

I PHOSPHORUS-CONTAINING COMPOUNDS IN NEUROSPORA

II SYNTHETIC SUBSTRATES FOR CHYMOTRYPSIN

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## A B S T R A C T

### I Phosphorus-containing Compounds in Neurospora

A paper chromatographic method is described for the separation of the water soluble phosphorus compounds found in tissue extracts. Results with known compounds are given. The method has been applied to the separation of the phosphorus compounds in extracts of Neurospora. The presence of the following compounds is indicated: the adenosine phosphates, 3-phosphoglycerate, glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate. The presence of at least one major unknown compound is also indicated. The significance of these results is discussed.

Modifications of the usual methods for the study of the phospholipids were also devised. The phospholipids were separated into the lecithins and cephalins. The cephalins were converted into the N-2,4-dinitrophenyl derivatives. These were chromatographed on silicic acid-celite columns. The fractions collected were hydrolyzed. Titration of the fatty acids indicate the presence of some phospholipids which do not correspond to the classical formulae. Chromatography of the phosphate esters present in these hydrolyzates revealed glycerophosphate and one unknown phosphorus compound. Chromatography of the DNP-amines showed the presence of eight components. The DNP derivatives of ammonia, ethanolamine, serine, and 1-amino-2-methyl-2-propanol were identified. The techniques used and the significance of the results are discussed.

## II Synthetic Substrates for Chymotrypsin

A general synthesis of methyl acyl-phenylalanyl-anthranilates has been devised. The acetyl derivative serves as a substrate for chymotrypsin. Methyl anthranilate is produced when this compound is hydrolyzed and it is detected by its fluorescence. The succinyl and maleyl derivatives were also prepared, but they are not hydrolyzed by the enzyme. These results are discussed.



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## Water Soluble Phosphorus Compounds

### Introduction

The recognition of the occurrence of organic phosphorus compounds in tissue extracts was made before the beginning of this century. However, it was not until 1907 that it became apparent that these compounds are extremely important in intermediary metabolism (1). Kay (2) in 1932, stated,

"Compounds of phosphorus are present in every cell and fluid of the body; they are concerned with carbohydrate metabolism, with lipins and lipin metabolism, with certain types of protein synthesis, with synthesis and breakdown of nucleic acids, with calcium metabolism, with the buffering power of the blood and other tissues, with growth and maintenance of bone, with muscular contraction, with the functioning of the central nervous system, and suffer changes in a large variety of diseased conditions."

In the scheme of carbohydrate degradation proposed by Embden and Meyerhof, and now known to be essentially correct, the majority of intermediates are phosphorus compounds. Furthermore, the only well understood method of energy transport in vivo, is by means of "high energy" phosphate compounds. Thus, phosphorus compounds play a central role in metabolism.

In the last decade, the fungus, Neurospora crassa, has become increasingly useful in genetics and biochemistry. Fundamental problems of genetics have been studied using Neurospora. The organism can be grown vegetatively to produce a large amount of cell material and such material is valuable for biochemical investigations because the genetic constitution of the cells is known, and because the organism can be grown under controlled

conditions. Perhaps the most important property that makes Neurospora useful for biochemical studies is that many mutants can be grown by the addition of single compounds to the medium. Genetically these mutants are lethals, but the compounds added to the medium permit the growth of the mutants. These mutants may accumulate compounds which are related to the added substances. A large amount of tissue in which a particular metabolic system is disturbed can be obtained, and compounds or enzyme systems isolated from it. By these methods, it may be possible to surmise the chain of events in the biosynthesis of a metabolite.

Although the phosphorus-containing compounds of many organisms are known to be involved in intermediary metabolism, very little is known about their significance in the metabolism of Neurospora. A knowledge of those compounds present in an organism is valuable as a preliminary to any study of its metabolism. Since only one report concerning the phosphorus-containing compounds in Neurospora (3) has appeared, a general survey of these compounds was begun.

The method most widely used for the separation and identification of water soluble organic phosphorus compounds is based on the solvent fractionation of the barium salts of these substances. Such a procedure has been described in great detail by LePage (4). An outline of this procedure is given in Fig. 1. The three fractions shown are analyzed for known components. When this is done, a balance sheet is prepared to determine how much of the organic phosphorus is accounted for by known

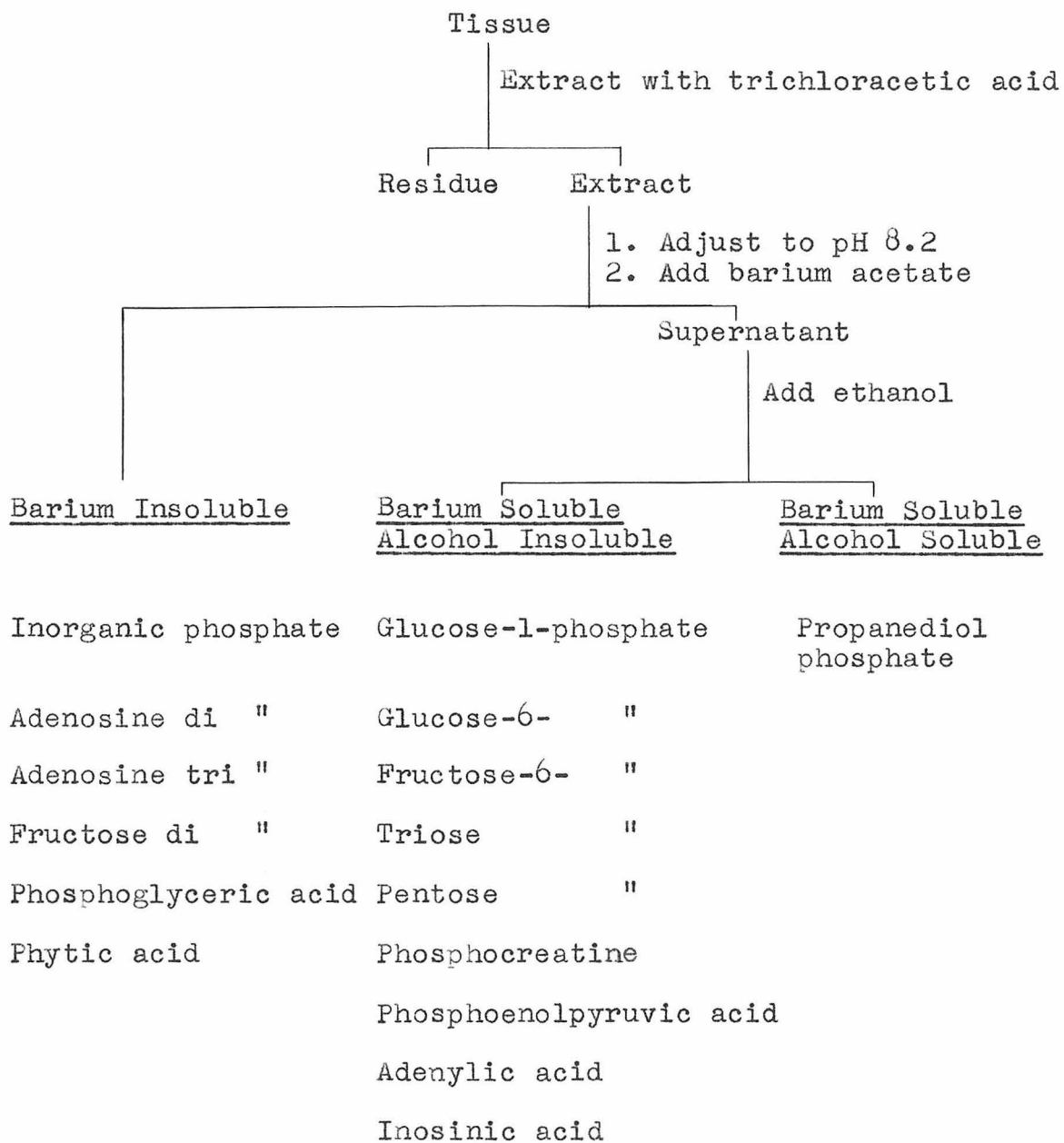


Fig. 1. Outline of a method for extraction and fractionation of certain tissue phosphorus compounds.

components. In some tissue extracts (especially from animal organs), this procedure will account for most of the organic phosphorus; however, the method is wholly inadequate for many tissue extracts (5). This procedure requires careful control of the pH, concentrations, temperature, etc., and it is a tedious and time-consuming method. The numerous modified procedures which have been suggested (6) imply that the workers were not satisfied with the method as outlined by LePage. In fact, it is necessary to know the relative concentrations of the phosphorus-containing compounds before the procedure is applied in order to know which modification to use.

Because of these difficulties with the barium fractionation procedure, an attempt was made to apply the methods of paper chromatography to the problem of separation of the phosphorus compounds in tissue extracts. The following factors were considered particularly important.

1. The method for detecting the phosphorus compounds on paper should be sensitive to microgram quantities of phosphorus.
2. The chromatographic solvents should provide adequate separation of the numerous phosphorus compounds that are present in tissue extracts. In order to be used for two dimensional paper chromatography, more than one solvent should be obtained.
3. It would be desirable that the solvents be relatively inert in order to avoid hydrolysis of labile compounds.

### Experimental

The first problem was to devise a method which could be used for detecting microgram quantities of phosphorus-containing compounds on paper sheets or strips. The reduction of phosphomolybdate to form a blue material was adapted for this purpose. The following solution was sprayed onto strips of filter paper on which were spots containing 2, 1, 0.5, and 0.2  $\mu$ g. of sodium phosphate: ammonium molybdate, 5 g., water, 75 ml., nitric acid (conc.), 25 ml. After the paper had dried, it was sprayed with a 5% solution of ferrous sulfate. All the spots turned blue immediately. When sodium glycerophosphate spots were treated in this way, no color was observed. Consequently, it was necessary to find some way of hydrolyzing these esters on paper. The following experiments were performed in order to test various methods.

1. Dilute sulfuric acid (0.5 N) was sprayed on a strip of paper on which there were spots containing 5, 2, and 1 g. of potassium glycerophosphate. After two hours at 80°, no inorganic phosphate could be detected. However, the paper had become extremely fragile, and therefore this method was abandoned.
2. Sodium hydroxide (1 N) was sprayed on a strip of paper which had been spotted with potassium glycerophosphate. After two hours at room temperature, considerable inorganic phosphate could be detected. However, the glycerophosphate had diffused from its original location. Therefore, sodium hydroxide dissolved in propa-

nol was tried instead of the aqueous solution. The solvent evaporated rapidly, and it was necessary to place the strip of paper in a chamber containing water vapor. Although inorganic phosphate could be detected, the hydrolysis was so slow that this method was abandoned.

3. A phosphatase preparation\* (2 mg.) dissolved in 5 ml. of ethanolamine buffer (pH 9.0, 0.1 M) was sprayed on the glycerophosphate spotted strip. Inorganic phosphate could be detected after 15 minutes. This technique was found to be useful since the enzyme will hydrolyze most phosphate esters and polyphosphates. Although adequate, this procedure was abandoned after the following technique was found to be more convenient.
4. The paper containing the phosphorus compounds was sprayed with the following solution (7): 4% ammonium molybdate, 25 ml., 60% perchloric acid, 5 ml., 1 N hydrochloric acid, 10 ml., and water, 60 ml. After being dried for one minute at 80°, the paper was placed 10 cm. from an ultraviolet lamp for 10 minutes (8). The ultraviolet lamp was a General Electric germicidal lamp (rating: 25 microwatts of 2537 Å. radiation per sq. cm. at 1 meter). The phosphorus compounds appear as blue to blue-green spots. Using

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\*Intestinal Phosphatase, Lot 905 P, Armour Laboratories, Armour and Co., Chicago, Ill.



this procedure, 0.2 $\mu$ g. of phosphorus per sq. cm. can be detected. This procedure is simple, rapid, and quite general in its applicability.

While testing various methods for locating the phosphate esters on paper, it was found that some papers gave a considerable background color, and these were therefore useless for the chromatography of phosphorus compounds. Other papers were found to contain small amounts of the alkaline earth metals, and while these do not interfere with the color development, they prevent movement of the phosphorus compounds in certain chromatographic solvents. Such papers as Whatman No. 1, and 4, Schleicher and Schüll No. 598 and 595 were not used for these reasons. The most satisfactory paper for general use was found to be Schleicher and Schull No. 589 Blue Ribbon. Whatman No. 50, while more expensive, is also satisfactory. The latter is a more dense paper than is customarily used for chromatography, and solvents move slowly on it. However, this may be an advantage in the separation of compounds which lie close together in a given solvent.

The following procedure was used for screening possible chromatographic solvents. Strips of paper, 4.5 in. long, 5/8 in. wide at the top, and 1/2 in. wide at the bottom, were spotted 1 in. from the bottom with a mixture of sodium phosphate, calcium 3-glycerophosphate, and potassium glucose-1-phosphate (3 g. of each). The solvent to be tested (2 ml.) was placed in a 6 in. test tube which was supported in a rack. The paper strip was inserted and the solvent was allowed to rise until

it reached almost to the top of the strip. The strip was then dried and the positions of the phosphorus compounds were determined. Using this procedure (9) it was possible to survey over 200 solvents and solvent mixtures in a few weeks. The following substances did not move the phosphorus compounds used in the survey.

Alcohols	Ethers
n-propanol	diethyl ether
iso-propanol	di-iso-propyl ether
n-butanol	dioxane
iso-butanol	Amines
sec-butanol	benzylamine
tert-butanol	piperidine
iso-pentanol	pyridine
furfuryl alcohol	lutidine
Miscellaneous	collidine
acetone	
ethyl carbonate	
ethyl acetate	

These solvents were all commercial products and they were used without purification. The only solvents tried which moved the phosphorus compounds were water, methanol, ethanol,  $\beta$ -methoxyethanol, formamide, and acetonitrile. Mixtures of water and dioxane gave the following results. With low concentrations of water (5-10%) very low Rf values were observed, and no resolution of the compounds could be obtained. At higher water

concentrations (10-30%) more movement was evident, but the compounds tailed badly. In solvents containing over 35% water, the compounds all moved with the solvent front. Similar results were observed with mixtures of water and propanol, and water and acetone.

When mixtures of the commercial formamide and dioxane were tried, definite spots were observed. The three test compounds were separated by a 30% solution of formamide in dioxane. Examination of the commercial formamide disclosed that it contained water and formic acid. When the formamide was purified, it no longer moved the phosphorus compounds, and it showed the same behavior when mixed with water as did dioxane, propanol, and acetone. Thus, control of the water content and the acidity of the solvent were found to be necessary. Since the phosphorus compounds streaked badly in the absence of added acid or base, a series of methanol-water mixtures which contained formic acid or ammonium hydroxide were tried. It was found that the phosphorus compounds could be chromatographed in alkaline solvents if they were placed on the paper as the free acids. Methanol was chosen as the non-aqueous component, since it moves up the paper relatively rapidly. Formic acid and ammonium hydroxide were chosen because they can be easily evaporated. The most useful solvents obtained were the following.

Alkaline Solvent:

Methanol, 60 ml., water, 30 ml., ammonium hydroxide (conc.), 10 ml.

Acid Solvent:

Methanol, 80 ml., water, 5 ml., formic acid (90% aqueous soln.), 15 ml.

After these solvents had been chosen, the Rf values of the available phosphorus compounds was determined as follows. The chromatographic chamber used was a cylindrical jar (height, 14 in., diameter, 6 in.) covered with a glass plate and sealed with modeling clay. The solvent was placed in the jar to a depth of  $\frac{1}{4}$  in. The jar and its contents were placed in a cold room (5°) since it was found that the slight amount of hydrolysis that occurred when the chromatographs were run at room temperature could be prevented by running them in the cold. A pencil line was drawn approximately 1 in. from the lower edge of a sheet of Schleicher and Schüll No. 589 Blue Ribbon filter paper 8-10 in. square. Solutions of known phosphorus compounds (as the free acids) were placed on this line at intervals of about one inch. The amount of solution applied was regulated to give 2-5  $\mu$ g. of phosphorus in each spot. The paper was formed into a cylinder and stapled together so that the edges did not contact each other. An iron wire was inserted diametrically through the upper end of the paper cylinder, which was then suspended above the solvent by means of a magnet placed on top of the glass cover. After 1-2 hours equilibration in this manner, the paper cylinder was dropped into the solvent by removing the magnet. This period of equilibration was found to increase the reproducibility of the Rf values.

