

XLIII. THE PRECIPITATION OF THE BASIC AMINO-ACIDS OF PROTEINS WITH PHOSPHOTUNGSTIC ACID.

BY KENNETH VIVIAN THIMANN (Beit Memorial Research Fellow).

From the Biochemical Department, Imperial College of Science and Technology, London, S.W. 7.

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ALTHOUGH the fact has long been known that the diamino-acids are precipitated from solution by the addition of phosphotungstic acid, very little appears to be known as to the actual mechanism of the reaction. This is the more remarkable since the precipitation has been so widely used in biochemical research.

The effect of phosphotungstic acid on arginine was first studied by Gulewitsch [1899] who showed precipitation to be most complete in 5-7 % H_2SO_4 , in which the solubility amounted to 70 mg. arginine per litre. He pointed out that "the composition of the arginine phosphotungstate can be different if the conditions of precipitation are different, especially if a mineral salt is present." Advocated as an analytical method for the estimation of the bases by Hausmann [1899] and modified by Osborne and Harris [1903] and others, the method has thus been in use for some 30 years. Osborne, Leavenworth and Brautlecht [1908] showed that the phosphotungstic acid precipitate agreed approximately with the amount of diamino-acids obtainable from the protein hydrolysate by other means. There were, however, one or two notable exceptions, and the application of later refinements in the technique of the isolation of amino-acids would undoubtedly reveal many more. Thus, Gortner and Sandström [1925], working with a synthetic mixture of amino-acids, showed that precipitation was almost invariably too high, even after boiling the mixture, unless proline and tryptophan were both absent. Van Slyke's [1911] division of the nitrogen in the precipitate into arginine, histidine, cystine and lysine does not, of course, take account of the possible presence of other amino-acids in the precipitate; proline, for example, would be estimated as histidine.

Wechsler [1911] and Drummond [1918] carried out analyses of some of the phosphotungstates, whose general formula was shown to be $A_3, 2\text{H}_3\text{PO}_4, 24\text{WO}_3, + \text{H}_2\text{O}$ in some cases, where A is the molecule of basic amino-acid. Drummond found the conditions of optimum precipitation for a dilute solution of histidine as being with 5 % H_2SO_4 (confirming Gulewitsch), and with

6–9 g. phosphotungstic acid per 100 cc. of resulting solution. Even under these conditions, however, 7 mg. N per 100 cc. remained unprecipitated, a solubility almost as high as that quoted in the same paper as the solubility in water, and four times as high as the minimum found by the present author (p. 374). It is evident that optimum precipitation conditions had not been attained. This may have been due to his use of an impure sample of histidine, or to failure to wash the precipitate with 2.5 % phosphotungstic acid solution.

Rôle of the basic amino-acids and proline.

There seems little doubt that the basic amino-acids are of the greatest importance in the structure of the protein molecule. Changes occurring in the latter appear to be reflected in, if not due to, changes in the content and proportion of these bases. Examples of such changes have been given by Schryver and Thimann [1927], Thimann [1926], Thornley [1927], Linderstrøm-Lang [1929], Knaggs [1929] and others.

Arginine has been considered by Kossel [1896] to act as the nucleus of the protein molecule, and a number of existing figures for the arginine content of proteins has been collected by Larmour [1928] to show the dependence of the amount of basic nitrogen on the amount of arginine in the molecule, and hence the governing importance of the latter. This is held to confirm Kossel's theory. Determinations of the amount of arginine are of necessity somewhat inaccurate, where protein hydrolysates are concerned, for two reasons. In the first place its precipitation by phosphotungstic acid is incomplete, as was pointed out by Gulewitsch [1899] and again by Plimmer and Rosedale [1925]. In the second place, in experimental procedure involving treatment with alkali, as in the determination of the amide-nitrogen of hydrolysates, the possibility of its decomposition has to be considered. Treatment with 50 % alkali, as in the Van Slyke procedure, yields ammonia, but only after 6 hours' boiling, so that this reaction need hardly be taken into account. Schulze and Winterstein [1901], however, showed that after 1 hour's hydrolysis with dilute alkali a 40 % conversion to ornithine and urea takes place. Although both these substances are precipitated by phosphotungstic acid, their precipitation in all probability takes place under different conditions from that of arginine, and the solubility of their phosphotungstates is certainly different, so that not only the arginine figures, but also the total phosphotungstic acid precipitate, would be affected. Since the hydrolysate is in fact treated with warm alkali for a short time the occurrence of this reaction is probable. This may perhaps act as a subsidiary cause for the variation of the ammonia-nitrogen also, though Henriques and Gjaldbæk [1910] attribute this mainly to deamination of some of the amino-acids, notably cystine.

