic acid in toto varied from 23.6 to 24.6. However, the data (Table XVIII) show that quite different fractions can be recovered from a nucleoprotein which has already been reprecipitated twice at pH 4. In more alkaline solution the nucleoproteins precipitated contain less nucleic acid. The marked difference between a freshly prepared nucleoprotein, which has not been exposed to a pH higher than 7, and one stored at pH 8.8 for one week indicates that denaturation facilitates the separation of the protein from the nucleic acid and also that denaturation is a major factor in the formation of various nucleoprotein fractions. However, the possibility that a variety of nucleoproteins are present in the living cell is not eliminated.

From this and other similar experiments it is apparent that to obtain maximum precipitation at pH 4 it is advisable to extract and reprecipitate under as mild conditions as possible in order to avoid disrupting the link between nucleic acid and the protein. The protein is actually used here as a precipitant for the nucleic acid in the

process of washing out other substances which would be considered contaminants. As the wheat germ becomes stale and the isolation procedure more drastic, the nucleo-protein bond is ruptured so that lower recoveries of nucleic acid are obtained. A hydrogen ion concentration of pH 4 appears to be a mixed isoelectric point of the nucleic acid and the protein. Obviously the protein has a higher isoelectric point, which is reasonable for an albumin, and the nucleic acid a lower one. If the two are linked together, then optimum precipitation occurs at pH 4. During the process of splitting the nucleic acid from the protein, the nucleic acid seems to become more soluble and recoveries diminish.

To summarize this section on the isolation of nucleo-proteins from a water extract of wheat germ with the object of obtaining a good recovery of nucleic acid, it has been shown that when a fresh sample of wheat germ is used and the best procedures followed, 2.5 per cent of the wheat germ can be recovered as a nucleoprotein containing about 20 per cent nucleic acid. Most of these nucleoproteins give a negative test with Dische's diphenylamine reagent (86) indicating that no detectable amounts of desoxyribose nucleic acid are present.

III. PREPARATION OF CRUDE NUCLEIC ACID SOLUTIONS

(a) Using Barium Acetate.

Jorpes (55) and other workers (37, 79) have used barium acetate to precipitate ribonucleic acids. Aliquots of a nucleoprotein solution, containing 20.9 per cent nucleic acid, were adjusted to various hydrogen ion concentrations in the range of pH 6.5 - 11.5 and the molar concentration of barium acetate was varied from 0.02 to 0.3 M. After centrifuging, the supernatant liquids were analyzed for nitrogen and pentose. The best result was obtained at pH 7 and 0.3 M barium acetate, where 80 per cent of the nucleic acid was precipitated as a nucleoprotein containing 25 per cent nucleic acid. In a similar experiment a nucleoprotein solution, containing 18.9 per cent nucleic acid, was used. However, this nucleoprotein had been exposed to an excess of alkali (pH 9.5). At pH 9.5 and 0.3 M barium acetate, 90.5 per cent of the nucleic acid was recovered as a nucleoprotein containing 27.4 per cent nucleic acid.

Two nucleoprotein solutions were prepared, one by a "mild" and the other by a "drastic" procedure. Two 100 g. portions of wheat germ were extracted for 10 minutes with 800 ml. of water and, after centrifuging, the residues were again extracted with 700 ml. of water, giving an overall ratio of one part of wheat germ to 15 of water.

The first and second extracts were combined in each case and one solution adjusted to pH 4 ("mild procedure") and the other to pH 3.6 ("drastic procedure"). The resulting precipitates, separated by centrifuging, were redissolved to give 650 ml. each of solutions of pH 6.6 and 9.5, respectively. They were precipitated again in a similar manner at pH 4.2 and 3.7 and redissolved to give two solutions of 300 ml. each and of pH 6.3 and 9.5. For convenience the two solutions will be referred to as "mild" and "drastic". The "mild" solution contained 3.19 mg. of nitrogen and .75 mg. of pentose per ml., indicating that the nucleic acid represented 16.6 per cent of the nucleoprotein. The "drastic" solution contained 3.26 mg. of nitrogen and .74 mg. of pentose per ml., or 15.9 per cent nucleic acid. Each solution was divided into two portions, one was adjusted to pH 9.5 and the other to pH 6.6. These hydrogen ion concentrations were suggested by Jorpes for barium acetate precipitation. To 10 ml. portions of each of the four solutions, 10 ml. aliquots of various barium acetate solutions of the corresponding hydrogen ion concentration were added to give concentrations ranging from 0.1 to 0.8 Molar. The results are given in Table XIX and Figures 10 and 11.

TABLE XIX - Effect of hydrogen ion concentration and amount of barium acetate on the precipitation and purification of nucleic acid.

Procedure for nucleoprotein preparation.	Barium acetate concentration (M)	Precipitation at pH 6.6			Precipitation at pH 9.5		
		Precipitated N. (per cent)	Precipitated pentose (per cent)	Nucleic acid in precipitate (per cent)	Precipitated N. (per cent)	Precipitated pentose (per cent)	Nucleic acid in precipitate (per cent)
	0.1	77.3	80.4	17.0	78.2	83.4	17.5
	0.2	71.1	77.4	17.9	75.6	82.9	18.0
"Mild"	0.4	65.2	74.9	18.8	71.9	82.1	18.6
	0.6	64.1	74.6	19.0	70.8	81.9	18.8
	0.8	65.6	74.5	18.6	73.5	82.2	18.4
	0.1	81.0	94.4	18.3	82.7	95.3	18.1
	0.2	74.0	93.6	19.8	77.2	95.2	19.4
"Drastic"	0.4	70.3	93.6	20.9	71.5	94.8	20.7
	0.6	70.0	92.9	20.9	71.5	94.1	20.6
	0.8	73.0	92.8	20.0	74.0	93.6	19.9

The supernatants obtained in the above precipitations were analyzed, the percentages of nitrogen and pentose in the precipitates were calculated and from this the amount of nucleic acid in the precipitate was estimated. The results illustrated in Figure 10 show that a much greater recovery of pentose is obtained in the barium precipitate from the

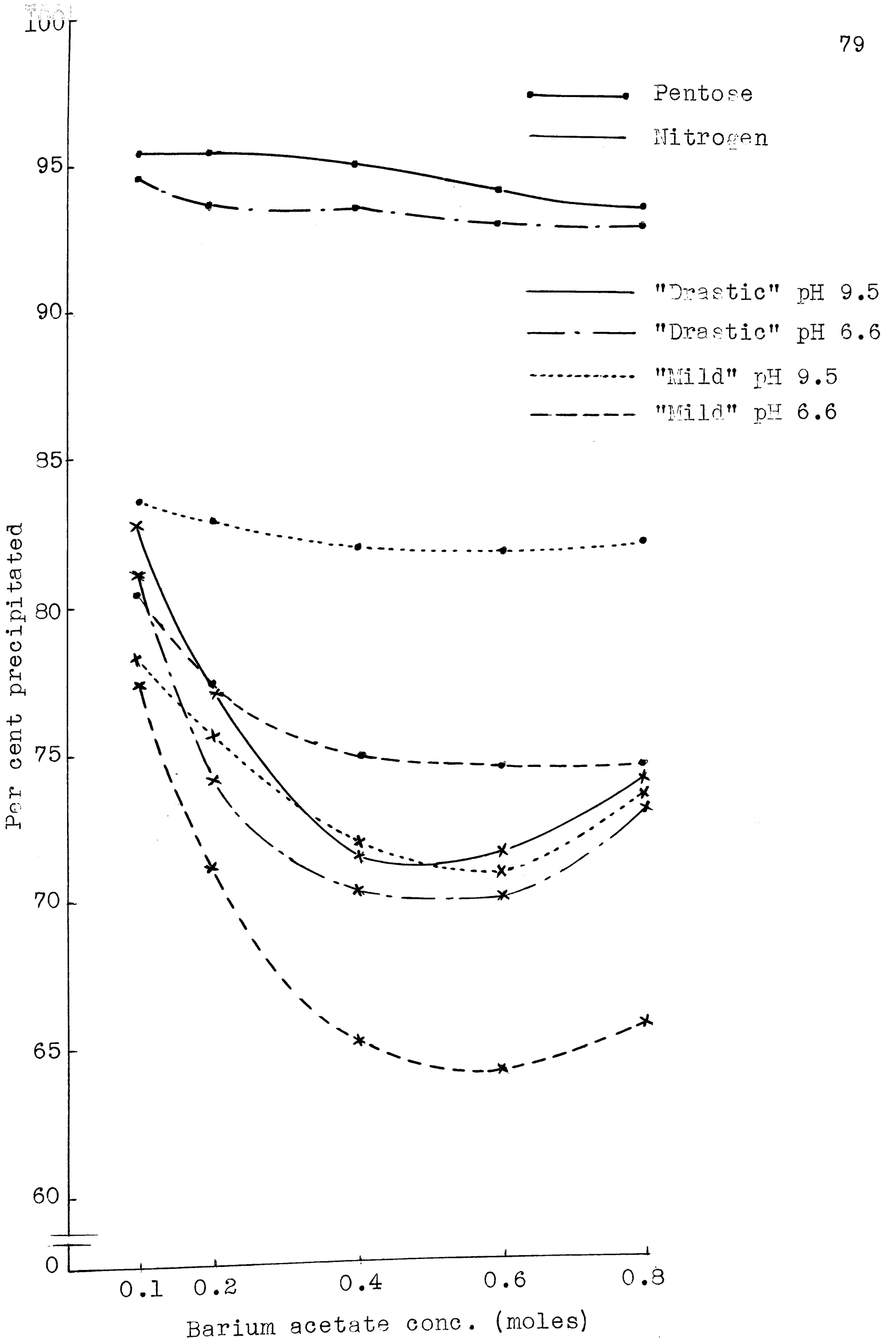


Figure 10 - Effect of hydrogen ion concentration and amount of barium acetate on nitrogen and pentose content of the precipitation.