These procedures were adapted to the performance of two dimensional chromatography. Only one spot was placed at the lower corner of the sheet (about one inch from each edge) and the chromatograph was developed with one solvent as described above. This solvent was removed by drying the sheet in an oven at 80°C. The paper cylinder was then reformed so that the second solvent moved at right angles to the direction of the first solvent. The equilibration was repeated as described before, and the chromatograph developed in the second solvent. The acid solvent was usually used first.

In this way, either one or two dimensional paper chromatography was performed. The Rf values found for known compounds are listed in Table 1. The values are reproducible to about 0.08. Figure 2 illustrates the relative positions of these compounds after separation by two dimensional chromatography. In the separation of complex mixtures of compounds by two dimensional paper chromatography, the relative positions are usually more significant than the Rf values (10).

Wild type Neurospora 5256A was grown for 6 days on Fries medium containing 2% sucrose. The mycelia were harvested and frozen in liquid nitrogen. The frozen pieces were ground in a meat grinder which was kept cold by a dry ice pack. The finely powdered mycelia were suspended in a 0.5% solution of sodium fluoride, which prevented phosphatase activity. This suspension was adjusted to pH 6.2 with potassium hydroxide and was placed in a large Mason jar. Several dialysis bags containing 10-15 ml. of the sodium fluoride solution were introduced. The jar

TABLE 1

Rf Values of Some Phosphate-containing Compounds

Compound	Acid Solvent*	Alkaline Solvent*
Adenosine triphosphate	0.12	0.15
Adenosine-3-phosphate	0.17	0.32
Fructose-1,6-diphosphate	0.40	0.24
Glucose-1-phosphate	0.27	0.60
Glucose-6-phosphate	0.38	0.48
Fructose-6-phosphate	0.34	0.44
3-Phosphoglycerate	0.50	0.35
2-Phosphoglycerate	0.46	0.18
Phosphoenolpyruvate	0.52	0.46
Ortho-phosphate	0.63	0.28
Pyro-phosphate	0.46	0.05
Tripoly-phosphate	0.37	0.06

\* See text.

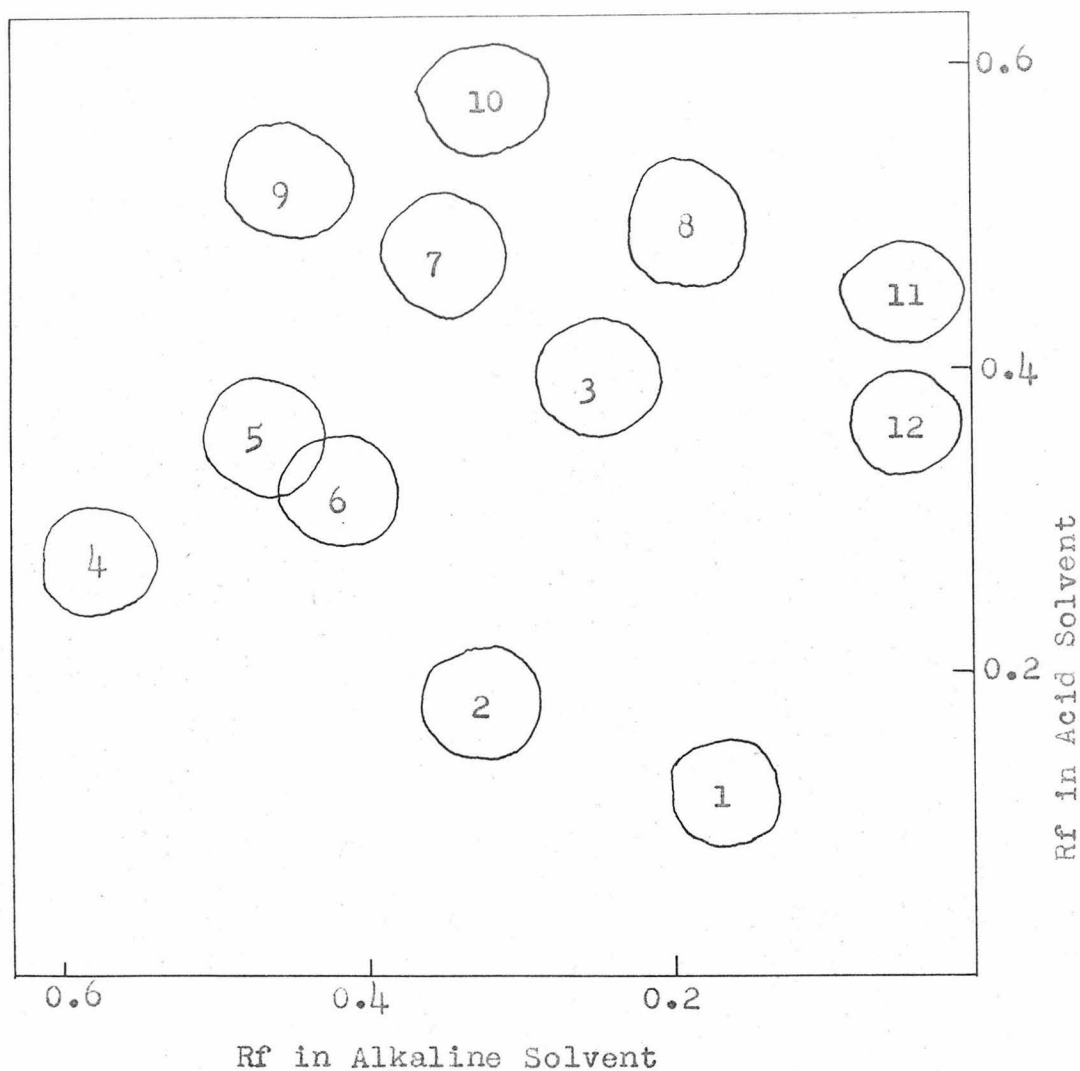


Fig. 2. Relative positions of spots obtained with known compounds after two dimensional chromatography.

- |                             |                        |
|-----------------------------|------------------------|
| 1. Adenosine triphosphate   | 7. 3-Phosphoglycerate  |
| 2. Adenosine-3-phosphate    | 8. 2-Phosphoglycerate  |
| 3. Fructose-1,6-diphosphate | 9. Phosphoenolpyruvate |
| 4. Glucose-1-phosphate      | 10. Ortho-phosphate    |
| 5. Glucose-6-phosphate      | 11. Pyro-phosphate     |
| 6. Fructose-6-phosphate     | 12. Tripoly-phosphate  |

was sealed and placed on a shaker in a cold room. At 2-3 hour intervals the dialysis bags were removed and replaced with others containing fresh sodium fluoride solution. This process was continued for two days. The dialysate (350 ml.) was yellow and highly fluorescent and contained 252 mg. of phosphorus\*. Most of the inorganic phosphate was removed from this solution by the addition of magnesia mixture ( $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$ , 5 g.,  $\text{NH}_4\text{Cl}$ , 10 g., water, 100 ml.) and ammonium hydroxide. After an hour the precipitate which formed was removed by filtration.

The filtrate (containing 25 mg. of phosphorus) was adjusted to pH 8.2 by the addition of 1 N hydrochloric acid; then 3 ml. of barium acetate (1 M) was added, whereupon a curdy precipitate formed. This material was separated into the barium insoluble (BI), barium soluble alcohol insoluble (BSAI), and barium soluble alcohol soluble (BSAS) fractions exactly as described by LePage (4). The phosphorus content of these fractions was found to be 4, 4, and 17 mg. respectively.

Each of these fractions was chromatographed by the two dimensional procedure described before. The results of these separations are shown in Fig. 3. The BI fraction showed spots in the positions normally occupied by inorganic phosphate, 3-phosphoglycerate, the adenosine phosphates, glucose-1-phosphate, and glucose-6-phosphate. Spots corresponding to the

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\*Samples were digested with perchloric acid and hydrogen peroxide. After digestion, the phosphorus was determined by the method of Houlahan and Mitchell (3).



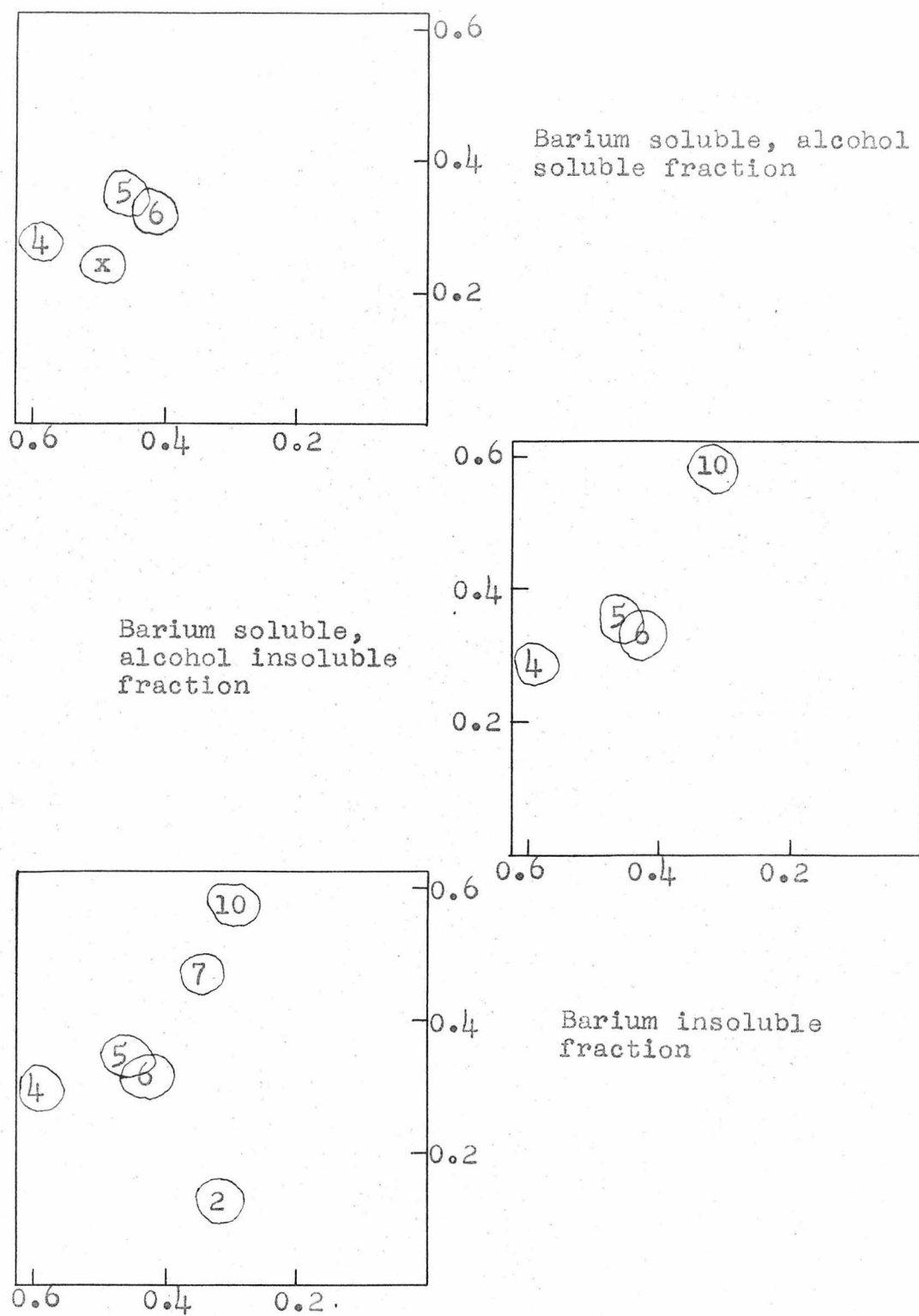


Fig. 3. Results of the two dimensional chromatography of the fractions indicated. Spots are numbered as in Fig. 2.

positions of glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate were found in the BSAI fraction. Glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate were found in the BSAS fractions. In addition, an unknown phosphorus compound was present in the BSAS fraction. Several two dimensional chromatographs of the BSAS fraction were prepared. These were sprayed with solutions of naphthores-orcinol, ninhydrin, and orcinol. These reagents react with sugars, amines, and pentoses, respectively. However, none of them reacted at the spot where the unknown phosphorus compound was located. This compound was not investigated further.

### Discussion

The chromatographic procedures which were devised are satisfactory in two respects. The method for detecting the phosphorus compounds is quite sensitive, and the solvents described are capable of separating the ordinary tissue phosphorus compounds into single spots in many instances, and into groups of related compounds in others. Thus, fructose-1,6-diphosphate is separated from other compounds, whereas the hexose monophosphates move to similar positions on the chromatographs. Unfortunately a satisfactory inert solvent has not been found. In neutral, or only slightly acid or basic solvents, none of the phosphorus compounds could be made to move in a reproducible manner. Although it was not possible to use non-acidic or non-basic solvents, operating at low temperatures was quite effective in reducing hydrolysis.

These studies of the chromatography of phosphorus compounds bring out a number of facts that are probably quite general. When working with compounds that can exist in several different charge forms (anions, cations, uncharged forms, zwitterions) the desired form is obtained by control of the acidity of the solvent. With such compounds, the relative positions are determined by the charge form. Thus, using a strongly acid solvent, the phosphorus compounds always moved in this order: phosphoric acid moved most rapidly, 3-phosphoglycerate next, and glucose-1-phosphoric acid last. The  $R_f$  values of these compounds can be reduced or increased at will by varying the water content of the solvent, but the order of the movement of these compounds is always the same, as long as the solvent is strongly acid. On the other hand, in a strongly alkaline solvent, the compounds being chromatographed exist as salts. The order of movement is changed to glucose-1-phosphate first, 3-phosphoglycerate next, and inorganic phosphate last. Whenever a strongly alkaline solvent is used, this is the order, although varying the water content of the solvent changes the  $R_f$  values of these compounds. Thus, if it were desirable to move pyrophosphate away from the origin in an alkaline solvent, this could be done by increasing the relative amount of water in the solvent, but this would also increase the  $R_f$  values of all the other compounds, so that pyrophosphate would still move slower than orthophosphate, and faster than tripolyphosphate.

The results of the chromatography of the phosphorus

compounds also focus attention on the lack of efficacy of the barium fractionation procedure for the separation of the phosphorus compounds. Some of the compounds appeared in fractions where they were not expected, and others appeared in all fractions. Therefore, it is suggested that the barium fractionation procedure is unnecessary. After the isolation of the barium salts, the phosphorus compounds could be chromatographed directly. The outline of such a procedure is given in Fig. 4.

The finding of the various phosphorus compounds mentioned throws some light on the intermediary carbohydrate metabolism of Neurospora. The known pathways of carbohydrate metabolism (as deduced primarily from yeast preparations, muscle breis, and liver slices) are briefly summarized in Fig. 5. The phosphate compounds whose presence was indicated by the two dimensional chromatographs are enclosed in rectangles. Two important pathways are shown. In one pathway (originally suggested by Embden and Meyerhof), glucose is eventually converted to fructose-1,6-diphosphate, which is split into two three carbon compounds, both of which are eventually converted to pyruvic acid. In the other pathway (suggested by Warburg, Lipmann, & Dickens), glucose is converted to 6-phosphogluconic acid via glucose-6-phosphate. The gluconic acid is eventually decarboxylated to form a phosphopentose, which is split into a three and a two carbon compound. The pathway of further metabolism of the two carbon compound is still uncertain, but the three carbon compound is converted to pyruvic acid (11).