Lysine is the seat of the bulk, at any rate, of the free amino-nitrogen of proteins, its ϵ -amino-group, which is free, being readily removed by nitrous acid, as was shown by Dunn and Lewis [1921]. It is, however, improbable that such deamination ever occurs in the ordinary way, since the product

which would result, α -amino- ϵ -hydroxycaproic acid, has not been isolated from the products of protein hydrolysis. It may, of course, if present in small amount, have been overlooked, and its isolation in the future would be a matter of interest. Whether deamination occurs or not, the free nature of the ϵ -amino-group gives to lysine a unique importance in the protein molecule, and renders it probable that this amino-acid would be associated in some way with any intramolecular change that takes place. Its appearance in increased amount in gelatin which has been treated with acid [Schryver and Buston, 1927], supports this view. These workers obtained the bulk of the increase in the racemised or *dl*-form, but this, of course, may be merely a result of the method of isolation, which involved treatment with baryta and other possible racemising agents. Such racemisation is not without precedent, moreover, since Gulewitsch [1923] obtained racemic arginine among the products of gelatin hydrolysis, and ascribed the racemisation to the sulphuric acid used for hydrolysis.

Further evidence in support of the importance of this free NH_2 -group is adduced by Hofmann and Gortner [1925] and Gortner [1927], who have shown that the combination between protein and acid dye takes place in stoichiometrical relation to the lysine-N + $\frac{1}{4}$ of the arginine-N, between certain p_{H} limits. Greenberg and Schmidt [1924] have in the same way compared the amount of combination with acid, calculated on this basis, with that actually found, for caseinogen, gelatin and gliadin, and obtained satisfactory agreement. Hitchcock [1923] showed that when gelatin was deaminised the reduction in combining capacity was exactly equivalent to the loss of amino-N. Much has been published on this point. Since the free amino-N, and therefore the lysine, of gelatin is thus responsible for its chemical combination with acids, it is to be expected that the results of acid treatment of gelatin would be closely connected with the lysine content. It is therefore not surprising to find, as will be shown herein, that a large part of the variations in the amount of nitrogen precipitated by phosphotungstic acid in gelatin which has been treated with acid may be ascribed to variations in the lysine content.

The consideration of cystine has been deferred to a later date, since the bulk of these researches have been concerned with gelatin, from which cystine is absent.

Proline is of importance because under such conditions it may be precipitated by phosphotungstic acid, as was pointed out by Gortner and Sandström [1925]. Very few data relating to proline are to be found in the literature, and even those are chiefly derived from impure preparations, since it has only recently been obtained in a pure state by Kapfhamer and Eck [1927] and by Town [1928]. Its phosphotungstic acid precipitate is readily soluble in excess of phosphotungstic acid, and a permanent precipitate can only be obtained with concentrated solutions, or if the phosphotungstic acid is taken up by diamino-acids in the solution, so as to leave just sufficient to precipitate

the proline. If the amount of diamino-acids were increased beyond this point, the total percentage precipitation obtained would decrease again, since there would not then be sufficient excess of phosphotungstic acid to precipitate these acids completely. For complete precipitation of the basic amino-acids an excess of the order of ten times their weight of phosphotungstic acid is required. Gulewitsch [1899] gives 12-14 times. This amount would suffice to redissolve the bulk, at any rate, of the proline, so that if the diamino-acids are completely precipitated, the proline is probably not, and *vice versa*.

The precipitation of any considerable percentage of proline would therefore take place only within certain limits of nitrogen concentration and at a certain ratio of proline to other amino-acids. Slight precipitation, however, probably takes place at most concentrations.

Variations in the precipitation of histidine with phosphotungstic acid do not seem to have been mentioned in the literature. No evidence of variation was obtained in the present work. Its phosphotungstate is more soluble in water than that of the other two bases.