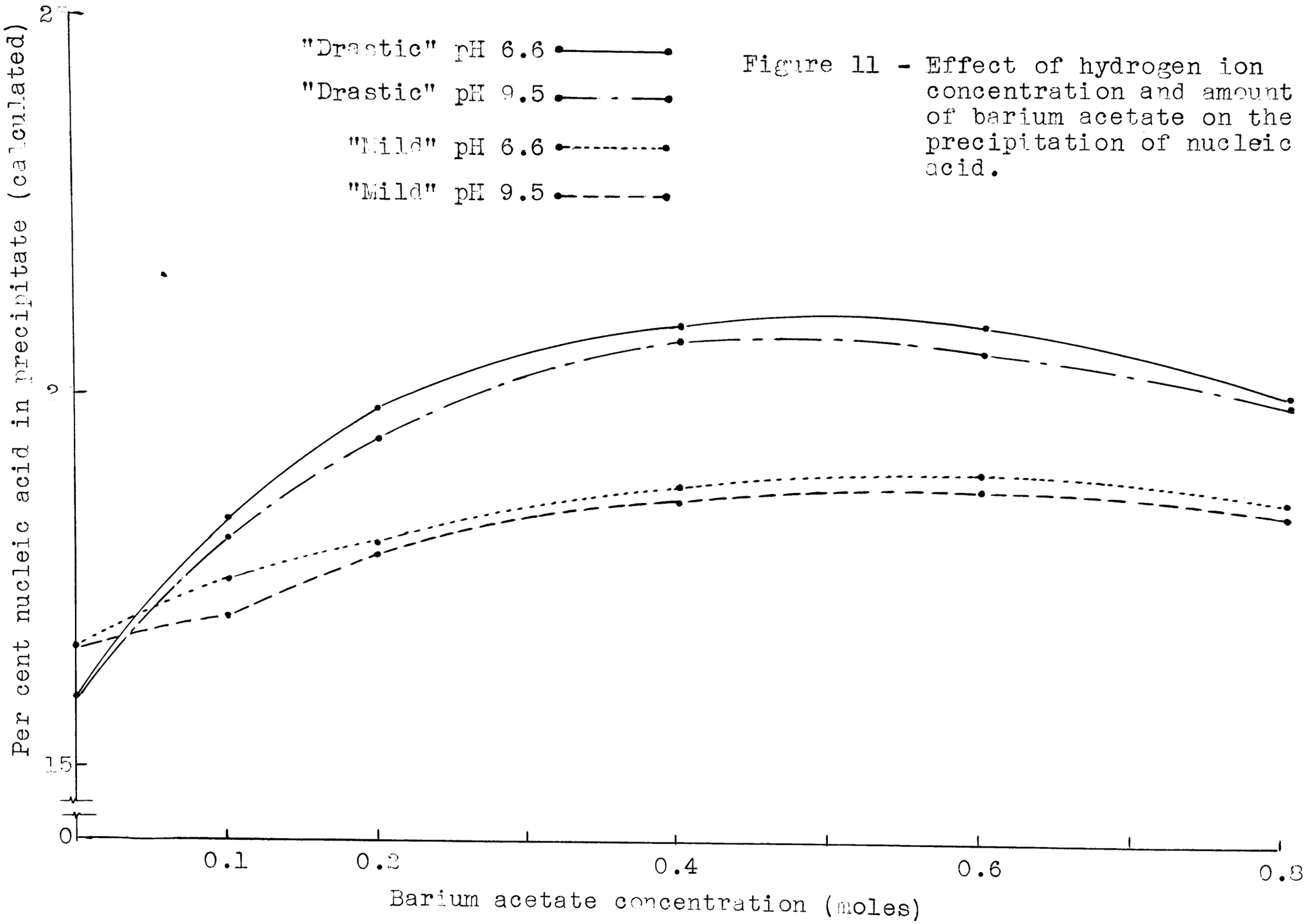


Figure 11 - Effect of hydrogen ion concentration and amount of barium acetate on the precipitation of nucleic acid.

"drastic" as compared to the "mild" solution. The hydrogen ion concentration at which the precipitation is carried out has slight effect in the case of the "drastic" solution, whereas with the "mild" solution high pH tends to give results which are intermediate. The barium concentration has little effect on the amount of pentose in the precipitates once the nucleic acid-protein bond is weakened, but it seems to have a marked effect on the solubility of the nitrogen, exhibiting a salting-in effect up to 0.6 M when a salting-out effect becomes apparent. Jorpes recommends a barium acetate concentration equivalent to 0.147 M. Figure 11, in which the per cent nucleic acid in the precipitates obtained from the four solutions is plotted against barium concentrations, shows a very marked difference between the "drastic" and "mild" procedures, and a very slight difference due to hydrogen ion concentration of precipitation. To underline the significance of the results in Figure 11 it should be added, for example, that with 0.4 M barium acetate from the "drastic" solution 93.6 per cent of the nucleic acid is recovered at pH 6.6 in the form of a nucleoprotein containing 20.9 per cent nucleic acid and 94.8 per cent of the nucleic acid is recovered at pH 9.5 in the form of a nucleoprotein containing 20.7 per cent nucleic acid; whereas from the "mild" solution 82.1 per

cent of the nucleic acid is recovered at 9.5 in the form of a nucleoprotein containing 18.6 per cent nucleic acid and 74.9 per cent of the nucleic acid is recovered at pH 6.6 in the form of a nucleoprotein containing 18.8 per cent nucleic acid.

Obviously alkaline treatment hydrolyzes the bond in the nucleoprotein or denatures the protein so that the nucleic acid is liberated. A nucleoprotein solution freshly prepared by the "mild" procedure was divided into six fractions, adjusted to pH 6.6, 7.5, 8.5, 9.5, 10.5 and 11.5. These solutions were then stored in the ice-box and at intervals up to 31 days the hydrogen ion concentration was readjusted and a sample of the supernatant solution taken for analysis. A portion of the supernatant was precipitated with 0.2 M barium acetate and the supernatant again analyzed. The results were compared with analyses done at zero time. The initial and final results are reported in Table XX. During the period of storage the solution at pH 11.5 was discarded because it showed a higher nucleic acid concentration in the supernatant liquid than in the barium acetate precipitate. The significance of this was not recognized until later (see below). The data in the last line of Table XX which were obtained by subtracting the per cent nucleic acid in the precipitate from the per cent nucleic acid in

TABLE XX - Effect of storage at 5°C. on precipitation of nucleic acid by barium acetate.

pH of precipitation and storage	6.6		7.5		8.5		9.5		10.5	
Time of standing before precipitation (days)	0	31	0	31	0	31	0	31	0	31
Nitrogen in solution (mg./ml.)	1.652	1.820	1.755	2.128	1.792	2.224	2.156	2.240	2.296	2.164
Pentose in solution (mg./ml.)	0.517	0.509	0.548	0.545	0.561	0.561	0.621	0.623	0.620	0.632
Per cent nucleic acid in solution	21.8	19.6	21.8	17.9	21.9	17.7	20.1	19.4	18.9	20.4
Nitrogen precipitated (per cent)	62.1	45.4	61.8	50.1	65.6	55.7	68.8	60.8	74.4	60.3
Pentose precipitated (per cent)	79.2	76.8	80.8	80.9	81.7	87.5	84.9	83.5	84.3	71.3
Per cent nucleic acid in precipitate	27.9	33.0	28.6	29.0	27.1	27.8	24.8	26.7	22.3	23.4
Increase in per cent nucleic acid	6.1	13.4	6.8	11.1	5.2	10.1	4.7	7.3	3.4	3.0

the nucleoprotein solution, show that, on increasing the pH of storage, the per cent of nucleic acid in the nucleoprotein decreases and the effect of storage on the separation of nucleic acid is less beneficial at higher pH:. actually, at pH 10.5 there is less separation of nucleic acid after 31 days' storage. Apparently, during the splitting of the nucleic acid-protein bond some other changes occur in the protein which eventually cause the protein and nucleic acid to be co-precipitated. At pH 11.5 the protein was preferentially precipitated. It also seems that these changes in the protein occur before the nucleic acid is completely liberated from the protein. The alkali treatment might be prolonged so as to permit preferential precipitation of the protein while most of the nucleic acid would stay in solution.