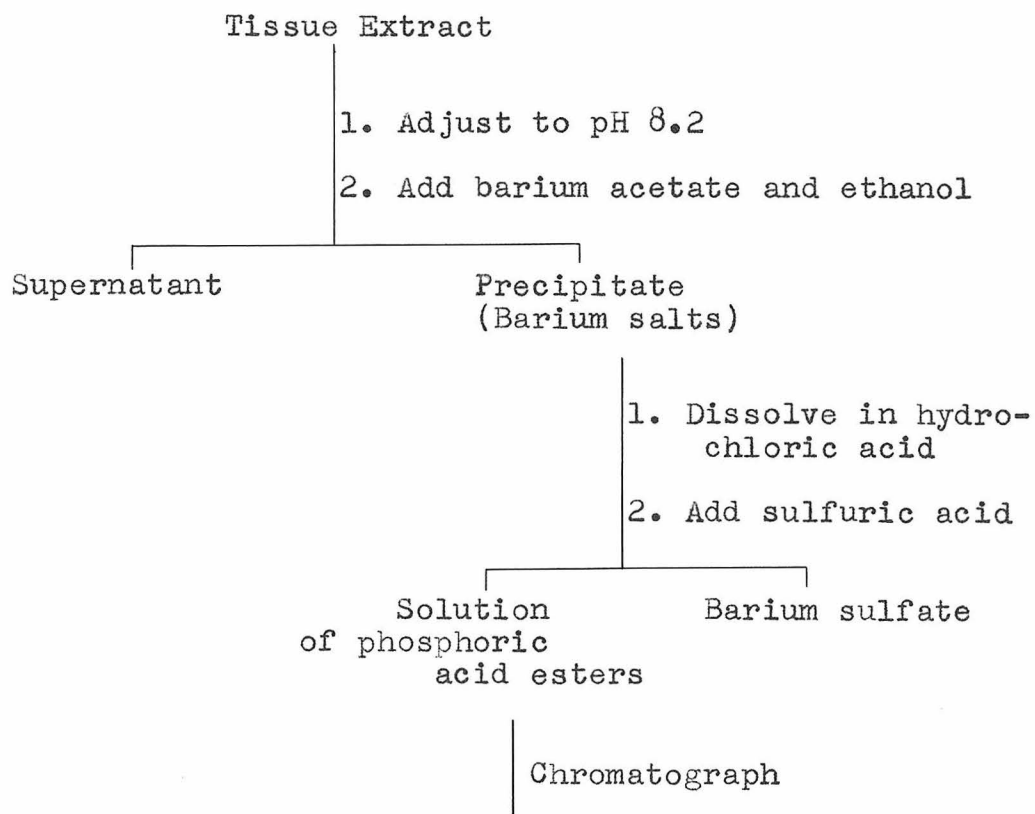


Fig. 4. A suggested procedure for the separation and identification of phosphorus compounds.

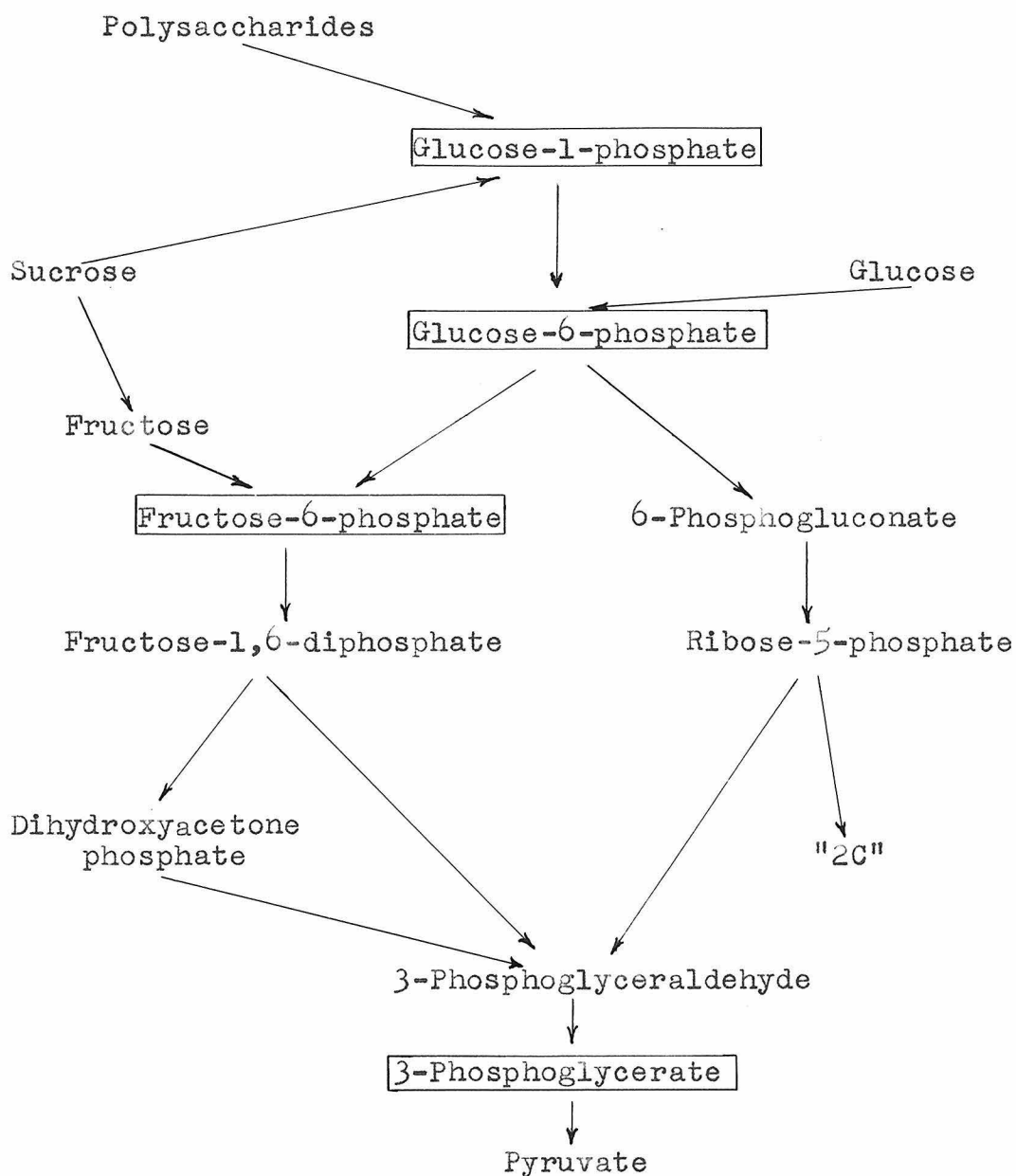


Fig. 5. The known pathways of carbohydrate metabolism.

(The arrows are not intended to imply that the reactions are not reversible.)

The Neurospora was grown on sucrose; therefore, the finding that there is fructose-6-phosphate in the tissue extract does not necessarily imply that Neurospora degrades carbohydrates via the Embden-Meyerhof pathway, since the fructose could come from the sucrose. In order to establish the existence of either of the two pathways in an organism, it is necessary to demonstrate the existence of fructose-1,6-diphosphate and/or 6-phosphogluconic acid in that organism. No rigorous search was made for these compounds so it is not possible to conclude that either of these compounds does or does not occur in Neurospora.

An alternative and complementary method of establishing the existence of either of these pathways would be to establish the existence of the enzymes involved in the conversions. This study could be assisted greatly by the chromatographic procedures described in this thesis. For example, if an enzyme preparation split fructose-1,6-diphosphate, and the enzyme system were poisoned with bisulfite, the triose phosphates which are produced would be accumulated in the reaction mixture. These could easily be demonstrated by the chromatographic method described.

## Lipid Soluble Phosphorus Compounds

### Introduction

An important portion of the phosphorus in many tissues is lipid soluble. These compounds are called the phospholipids. They have been studied extensively in some tissues, notably mammalian nerve, muscle, lung, and liver tissue, and also in the seeds of certain higher plants. However, the phospholipids of the lower organisms (with the exception of E. coli) have not been studied extensively.

A typical procedure for the isolation and separation of the components of the phospholipids is illustrated in Fig. 6 (12). While some workers prefer to isolate the phospholipids from heat, air, or acetone dried tissue, others prefer to isolate them from the fresh moist tissue. As yet, no qualitative difference has been noted between the products isolated from dry and moist tissue. Dry tissue is usually extracted for a prolonged period in a Soxhlet apparatus with ligroin, chloroform, or ethanol. Moist tissue is usually ground with ethanol, ether, and finally ligroin but complete extraction of all the phospholipids from tissue is difficult. The extracts obtained by these methods are poured into acetone. These crude acetone precipitated phospholipids are usually "purified" further. One method of purification is to dissolve the crude material in ether, chloroform, or ligroin, remove any insoluble material, and reprecipitate the phospholipids by the addition of acetone. This whole procedure is repeated several times. Alternately,



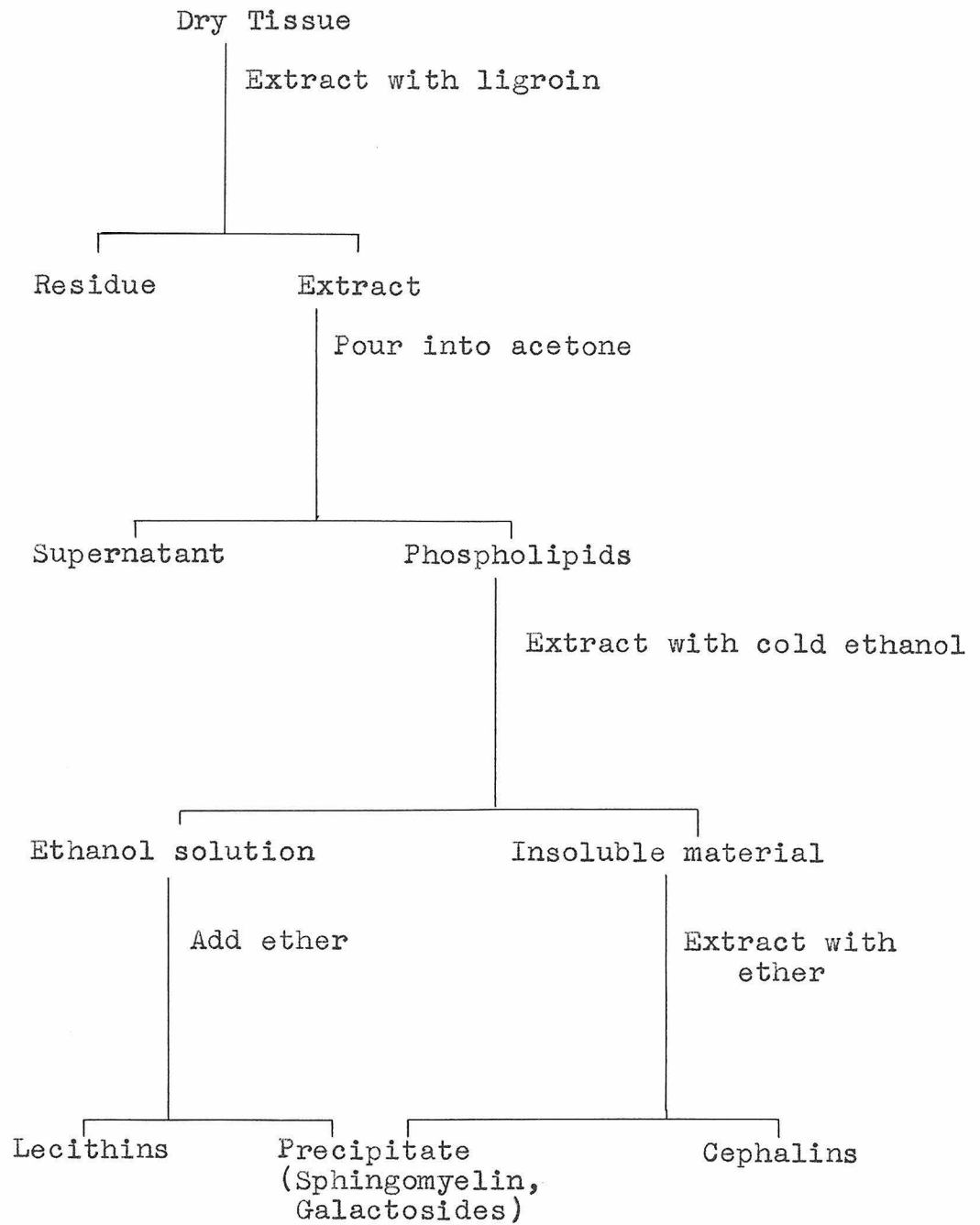


Fig. 6. A typical procedure for the isolation and separation of phospholipid components (12).

the crude phospholipids are suspended in water and the suspension is dialysed to remove small water soluble components. Another procedure involves the extraction of the phospholipids from the aqueous suspension with chloroform. This purified material usually contains, besides phosphorus, both primary and non-primary amino nitrogen, sulfur, carbon, hydrogen, oxygen, and traces of sodium, potassium, calcium, and magnesium.

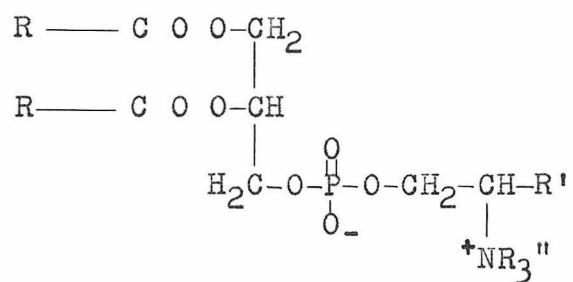
When freshly isolated, the phospholipids are usually colorless, flocculent materials, which soon coalesce to form a waxy, intractable, soap-like substances. On standing in air, the phospholipids rapidly darken, and eventually change into a black tarry mass. These changes are due to oxidation, peroxidation, decomposition, and polymerization of the unsaturated fatty acid residues in the phospholipid molecules. The phospholipids are quite soluble in chlorinated hydrocarbons, moderately soluble in ether and ligroin, and practically insoluble in acetone. However, they can be dispersed in water to form a thick gel, in a fashion characteristic of many other detergents.

The phospholipids are separated into two large classes depending on their solubility in cold ethanol. Those which are soluble in ethanol are called the lecithins, and those which are insoluble are called the cephalins (or kephalins). Recently, efforts have been made to separate these components by chromatography. Two reports of the failure of this method have appeared (13). However, Bevan, et al., (14) were able to free lecithin of all traces of primary amino-containing compounds by chromatography of the crude phospholipids on a cellulose

column, using as a solvent a 10% solution of ethanol in chloroform. Hanahan, et al. (15), purified egg lecithin similarly on an alumina column.

Most workers have used the nitrogen/phosphorus ratio as the only criterion of purity of these materials. The lecithins or cephalins are repeatedly precipitated until this ratio is 1.0. Further studies of the lecithins and cephalins usually depends on degradative procedures. Basic or acidic hydrolyses in aqueous or non-aqueous media have been used for this purpose. For example, the phospholipids are hydrolyzed by refluxing them in a dry methanolic solution of hydrogen chloride, or they are hydrolyzed by treatment of a benzene solution of the phospholipids with sodium ethoxide solution at room temperature, or by refluxing an aqueous dispersion of the phospholipid with acid. When they are degraded in any of these ways, the components which are usually identified are fatty acids, glycerophosphoric acid, and one or more amines. The lecithins contain predominantly choline, while the cephalins have been shown to contain ethanolamine, and serine. By milder degradation procedures, it is possible to obtain larger component parts of the phospholipid molecules. From such evidence, the structures of the principal phospholipids have been determined. These structures are illustrated in Fig. 7.

Although the amines mentioned above account for much of the nitrogen in most phospholipids, there is ample evidence that other amines occur in such extracts (12). For example, Chargaff, et al. (16) analyzed the phospholipids from various



I

R = fatty acid residue

when  $\text{R}' = \text{H}$ ,  $\text{R}'' = \text{CH}_3$ , I is phosphatidyl choline

$\text{R}' = \text{H}$ ,  $\text{R}'' = \text{H}$ , I is phosphatidyl ethanolamine

$\text{R}' = \text{COOH}$ ,  $\text{R}'' = \text{H}$ , I is phosphatidyl serine

Fig. 7. The principal components of phospholipids.

sources by the isotope dilution technique and obtained the following results.

