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ELEUSININ, THE ALCOHOL-SOLUBLE PROTEIN.

BY

Nuggehalli Narayana and Roland V. Norris.

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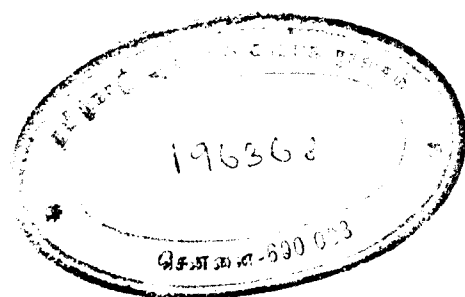
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1.—THE OXIDATION OF SULPHUR IN SUSPENSIONS OF ACTIVATED SLUDGE AND ITS INFLUENCE ON THE SOLUBILISATION OF MINERAL PHOSPHATES.

By C. V. Ramaswami Ayyar, T. S. S. Perumal and Roland V. Norris.

In recent years much work has been done to render the insoluble mineral phosphates available for plant nutrition. A number of methods have been devised for this purpose and of these, the one developed by Lipman and his associates (*Soil Sci.*, 1915-1923) in America has given the most encouraging results. It consists in composting raw phosphate with sulphur and soil under carefully controlled conditions of moisture and aeration, when the sulphuric acid produced by the biological oxidation of the sulphur acts upon the phosphate and renders it soluble. The amount of 'available' phosphate thus obtained being quite considerable, the application of this process warrants extended trial in agricultural practice.

Very little attention has been paid to utilising the large deposits of high grade mineral phosphates found in India, though useful work has been done by Hutchinson (*Agric. J. India*, 1924, 19, 4) and by Sivan (*Mem. Dept. Agric. in India, Chemistry Series*, 1925, 7, 145) who have studied the problem from two different aspects. The former investigated the solubilisation of mineral phosphate in culture media by pure strains of sulphur-oxidising organisms and observed that 89 per cent. of the total phosphate became soluble in less than 10 weeks. Sivan examined the influence of decomposing green manure on the solubilisation of mineral phosphates in paddy soil and noticed a gradual production of 'available' phosphate both in the laboratory and the field experiments. These results only emphasise the necessity for more extended investigations of the problem by other methods also.

One of such methods consists in the utilisation of activated sludge, which is rich in oxidising bacteria, and was also found to contain organisms capable of oxidising sulphur rapidly to sulphuric acid; it was therefore considered to be an excellent medium for effecting solubilisation of insoluble phosphates in presence of sulphur and the necessary supply of air.

Experiments showed that under such conditions considerable acidity soon developed and resulted in a marked selection of the microflora, most organisms other than the sulphur oxidisers being eliminated. This observation led to the study of the influence of this reaction, under various conditions, on the solubilisation of pure tricalcium phosphate

and the mineral phosphate obtained from Trichinopoly deposits containing 44 per cent. and 26 per cent. of total P_2O_5 respectively in the insoluble condition.

EXPERIMENTAL.

Suspensions of activated sludge containing varying amounts of sulphur, phosphates and other mineral salts, as indicated in the following scheme, were aerated vigorously with compressed air for about twelve weeks; samples were taken at weekly intervals, and their titratable acidity, water-soluble phosphates and sulphates along with P_H determined. To 100 c.c. lots of a 15 per cent. suspension of activated sludge were added the phosphates (1 gm.), flowers of sulphur (5 gms.) and in those cases where certain salts were used, 0.025 gm. of the salt in question, these mixtures being aerated in jars, using compressed air.