A similar experiment was carried out using a nucleoprotein solution prepared by a "drastic" procedure. Aliquots were stored at room temperatures at pH 6.5, 7.5, 8.5 and 9.5 and were protected by a layer of toluene. During storage the hydrogen ion concentration changed quite rapidly, tending to drop, the change being greatest at high pH, but the solutions were readjusted daily to the original value. Table XXI gives the data for zero and 15 days' storage. Before taking the sample for analysis and precipitation, the solutions were centrifuged to remove

TABLE XXI - Effect of storage at room temperature on precipitation of nucleic acid by barium acetate.

pH of storage	6.5		7.5		8.5		9.5	
Time of standing before precipitation (days)	0	15	0	15	0	15	0	15
Nitrogen in solution (mg./ml.)	1.54	.756	1.57	1.51	1.57	1.55	1.54	1.56
Pentose in solution (mg./ml.)	0.435	0.361	0.447	0.464	0.435	0.464	0.435	0.464
Per cent nucleic acid in solution (calculated)	19.8	33.4	19.9	21.4	19.4	20.9	19.4	20.8
Nitrogen precipitated (per cent	76.8	24.7	78.6	65.3	77.9	63.8	76.1	72.3
Pentose precipitated (per cent)	83.7	3.31	86.4	28.5	86.2	30.8	87.2	50.5
Per cent nucleic acid in supernatant.	12.0	43.2	12.6	43.7	12.2	40.2	10.6	22.7
Change in per cent nucleic acid in supernatant.	-7.8	+9.8	-7.3	+22.3	-7.2	+19.3	-8.8	+1.9

flocculated material and this accounts for the slight differences in the analytical results prior to precipitation. Under the conditions of this experiment and after 15 days' storage, protein was preferentially precipitated by barium acetate at a lower pH. At a higher pH nucleic acid is also precipitated so there is very ineffective separation of nucleic acid from protein. Barium acetate is not, under these conditions, an efficient reagent for the preparation of nucleic acid from an extract of wheat germ. However, these studies were advantageous in planning subsequent experiments.

(b) Denaturation and isoelectric precipitation of the proteins.

The results in Table XVIII indicated that after 8 days' storage at 5°C. and pH 8.8 it is possible to prepare a nucleoprotein containing 62.0 per cent nucleic acid from a nucleoprotein containing 23.6 per cent nucleic acid, but the recovery of nucleic acid is only 20.9 per cent. From the preceding experiment using barium acetate it was also realized that the removal of the protein after denaturation might be effected by isoelectric precipitation. The four solutions stored for 15 days at room temperature when mixed together and centrifuged gave a solution of pH 7.15 containing 1.41 mg. nitrogen per ml. and 0.439 mg. of pentose per ml. (i.e. 21.8 per cent ribonucleic acid in the nucleoprotein). Aliquots of this solution were adjusted to

different hydrogen ion concentrations and after centrifuging the supernatant liquids were analyzed for nitrogen and pentose. Results are shown in Table XXII and Figures 12 and 13, which also give, for comparison, the data of Table XVII obtained from a "mild" preparation without storage.

TABLE XXII - Solubility of denatured nucleoprotein at various hydrogen ion concentrations.

pH	Total nitrogen in solution (per cent)	Pentose in solution (per cent)	Calculated protein nitrogen in solution (per cent)	Calculated purity of nucleic acid in solution (per cent)
6.55	98.9	100.0	97.5	22.0
6.00	97.7	99.0	96.2	22.1
5.40	34.5	86.4	19.8	54.7
5.15	30.7	84.4	15.5	60.3
4.65	27.1	77.1	13.0	61.9
4.45	28.8	74.5	16.0	56.4
3.95	54.3	68.0	50.0	26.8
3.60	71.2	76.5	68.9	23.5
3.35	97.3	94.3	97.2	21.2
2.85	100.0	98.8	99.3	21.5

After denaturation the solubility of the nucleic acid is greatly increased but the hydrogen ion concentration for minimum solubility is still close to pH 4. The amount of

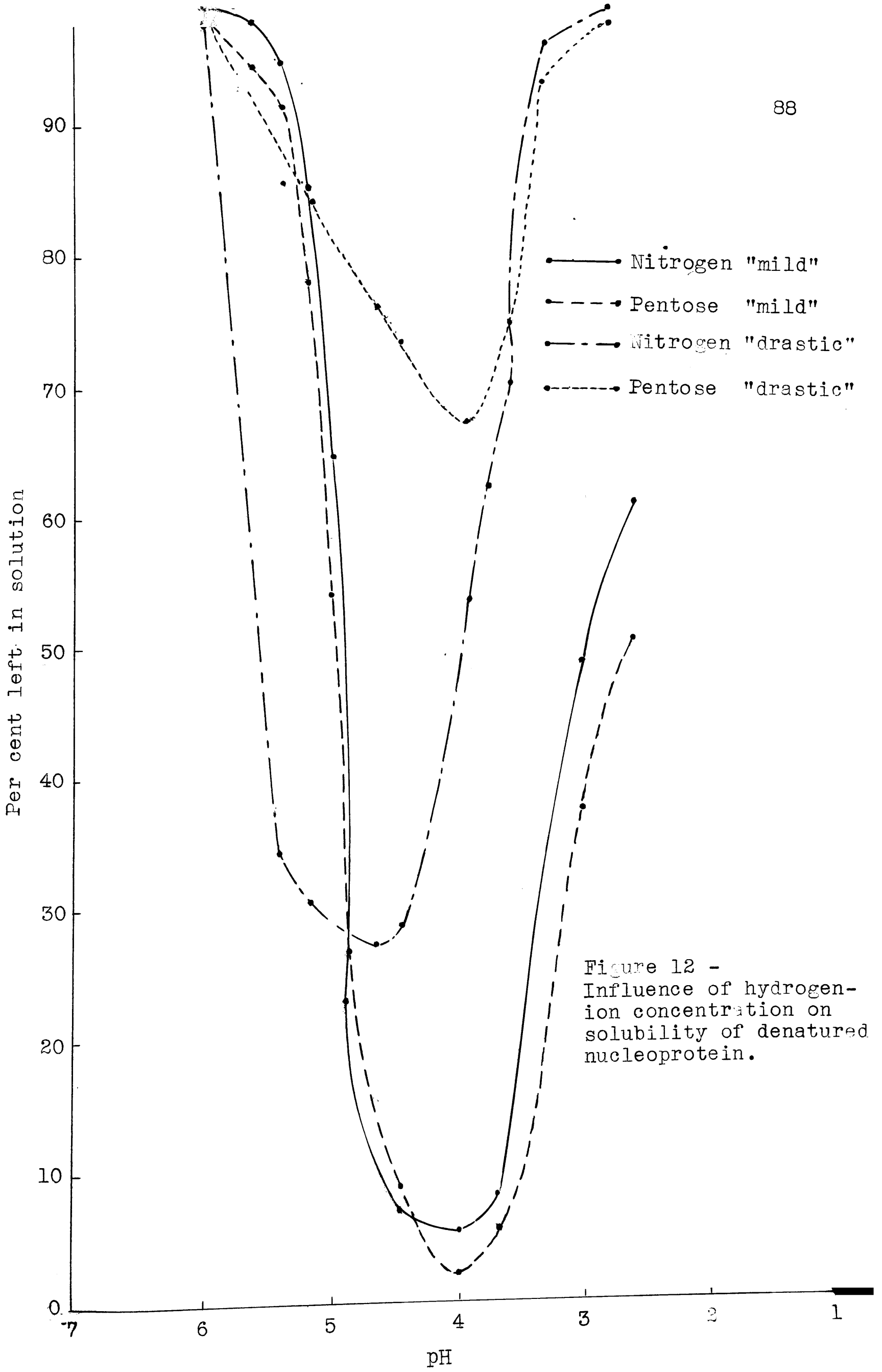


Figure 12 - Influence of hydrogen-ion concentration on solubility of denatured nucleoprotein.