	Pig Liver (as % of the	Pig Heart original phospholipid)	Beef Brain
Total amino N	0.87	0.83	1.13
Ethanolamine N	0.45	0.61	0.76
Serine N	0.11	0.11	0.37
Other amino N	0.31	0.11	0.00

It is evident that the ethanolamine and serine do not account for all the amino nitrogen in some phospholipids. In the pig liver, for example, over 35% of the amino nitrogen is unaccounted for. As will be shown later in this thesis, at least four unknown primary amino compounds occur in Neurospora phospholipids.

Research on the phospholipids of fungi has generally been limited to the identification of choline in the phospholipid extracts. Sumi (17) identified lecithin as a component of Aspergillus spores, and Thomas showed that the mycelia of Sclerotinia contained lecithin (18). Takata (19) isolated the phospholipids of Aspergillus oryzae. By the procedure outlined in Fig. 6, these phospholipids were separated into three fractions, the lecithins, the cephalins, and the sphingomyelins. Nitrogen and phosphorus were the only elements chemically determined. A more detailed study of the phospholipids of Aspergillus sydowi was described by Woolley et al. (20). The phospholipids were hydrolysed and the following components

were identified. Glycerophosphoric acid was isolated as the barium salt. Choline was identified as the chloroplatinate, and ethanolamine was identified as the picrolonate. Ammonia was also produced on hydrolysis of these phospholipids and it was identified as hexamethylenetetramine. The fatty acids were fractionated by the lead soap method and it was shown that they are mainly palmitic and stearic acids. Some evidence for the occurrence of oleic acid was also obtained.

### Experimental

The Neurospora phospholipids were isolated and separated into cephalins and lecithins as outlined in Fig. 8. In order to aid in the identification of the component containing the free amino group, the cephalins were treated with 2,4-dinitrofluorobenzene, a very reactive alkylating agent, which attacks primary and secondary amino groups and alcohols. The dinitrophenyl (DNP) derivatives of amines are deep orange-yellow, while those of alcohols are pale yellow. The DNP-cephalins, being colored, were convenient for chromatography, and therefore, an attempt was made to separate the various DNP-cephalins in this way.

Wild type Neurospora 5256A was grown for 6-7 days on Fries medium containing 2% sucrose. The mycelia were collected, pressed as dry as possible by hand, ground in a meat grinder, and suspended in ethanol-ether (3:1) at room temperature. After two days, the suspension was filtered, and the mycelia were re-extracted with ethanol-ether overnight. The residual

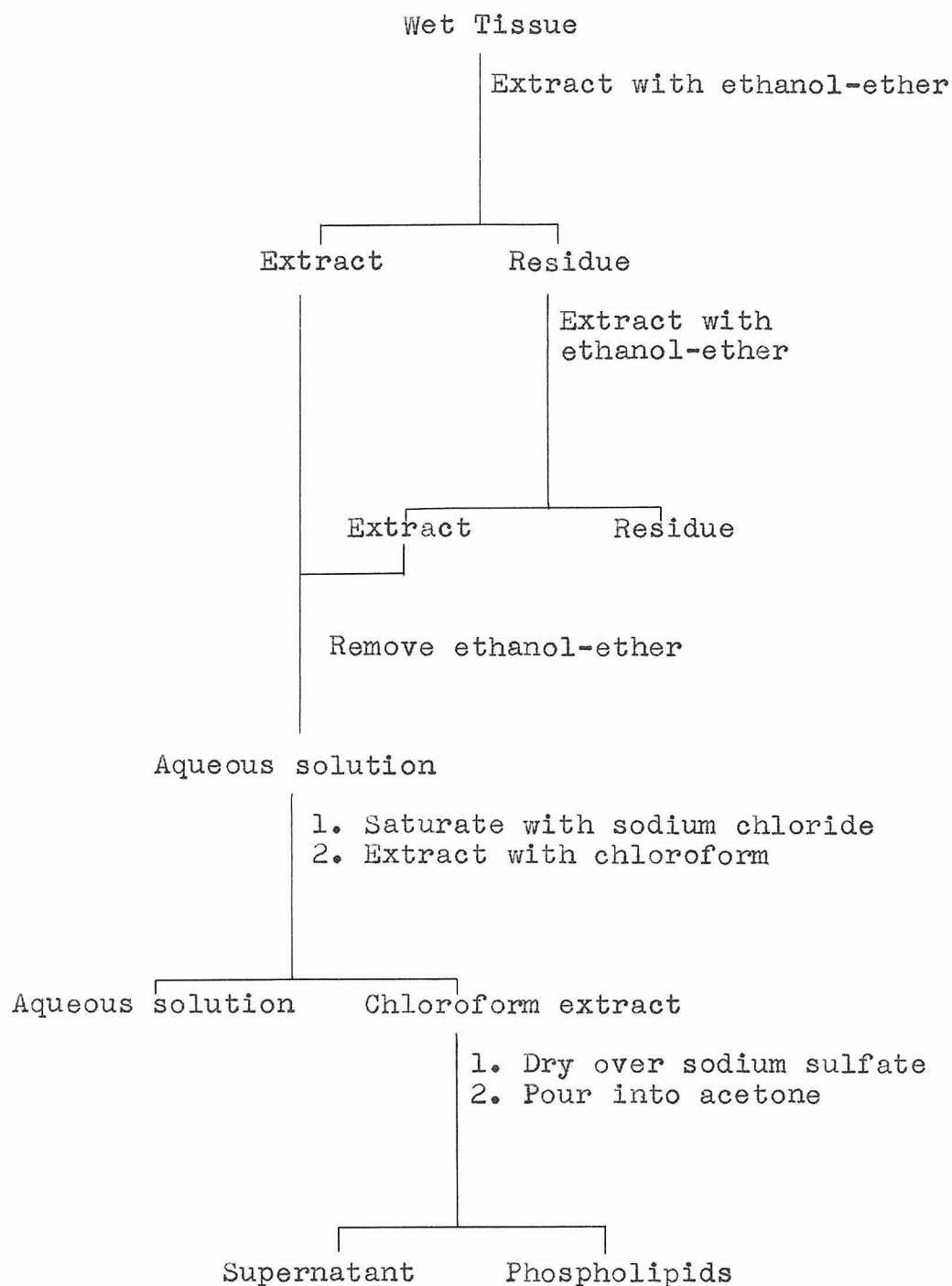


Fig. 8. Outline of the procedure used to extract and isolate phospholipids from Neurospora.

mycelia were dried and weighed. The combined extracts were evaporated to a small volume in a flash evaporator, and the aqueous solution so obtained was saturated with sodium chloride and extracted repeatedly with chloroform. This whole procedure was repeated until 1.5 kg. (dry weight of the residues) had been extracted. The chloroform extracts were combined, dried over anhydrous sodium sulfate, and evaporated (in vacuo) to 350 ml. This solution was poured into 3500 ml. of acetone. The mixture was placed in the refrigerator for three days. About 16 g. of phospholipid (containing 830 mg. of phosphorus) precipitated. This material was collected by centrifugation and it was then fractionated as shown in Fig. 9. The phospholipids were dissolved in 150 ml. of chloroform. Absolute ethanol (600 ml.) was poured into this solution to precipitate the cephalins. These were removed by centrifugation. The supernatant solution was poured into 3000 ml. of acetone in order to precipitate the lecithins.

The cephalins (containing 140 mg. of phosphorus) were dissolved in 100 ml. of chloroform. To this solution was added 1.5 g. of 2,4-dinitrofluorobenzene dissolved in 20 ml. of ethanol, and 70 ml. of a 4% solution of sodium carbonate. The mixture was shaken occasionally during 16 hours. The chloroform layer was removed and the aqueous layer (adjusted to pH 2.5 with conc. hydrochloric acid) was extracted with three 20 ml. portions of chloroform. The aqueous layer was discarded, since it contained only 5 mg. of phosphorus.



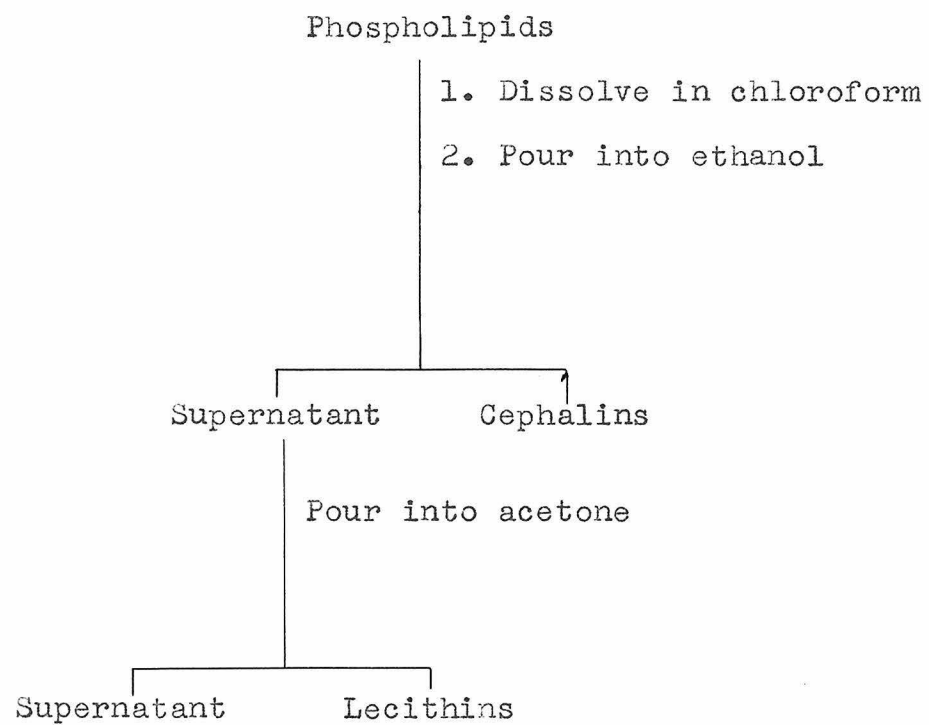


Fig. 9. Outline of the procedure used to separate the phospholipids into lecithins and cephalins.

The chromatography of the DNP cephalins was performed on silicic acid-celite columns. The adsorbent consisted of two parts (by weight) of Mallinckrodt silicic acid (100 mesh, specially prepared for chromatographic analysis) and one part of Johns Manville Hyflo Supercel filter aid. Chromatographic columns were prepared as follows. The dry adsorbent was poured into the glass columns (2 cm. X 13 cm.). Suction was applied and the adsorbent was washed with acetone, acetone-ligroin, and ligroin (40-60 ml. each). When the last few ml. of ligroin remained above the column, the vacuum pump was turned off.

The chloroform solution of the DNP-cephalins was evaporated (in vacuo) to 25 ml., 5 g. of the chromatographic adsorbent was added and the rest of the chloroform was removed. The adsorbed material was shaken with 10 ml. of acetone and the mixture was filtered. This treatment was repeated three more times and it removed much orange-yellow material which contained only 6 mg. of phosphorus. This material was discarded. Further elution in this manner removed the following amounts of phosphorus.

Fraction	Eluent	Phosphorus eluted(mg.)
L2	10 ml. Acetone-methanol(4:1)	25
L3	25 ml. Methanol	71
L4	20 ml. 0.1 N $\text{NH}_4\text{OH}$	33

Each of these fractions was adsorbed on 2.5 g. of silicic acid-celite as described before. A slurry of this material in ligroin was poured onto a chromatographic column prepared as

described above. In this manner, the width of the original band was limited to a few millimeters. Air pressure rather than suction was used to force the solvents through the column as the solvents were volatile and evaporated from the lower part of the column when suction was used. The DNP-cephalins were separated from each other using acetone, and mixtures of acetone and methanol as chromatographic solvents. The eluate was collected in fractions corresponding to the colored zones that formed. The phosphorus content of each fraction was determined. The results of these procedures are illustrated in Fig. 10.

If a fraction was found to contain phosphorus, it was treated by the procedures outlined in Fig. 11. The chromatographic solvent was removed, and the residue was dissolved or suspended in about 25 ml. of ligroin. This was added to an equal volume of 5 N hydrochloric acid and the mixture was refluxed for 16-20 hours. This two phase hydrolysis produced less tarry material than was formed when acid alone was used for the hydrolysis. After hydrolysis, the ligroin layer was removed and the aqueous layer was extracted with two 10 ml. portions of ligroin. The combined ligroin solution of the fatty acids (fraction L, Fig. 11) was evaporated (in vacuo) and dried in a vacuum desiccator over sodium hydroxide pellets. The aqueous solution (containing DNP-amines and the phosphate esters) was evaporated (in vacuo) and the residue was taken up in 5-10 ml. of water. This was extracted with three 5 ml. portions of ethyl acetate, which removed the DNP-amines

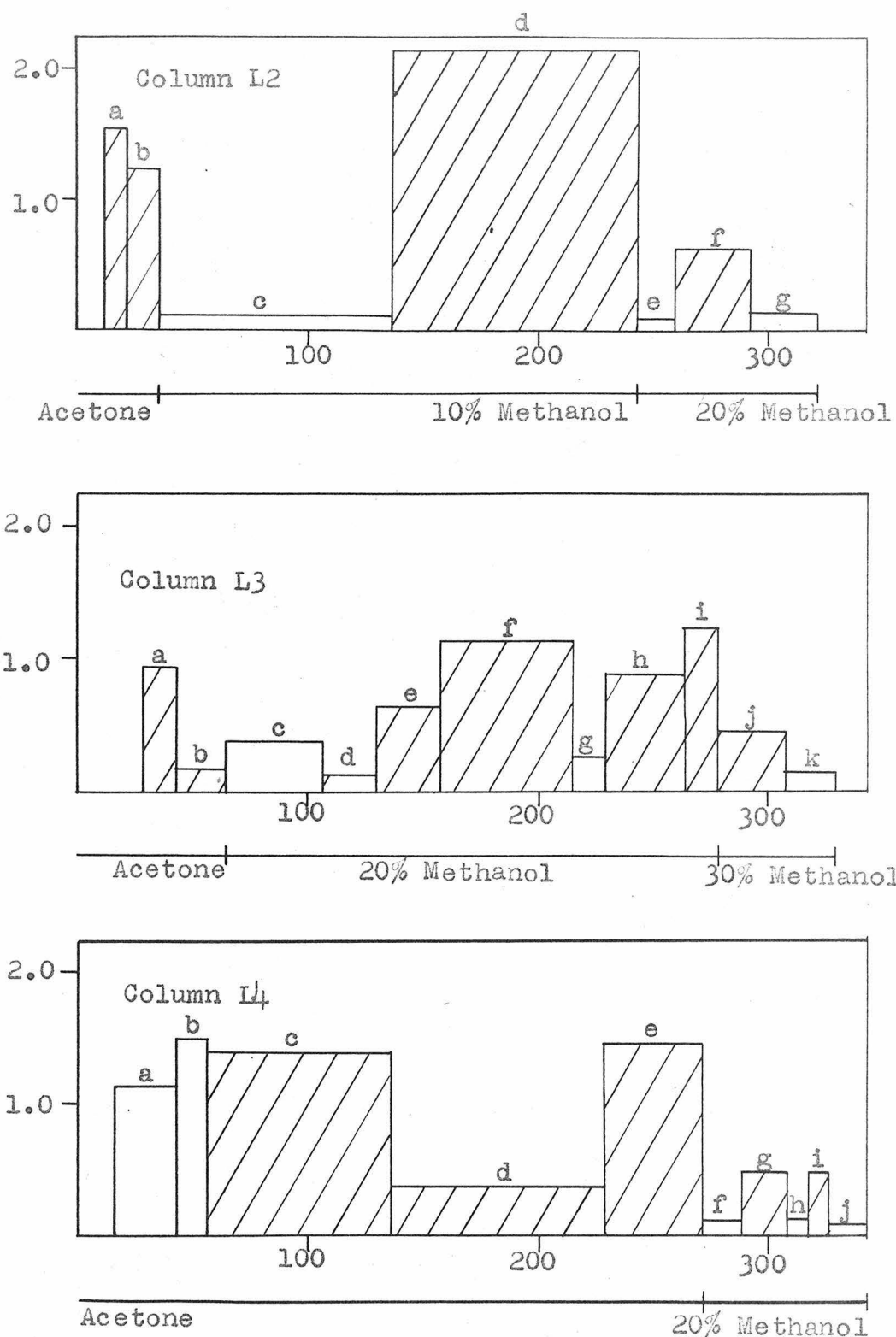


Fig. 10. Results of the chromatography of the DNP-cephalins. Absciss, volume (ml.) eluted from the column; ordinate, optical density at  $\lambda = 420 \text{ m}\mu$ . The shaded rectangles represent fractions which contained phosphorus.