It was shown in the previous communication [Thimann, 1930], that the amount of protein hydrolysate precipitated by phosphotungstic acid varies with the concentration of nitrogen in the solution. The experiments were therefore directed to determining the following points.

(a) The variation in the amount of single amino-acids precipitated by phosphotungstic acid with concentration of nitrogen.

(b) The constancy or otherwise of the solubility of the phosphotungstates.

(c) The behaviour of mixtures of amino-acids, and the interpretation of the curves given by gelatin hydrolysates.

EXPERIMENTAL.

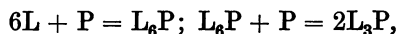
Arginine was prepared *via* the flavianate by the method of Kossel and Staudt [1926], purified by way of the carbonate and obtained as a dry crystalline mass containing one molecule of water. Through the kindness of Drs Vickery and Leavenworth, samples of arginine, histidine, and lysine hydrochloride prepared by them [1928, 1, 2] were obtained. The arginine agreed closely with the above. Both the histidine and the lysine hydrochloride were finely crystalline specimens, containing 27.04 and 10.99 % N respectively; calculated 26.96 (free base) and 11.04 (hydrochloride + 4H₂O). A sample of pure crystalline proline was very kindly supplied by Dr Town.

Solutions of weighed quantities of the amino-acids were made up, the nitrogen was estimated, and small volumes were measured with calibrated outflow pipettes. 0.3 cc. concentrated N-free sulphuric acid was added, the volume made up to 10 cc., and 3 cc. 20 % phosphotungstic acid in 5 % sulphuric acid solution were added. The tubes were allowed to stand in ice overnight, and the precipitates centrifuged, washed, redissolved, and incinerated as previously described for the determination of the Hausmann numbers

[Thimann, 1926]. The nitrogen of both precipitate and filtrate was determined on aliquot parts.

Two slight difficulties were encountered. Arginine phosphotungstate, when precipitated from dilute solutions, was fibrous and glairy, resembling egg-white, and therefore difficult to wash satisfactorily. It was eventually broken up with a fine glass rod of triangular section, which could also be used to cut the fibres. The rod was carefully preserved overnight and the adhering particles of fibre washed down into the centrifuge cup.

Lysine was not completely precipitated from the more concentrated solutions, and a secondary precipitate appeared in the filtrate when the wash-liquid was added. A further 1 cc. of 20 % phosphotungstic acid was therefore added to each and they were again centrifuged. Additional phosphotungstic acid added to the resulting filtrate produced no further precipitate. To keep the volume constant only 9 cc. wash-liquid were used. It is not possible that insufficient precipitant could have been added, since there was in these solutions a maximum of 39 mg. lysine to which 600 mg. phosphotungstic acid were added, *i.e.* a 15-fold excess. The probability is indicated that some type of soluble double salt was formed, and that this was decomposed by further phosphotungstic acid, thus:



where L_3P is the normal insoluble salt (as given by analysis), and L_6P the soluble double salt.

This was the only amino-acid with which such a phenomenon was observed, and it is significant that the same phenomenon also occurs in the case of acid-treated gelatin. The formation of such a secondary precipitate, with incomplete initial precipitation, therefore points in such cases to a larger percentage of lysine (see below).

Table I. *Arginine (b). (Results typical of all experiments.)*

487.3 mg. in 50.01 cc.; *i.e.* 2.909 mg. N per cc.

cc. sol. taken	Total N mg.	Precipitate-N		Filtrate-N		Total recovery %
		%	mg.	%	mg.	
0.200	0.575	0.290	50.36	0.292	50.71	101.07
0.539	1.568	1.106	70.54	0.430	27.50	98.04
0.988	2.873	2.397	83.40	0.447	15.54	98.94
1.977	5.750	5.089	88.50	0.669	11.64	100.19
2.965	8.628	7.834	91.84	0.781	9.15	99.99

All values are the mean of two determinations.