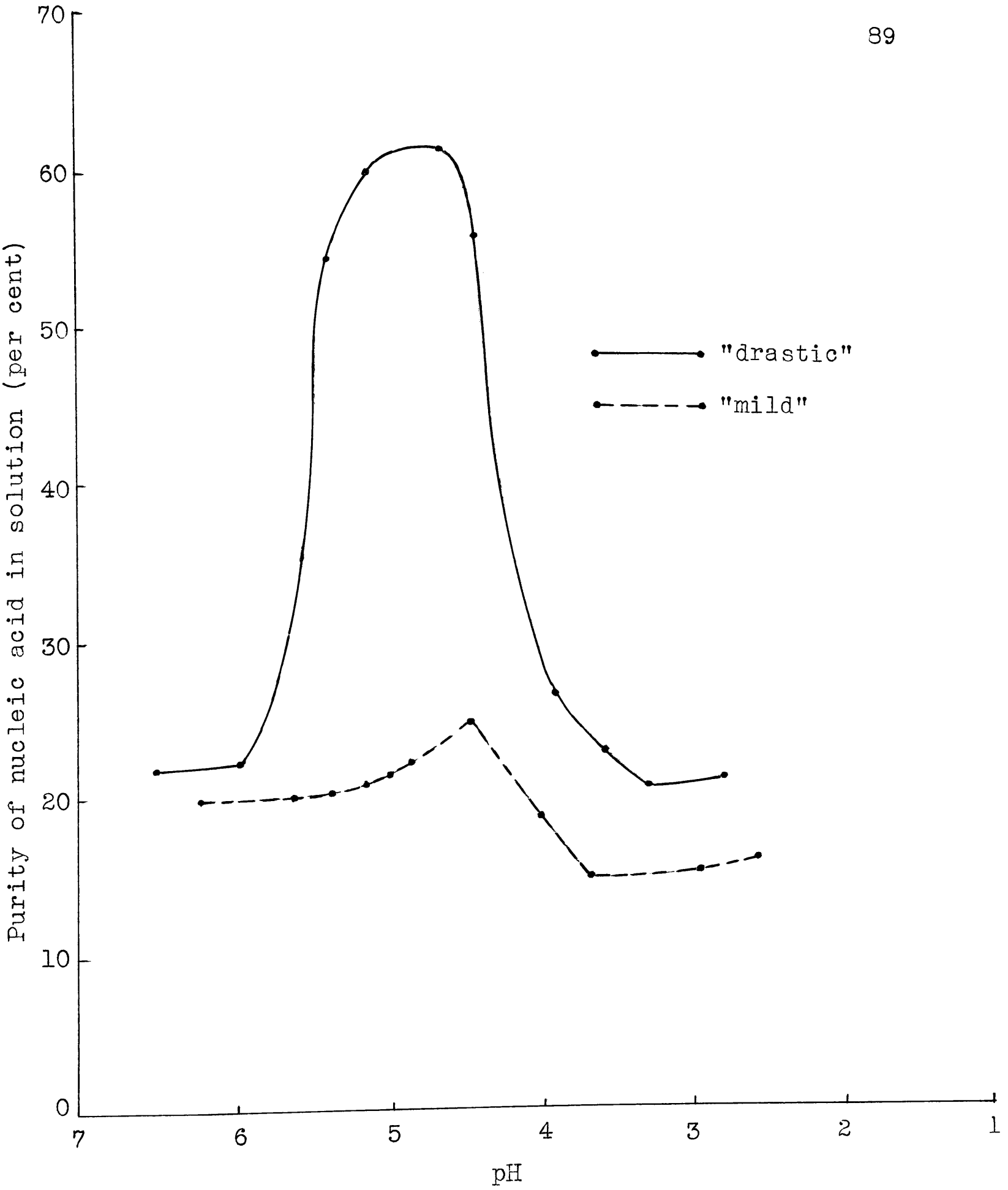


Figure 13 - Purity of nucleic acid in denatured and undenatured nucleoprotein solutions at various hydrogen ion concentrations.

nitrogen precipitated in the case of the "drastic" preparation is almost as great as with the "mild" preparation, particularly if correction is made for the nitrogen content of the soluble ribonucleic acid (see column 4, Table XXII), but the hydrogen ion concentration for minimum solubility is shifted to a higher value about pH 4.65. This experiment indicates that by adjusting the hydrogen ion concentration of a denatured nucleoprotein solution (21.8 per cent nucleic acid) to pH 4.65 and centrifuging, the supernatant liquid contains 77.1 per cent of the original nucleic acid in a nucleoprotein containing 61.9 per cent nucleic acid.

(c) Using ethyl alcohol.

The hydrogen ion concentration for maximum precipitation of the protein fraction (about pH 4.65) and of the nucleic acid fraction (about pH 4) are too close to give a sharp separation. Perhaps the addition of ethyl alcohol would shift the titration curve of the protein towards higher hydrogen ion concentrations without affecting markedly the solubility of nucleic acid and, therefore, permit a better separation. It was difficult to measure the hydrogen ion concentrations of alcoholic solutions. The values reported were taken after the readings were constant for some time.

An experiment was carried out with aliquots of a nucleoprotein solution prepared by the "mild" procedure. Six solutions of ethyl alcohol of different concentrations were prepared. Two aliquots of each of these solutions were taken, the hydrogen ion concentration of the first was adjusted to give a clear supernatant liquid and the second to give a slightly higher pH value. The results are shown in Table XXIII and they indicate that the bond between the protein and the nucleic acid has to be weakened before a successful separation can be obtained. It is also evident that higher alcohol concentrations and lower pH values give nucleic acid of a higher purity and that at lower alcohol concentrations and higher pH values more nucleic acid is recovered. The results with alcohol concentrations lower than 40 per cent do not differ markedly from water. Actually these results are not very conclusive because a better hydrogen ion concentration for precipitation might exist for the same concentration of alcohol.

The same nucleoprotein solution was divided into aliquots which were adjusted to pH 9, 10 and 11, made up to different concentrations of alcohol and heated for one hour at 80°C. A minimum amount of hydrochloric acid was then added to give, on centrifuging, a clear supernatant liquid which was analyzed. The hydrogen ion concentration

TABLE XXIII - Isoelectric and ethyl alcohol precipitation of a nucleoprotein solution prepared by the "mild" procedure.

Ethyl alcohol concentration (per cent)	pH of precipitation (approx.)	Supernatant			
		Nitrogen (per cent)	Pentose (per cent)	Purity of nucleic acid (per cent)	Appearance.
90	8.0	9.7	22.6	40.9	Clear.
80	8.1	54.3	96.1	34.1	almost clear.
	7.6	3.0	14.8	96.1	clear.
70	8.0	93.8	100.0	20.7	turbid.
	7.75	3.5	16.7	91.1	clear.
60	7.7	100.0	100.0	19.4	turbid.
	7.0	4.8	19.8	82.4	clear.
50	7.6	100.0	100.0	19.4	turbid.
	6.8	7.0	22.8	64.2	slightly turbid.
40	7.6	100.0	100.0	19.4	turbid.
	6.8	18.9	37.2	39.8	slightly turbid.

was recorded. Table XXIV shows the results. A concentration of forty per cent alcohol and pH 11 at time of heating gives the best recovery, i.e. 73.2 per cent of the nucleic acid present was recovered as a preparation containing 47.6 per

TABLE XXIV - Isoelectric and ethyl alcohol precipitation of denatured nucleoproteins.

Ethyl alcohol concentration (per cent)	pH at time of heating.	pH of precipitation (approx.)	Drops 0.25 N HCl. added	Supernatant		
				Nitrogen (per cent)	Pentose (per cent)	Purity of nucleic acid (per cent)
0	11	6.5	21	24.9	46.9	36.6
40	11	7.4	15	28.7	73.2	47.6
	10	7.2	11	20.9	38.9	36.2
	9	7.1	10	14.6	23.2	30.9
60	11	7.8	16	20.6	42.7	40.4
	10	7.6	11	14.1	22.9	31.6
	9	7.4	9	12.5	18.7	29.3
80	11	9.9	2	16.5	32.9	38.8
	9	9.4	0	8.4	11.8	23.8

cent nucleic acid. A higher alcohol concentration and a higher hydrogen ion concentration at the time of heating gives lower recoveries. The optimum hydrogen ion concentration for precipitation is lower at lower alcohol concentrations. Heating for one hour at 80°C. and at pH 11 does not sufficiently weaken the nucleoprotein bond.

The next experiment was designed to determine the optimum time of heating. In Table XXV data are shown

TABLE XXV - Effect of time of heating on nucleoprotein precipitation.

Ethyl alcohol concentration (per cent)	Heating time (hrs.)	Drops 0.25 N HCl. added	pH of precipitation.	Supernatant		
				Nitrogen (per cent)	Pentose (per cent)	Purity of nucleic acid (per cent)
0	1	21	6.5	24.9	46.9	36.6
	2	17	6.3	12.5	23.2	36.3
	4	13	6.5	44.2	81.0	35.7
40	1	15	7.4	28.7	73.2	47.6
	2	12	7.4	31.1	80.7	50.3
	4	8	8.1	46.7	96.6	40.2
60	1	16	7.8	20.6	42.7	40.4
	4	7	8.4	50.8	81.0	30.9
80	1	2	9.9	16.5	32.9	38.8
	2	0	10.0	22.1	45.1	39.7
	4	0	10.1	23.8	56.2	46.0

which were obtained from aliquots of the same solution, all at pH 11, but with concentrations of alcohol of zero, 40, 60 and 80 per cent, and which were heated for 1, 2 and 4 hours at 80°C. After the addition of a minimum amount of hydrochloric acid to obtain a clear supernatant solution, these aliquots were centrifuged and analyzed. A concen-

tration of 40 per cent alcohol again gave the best recovery and highest purity; heating for two hours was definitely better than one hour and four hours' heating was unsatisfactory. These differences, however, were due in part to the fact that the optimum hydrogen ion concentration for precipitation was not yet determined and adjustments were made visually relying on the point at which a clear solution was obtained.

In all previous experiments the ethyl alcohol was always added before heating, assuming it would facilitate denaturation of the nucleoprotein. The addition of the alcohol after heating was next tried. The results in Table XXVI show that the presence or absence of alcohol during heating has little effect on the final results. Addition of the alcohol after heating produces the same degree of hydrolysis of the protein-nucleic acid bond and the protein is more easily precipitated. The same amount of hydrochloric acid was added to the four solutions and, therefore, it is interesting to note the difference in hydrogen ion concentration. Heating was carried out for 1.5 hours at 80°C. and pH 11. A 30 per cent alcohol concentration was tried with no improvement over an aqueous solution.

A more detailed study of the optimum time of heating and hydrogen ion concentration for precipitation was carried out on solutions of pH 11 which were heated at 80°C. for

TABLE XXVI - Effect of order of addition of ethyl alcohol on nucleoprotein precipitation.

Ethyl alcohol concentration (per cent)	Time of alcohol addition	Supernatant			
		pH	Nitrogen (per cent)	Pentose (per cent)	Purity of nucleic acid (per cent)
40	before heating	9.1	42.6	88.6	40.4
	after heating	8.7	38.7	88.2	44.2
50	before heating	9.2	45.8	84.0	34.8
	after heating	8.8	36.1	83.0	44.8

1.5, 2, 2.5 hours. To aliquots of these solutions different volumes of 0.25 N hydrochloric acid were added and the hydrogen ion concentration measured. Ethyl alcohol was then added to give a concentration of 40 per cent. After one hour the solutions were centrifuged and the supernatant solutions analyzed. The results in Table XXVII show that heating for 2.5 hours gives a higher recovery and greater purity. It is difficult to decide which hydrogen ion concentration is best for precipitation, because there is not a sharp change. Probably the maximum recovery of nucleic acid is the most important consideration.