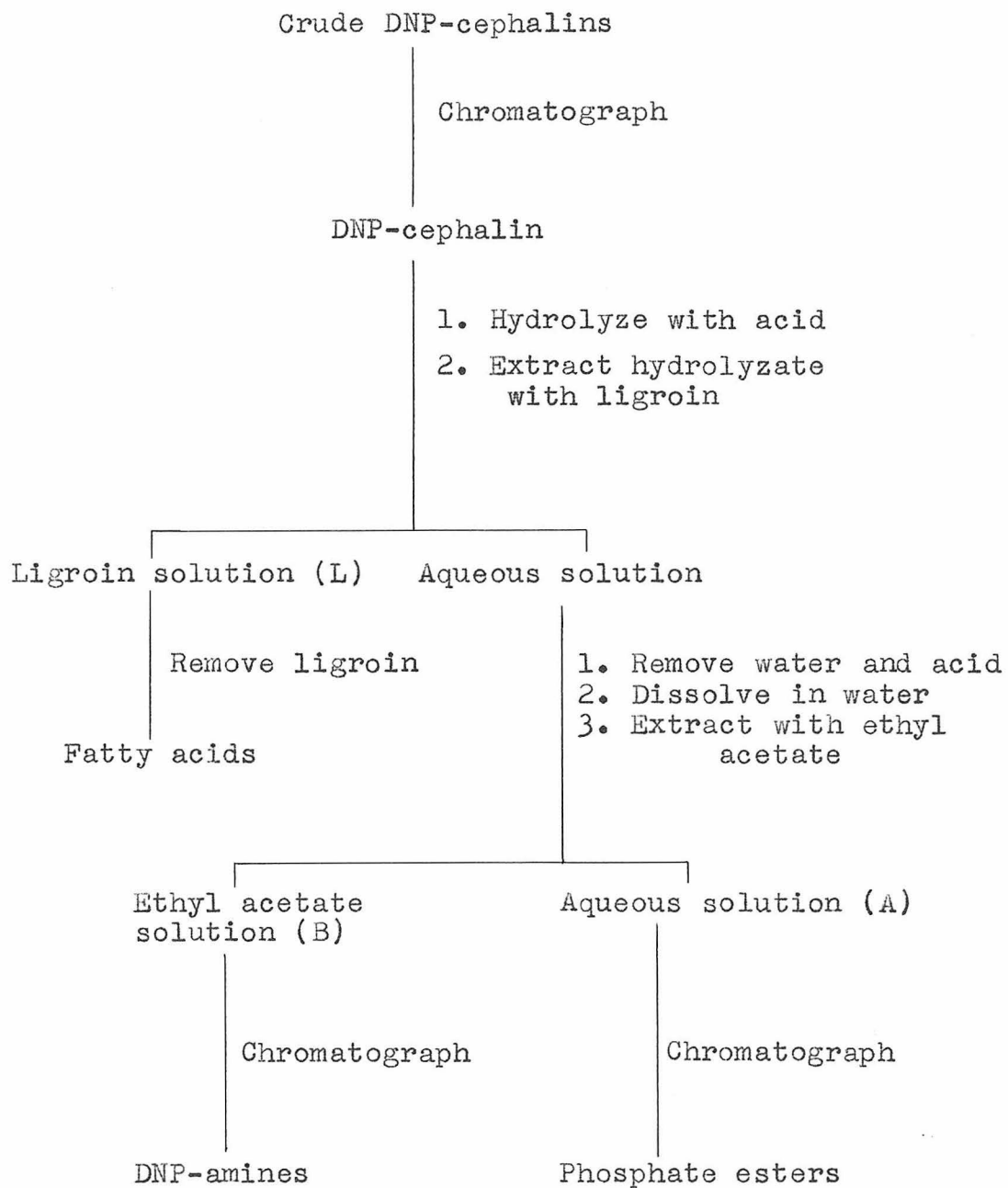


Fig. 11. Outline of the procedures used to identify the components of the DNP-cephalins.

(fraction B, Fig. 11). The aqueous solution (fraction A, Fig. 11) contained the phosphate esters.

The fatty acids (fraction L, Fig. 11) were weighed, then dissolved in propanol (5-10 ml.), and titrated with 0.1 N sodium hydroxide. When known amounts of palmitic and stearic acids were titrated in this fashion the amount of base required was 98-103% of the theoretical amount. From the weight of the fatty acid and the number of moles titrated, an average molecular weight was calculated, assuming that all the acids were monobasic. The number of moles of fatty acid was divided by the phosphorus content (in moles) of the fraction, and these ratios, as well as the average molecular weight of the fatty acids are given in Table 2.

The aqueous solution of the phosphate esters (fraction A, Fig. 11) was evaporated to about 0.5 ml. and an aliquot was chromatographed on paper to identify the phosphate ester component. The paper chromatography was performed two dimensionally as described on Page 10 of this thesis. Most of the fractions showed a definite spot where glycerophosphate was expected; however, three of the fractions (L3f, L4c, L4d, Table 2) showed another spot besides. No further work was done on the characterization of this second component.

The ethyl acetate solution of the DNP-amines (fraction B, Fig. 11) was evaporated and adsorbed on silicic acid-celite and chromatographed as described for the DNP-cephalins. The composition of the solvents used and the results of the chromatographic separation are given in Tables 3, 4, and 5. Several

Table 2

Fraction (see Fig. 10)	Eluent	<u>Fatty acid</u> <u>Phosphorus</u>	Av. Molecular weight of Fatty Acids
L2a	1	1.92	187
b	1	0.47	202
d	2	2.31	242
f	2	2.12	283
L3a	3	0.74	287
b	3	0.95	168
e	3	1.47	249
f	3	6.01	298
h	3	0.95	210
i	3	0.55	173
j	3	1.08	250
L4c	1	1.56	305
d	1	0.43	311
e	1	1.94	193
g	3	0.68	221
i	3	0.41	291

Eluents: 1: Acetone  
 2: Acetone:methanol, 9:1  
 3: Acetone:methanol, 4:1

Table 3

Chromatography of the DNP-Amines

Fraction (see Fig. 10)	Eluent	DNP-Amines
L2a	1	A
b	2	A, D
d	2	A, D
f	2	A, D
L3a	1,3	A, C, D
b	2	D
e	2	A
f	2	A
h	1,3	B, C, D
i	2,3	D
j	2	D
L4c	1,3	A
d	1,2	A, D
e	1,2	D
g	1,2	D
i	1,2	D

Eluents: 1. Ethyl acetate, acetic acid, ligroin, 5:1:94  
 2. Ethyl acetate, acetic acid, ligroin, 10:2:88  
 3. Ethyl acetate, acetic acid, ligroin, 15:3:82

DNP-amines: A: 2,4-Dinitroaniline, 1-DNPamino-2-methyl-2-propanol, DNP-ethanolamine, one unknown  
 B: Two unknowns  
 C: 1-DNPamino-2-methyl-2-propanol, DNPethanolamine  
 D: DNP-ethanolamine, DNP-serine, one unknown



Table 5

Rf Values for DNP-amines Chromatographed on  
Alumina-impregnated Paper

DNP-amine	Solvent		
	1	2	3
A1	0.25	1.0	1.0
A2	0.0	0.86	1.0
A3	0.0	0.71	1.0
A4	0.0	0.27	1.0
B1	0.12	1.0	1.0
B2	0.0	0.0	0.60
C1	0.0	0.70	1.0
C2	0.0	0.28	1.0
D1	0.25	1.0	1.0
D2	0.0	0.27	1.0
D3	0.0	0.0	0.36
DNP-ethanolamine	0.0	0.27	1.0
Dinitroaniline	0.0	0.86	1.0
DNP-serine	0.0	0.0	0.36
1-DNPamino-2-methyl- 2-propanol	0.0	0.71	1.0
1 DNPamino-2-propanol	0.0	0.63	1.0

Solvents: 1. 0.1% morpholine in ligroin  
2. 10% acetone in ligroin  
3. Propanol, 0.1 N ammonium hydroxide, 3:1

of the fractions described in Table 3 crystallized after the solvent had been removed. These were identified as follows.

C2(Table 4). This material was recrystallized from benzene-ligroin (7:3); yield, 25 mg.; m.p., 82-84°. A mixed melting point with DNP-ethanolamine, m.p., 83-84°, prepared by the method of Porter and Sanger (21) showed no depression. The DNP-amines could be chromatographed on alumina impregnated paper. Whatman No. 1 filter paper sheets were thoroughly rubbed with alumina\*. Using the ascending chromatographic technique of Kirby and Williams (22), and a 10% solution of acetone in benzene as the solvent, both DNP-ethanolamine and the compound in fraction C2 moved to Rf 0.27.

D3(Table 4). The compound contained in this fraction was recrystallized from benzene-ligroin(7:3); yield, 17 mg.; m.p., 196-7°. A mixed melting point with DNP-serine, m.p., 196-7°, showed no depression. Chromatography on alumina treated paper (as described above), using a mixture of 30 ml. of propanol and 10 ml. of 0.1 N ammonium hydroxide as the solvent, moved both DNP-serine and the compound in fraction D3 to Rf 0.36.

A2(Table 4). The compound contained in this fraction was recrystallized twice from benzene-ligroin(7:3); yield, 14 mg.; m.p., 175-6°.

Anal. Calcd. for  $C_6H_5N_3O_4$ : C, 39.34; H, 2.75; molec. wt., 183. Found: C, 39.41; H, 2.77; molec. wt.\* , 206  $\pm$  30.

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\*Grade A, Minus 80 mesh alumina, Aluminum Ore Co., E. St. Louis, Ill.

\*Determined from the optical density at  $\lambda = 355$  m $\mu$ , using  $\epsilon = 21,000$  per dinitrophenyl group, determined from DNP-serine.

A sample of 2,4-dinitroaniline\*, m.p., 175-6° showed no depression in a mixed melting point with the compound in fraction A2. Both compounds moved to Rf 0.86 when chromatographed on alumina impregnated paper, using 10% acetone in benzene as the solvent.

A3(Table 4). The compound contained in this fraction was recrystallized from benzene-ligroin (7:3); yield 12 mg.; m.p., 106-8°.

Anal. Calcd. for  $C_{10}H_{13}N_3O_5$  : C, 47.05; H, 5.13; molec. wt., 255. Found: C, 47.25; H, 4.86; molec. wt.,  $221 \pm 35$ .

The DNP group was removed by the method of Lowther (23), as follows. Six milligrams of this compound was placed in a tube with 2 ml. of conc. ammonium hydroxide. The tube was sealed and placed in a boiling water bath for two hours, then it was cooled, opened, and placed in a vacuum desiccator over sulfuric acid. After the aqueous solution had evaporated, the contents of the tube were dissolved in 2 ml. of 1 N hydrochloric acid, and this solution was extracted with butanol to remove dinitrophenol and any unhydrolysed DNP-amine.

A portion of the solution of the amine (1.5 ml.) was neutralized with 0.5 N potassium hydroxide. This solution was cooled in ice and 10 ml. of phosphate buffer (pH 7.12, 0.1 M), 5 ml. of sodium arsenite (0.1 M), and 1 ml. of periodic acid (0.1 M) were added to it. Nitrogen was bubbled through the

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\* Eastman Kodak Co.

solution and the exit gases were led through a trap containing 20 ml. of a 2,4-dinitrophenylhydrazine solution (24). After 45 minutes the dinitrophenylhydrazine solution was removed and extracted three times with a total of 20 ml. of benzene. This benzene solution was poured through an alumina column (1 cm. X 10 cm.). The excess reagent was adsorbed at the top of the column and a zone of dinitrophenylhydrazone passed through the column and was collected in the eluate. The benzene was removed, and the solid was recrystallized from 0.5 ml. of ethanol; yield, 2 mg.; m.p., 124-5°. A mixed melting point with acetone dinitrophenylhydrazone, m.p., 125-6°, showed no depression. The dinitrophenylhydrazones were chromatographed on alumina treated paper, using 10% ether (anhydrous) in ligroin as a solvent; both derivatives moved to Rf 0.65\*.

The periodate reaction mixture was treated with 2 ml. of conc. sulfuric acid and 10 ml. of dinitrophenylhydrazine solution. After an hour at room temperature, this solution was extracted with three 10 ml. portions of benzene. The benzene solution was poured through an alumina column to remove the dinitrophenylhydrazine. The derivatives were chromatographed on alumina impregnated paper. Two hydrazones were

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\*Rf values of other dinitrophenylhydrazones are as follows:

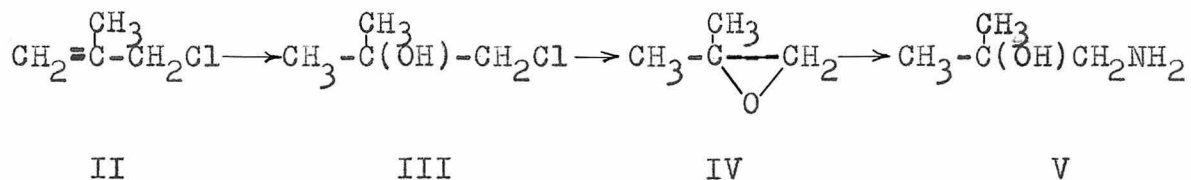
Formaldehyde	0.27	Butyraldehyde	0.76
Acetaldehyde	0.40	2-Hexanone	0.83
Propionaldehyde	0.70	Keto-acids	0.0

observed. One moved with the Rf of acetone dinitrophenylhydrazone (0.65) and the other moved with the Rf of formaldehyde dinitrophenylhydrazone (0.30). The remainder of the benzene solution was evaporated in the presence of 1.5 g. of alumina. This material was placed on top of a column of alumina, and the column was developed with 10% ether in ligroin. After the zones had been eluted, the solvents were evaporated. The first fraction contained acetone dinitrophenylhydrazone (less than 1 mg.) and the other fraction contained formaldehyde dinitrophenylhydrazone; yield, about 1 mg.; m.p., 165-6°. The latter was not depressed in a mixed melting point with known formaldehyde dinitrophenylhydrazone.

A portion of the amine solution which resulted from the hydrolysis of the DNP group was chromatographed on Whatman No. 1 filter paper, using butanol saturated with 1% ammonium hydroxide as the chromatographic solvent. The amine was located by spraying the paper with ninhydrin. A spot was noted at Rf 0.58. A similar chromatograph was treated with acidified potassium permanganate (25) before development with the solvent. After development no ninhydrin reactive material was observed. This procedure was used by Ellis et al. (26) to show that the amine present in vitamin B<sub>12</sub> was 1-amino-2-propanol and not 2-amino-1-propanol. When the latter compound was treated with acid permanganate, alanine was observed on the chromatographs. However, 1-amino-2-propanol no longer reacts with ninhydrin after treatment with acid permanganate. When ethanolamine was treated in this way, glycine could be detected

on the chromatographs.

From the analysis, degradation products, and the results of the permanganate oxidation, it appeared probable that the unknown amine is 1-amino-2-methyl-2-propanol(V). The final proof of the identity of this amine with the isolated compound was made by comparison of the properties of the synthetic and isolated amines. The amine was synthesized by the method outlined below:



Isobutylene chlorohydrin (III). This compound was prepared from methallyl chloride\* (II) as described by Burgin et al. (27). The fraction boiling from 120-8° was collected; lit. b.p., 124-5°.

Isobutylene oxide (IV). This was prepared by dehydrochlorination of isobutylene chlorohydrin (III) by the method of Wilson and Lucas (28). The product boiled at 58-9°; lit. (29), b.p., 57-8°.

1-amino-2-methyl-2-propanol (V). This compound was prepared by the method of Cairn and Fletcher (30); b.p., 148-51°; lit. (30), b.p., 150°.

1-DNPamino-2-methyl-2-propanol. This compound was prepared from V by the method of Porter and Sanger (21). Two recrystal-

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\*We thank the Shell Chemical Corp. for a generous sample of this compound.

lizations from benzene-ligroin(7:3) gave orange-yellow crystals; m.p., 107-8°.