Similar results were obtained with the other sample of arginine (*a*) (Vickery and Leavenworth). The curves (Fig. 1) show the precipitation in percentage at the different nitrogen concentrations for arginine (samples *a* and *b*), histidine, lysine and proline. It will be observed:

(1) that lysine shows the steepest rise, and is therefore the most insoluble;

(2) that precipitation is not complete in any of the cases, reaching 92 % for arginine, 91.6 % for histidine, and 98.4 % for lysine;

(3) that proline is only precipitated at very high concentrations, and then only in part;

(4) that the amount of nitrogen remaining in the filtrate, which should be constant if ordinary solubilities are involved, increases steadily with the concentration (see Table I).

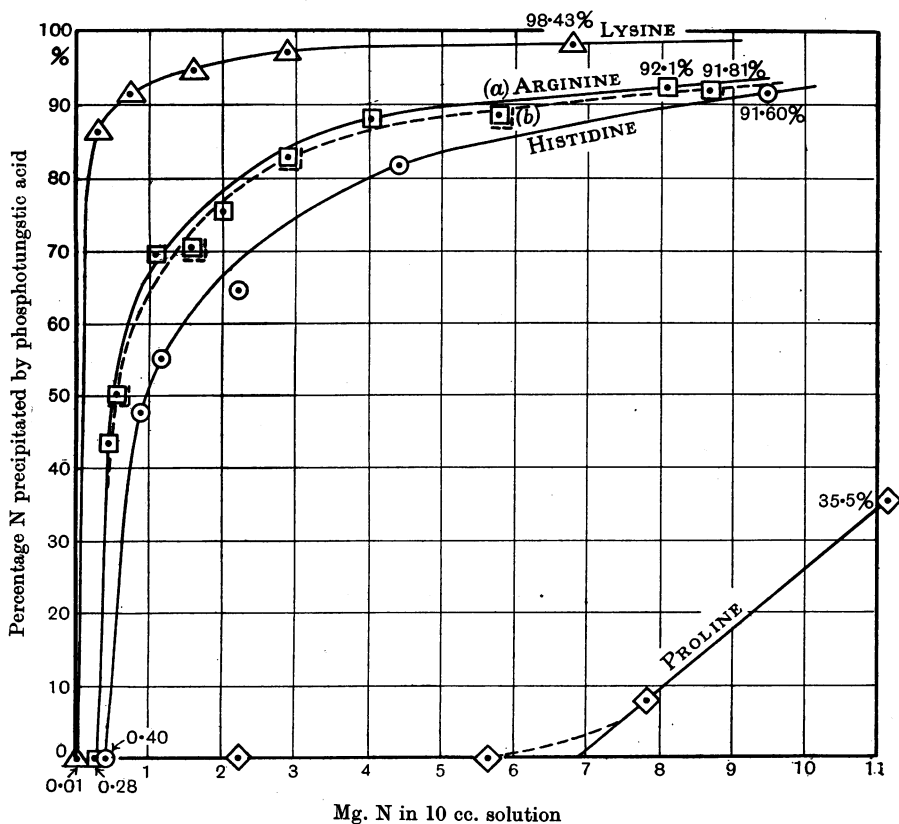


Fig. 1. Precipitation curves of basic amino-acids.

This last phenomenon may be due to two factors, the first of which is a true solubility in excess of amino-acid, and the second the presence of a quantity of non-precipitable impurity, such as a mono-amino-acid. This seemed improbable in view of the purity of the samples used, and its improbability was further confirmed in the following manner. Curves were plotted showing the amount of nitrogen in solution at different concentrations (see Fig. 2). Now the point at which the vertical axis is cut may be taken to represent the true minimum solubility of the phosphotungstate in 23 cc. of approximately 3.7 % phosphotungstic acid, 5 % sulphuric acid solution

(0.85 g. phosphotungstic acid having been added in all to 23 cc.). A line through this point parallel to the x -axis would represent a case of simple solubility.

In the case of arginine, however, the curve rises to an extent corresponding approximately with 0.45 mg. in 10 mg., so that 4.5 % of soluble impurity would have to be present to account for this slope. This quantity is altogether too large to escape detection and moreover would considerably alter the nitrogen figures, so that its presence, and hence this explanation, must be ruled out. It follows that the phosphotungstates of arginine and histidine, and to a much less extent that of lysine, are soluble in excess of the amino-acid. It may be that some kind of equilibrium between two salts of different solubility is established. In any case the precipitation is not simple.