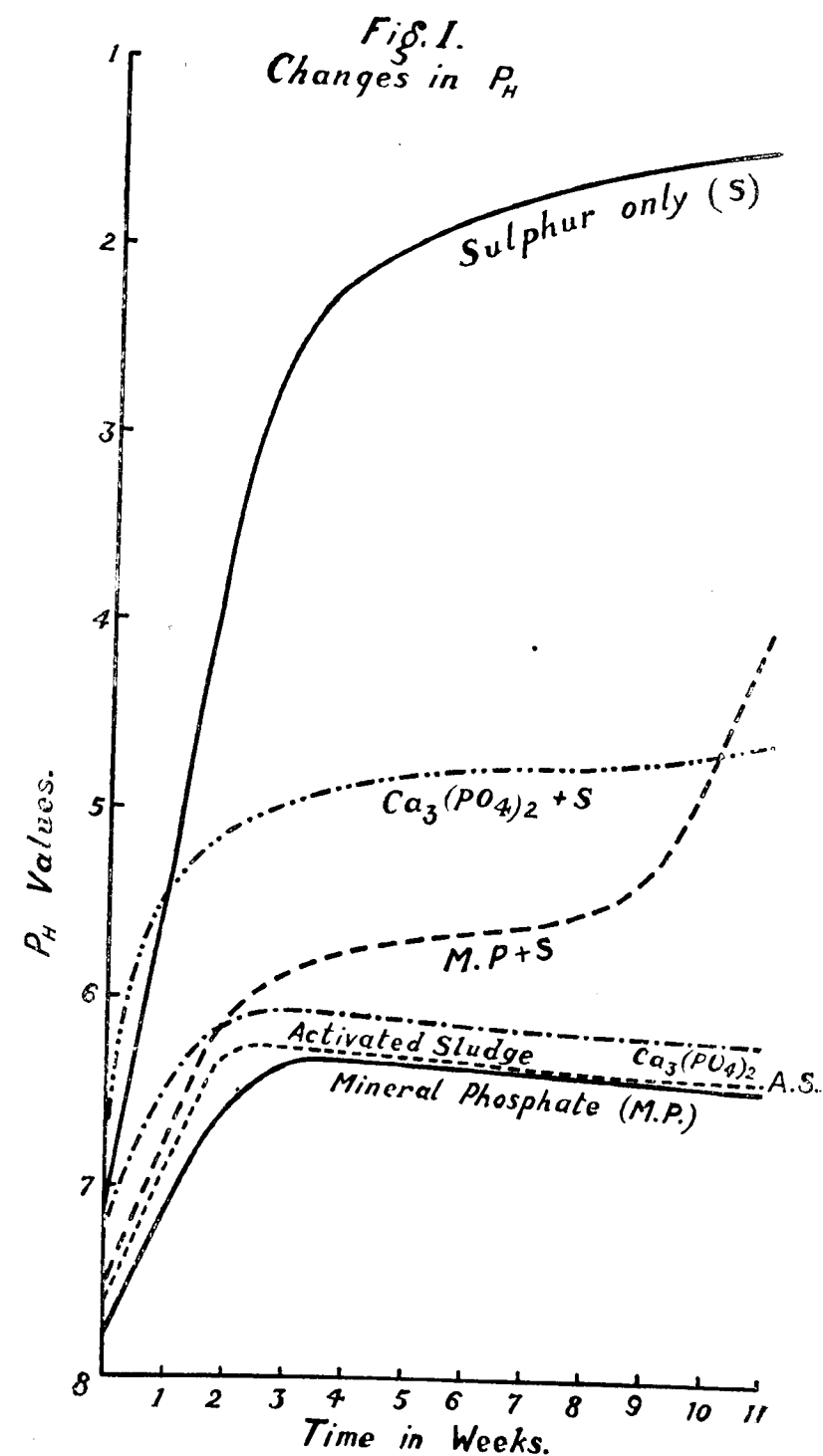
The scheme of experiments involved the following series:—

1. Activated sludge only.
2. " " with 5 gms. sulphur.
3. " " " 1 gm. tricalcium phosphate.
4. " " " 1 gm. mineral phosphate.
5. " " " $Ca_3(PO_4)_2$ and sulphur.
6. " " " Mineral phosphate and sulphur.
7. " " " " " $MnSO_4$.
8. " " " " " $FeSO_4$.
9. " " " " " $Al_2(SO_4)_3$.

Water-soluble phosphoric acid was determined by the volumetric method according to A. O. A. C. methods (1925, 3). Water-soluble sulphate was estimated by the usual gravimetric method of precipitation with barium chloride. Acidity or alkalinity was estimated by boiling the solution for 5 minutes to remove carbon dioxide and titrating with methyl-red as indicator using $N/50$ sodium hydroxide or sulphuric acid, the results being expressed for 100 c.c. of solution, in all cases. P_H values were determined colorimetrically by employing Clark and Lubs (*J. Bact.*, 1917, 2, 109, 191) series of indicators using the colour standards prepared after the method recommended by Medalia (*J. Bact.*, 1920, 5, 441; 1922, 7, 589), for comparison.

The P_H values obtained are represented graphically in Fig. 1. A study of these curves will show that treatment with sulphur has produced marked acidity.