A mixed melting point of the isolated derivative (A3) and the synthetic derivative was not depressed; m.p., 106-8°. These derivatives were chromatographed on alumina treated paper as described before, both moving to Rf 0.71. The rates of movement of the synthetic and isolated derivatives on a silicic acid-celite column were the same. The free bases were chromatographed on Whatman No. 1 filter paper, and moved to the following Rf's: 0.54, in butanol saturated with 1% ammonium hydroxide, and 0.87 in isobutyric acid saturated with water.

#### Discussion

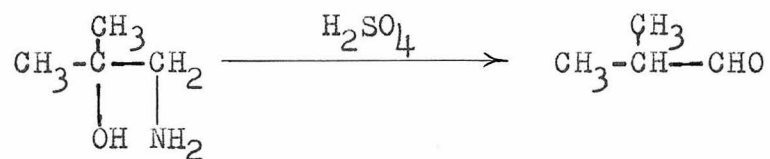
It is evident that many facets of these techniques described in the above experimental section require further elaboration. For example, the chromatography of the DNP-cephalins appeared quite crude. However, the results of the fatty acid titrations show that the separation that was obtained is based on the chromatographic behavior of the whole phospholipid molecule, and not on the amine component. Consequently, it is not entirely surprising that many of the fractions contained several bases. The purification of the DNP-amines was only partly achieved. Some of the DNP-amines isolated from the chromatographic columns were found to be grossly contaminated when chromatographed on alumina treated paper. Nevertheless, these techniques could be elaborated to yield refined analytical procedures. The separation of the DNP-cephalins might be effected on a "reversed phase" powdered rubber column, such as

was used to separate the long chain fatty acids from each other (31).

The fatty acid/phosphorus ratios that have been presented are considered approximate values. The determination of the phosphorus in lipids was difficult and required prolonged periods of digestion. In order to get consistent results, it was often necessary to repeat a phosphorus determination 5 or 6 times. Consequently the error of the fatty acid/phosphorus ratio is probably of the order of 20%. Despite this fact, the variation of this ratio, as observed experimentally, is large enough to suggest that many of the cephalins do not have the structures expected of them (Fig. 7).

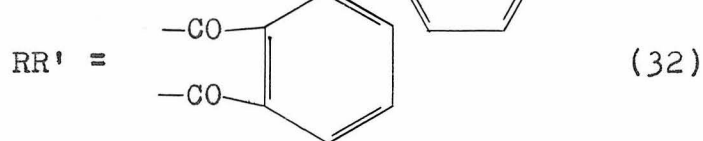
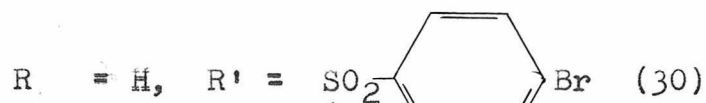
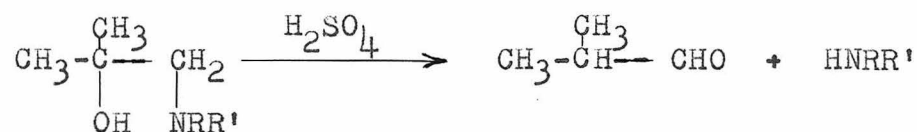
Since serine and ethanolamine are known to occur in the phospholipids of other organisms, the isolation of DNP-serine and DNP-ethanolamine from Neurospora phospholipids is not surprising, but it serves to confirm the validity of the techniques used.

Many derivatives of 1-amino-2-methyl-2-propanol have been prepared (32). Most of its reactions are those expected of either an amine or an alcohol, or an amino-alcohol. However, an interesting rearrangement is brought about by treating the compound with strong acid (30,32,33):

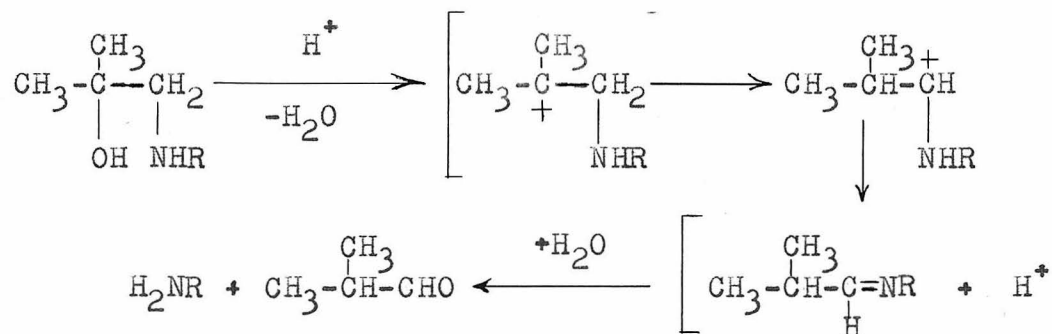




This rearrangement is not prevented by acylation of the amino group:



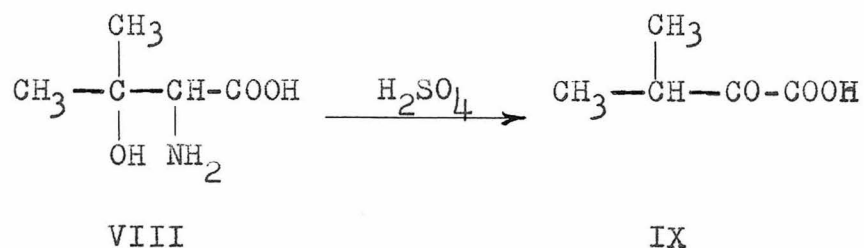
Cairn and Fletcher (30) have proposed the following mechanism for this reaction.



Since no evidence for the presence of any of the intermediates shown in brackets was presented, this mechanism must be regarded as speculative. Furthermore, this mechanism is not entirely compatible with the results obtained by the decomposition of the phthalyl derivative (32). Although no investigation of this reaction was performed, the analogous reaction of 1-DNPamino-2-methyl-2-propanol would account for the presence of 2,4-dinitroaniline in the DNP-cephalin hydrolysate.

Biologically, ethanolamine is formed by the decarboxylation of serine (34). By analogy, 1-amino-2-methyl-2-propanol might

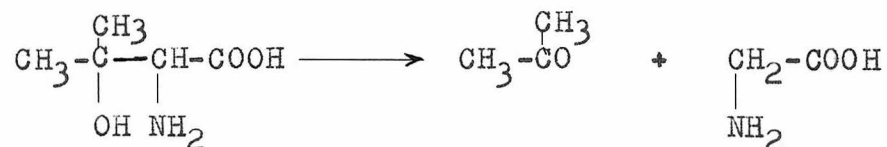
be formed by the decarboxylation of  $\beta$ -hydroxyvaline(2-amino-3-hydroxy-3-methyl-butanoic acid, VIII). Very little is known concerning the natural occurrence of this amino acid. Although several isolations of this substance have been reported (35), other workers have questioned these findings (36), especially the occurrence of  $\beta$ -hydroxyvaline as a protein constituent. Most of this work was done prior to the general use of paper chromatography, and these arguments might be resolved by application of this technique. The structure of  $\beta$ -hydroxyvaline is analogous to 1-amino-2-methyl-2-propanol, and might be expected to rearrange to  $\beta,\beta$ -dimethylpyruvic acid ( $\alpha$ -keto-methylbutyric acid), on treatment with acid.



In fact, this amino acid is unstable under conditions commonly used to hydrolyze proteins (37). Thus, one possible way of showing the presence of  $\beta$ -hydroxyvaline in proteins, would be to demonstrate the occurrence of  $\beta,\beta$ -dimethylpyruvic acid in protein hydrolysates. In addition, decarboxylation may occur, so that the occurrence of isobutyraldehyde might also be expected. Despite this lability of  $\beta$ -hydroxyvaline several investigators have used acid to hydrolyze proteins in which they hoped to find  $\beta$ -hydroxyvaline (36). Other studies of

protein hydrolysates by the method of periodate oxidation have found that there are more hydroxyamino acids in the hydrolysate than can be accounted for by the serine and threonine content (38), leading the investigators to suggest that  $\beta$ -hydroxyvaline may possibly occur in the protein.

Perhaps the most interesting evidence concerning the possible natural occurrence of  $\beta$ -hydroxyvaline is a report (39) of an activity of guinea pig liver slices, which is referred to as glycinogenase. Several  $\beta$ -hydroxy- $\alpha$ -amino acids are cleaved to glycine and a carbonyl compound. Hydroxyvaline was found to give glycine and acetone.



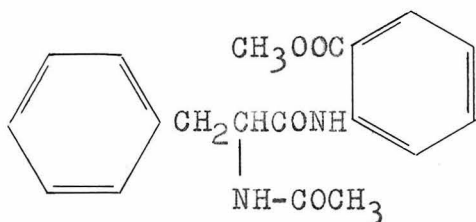
If 1-amino-2-methyl-2-propanol is formed from  $\beta$ -hydroxyvaline, Neurospora should contain the necessary enzyme to catalyze this reaction. Neurospora has an active decarboxylase (40), and it would not be surprising if  $\beta$ -hydroxyvaline was decarboxylated. Consequently, such evidence could not be used to prove unequivocally that  $\beta$ -hydroxyvaline was the origin of 1-amino-2-methyl-2-propanol. An important point in proving this would be the demonstration of the occurrence of  $\beta$ -hydroxyvaline in the tissues of Neurospora.

It should be noted that  $\alpha, \beta$ -dihydroxy-isovaleric acid is accumulated by certain Neurospora mutants (48). The possibility that it may be related to  $\beta$ -hydroxyvaline would also be worth investigating.

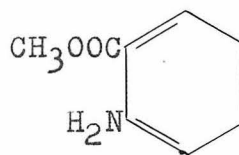
## Synthetic Substrates for Chymotrypsin

### Introduction

Some investigations carried on prior to those described here (41) demonstrated that the enzyme chymotrypsin will catalyze the hydrolysis of the peptide methyl acetylphenylalanyl-anthranilate (X) yielding as one of the products, methyl



X



XI

anthranilate (XI). This product is strongly fluorescent even in very dilute solutions, in contrast to the peptide which is practically non-fluorescent. Thus, this synthetic peptide provides a basis for a sensitive fluorometric method for the determination of chymotrypsin activity. Although the acetyl derivative is satisfactory as a substrate its general usefulness is limited by its relative insolubility. In addition the yield obtained from the original synthesis was poor. Therefore, an attempt was made to devise a general procedure for synthesis that would permit the preparation of this and related compounds in good yields. A number of methods were designed and investigated and the primary goal was attained by an application of the series of reactions shown in Fig. 12. The acetyl, succinyl, and maleyl derivatives were prepared. The last two substances

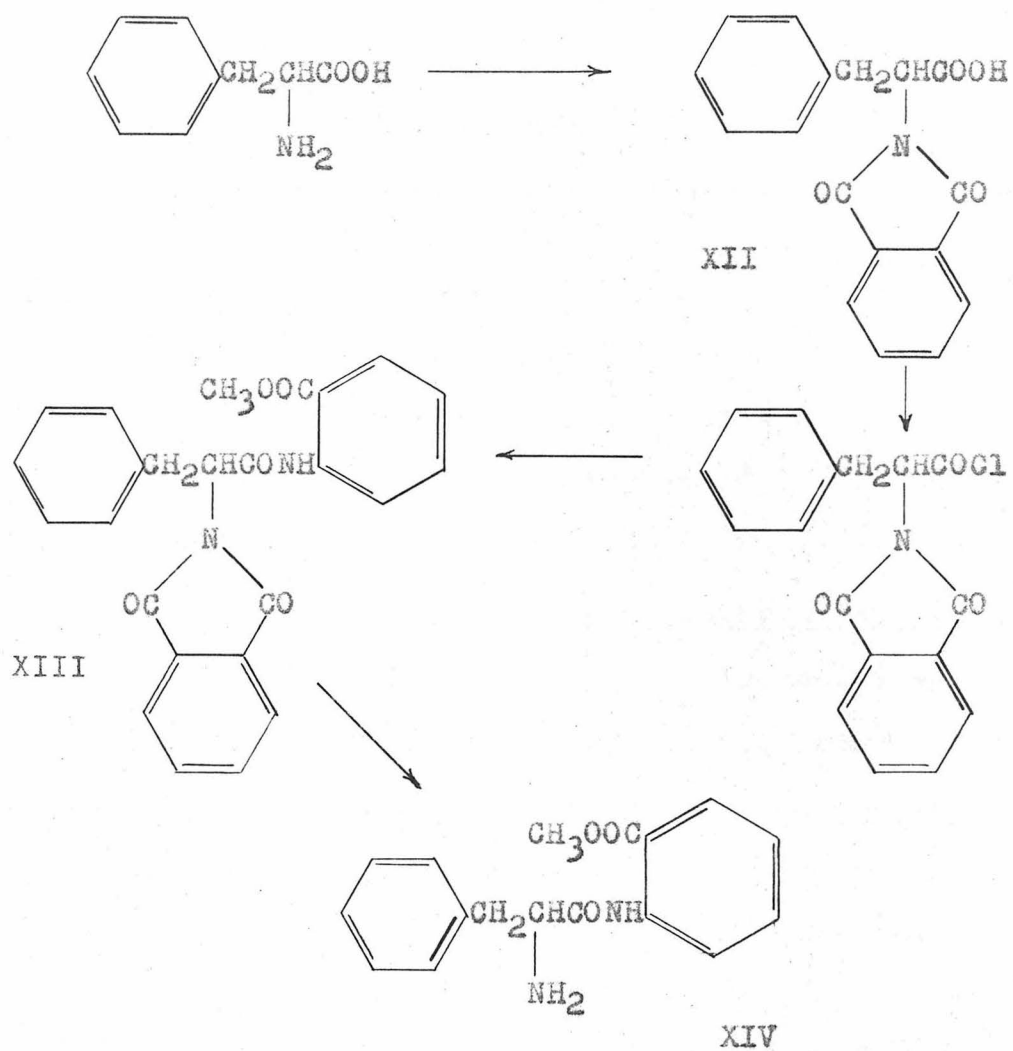


Fig. 12. Preparation of methyl phenylalanyl-anthranilate

have satisfactory solubility properties but they are not hydrolyzed by chymotrypsin. Nevertheless, the general method of synthesis has been shown to be satisfactory and it should be applicable in the preparation of a variety of peptides that may serve as substrates for this enzyme. Some of these possibilities will be considered in the discussion.

### Experimental

Phthalyl-DL-phenylalanine (XII). This compound was prepared by the method of Sheehan (42). It was recrystallized from water; m.p., 176-8°; lit., 178°; neut. eq., calcd., 295; found, 302.

Methyl phthalyl-DL-phenylalanyl-anthranilate (XIII). Phthalyl-DL-phenylalanine (4.55 g., 0.0154 mole) was refluxed with 20 ml. of thionyl chloride for half an hour. The excess thionyl chloride was removed by distillation in vacuo and 20 ml. of benzene was added. The benzene was removed by distillation in vacuo and the process was repeated twice to remove all thionyl chloride. The resulting acid chloride was dissolved in 10 ml. of dioxane and the solution was added with continual shaking to a suspension of 2.48 g. (0.154 mole) of methyl anthranilate (XI) in ice cold water. A yellowish oil which formed, solidified on standing in the refrigerator. The solid was suspended by shaking and the suspension was extracted with benzene (two 20 ml. portions). The benzene solution was washed successively with a 10% solution of sodium carbonate, water, 2 N sulfuric acid, and water. After removal of the benzene by evaporation the residual mass was recrystallized from ethanol.

The yield of white needles, m.p., 161-2°, was 4.26 g. (79%). Another crystallization from ethanol raised the melting point to 162-3°.

Anal. Calcd. for  $C_{25}H_{20}O_5N_2$ : C, 70.12; H, 4.67; N, 6.54. Found: C, 70.48; H, 5.02; N, 6.91.