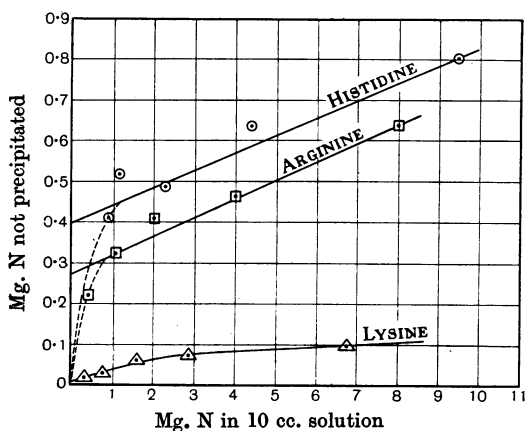


Fig. 2. Variation of the solubility of the phosphotungstates with nitrogen-concentration.

It seems at first sight a curious anomaly that lysine, which is known to give a soluble double salt in strong solutions, appears here as the least soluble of the three. This, however, is explained by the fact that in the two solutions of highest concentration, additional quantities of 1 cc. phosphotungstic acid solution were added in order to ensure complete precipitation. Apparently the formation of the soluble compound of lysine is on a somewhat different scale from those of the other bases, involving larger amounts when it occurs, but being more amenable to modified precipitation conditions.

The minimum solubilities as extrapolated from these curves are as follows (Table II):

Table II.

Minimum solubilities of phosphotungstates in 3.7 % phosphotungstic acid, 5 % H_2SO_4 .				
	Arginine (a)	Arginine (b)	Histidine	Lysine
mg. N per 23 cc. from curve	0.29	0.28	0.41	0.01
mg. amino-acid per 100 cc.	0.0072	0.0070	0.0115	0.0003
mg. N per 23 cc. given by Van Slyke	0.37	0.37	0.44	0.06

INTERPRETATION OF PRECIPITATION CURVES OF ACID-TREATED GELATIN.

Comparison of the curves for successive acid-flocculations of bone gelatin, given in the preceding communication [Thimann, 1930, p. 365] shows that they possess the following principal characteristics.

(1) A successive steepening of the rise from the origin, from the first to the fourth treatment.

(2) A rapid fall to very low values in the gelatin treated twice with acid (curve C).

(3) The occurrence of a peak in the curve for gelatin treated once with acid (curve B) or once with alkali (curve F), particularly the former.

It was shown above that the lysine curve is considerably steeper than that of the other amino-acids, so that the increase in steepness may be ascribed to increasing quantities of lysine. Confirmation of this is afforded by the steep fall. In all the points on the low portion of the curve, a considerable further precipitate separated when the wash-liquid was added to the filtrate, and 1 cc. additional phosphotungstic acid was therefore added

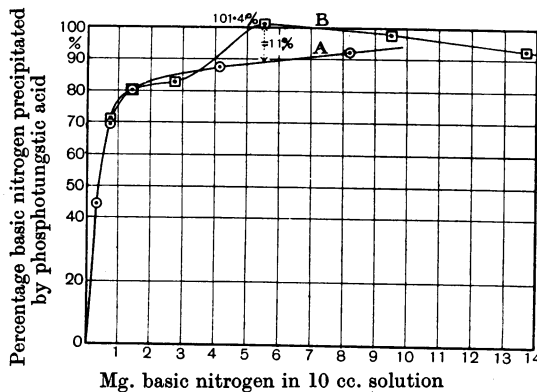


Fig. 3. Precipitation of amino-acid mixture with and without proline.

and the precipitate again centrifuged. The addition of further phosphotungstic acid to the filtrate again caused a precipitate, and even after three additions of 1–2 cc. a precipitate was still produced on further addition of phosphotungstic acid to the centrifugate. This phenomenon is produced only by lysine, as shown above, and these facts therefore fall into line with the theory that relatively large quantities of lysine were present, and hence precipitation was incomplete throughout. Acid treatment therefore increases considerably the lysine content of these gelatin hydrolysates.