The series without sulphur was characterised by a more or less constant P_H which in the sulphur series without added phosphate fell to 1.6. The curves thus arrange themselves in two groups, (1) controls without sulphur and (2) those containing sulphur with or without added phosphates. It will be clearly seen that in the former the P_H remained



in the range 7·8 to 6·0 and it may be noted that this is the range in which nitrification proceeds most vigorously (Ranganathan and Norris, *This Journal*, 1927, **10A**, 114).

In the sulphur series without added phosphates the acidity increased to a P_H of 1·6 and nitrification was inhibited; there was thus an accumulation of ammonia which was fixed as ammonium sulphate. This rise in acidity naturally had a marked influence on the bacterial flora and only a few limited types were able to survive.

In the presence of pure tricalcium phosphate, a buffer action was noticed, the P_H remaining at about 4·6 and more phosphate being steadily dissolved as further acid was produced. The course of events was not quite the same when mineral phosphate was employed. In this case there were two stages at which the buffer action became noticeable, one at P_H 5·8 and the other at 4·9. The first might be traced to the temporary influence of the calcium carbonate present as impurity. Subsequently the real buffer effect due to the phosphate was experienced. This buffer effect naturally did not persist indefinitely and it will be noticed that in the series to which manganous sulphate, ferrous sulphate and aluminium sulphate were added the P_H values were 1·80, 1·95 and 1·86 respectively after 16 to 20 weeks of aeration with a corresponding increase of water-soluble phosphate and sulphate. The chemical changes involved might be presumed to be of the normal type resulting in the continuous production of sulphuric acid and the action of this on the tricalcium phosphate leading to the production of di- and monocalcium phosphates, or even in extreme cases to the accumulation of free acid. This alone would explain such a fall in P_H shown by the crude phosphate series with sulphur and salts.

TABLE I.

Titratable Acidity in 100 c.c.; Acidity (+), Alkalinity (-).

Time	Sludge only	Sludge + Sulphur	Sludge + $Ca_3(PO_4)_2$	Sludge + Mineral phosphate	Sludge + S + $Ca_3(PO_4)_2$	Sludge + S + Mineral phosphate
	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
At beginning	- 9·6	- 6·8	- 13·2	- 14·8	- 10·4	- 13·2
1 Week	- 6·8	+ 0·8	- 6·0	- 14·8	- 1·2	- 6·8
2 Weeks	- 3·6	+ 4·0	- 6·0	- 14·0	+ 1·2	- 1·2
3 "	- 3·6	+ 11·2	- 5·2	- 12·0	+ 2·8	+ 0·8
4 "	- 3·6	+ 42·4	- 4·8	- 11·0	...	+ 1·2
5 "	- 3·6	+ 64·0	- 3·8	- 10·0	+ 3·6	+ 1·2
6 "	- 3·6	+ 138·4	- 3·6	- 9·0	...	+ 1·6
7 "	- 3·6	+ 147·8	- 3·6	- 9·0	+ 5·4	+ 1·6
8 "	- 3·6	+ 170·0	- 3·2	- 8·0	+ 11·6	+ 8·6
9 "	- 3·6	...	- 3·2	- 8·0	+ 12·0	+ 8·6
10 "	- 3·6	+ 182·8	- 3·2	- 8·0	+ 12·0	+ 8·6
11 "	- 3·6	+ 201·6	- 3·2	- 8·0	+ 12·0	+ 8·6