TABLE XXVII - Optimum time of heating and hydrogen ion concentration for precipitation.

Heating time (hrs.)	Hydrochloric acid added (ml. 0.25 N)	Supernatant pH approx.	Nitrogen (per cent)	Pentose (per cent)	Purity of nucleic acid (per cent)	Appearance
1.5	0.75	8.8	47.7	91.3	35.7	turbid
	0.80	8.5	38.0	91.9	45.1	slightly turbid.
	0.85	8.3	38.0	91.9	45.1	clear
	0.90	8.1	36.4	92.5	47.6	clear
	0.95	7.8	35.3	91.3	48.3	clear
	1.00	7.6	35.3	90.7	48.0	clear
2.0	0.75	8.5	44.1	95.0	40.2	slightly turbid.
	0.80	8.4	42.4	95.0	42.4	clear
	0.85	8.1	37.3	95.0	48.3	clear
	0.90	7.8	35.3	94.4	50.0	clear
	0.95	7.6	35.3	92.5	49.3	clear
	1.00	7.4	35.3	93.3	49.6	clear
2.5	0.70	8.6	41.8	100.	44.6	turbid
	0.75	8.4	42.2	100.	44.3	slightly turbid.
	0.80	8.2	40.4	99.1	45.7	slightly turbid.
	0.85	7.9	38.0	98.3	48.3	clear
	0.90	7.7	35.3	93.5	49.6	clear
	0.95	7.5	35.3	96.6	50.4	clear

Since this experiment has shown that heating for 2.5 hours gives the best recovery, the heating time was further extended to 3 hours at 80°C. and pH 11, followed by addition of alcohol to 40 per cent concentration and adjustment of the hydrogen ion concentration to various levels ranging from pH 4.1 to pH 8.6. The data are given in Table XXVIII.

TABLE XXVIII - The effect of hydrogen ion concentration on the precipitation of a denatured nucleoprotein.

	Supernatant		
pH	Nitrogen (per cent)	Pentose (per cent)	Purity of nucleic acid (per cent)
8.6	43.3	99.0	41.8
7.8	39.6	100.1	47.6
7.3	36.5	100.1	51.5
6.6	30.2	96.3	58.3
5.7	25.5	89.7	64.9
4.8	22.2	72.2	59.9
4.1	20.9	56.6	49.6

Three hours' heating gives considerably higher recovery and a purer product than in the previous experiments. The optimum hydrogen ion concentration for precipitation must be between pH 7.3 and pH 6.6.

In all previous experiments the tests were done on

small aliquots (usually 10 ml.) and no particular care could be taken to avoid local concentration of acid while adjusting the hydrogen ion concentration. Therefore, the results cannot be quite the same if local concentration of acid is reduced to a minimum as when dilute acid (0.25 N hydrochloric acid) is added very slowly through a fine bore pipette immersed in a fairly large volume of solution and with vigorous stirring. This was demonstrated with an 860 ml. aliquot of the solution employed to obtain the data in Table XXVIII which had been denatured and contained 40 per cent alcohol. This solution was carefully adjusted to pH 7, and gave complete recovery of nucleic acid in the supernatant solution. The purity of the nucleic acid was 62 per cent. It would appear, therefore, that when nucleic acid is precipitated by local concentration of acid, it redissolves with difficulty and this reduces the yield. In addition, the stirring may speed the attainment of electric balance between the protein and the fluid, thus facilitating precipitation and improving the purity.

(d) Method of preparing a crude nucleic acid solution.

A typical procedure was as follows:-

A 1800 g. portion of finely ground wheat germ was extracted for 15 minutes with 18 l. of water (1:10) and

immediately centrifuged for 10 minutes at 3000 r.p.m. Only 16 l. of supernatant solution of pH 6.65 were recovered and more than 11 per cent of the extract was retained in the residue. The solution was mechanically stirred and 1600 ml. of 0.25 N hydrochloric acid were added which caused the hydrogen ion concentration to drop to pH 4.0. The resultant suspension, after standing at least one hour, was centrifuged for 10 minutes at 3000 r.p.m. and the supernatant liquid was discarded. The precipitate was suspended in 6400 ml. of water plus 50 ml. of 10 per cent sodium hydroxide giving a hydrogen ion concentration of pH 7.0. The opaque solution was centrifuged to remove undissolved particles which resulted in a loss of approximately 2.5 per cent. To the 8440 ml. of opaque solution 585 ml. of 0.25 N hydrochloric acid was added,while stirring, to bring to pH 4.0. After centrifuging for 10 minutes the supernatant liquid was discarded and the residue suspended in 4 l. of water plus 40 ml. of 10 per cent sodium hydroxide giving pH 6.9. On centrifuging this solution some undissolved material was collected which represented a loss of about 1.3 per cent. To 5750 ml. of supernatant solution at pH 6.85 a 410 ml. aliquot of 0.25 N hydrochloric acid was added to give pH 4.2. After centrifuging 10 minutes the supernatant liquid was again discarded and the residue suspended in 3 l. of water plus 90 ml. of 10 per cent sodium

hydroxide to give 4460 ml. of a nucleoprotein solution at pH 11.1, containing 5.22 mg. of nitrogen and 1.71 mg. of pentose per ml., in the form of a nucleoprotein containing 23.4 per cent ribonucleic acid. The solution contained approximately 34 g. of nucleic acid, thus 1.9 per cent of the wheat germ was recovered as ribonucleic acid in the nucleoprotein solution. Correcting for the measured losses incurred during the preparation, the yield becomes about 2.2 per cent. This yield is somewhat lower than was obtained when working on a smaller scale where 2.5 per cent of the wheat germ was recovered as nucleic acid from a similar nucleoprotein solution. It must be remembered that the recovery depends chiefly on the freshness of the wheat germ and the exposure to denaturation during preparation. In carrying out the procedure described above, the solutions were kept at 5°C. when not being handled.

An aliquot of the nucleoprotein solution prepared as described above, and corresponding to the extraction of approximately 1600 g. of wheat germ, was heated for 3 hours at 80°C. and pH 11. Absolute ethyl alcohol was added to give a concentration of 40 per cent, correcting for the 0.25 N hydrochloric acid required to bring the whole solution to pH 7.35. All these additions were made quite slowly with vigorous stirring in order to avoid any local concentration. After standing one hour the solution was

centrifuged and from the decanted clear supernatant liquid, 78.5 per cent of the nucleic acid was recovered as a nucleoprotein containing 79.5 per cent ribonucleic acid. As the residue contained quite a lot of liquid it was again extracted with water. Alcohol was added to a 40 per cent concentration and the hydrogen ion concentration adjusted to pH 7.1. The supernatant solution, after centrifuging, contained 14.5 per cent of the original nucleic acid with a purity of 77 per cent. Summing the components of the two supernatant solutions, after precipitation of the protein fraction, the actual recovery is 93 per cent of a product which was 78 per cent pure, while the original nucleoprotein contained only 23 per cent nucleic acid. This constitutes a loss of 7 per cent in preparing a product of over three times the original purity. Such solutions will be referred to as crude nucleic acid solutions.

During a few trials to obtain crude nucleic solutions following similar procedures, the recovery of nucleic acid from the nucleoprotein solutions varied from 85 to 97 per cent with the purity ranging from 74 - 90 per cent. The major variables involved are:

(1) The extent of hydrolysis of the nucleic acid from the protein, which is dependent on the state of the nucleoprotein before heating and/or the exact conditions

of heating. Insufficient hydrolysis would cause a low recovery, because the nucleic acid would tend to precipitate with the protein. Too much hydrolysis would tend to solubilize the proteins and would give a cruder nucleic acid solution.

(2) The optimum hydrogen ion concentration for the precipitation of the protein in a 40 per cent alcohol solution is actually dependent on the extent of hydrolysis. More hydrolysis requires a lower pH. At a pH higher than needed there is not as complete precipitation of the protein and the residue traps more liquid. At a lower pH there is a greater loss of nucleic acid in the precipitate, but less liquid is lost and a greater purity is achieved. In general, higher purity means lower recovery.

IV. PREPARATION OF NUCLEIC ACID FROM A CRUDE NUCLEIC ACID SOLUTION.

(a) Precipitation of nucleic acid.

The concentration of nucleic acid in the crude nucleic acid solution is low, at the most 0.4 per cent, but often as low as 0.05 per cent, particularly in the solutions obtained by extracting the protein precipitate for a second time. Therefore, these solutions are concentrated down under vacuum at 50 - 60°C. to give a syrupy solution containing more than 2 per cent nucleic acid plus the protein

contamination. The small amount of insoluble material which separates on concentrating is centrifuged off and is mostly protein. The hydrogen ion concentration is then adjusted to pH 4.0 and the amount of precipitate obtained depends on the purity of the crude nucleic acid solution. A study was made of this hydrogen ion concentration, but this precipitation is not as sensitive to hydrogen ion concentration as those previously described. The residue consists largely of a nucleoprotein which has a fairly high content of nucleic acid (25 to 50 per cent), thus as much as 30 per cent of the nucleic acid might be lost at this stage. When alcohol is added to a concentration of 60 per cent in the clear supernatant solution, containing nucleic acid almost exclusively, a precipitate is recovered which represents about 90 per cent of the nucleic acid and which can be called wheat germ ribonucleic acid. After washing the precipitate three times with absolute ethyl alcohol and ethyl ether a light white powder is obtained. The yield varies from 0.8 to 1.4 per cent (generally 1.1 per cent) by weight of the wheat germ. The purity was always abowe 95 per cent. Some typical analytical data will be given later.