The peak. It was shown above that the precipitation of any considerable amount of proline takes place only at certain nitrogen concentrations and at certain proline-diamino-acid ratios. The suspicion that this maximum was due to proline was confirmed as follows. A mixture of the three bases was made up in approximately the same proportions as in gelatin, and its precipitation curve obtained (curve A, Fig. 3). To the same solution was added

proline in corresponding proportions, and the precipitation curve again obtained (curve B, Fig. 3).

Mixture of bases.

	mg.	Proportions
Solution A. Arginine	35.4	} in 100 cc. water.
Lysine (hydrochloride)	286.4	
Histidine	73.5	
Solution B. Proline	237.5	3.5 in 30 cc. solution A.

A close similarity may be observed between curve B and that obtained for acid-flocculated gelatin (curve B, Fig. 2, of the preceding communication [Thimann, 1930, p. 365]). It is clear from these curves that proline is precipitated at certain concentrations only, and then only to the extent of about 11 %, but it is also abundantly clear that the peak of the gelatin curves is due to proline and can be imitated by addition of proline to the bases. The acid treatment of gelatin therefore increases the proline and lysine content.

All these curves show a general falling off at high concentrations, due to the slight solubility of the phosphotungstates in excess of amino-acid.

The phosphotungstic acid precipitation curves are therefore not merely useful as indicators of intramolecular lability, but are also amenable to interpretation and can yield valuable knowledge of the amino-acids present.

CRYSTALLISATION OF THE PHOSPHOTUNGSTATES.

During the course of this work opportunity was taken to crystallise the phosphotungstates in pure condition. Excess of phosphotungstic acid was not used in their preparation, in order to eliminate the possibility of the presence of the free acid.

Phosphotungstic acid itself crystallises well in large rhombohedral prisms¹ which quickly dehydrate and become opaque and friable on drying. The double refraction shown is very low. The angle between the well-developed rhombohedron and the basal plane is about 58°, giving an axial ratio of 1.6. The small crystals are shown in Plate I, Fig. 1, and a well-developed pyramid in Fig. 2.

Arginine phosphotungstate crystallises from water in 4- or 6-sided tablets, invariably twinned, of the monoclinic or triclinic system. They were erroneously described by Drummond [1918] as rhombohedral. The angle of extinction makes 27° with the composition plane. The refractive index is greater than 1.5 (Plate I, Fig. 3).

Histidine phosphotungstate may be obtained from water in the same form as arginine, but with greater difficulty and in smaller crystals. From acetone-water it separates in clustered prisms, also probably monoclinic (Plate I, Fig. 4).

¹ For assistance with the crystallography I am greatly indebted to Mr M. H. H. Hay, of the Natural History Museum, South Kensington.

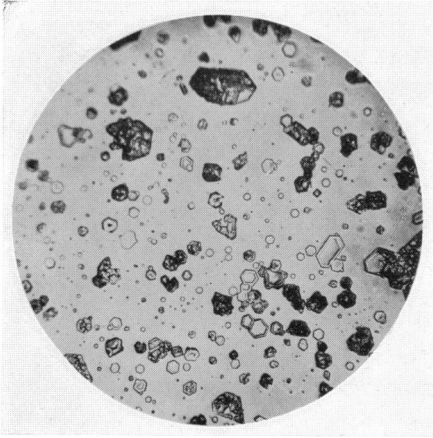


Fig. 1. Phosphotungstic acid.

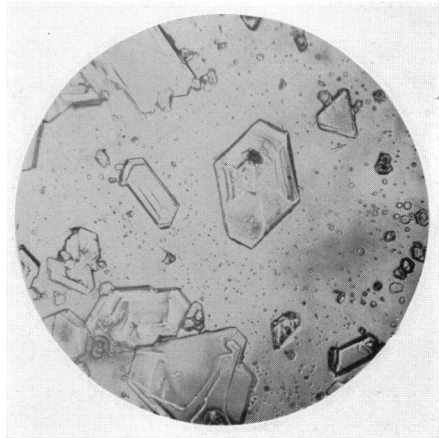


Fig. 2. Phosphotungstic acid.