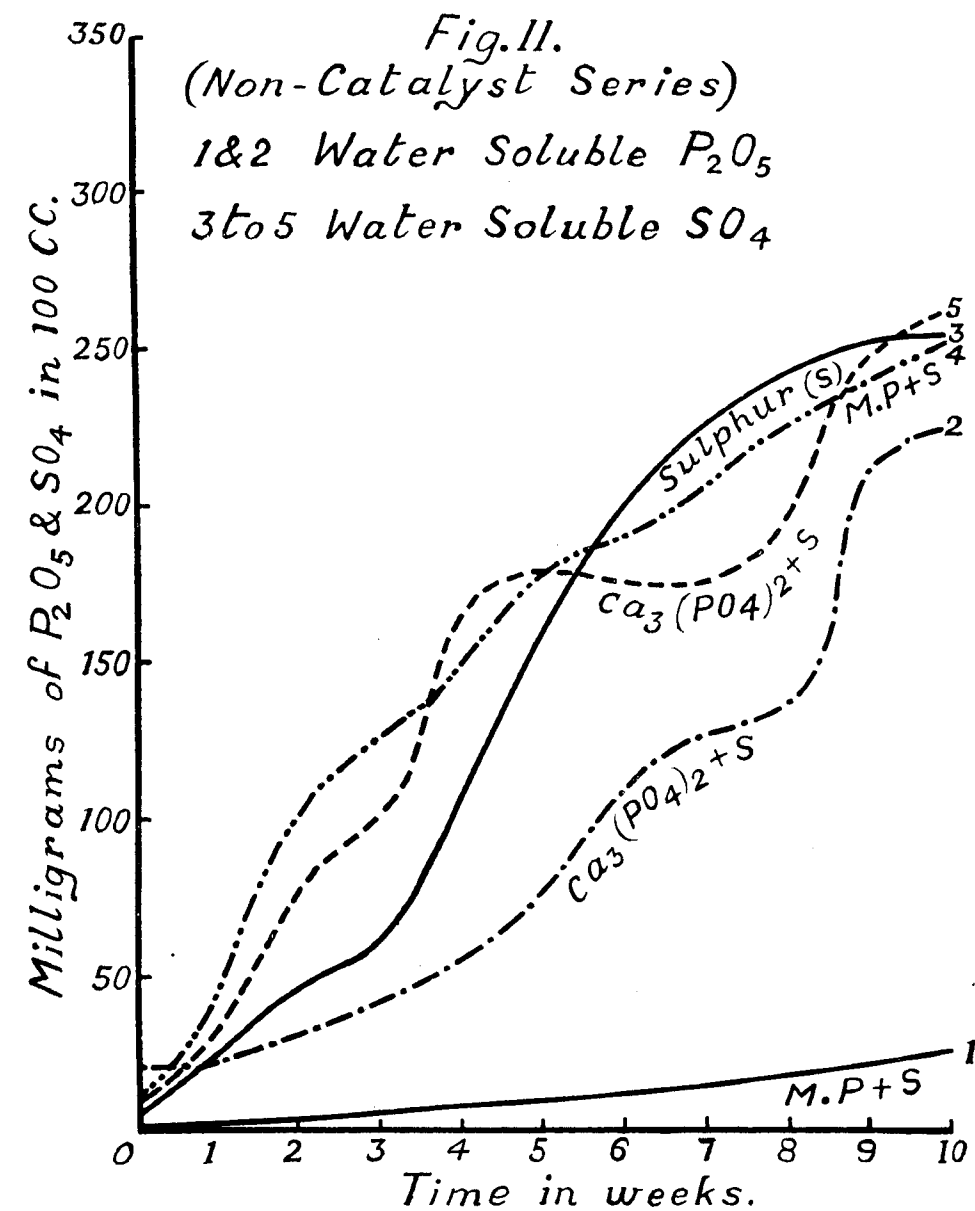
The titratable acidity produced is shown in Table I. The initial reaction was usually alkaline in all cases and it is to be noted that the titration figures for series 1, 3 and 4 exhibited relatively little change, whereas the introduction of sulphur led to a progressive development in titratable acidity up to the final analysis. There has, in fact, been an increase from an initial alkalinity equivalent to 6.8 c.c of $N/50$ sulphuric acid to an acidity equivalent to 201.6 c.c of $N/50$ sodium hydroxide. It is safe to assume therefore that, in a medium such as activated sludge, fairly well supplied as it is with nitrogen and phosphate compounds, sulphur-oxidising organisms multiply sufficiently to convert an appreciable amount of sulphur into sulphuric acid. With addition of the phosphate, even though there was a general production of acid as indicated by the formation of water-soluble sulphates shown in Figs. II and III given later, there was little development of free acidity, however, the acid as produced being utilised for the production of soluble phosphate.

Solubilisation of the phosphate was much less marked in the case of impure phosphate, for the impurities present, viz., 17 to 18 per cent. of calcium carbonate and 5 per cent. of iron and alumina, consumed the acid developed, thus reducing to a minimum the amount of acid available for action on the phosphate. In other words, no acid would be available for the production of soluble phosphate in such cases until all the impurities were neutralised. This doubtless explains the slow availability of mineral phosphates when used as manure.