(b) Purification of nucleic acid.

Nucleic acid preparations were purified with variable

yields depending on the original purity of the material and the degree of purification achieved. The method was very similar to the one used in the actual precipitation of nucleic acid from a crude nucleic acid solution. The following experiment is given as an example.

A 1.5 g. portion of a nucleic acid preparation, equivalent to about 1.2 g. of pure nucleic acid, (allowing for moisture, sodium and impurities) was suspended in 10 ml. of 0.1 N sodium hydroxide and 30 ml. of water added. The resulting turbid solution at pH 5.7 was brought to pH 4.0 and centrifuged after standing one hour. Sixty ml. of absolute ethyl alcohol were added to the clear supernatant solution and, after two hours, the precipitate was recovered by centrifuging. The nucleic acid was then suspended in 6 ml. of 0.1 N sodium hydroxide, 20 ml. of water added and adjusted to pH 4.0. Very little residue was obtained. 40 ml. of absolute alcohol were added to the clear supernatant solution. After two hours the precipitate was collected by centrifuging, washed three times with absolute ethyl alcohol and ethyl ether and dried under high vacuum at 50°C. A 0.90 g. portion of a white powder was recovered, containing over 99 per cent ribonucleic acid (correcting for moisture and sodium); this represents about 70 per cent of the original nucleic acid before repurification.

(c) Analyses of nucleic acids.

(1) Chemical analyses:

The theoretical values were calculated assuming that the basic unit is the tetranucleotide structure as postulated by Levene (16) for yeast nucleic acid. This tetranucleotide contains four phosphoric acid molecules, four ribose molecules and one molecule each of the following: adenine, guanine, uracil and cytosine. The total molecular weight is 1303.6 with an empirical formula $C_{38}H_{49}O_{29}N_{15}P_4$. In the analytical method used, only fifty per cent of the pentose in the tetranucleotide reacts with orcinol in the presence of ferric chloride. The pentose value which should be obtained by analysis is thus only one half of the theoretical value. For comparison, analyses were carried out on yeast ribonucleic acid prepared by Schwarz Laboratories Inc. (lot HN 4531) and claimed to be 97 - 99 per cent pure. Analytical results are reported in Table XXIX for two nucleic acid preparations (RNAI and RNAIV) and one purified nucleic acid preparation (purified RNAI) prepared from wheat germ by the described procedure. RNAI was prepared from 1800 g. of wheat germ with a yield of 1.2 per cent. RNAIV was prepared from 1000 g. of wheat germ with a yield of 1.1 per cent. Purified RNAI was obtained by reprecipitating a portion of RNAI twice with

TABLE XXIX - Chemical analyses of some nucleic acid preparations.

Ribonucleic acid sample	RNAI	RNAIV	Purified RNAI	Schwarz	Theoretical
Moisture (per cent)	7.9	8.5	2.5	8.7	0
Sodium (per cent)	2.8	3.2	2.6	1.0	0
Nitrogen (per cent dry & Na free)	15.33	15.52	16.01	15.96	16.12
Phosphorus (per cent dry & Na free	8.91	9.08	9.49	9.40	9.52
N : P ratio	1.72	1.71	1.69	1.70	1.69
Purity (per cent) using Nitrogen as base	95.10	96.27	99.32	99.00	100.00
Purity (per cent) using Phosphorus as base	93.59	95.38	99.68	98.74	100.00
Reacting pentose (per cent dry & Na free)	21.74	22.07	22.96	22.86	23.01 *
N : pentose ratio	0.705	0.703	0.697	0.698	0.70

* one half theoretical value as explained in text.

a recovery of 75 per cent, (i.e. it represents about 0.75 per cent of the wheat germ). The per cent moisture and sodium can be reported to the first decimal only, because their estimation involves quite a large error. Phosphorus and nitrogen determinations were done quite accurately but the accuracy of the values reported is dependent on the moisture and sodium content. The purified RNAI and Schwarz samples gave values agreeing closely with the theoretical. The differences can be accounted for by the precision and accuracy of the methods employed. The analyses on samples RNAI and RNAIV indicate that most of the impurities present are not nitrogenous. The fact that the per cent reacting pentose is in agreement with the other results (i.e. nitrogen pentose ratio) is indication that the assumptions made were justified. All these preparations gave a negative test with Dische diphenylamine reagent (86).

(2) Spectrophotometric analyses:

The samples which were analyzed chemically were also analyzed spectrophotometrically and in addition a solution of crude nucleic acid which was diluted to the required concentration in terms of reacting pentose. From its nitrogen pentose ratio this crude nucleic acid was found to contain 20 per cent nitrogenous impurities, but possibly also other contaminants not containing nitrogen were present. A Beckman photoelectric quartz spectrophotometer was used

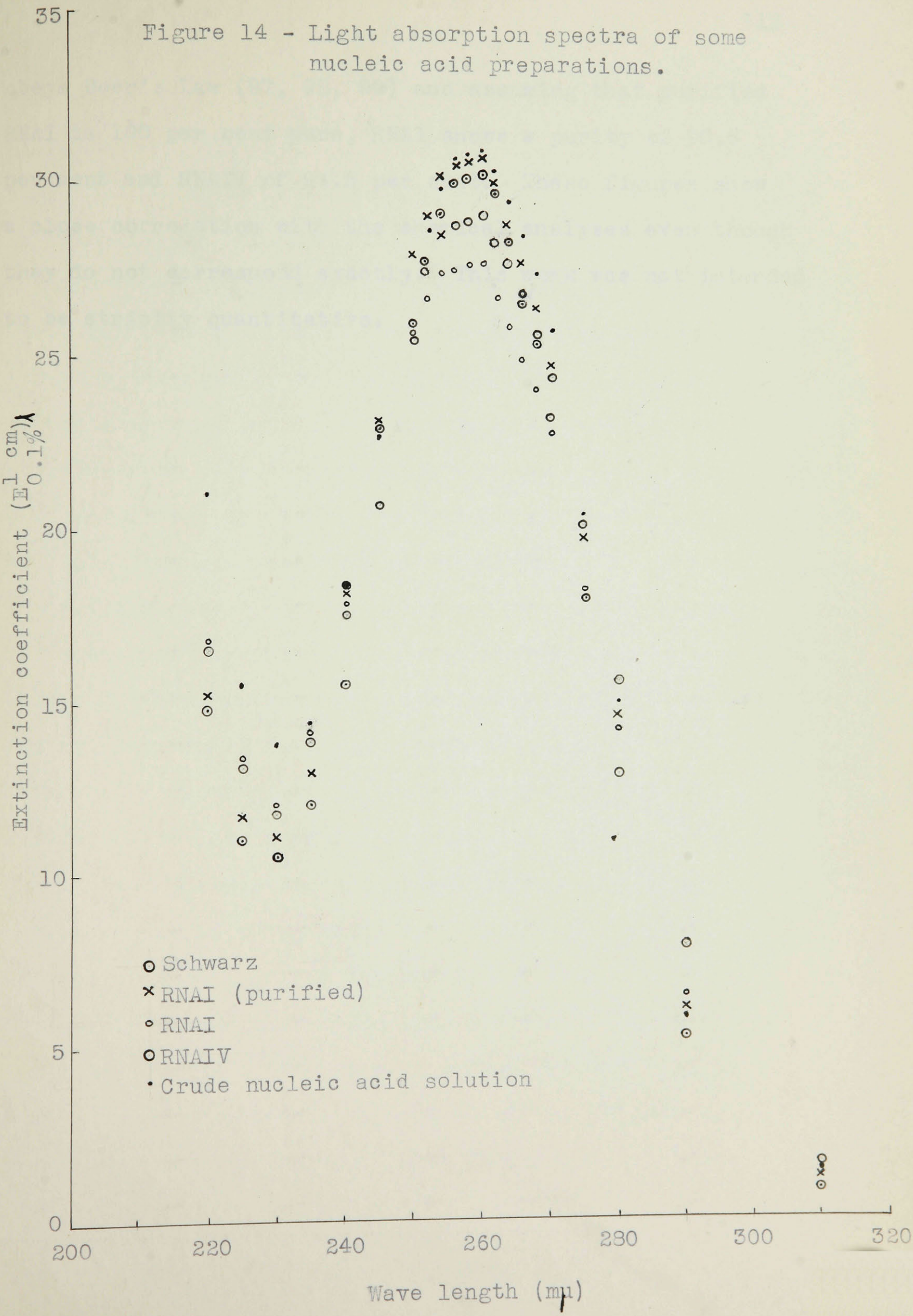
with square cuvettes one cm. wide. The slit was kept at a constant aperture of 15 Å. The solutions were adjusted to contain 10 µg. of nucleic acid per ml. (after corrections for moisture and sodium) in 0.005 N phosphate buffer at pH 7 and 0.0001 N sodium hydroxide (sodium hydroxide was used to dissolve the weighed nucleic acid before diluting). The actual hydrogen ion concentration of the solutions varied between pH 7.12 and 7.15. Readings were taken between 220 and 310 mµ. and recalculated as extinction coefficients for 1 cm. layer of a 0.1 per cent solution (1 mg./ml.) of the moisture and sodium free sample ($E\,\frac{1\ cm}{0.1\%} = \frac{1}{cx} \log \frac{Io}{I}$ where x = thickness in cm. and C = concentration in mg/ml).