Methyl DL-phenylalanyl-anthranilate (XIV). Methyl phthalyl-DL-phenylalanyl-anthranilate (4.03 g.) was dissolved in 40 ml. of methanol. To this was added 0.82 g. of hydrazine solution (aqueous, 85%) and the mixture was refluxed for 6 hours. The methanol was removed by evaporation (in vacuo) and the residual mass was washed out of the flask with about 10 ml. of 2 N hydrochloric acid. It was triturated in a mortar with 10 ml. more of the hydrochloric acid and the acid solution was separated from the phthalylhydrazide by filtration. The acid in the filtrate was neutralized with a 10% solution of sodium carbonate. A gummy intractable solid precipitated. This suspension was extracted with four 30 ml. portions of benzene and the benzene solution was dried over calcium chloride. Titration of an aliquot of the benzene solution in methanol with 0.1 N hydrochloric acid indicated that the yield of amine was 2.1 g. (76%).

Methyl acetyl-DL-phenylalanyl-anthranilate (X). An aliquot of the benzene solution (40 ml.) containing 0.002 mole of methyl DL-phenylalanyl-anthranilate was refluxed with 0.23 g. (0.0022 mole) of acetic anhydride for five hours. The benzene was removed by distillation in vacuo. The residual mass was crystallized from 50% ethanol; m.p., 121-2°; yield, 0.52 g. (82%).

Anal. Calcd. for  $C_{19}H_{20}O_4N_2$ : C, 67.04; H, 5.92; N, 8.23.  
Found. C, 67.10; H, 5.86; N, 8.47.

Methyl succinyl-DL-phenylalanyl-anthranilate. This substance was prepared from methyl DL-phenylalanyl-anthranilate and succinic anhydride by a procedure analogous to that described above for the acetyl derivative. This peptide was recrystallized from benzene-ligroin (4:6); m.p., 157-8°; neut. eq., calcd., 398; found, 406; yield, 60-65%.

Anal. Calcd. for  $C_{21}H_{22}O_6N_2$ : C, 63.31; H, 5.56; N, 7.03.  
Found. C, 63.47; H, 5.71; N, 7.73.

Methyl maleyl-DL-phenylalanyl-anthranilate. This compound was prepared exactly as described above for the acetyl derivative, except that maleic anhydride was used. This peptide was recrystallized from benzene-ligroin (4:6); m.p., 163-4°; neut. eq., calcd., 396; found, 395; yield, 52%.

Anal. Calcd. for  $C_{21}H_{20}O_6N_2$ : C, 63.62; H, 5.09; N, 7.07.  
Found. C, 63.82; H, 5.06; N, 7.08.

Enzymatic Hydrolyses. Ten milligrams of an enzyme preparation\* was dissolved in phosphate buffer (0.1 M, pH 7.87) in a glass stoppered flask, and placed in a water bath maintained at  $35 \pm 1^\circ$  C. The substrate (dissolved in buffer) was added and finally buffer was added to the flask to make the total volume 20 ml. At 5- 10 minute intervals thereafter, aliquots were withdrawn, diluted appropriately and their fluorescence determined. The fluorometer<sup>†</sup> was standardized with

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\*Chymotrypsin, Lot 90402, Armour Laboratories, Armour and Co., Chicago, Ill.

<sup>†</sup>Electronic Photofluorometer, Coleman Electric Co., Maywood, Ill., Model 12B.



solutions of methyl anthranilate in buffer. Under these conditions, the succinyl and maleyl derivatives were not hydrolyzed. The acetyl derivative was hydrolyzed, however. The rate of hydrolysis was determined by plotting the concentration of methyl anthranilate as a function of time, and measuring the slope of a line through these points. The error of each rate determination was estimated from the slopes of the steepest and flattest lines which were compatible with the data. A typical result of such a determination of the rate of hydrolysis of the acetyl derivative is shown in Fig. 13. The results of the rate determinations for several concentrations of the acetyl derivative are given in Table 6.

An estimate of the Michaelis constant was made (43) by plotting (Fig. 14 and Fig. 15)  $1/v$  vs.  $1/S$  and  $S/v$  vs.  $S$ , where  $S$  is the substrate concentration and  $v$  is the observed rate. The slope of the first plot is  $K_s/V$  and of the second,  $1/V$ , where  $K_s$  is the Michaelis constant and  $V$  is the maximum velocity. Therefore the slope of the line of the first graph divided by that of the second graph is equal to  $K_s$ . The value of  $K_s$  for the acetyl derivative, determined in this way is  $3.9 (\pm 1.7) \times 10^{-4}$  mole/l.  $V$  was estimated from the reciprocal of the slope of the second graph and was found to be  $3.8 (\pm 1.6) \times 10^{-7}$  mole/l/min.

The variability of the rates is primarily due to two causes: the instability of the fluorometer response, and the fluctuations of the water bath temperature. While it is difficult to estimate the error due to the fluorometer instability, it is possible to estimate the error due to small fluctuations in the

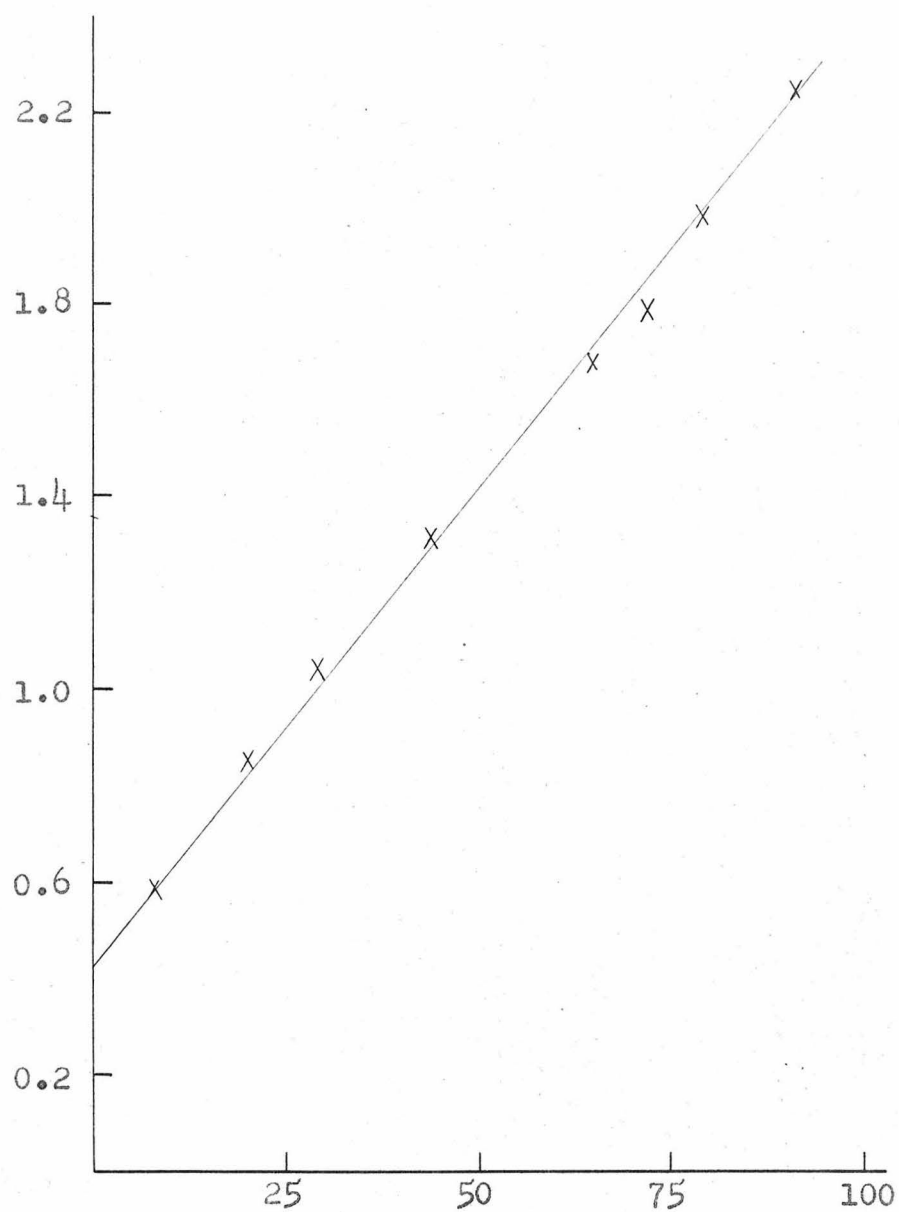


Fig. 13. Graph of results of run 26 B. Abscissa, time in minutes; ordinate, concentration of methyl anthranilate in  $\mu$ g./ml.

Table 6

S ( X 10 <sup>6</sup> mole/l.)	v ( X 10 <sup>6</sup> mole/l/min.)		
8.8	0.0086	±	0.0002
29.4	0.0253		0.0006
	0.0272		0.0006
73.5	0.0587		0.0020
	0.0636		0.0006
147.1	0.105		0.003
	0.116		0.005
294.1	0.195		0.010
	0.213		0.008
	0.218		0.013
	0.238		0.020
	0.228		0.020

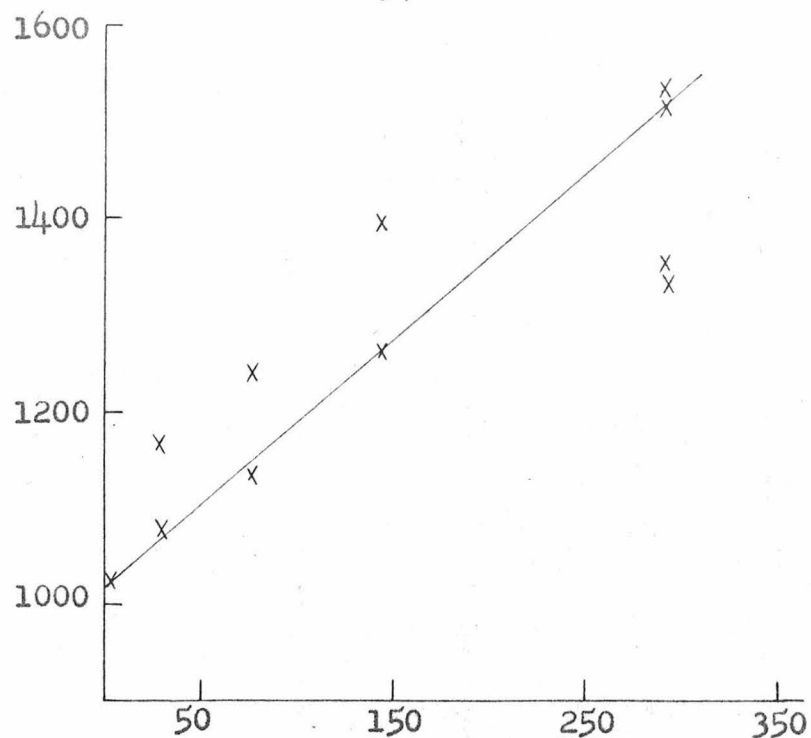


Fig. 14. Graph of  $S/v$  vs.  $S$ . Abscissa,  $S$  (mole/l.  $\times 10^{-6}$ ); ordinate,  $S/v$  (min.).

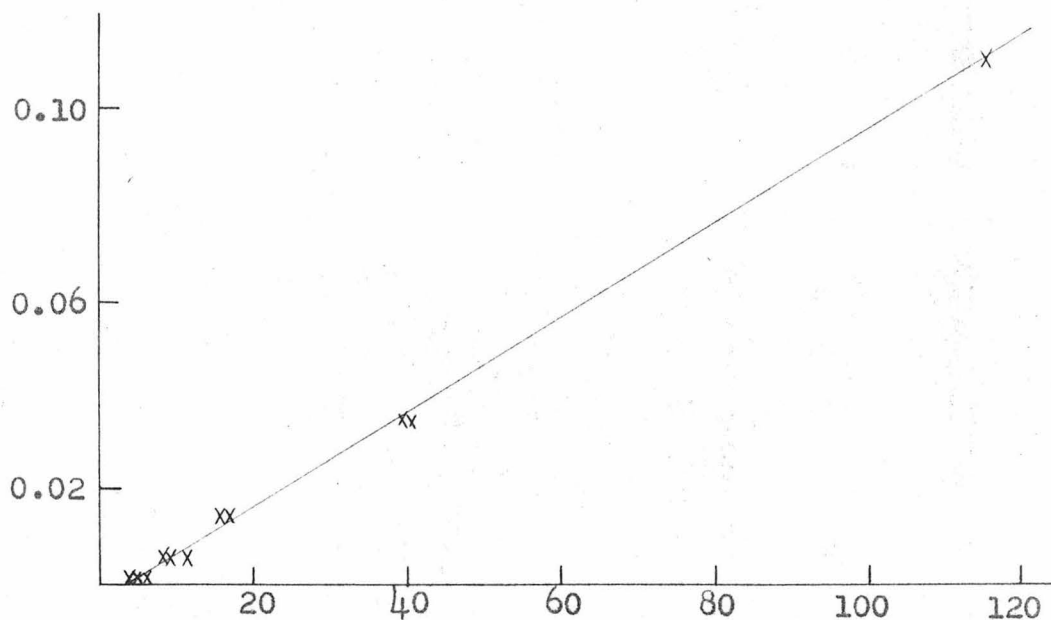


Fig. 15. Graph of  $1/v$  vs.  $1/S$ . Abscissa,  $1/v$  (min.  $\times$  l./mole  $\times 10^{-6}$ ); ordinate,  $1/S$  (l./mole  $\times 10^{-6}$ ).

temperature from the Arrhenius equation:

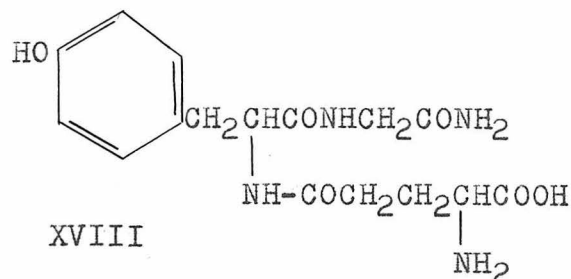
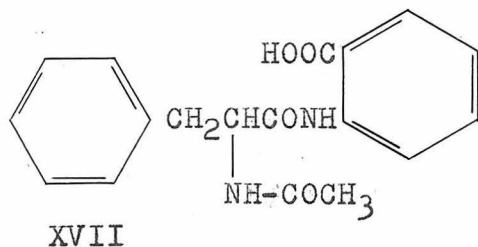
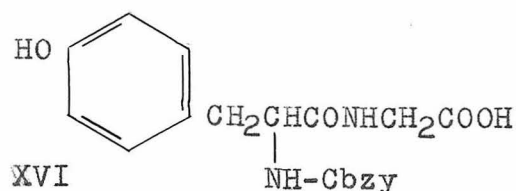
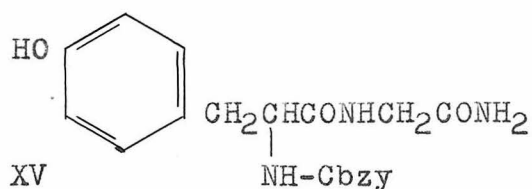
$$\ln k = \ln A - B/RT$$

where  $k$  is the rate constant,  
 $T$  is the absolute temperature,  
and  $A$ ,  $B$  are constants.

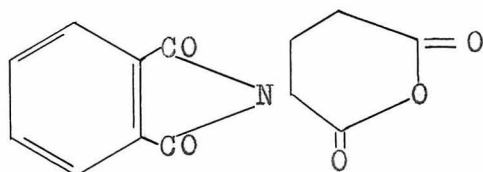
$B$  has been determined for chymotrypsin-catalyzed hydrolyses and is 16 to 18 kcal./mole (44). At 35°, the change of rate is then about 4% per degree.