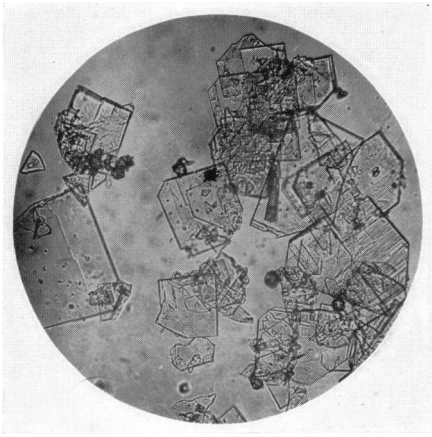


Fig. 3. Arginine phosphotungstate.

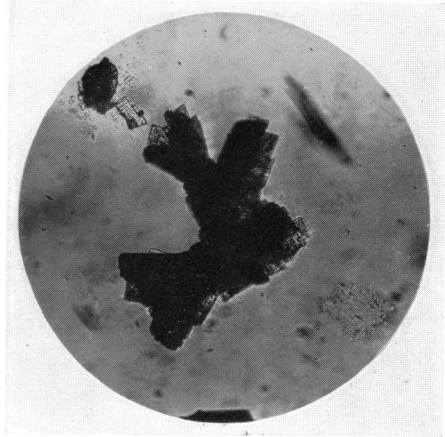


Fig. 4. Histidine phosphotungstate (prismatic form).

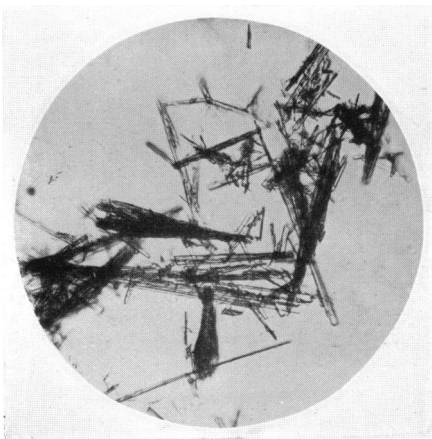


Fig. 5. Lysine phosphotungstate.

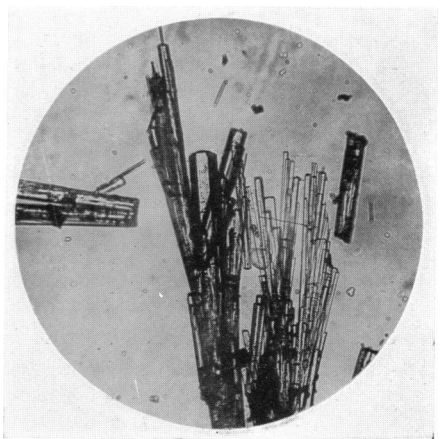


Fig. 6. Proline phosphotungstate.

Photomicrographs of phosphotungstates. Magnification $\times 187.5$.

Lysine phosphotungstate crystallises readily from water to which a few drops of acetone have been added in tiny clusters of fine orthorhombic needles, resembling wheatsheaves in the typical form. By slow evaporation needles 2-3 mm. long may be obtained (Plate I, Fig. 5).

Proline phosphotungstate separates from water to which a few drops of acetone have been added, in orthorhombic needles of large size, clustered together (Plate I, Fig. 6). The double refraction is high—about 0.04—and the refractive index greater than 1.5. By slow evaporation it can be obtained from water in small rhombs.

SUMMARY.

1. The rôle of the basic amino-acids and proline in the protein molecule is discussed, and the probable importance of lysine in intramolecular change is emphasised.

2. The precipitation of the individual amino-acids by phosphotungstic acid is determined at various nitrogen concentrations. It is not a simple phenomenon, the solubility of the phosphotungstates increasing with nitrogen concentration. In the case of lysine there is evidence for the formation of a soluble double salt. Proline is precipitated only at high nitrogen concentrations.

3. The minimum solubilities of the basic amino-acids in the final precipitating liquid are determined and found to be somewhat less than the solubility corrections given by Van Slyke. The actual correction to be employed in any experiment, however, will be considerably higher and will vary with the nitrogen content of the solution.

4. The precipitation curves of the individual amino-acids throw considerable light on the curves obtained with gelatin hydrolysates. In this case they show that the acid flocculation of gelatin produces an increased amount of proline and lysine.

5. The crystalline form of the phosphotungstates is described.

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