The results are shown in Table XXX and Figure 14. All absorption curves show a typical maximum at 260 mµ. and minimum at 230 mµ. On the longer wave length side of the maximum the curves have a steeper slope than on the other side. The depression of the minimum appears to be reduced by impurities containing nitrogen (protein) as is particularly well shown in the case of the curve for crude nucleic acid which otherwise follows the curves for the purest samples. RNAI and RNAIV have slightly lower maxima than the other samples, showing that they contain some contaminant which was weighed as nucleic acid. Assuming that the absorption of nucleic acid at 260 mµ.

TABLE XXX - Light absorption data of some nucleic acid preparations.

Wave length (mμ.)	Schwarz $(E)_\lambda$	Purified RNAI $(E)_\lambda$	RNAI $(E)_\lambda$	RNAIV $(E)_\lambda$	Crude nucleic acid solution $(E)_\lambda$
220	14.8	15.2	16.9	16.5	21.1
225	11.0	11.7	13.3	13.1	15.4
230	10.5	11.1	12.0	11.7	13.8
235	12.0	13.0	14.9	13.9	14.3
240	15.5	18.2	17.9	17.5	18.3
245	20.7	23.0	22.9	23.0	22.7
250	25.9	27.9	25.7	26.5	26.9
252	27.7	29.0	26.7	27.4	28.6
254	29.2	30.1	27.4	28.5	29.8
256	30.0	30.5	27.5	28.8	30.6
258	30.1	30.6	27.6	28.9	30.8
260	30.2	30.7	27.7	29.0	30.9
262	29.7	29.9	26.7	28.3	30.3
264	28.3	26.8	25.9	27.7	29.5
266	26.6	27.7	24.9	26.7	28.5
268	25.4	26.4	24.1	25.5	27.4
270	23.2	24.7	22.8	24.4	25.7
275	18.0	19.7	19.2	20.1	20.5
280	12.9	14.6	14.9	15.6	14.9
290	5.2	6.0	6.5	7.9	5.9
310	0.8	1.2	1.3	1.6	1.4

Figure 14 - Light absorption spectra of some nucleic acid preparations.



obeys Beer's Law (97, 98, 99) and assuming that purified RNAI is 100 per cent pure, RNAI shows a purity of 90.5 per cent and RNAIV of 94.5 per cent. These figures show a close correlation with the chemical analyses even though they do not correspond exactly. This work was not intended to be strictly quantitative.

DISCUSSION

I. ANALYTICAL RESULTS.

The analytical results are expressed on the moisture-free basis: the moisture estimation, as described, can be considered reliable and also account for the bound water. There might be a difference of opinion in regard to expressing the results on a sodium-free basis without taking into account any other ion. Sodium might also be considered to be an impurity. Wheat germ has quite a high ash content but in the course of the various precipitations and solutions practically none of it should be left in the preparations. On the other hand the only chemical reagents, used in the isolation procedure, are hydrochloric acid and sodium hydroxide, which are employed to adjust the hydrogen ion concentrations to the required levels. Therefore, practically the only metallic ion present is sodium, which readily forms sodium salts with the nucleic acid. The hydrogen ion concentration for nucleic acid precipitation is pH 4, which is close to, but above, the isoelectric point of the nucleic acid. Therefore, it is logical to assume that mixed sodium salts of nucleic acid are formed. The number of sodium ions for the "statistical" tetranucleotide is dependent on the sodium and hydrogen ion concentrations. Table XXXI shows the theoretical

sodium content of the possible "statistical" tetranucleotide salts.

TABLE XXXI- Theoretical sodium content of the possible "statistical" tetranucleotide salts.

Sodium atom present	Tetranucleotide molecular weight	Sodium (per cent)
0	1303.6	0
1	1325.6	1.74
2	1347.6	3.41
3	1369.6	5.02
4	1391.6	6.58

The sodium concentration for the samples of nucleic acid prepared varied from 2.4 per cent to 3.6 per cent, showing that the "statistical" tetranucleotide varied from the mono- to the tri- sodium salt. On this basis it seems justifiable not to consider the sodium ion an impurity. It is very unlikely that more than traces of sodium chloride are present because of the very high solubility of this salt and the very small amount that could have been formed during the adjustment of the hydrogen ion concentration. Further evidence that a sodium salt of nucleic acid was actually isolated is to be found in the greater solubility of the preparation obtained in this investigation, as compared with other nucleic acid samples obtained by

precipitation at a higher hydrogen ion concentration. The nitrogen and phosphorus content of the products obtained are much closer to the theoretical values than is reported in the literature for various ribonucleic acids. Phosphorus values varying from 7.5 to 9 per cent, instead of the theoretical value of 9.52, are generally accepted and are explained (37) on the basis of a loss of phosphoric acid. The best values are reported by two groups of French workers, who have developed two modern methods of purification: Khouvine and Grégoire (82) reported 9.3 per cent phosphorus for ribonucleic acid from Calliphora erythrocephala, and Vendrely et al. (83) 9.59 per cent from yeast. In this investigation a sample of a commercial product isolated from yeast by Schwarz Laboratories, when analyzed for phosphorus, was found to contain 99.4 per cent of the theoretical amount. However, the same company admits that normally their product contains about 95 per cent of the theoretical phosphorus which is considerably higher than the value reported for other commercial products. The nitrogen content is not indicative of purity because the theoretical value might be obtained from a sample contaminated by proteins, as protein has almost the same nitrogen content as nucleic acid. However, a low nitrogen value would indicate contamination by non-nitrogenous substances such as carbohydrates.

The orcinol test measures 50 per cent of the ribose present in ribonucleic acid if a "statistical" tetranucleotide structure is assumed. On this basis the intensity of the colour developed by yeast nucleic acid, arabinose and ribose is exactly the same. This fact is contrary to the opinion of Schmidt and Thannhauser (106) who consider that the use of the colour tests for carbohydrates to measure nucleic acids involves some serious difficulties and the results are very variable. Cori and Cori (108) found that on the basis of phosphorus content yeast nucleic acid gives only 40 per cent of the pentose colour of muscle adenylic acid. Davidson and Waymouth (37, 100, 107) prepare their calibration curves in terms of nucleic acid phosphorus as they could not find any reasonable explanation for the colour developed from a weighed amount of nucleic acid. Davidson and Waymouth in one instance (37) reported the phosphorus content of the nucleic acid used as only 7.7 per cent. All these workers apparently heated for an insufficient period of thirty minutes, as advised by Schlenk (109) for nucleotides and nucleosides. The incomplete colour development gave low results and probably explains the variability. Therefore, the findings presented herein are not necessarily in contradiction to the results in the literature, and they might even be considered as confirming the theory that the "statistical" tetranucleotide contains equal numbers of purine and pyrimidine bases,

since the ribose, tied up with the pyrimidine bases, does not react appreciably with orcinol. Purified wheat germ nucleic acid gives identical results.

Throughout the development of the procedure for the preparation of nucleic acid, solutions were analyzed for pentose and nitrogen content and a nitrogen-pentose ratio was calculated. Assuming that the nucleic acid and the protein contain the same per cent nitrogen, the per cent purity of the nucleic acid in solution was calculated from the nitrogen : pentose ratio. This is a rapid and useful method with which to compare various details of procedure, but it does not take into account the non-nitrogenous impurities which might be present in the solution. However, the amount of these contaminations was found to be quite low under the conditions of the proposed procedure. This will be discussed later when considering the data in Table XXXII.

The ultraviolet absorption spectrum of purified ribonucleic acid from wheat germ is identical with that of a very pure sample of yeast nucleic acid, prepared by Schwarz Laboratories (New York). However, the extinction coefficient at 260 mμ. is difficult to compare with other values reported in the literature (23, 37,114, 115), because the calculation of these values is not clearly expressed and

the purity of the sample is not reported. However, Kalckar (116) in a very recent paper on the differential spectrophotometry of purine compounds reports very accurate molar extinction coefficients for purine bases which vary from 0.8 to 1.58 x 10^{-4}. When the extinction coefficient $(E.\frac{1\ cm}{1\ \%})_{260}$ as shown in Table XXX and Figure 14 is recalculated to give an average molecular extinction coefficient for the purine and pyrimidine bases that make up the "statistical" tetranucleotide, a value of 0.98 x 10^{-4} is obtained which agrees with Kalckar's measurements.

II. NUCLEOPROTEINS FROM WHEAT GERM.

The main purpose of this investigation on the preparation of a nucleoprotein is the isolation of nucleic acid (i.e. as a technique for separating nucleic acid from all other water soluble components of wheat germ). The treatments employed are as mild as possible, because it was found that better recovery would be obtained. However, it cannot be claimed that a "natural" nucleoprotein is isolated: it might be a slight modification or a complete artifact created during the extraction and precipitation. This definite nucleoprotein fraction accounts for about 80 per cent of the water-soluble protein nitrogen extracted, as measured by trichloroacetic acid precipitation. The

reliability of these data might be questioned due to the variability with results obtained by trichloroacetic acid precipitation. However, as the conditions for the precipitation of water extracts of wheat germ were studied, this value can be relied on. When stale wheat germ is extracted or if more drastic conditions are used, the nucleoprotein fraction represents a smaller percentage of the protein nitrogen extracted indicating that some breakdown occurs.