### Discussion

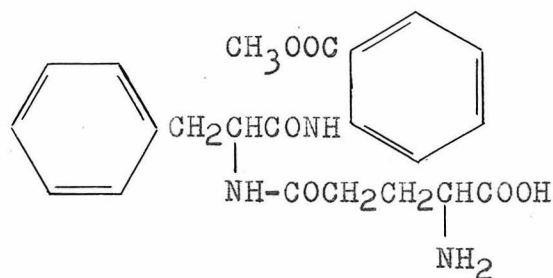
As expected, the solubility of the amides of methyl anthranilate in water at pH 7.87 was increased by the introduction of a carboxyl group. The fact that the enzyme did not hydrolyze these derivatives is not entirely unexpected. Bergmann and Fruton (45) observed that although carbobenzoxy-tyrosyl-glycinamide (XV) is hydrolyzed by chymotrypsin, the corresponding acid, carbobenzoxy-tyrosyl-glycine (XVI), is not hydrolyzed. We have observed the analogous behavior of methyl acetylphenylalanyl-anthranilate (X) and acetyl-phenylalanylanthranilic acid (XVII). However, glutamyl-tyrosyl-glycinamide (XVIII) is slowly hydrolyzed by chymotrypsin (46). Consequently, the carboxyl group was not expected to abolish all the substrate activity of the compounds prepared.



Several alternative acyl groups might have been used. Methyl phenylalanyl-anthranilate (XIV) could have been acylated with the acid anhydride of phthalyl-glutamic acid (XIX). After removal of the phthalyl group, the resulting peptide would be methyl  $\gamma$ -glutamyl-phenylalanyl-anthranilate (XX). By analogy with the corresponding tyrosine derivative (XVIII), this peptide should serve as a substrate for chymotrypsin.



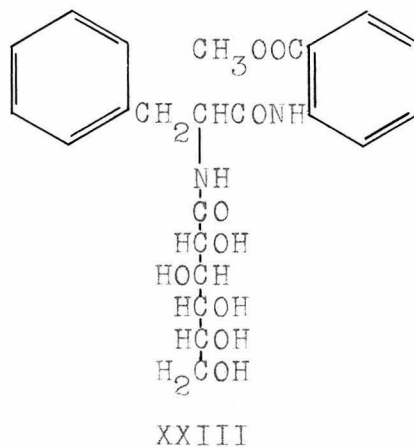
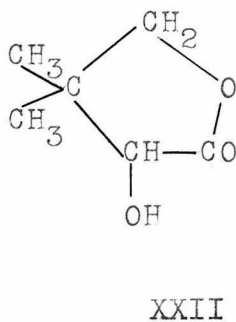
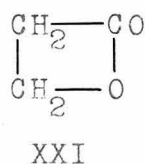
XIX



XX

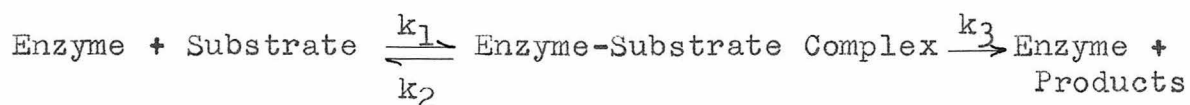
Another method of producing a more water soluble peptide from methyl phenylalanyl-anthranilate is to acylate it with a lactone. This produces a peptide which has an alcoholic

hydroxyl group on it. Many lactones would be suitable, but one which has the advantage of being commercially available is  $\beta$ -propiolactone (XXI). Another is pantoil lactone (XXII). However, the use of pantoil lactone has the advantage over  $\beta$ -propiolactone of introducing two hydroxyl groups into the peptide. Five hydroxyl groups could be introduced by the use of gluconyl lactone (also commercially available). The product of the treatment of methyl phenylalanyl-anthranilate with gluconyl lactone is methyl gluconyl-phenylalanyl-anthranilate (XXIII), a compound which would certainly be water soluble. Unfortunately, there are no data available on the effect of alcoholic hydroxyl groups on the activity of chymotrypsin. However, the alcoholic hydroxyl group bears no charge at pH 7.87, and would not be expected to be detrimental to the activity of the enzyme.



While kinetic studies were not a part of the original program, the results show that the peptides of methyl anthranilate may be used to study several problems related to the mechanics of enzymatic action. One problem that could be

studied is the significance of the Michaelis constant. The Michaelis constant has often been considered as a dissociation constant (47). If an enzyme-catalyzed reaction is represented by the following reaction sequence,



then the Michaelis constant  $K_s = (k_2 + k_3)/k_1$ , where  $k_1$ ,  $k_2$ , and  $k_3$ , are rate constants for the reactions denoted by the respective arrows. If  $k_3$  is much smaller than  $k_2$ ,  $K_s$  is very nearly equal to the dissociation constant for the enzyme-substrate complex. The evidence relating to this point is limited, and no definite statement in regard to the relative values of  $k_2$  and  $k_3$  can be made at present. However, methyl acetylphenylalanyl-anthranilate would be useful for obtaining information on this question. Since this compound is hydrolysed rather slowly (under the conditions used, the rate of hydrolysis was about 0.1% per minute), it could be used as a competitive inhibitor. A competitive inhibitor is assumed to react with the enzyme at the same site as the substrate, and thereby reduce the amount of enzyme left to react with the substrate. Thus, it would be possible to determine the inhibitor constant  $K_I = k_2/k_1$  for methyl acetyl-phenylalanyl-anthranilate. In this case, the inhibitor constant is the true dissociation constant for the enzyme-substrate complex. If the  $K_s$  and  $K_I$  values for the acetyl derivative were the same, or very nearly so, it follows that  $K_s$  is also a dissociation constant,



and that  $k_3$  is much smaller than  $k_2$ . However, if  $K_s$  and  $K_I$  are markedly different,  $K_s$  cannot be regarded as a dissociation constant.

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### Propositions

1. Urey (1) has calculated that the  $O^{18}:O^{16}$  ratio in carbonates deposited at  $0^{\circ}C$  is  $2.052 \times 10^{-3}$ , whereas at  $25^{\circ}$  the value of this ratio is  $2.044 \times 10^{-3}$  (if the ratio in the surrounding water is  $2.000 \times 10^{-3}$ ). Thus, the change of this ratio with temperature is appreciable. By the use of a sensitive mass spectrometer, this ratio can be determined accurately enough so that it is possible to determine at what temperature the carbonates were deposited.

I propose that the problem of deciding whether fossil remains are from warm or cold blooded animals could be answered by application of this method.

2. Certain N-acyl amides of methyl anthranilate show no (or negligible fluorescence (2)), whereas methyl anthranilate is strongly fluorescent. It has been shown that many proteolytic enzymes (papain, trypsin, chymotrypsin) can be made to synthesize anilides (3). If methyl anthranilate or its esters could be used in place of aniline, the kinetics of the peptide formation could be studied by the rate of disappearance of the fluorescence.

3. Attempts to understand the function and nature of gene action have led to certain proposals regarding the relationship between genes and enzymes in a given organism (6). The studies which have led to these proposals have been generally performed with organisms in which the gene under study has mutated, either naturally or with the assistance of the investigator.

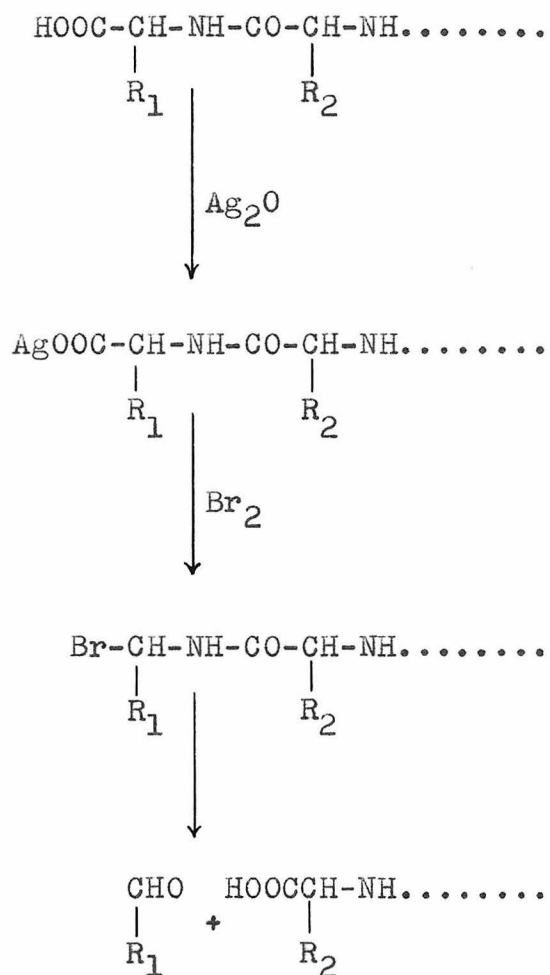
The exact nature of the mutation process is only vaguely understood. Therefore, I propose that studies of enzyme activity would be of interest if it were possible to do them in an organism where the gene involved was known to be deleted. The advantage of this organism is that no assumptions need be made concerning the nature of the mutation process, since the nature of the process is known in this case.

4. Risemann and Kirkwood (7) have proposed the following scheme to explain muscle contraction. The serine and threonine residues of the muscle protein fibers are phosphorylated, thereby producing a net change of the charge of the fiber surface. The electrostatic repulsion would extend the fiber. By an unspecified trigger mechanism, the phosphate esters are hydrolyzed, and the fiber contracts to a more stable configuration.

It has been shown that phosphorylated polyvinyl alcohol will exhibit properties somewhat similar to muscle. When placed in a neutral solution, the phosphorylated polyvinyl alcohol fibers become swollen and quite extended. If such a swollen fiber is placed in an acid solution, they contract violently, and their volume diminishes considerably (8).

I propose that muscle breis be examined for their ability to phosphorylate alcohols, and specifically, polyvinyl alcohol. A simple analytical procedure would be to measure the contraction of a fiber after it had been acted upon by the muscle brei, by placing it in an acid solution.

5. I propose the following method for the degradation of a peptide. Under the proper conditions, it may be possible to degrade the peptide one amino acid at a time.

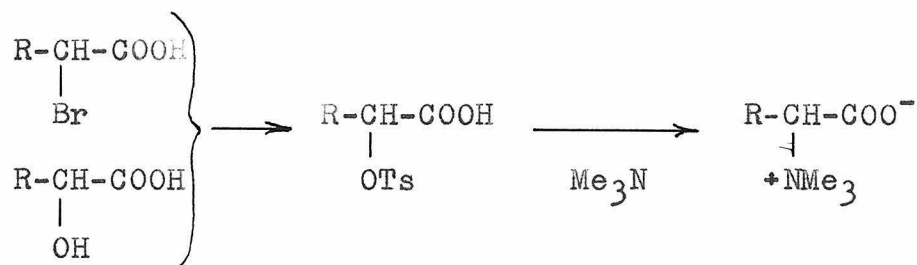


Identification of the aldehyde would serve to identify the terminal amino acid.

6. The recent work of Sanger (4) on the structure of certain peptides of the insulin molecule is very general in outline, and theoretically should be applicable to any pure peptide. Since many crystalline enzymes are available, I propose that the determination of the amino acid sequence in these peptides

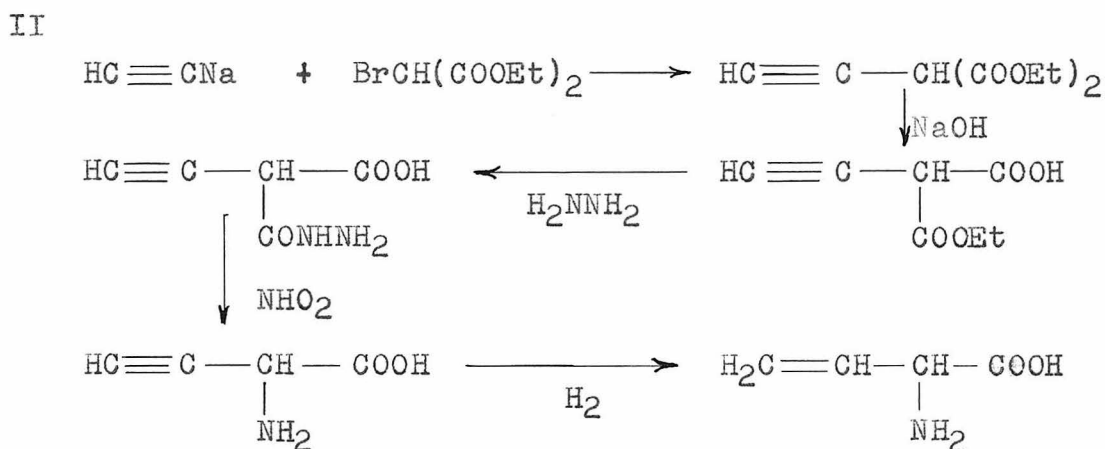
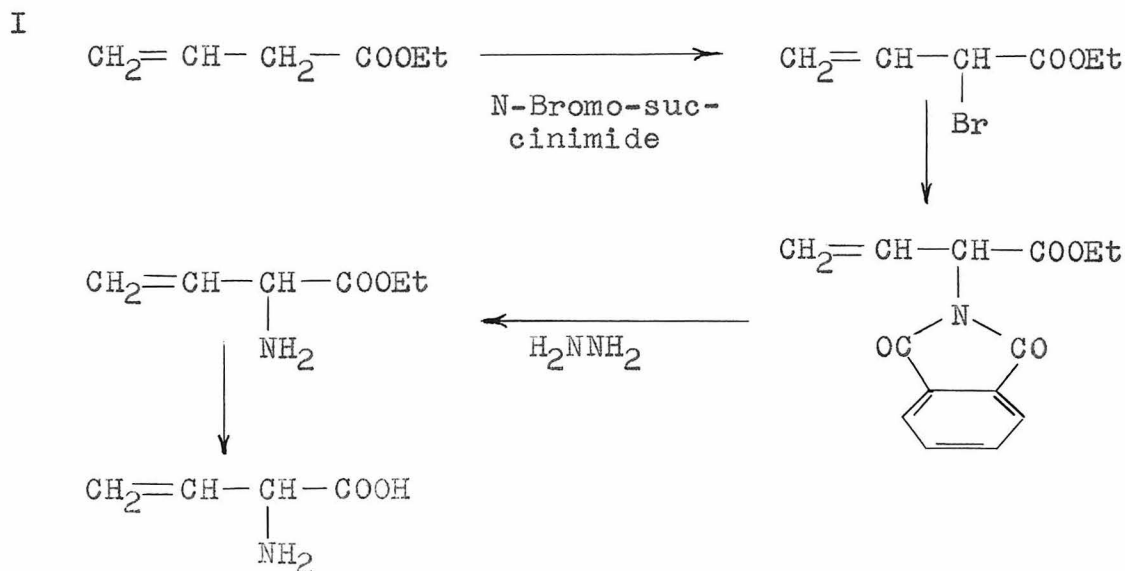
is a feasible and important project, especially if combined with the recent proposals of Pauling and Corey concerning the structure of the protein chain. By this method, it may be possible to obtain direct evidence concerning the nature of the "active site" of the enzyme.

7. I propose a general synthesis of betaines from bromo or hydroxy acids (10).

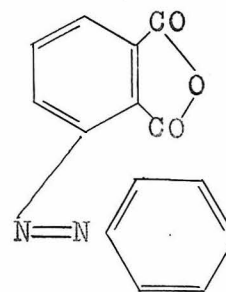
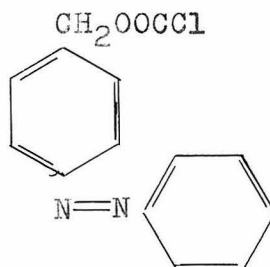
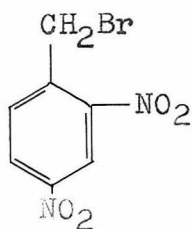


8. The biochemical interconversions of threonine, methionine, homocystine, and homoserine (9) have suggested the possibility that an intermediate common to all these conversions may be  $\alpha$ -amino-butene-3-oic acid (C-vinyl-glycine). Although several attempts to prepare this compound have been abortive (11), many possible syntheses can be designed. Two possibilities are outlined below.





9. All of the amine derivatives from which the amine can be easily regenerated are colorless. For chromatographic purposes, a colored compound is most convenient. I propose therefore, that the following reagents would be valuable for producing easily decomposed colored amine derivatives.



10. Some plants require a period of cold before they will flower. This treatment is called vernalization. Many studies of this phenomenon have not revealed the specific effect of the cold treatment. I propose that the tissues of such plants be examined before and after vernalization by procedures similar to those used by Hadorn and Mitchell in studies of the changes in *Drosophila* accompanying metamorphosis (12).

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