The nucleic acid-protein bond was found to be quite resistant to cleavage since at least 3 hours' heating at 80°C. and pH 11 is required. Nevertheless, nucleoproteins of various nucleic acid content can be isolated at various hydrogen ion concentrations, indicating that a complete gradation of nucleoprotein must be present. By choosing pH 4 for precipitation, a definite fraction is obtained, which at the third precipitation becomes sufficiently homogeneous that over 97 per cent is precipitated. However, this preparation can be further fractionated at various hydrogen ion concentrations. It is very hard to establish if this variability in the composition of the nucleoprotein reflects its natural state in wheat germ or whether the product is an artifact resulting from the method of isolation. Denaturation promotes the separation of these fractions. The importance of preparing the nucleoproteins under controlled conditions is also stressed

because the state of the nucleoprotein will alter the conditions for heat- and alkali-denaturation necessary for the separation of nucleic acid. Also, under the conditions described for the isolation of ribonucleic acid, no desoxyribonucleic acid is present as a contaminant. Probably the desoxyribonucleoprotein complex is not as easily extracted with water and/or it is more soluble at pH 4 (the hydrogen ion concentration at which the ribonucleoprotein is isolated) because the protein component is more basic.

III. RECOVERY AND PURITY OF RIBONUCLEIC ACID FROM WHEAT GERM AT DIFFERENT STAGES IN THE ISOLATION PROCEDURE.

The recovery and purity of the ribonucleic acid that can be expected at different stages in the procedure developed are summarized in Table XXXII. The maximum and minimum values reported are very extreme. The mode should be obtained without difficulty if the procedure is followed carefully. The recovery and the purity of the nucleic acid at the nucleoprotein stage after three precipitations is chiefly dependent on the freshness of the wheat germ and on the care taken to avoid denaturation during isolation (i.e. shortest possible period of handling, exact adjustment of hydrogen ion concentrations with stirring, use of small volumes). This is the only stage at which

TABLE XXXII - Recovery and purity of ribonucleic acid from wheat germ at different stages in the isolation procedure.

Material	Nucleic acid recovery (per cent of wheat germ)			Purity (per cent)		
	Maximum	Minimum	Mode	Maximum	Minimum	Mode
Nucleo-protein (3 times precipi-tated)	2.5	1.7	2.2	25	16	21
Crude nucleic acid solution	2.4	1.4	1.8	90	74	80
Nucleic acid solution	1.7	1.0	1.2	99	95	97
Nucleic acid pre-paration	1.4	0.8	1.1	97	94	95
Purified nucleic acid (two precipi-tations)	0.9	0.6	0.8	99.8	99.5	99.6

higher purity is associated with higher recovery, indicating that denaturation splits off some nucleic acid from the protein and then the nucleoprotein is precipitated with less nucleic acid. In the subsequent stages of the isolation procedure, higher recovery generally results in lower purity and vice versa. In the preparation of a crude

nucleic acid solution, the recovery and the purity are dependent on the extent of alkali denaturation and on the hydrogen ion concentration for precipitation of the protein from alcoholic solution. The loss in preparing nucleic acid from the crude nucleic acid solution is dependent on the amount of protein tied up with the nucleic acid and, therefore, on the extent of alkali denaturation. However, if alkali denaturation is carried too far, nucleic acid is destroyed (i.e. lower recovery) and extraneous soluble nitrogenous compounds are formed from the protein (i.e. lower purity). The same factors are involved in the isolation of nucleic acid and purified nucleic acid preparations.

The purity of nucleic acid in the nucleoprotein, crude nucleic acid and nucleic acid solutions is calculated from the nitrogen-pentose ratio and, therefore, they are only approximate values which do not take into consideration the possible presence of non-nitrogenous contaminations. The nucleic acid and the purified nucleic acid preparations were analyzed in the solid state for nitrogen, phosphorus and pentose. Therefore, their purity can be recorded with greater precision. That is why the purity recorded for the nucleic acid preparations is not as high as the purity of the nucleic acid solution from which it is isolated. This discrepancy, however, is not very great and, therefore,

for the purpose of comparing procedures the nitrogen-pentose ratio is a reliable criterion of purity. As in all preparations from biological materials, great variations in yield and purity are encountered in the preparation of ribonucleic acid from wheat germ. However, if the precautions outlined are followed, consistent results can be obtained.

No detailed study of the preparation of ribonucleic acid from wheat germ was found in the literature. Osborne and Harris (14) obtained the best results recording a yield of over one per cent of a product containing about 80 per cent nucleic acid as estimated from the phosphorus content. This product is comparable to the crude nucleic acid preparations which usually contain 1.8 per cent of the wheat germ as nucleic acid with a purity of about 80 per cent. The preparation obtained by Harris and Osborne (14) may have contained desoxyribose nucleic acid.

At various stages in the procedure for preparing nucleic acid from wheat germ, the required hydrogen ion concentration is stressed as being more important than the strength and volume of the reagent added. Hydrochloric acid and sodium hydroxide are always used and, therefore, relatively small quantities are necessary to change the hydrogen ion concentration. The concentration of sodium

chloride, resulting from the addition of acid and base, is usually below 0.02 N and very seldom above 0.05 N, but after concentrating the crude nucleic acid solution it might be as high as 0.15 N. The required quantities of acid and base depend on the buffering power of the solution, therefore, to describe conditions for general use it is more important to give the actual hydrogen ion concentrations required.

SUMMARY

1. Detailed conditions are given for the measure of hydrogen ion concentration in solutions and the estimation of nitrogen and pentose content of solutions and preparations. The pentose estimation is found to give consistent and quantitative measure of ribonucleic acid when it is assumed that it is a tetranucleotide and that orcinol does not react with the pentose combined with pyrimidine bases.

2. The determination of non-protein nitrogen in aqueous-extract of wheat germ by trichloroacetic acid precipitation has been studied and a reliable procedure is described.

3. The maximum extraction of protein is effected with one part of wheat germ to 10 to 20 parts of water and extracting for 10 to 45 minutes at room temperature (22°C.). Under these conditions 55 to 61 per cent of the nitrogen in wheat germ is extracted, depending on the freshness of the sample and the extent of protein denaturation during extraction. The extracted protein nitrogen varies from 45 to 50 per cent and the non-protein nitrogen from 10 to 12 per cent.

4. Maximum precipitation of nitrogen compounds in the above extract is obtained one hour after adjusting the

hydrogen ion concentration of the solution to pH 4. About 32 per cent of the extracted nitrogen remains in solution of which approximately 20 per cent is non-protein nitrogen and 12 per cent, by difference, is protein nitrogen not precipitated at pH 4.0. The material precipitated at pH 4.0 is a nucleoprotein fraction which on successive precipitations is more critically and completely precipitated at pH 4.0. After three precipitations about 2.2 per cent of the wheat germ is recovered as ribonucleic acid in the form of a nucleoprotein containing approximately 21 per cent nucleic acid. The use of stale wheat germ and conditions which cause more denaturation lowers the recovery of nucleic acid in the precipitate and to a greater degree than would be expected solely on the basis of decreased extraction. The variable composition of the nucleoproteins obtained under various conditions has been studied and is discussed.

5. The degree of denaturation necessary to permit the separation of ribonucleic acid from the protein has been investigated. The use of barium acetate does not facilitate the separation of nucleic acid under the conditions studied. Through denaturation the nucleic acid-protein bond is apparently broken and the hydrogen ion concentration for maximum precipitation of the protein is lowered. This shift is enhanced by the use of ethyl alcohol as

described in the procedure for the preparation of a crude nucleic acid solution. About 1.8 per cent of the wheat germ extracted is recovered as ribonucleic acid in this preparation which contains 20 per cent of other materials. Slight changes in procedure give higher purity and lower recovery or vice versa.

6. The crude nucleic acid solution is concentrated under vacuum and a small nucleoprotein fraction is removed by precipitation at pH 4.0. On addition of 60 per cent alcohol to the clear supernatant, nucleic acid is obtained as a white precipitate. This product comprises about 1.1 per cent of the wheat germ and is a ribonucleic acid contaminated by about 5 per cent of impurities. A procedure is given for the purification of nucleic acid and the yield is approximately 0.8 per cent of the wheat germ which gives about 99.5 per cent pure ribonucleic acid. Chemical and spectrophotometric analyses are given.

CLAIM TO ORIGINAL RESEARCH

1. The steps involved in the preparation of ribonucleic acid from wheat germ have not, heretofore, been studied in detail. A new procedure has been developed and is based on the following principles:-

(a) The careful isoelectric precipitation of a nucleoprotein fraction, avoiding excessive denaturation.

(b) The controlled denaturation of the nucleoprotein causing fission of the nucleic acid-protein bond.

(c) The use of ethyl alcohol to shift the hydrogen ion concentrations for the optimum precipitation of the protein, thus allowing the ribonucleic acid to be separated by precipitation.

(d) The purification of the nucleic acid by controlling the hydrogen ion concentrations and by the use of ethyl alcohol. These principles, even though they may have been applied in the preparation of nucleic acids from other sources have not been systematically investigated for any one product.

2. Employing the above mentioned procedure, the greatest yield of ribonucleic acid yet recorded has been obtained from wheat germ, and the high degree of purity of the product is comparable to the purity of ribonucleic acid

from other sources as reported in a few instances in the literature.

3. It has been found:

(a) That carefully defined conditions must be rigidly adhered to in the estimation of non-protein nitrogen in the aqueous extract of wheat germ by trichloro-acetic acid precipitation.

(b) That nucleoproteins of variable ribonucleic acid content can be isolated from wheat germ.

(c) That the absorption spectra of ribonucleic acid from wheat germ and yeast are identical.

(d) That the modified orcinol test can be used as a quantitative measure of ribonucleic acid if certain assumptions are made.

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