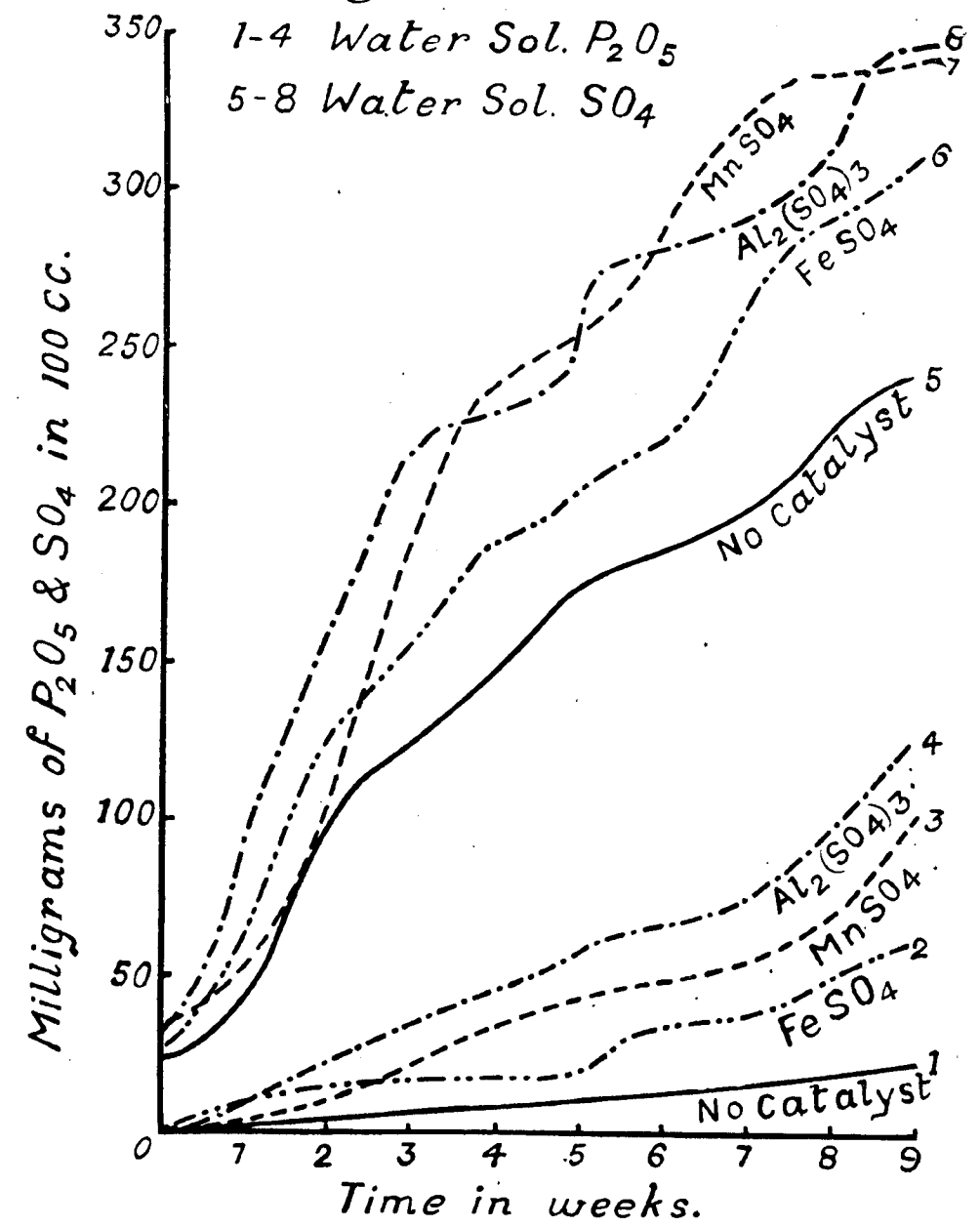
The water-soluble phosphoric acid found in the different mixtures with and without salts is recorded in Figs. II and III. It will be noted that the amount of water-soluble phosphate increased in all those mixtures which had received additions of both sulphur and phosphates; but the increase in the mineral phosphate series without salt did not reach the same value as when pure tricalcium phosphate was used, for the reasons already explained.

It was also found that only slow production of soluble phosphate (in the range 1 to 4 mgms. per 100 c.c.) took place in the non-sulphur series where vigorous nitrification proceeded, and supports the view that the nitrification process had but little influence on the solubilisation of phosphates. This confirms the observations of Ames and Richmond (*Soil Sci.*, 1918, 6, 351) who emphasise the fact that nitrification will have no effect on the solubility of mineral phosphate, or only acts to a limited extent.

As has been pointed out above, in the case of mineral phosphate the production of water-soluble phosphate was low. The addition however of certain salts such as manganous sulphate, ferrous sulphate and aluminium sulphate in very small quantities increased the production



1-4 Water Sol. P_2O_5
5-8 Water Sol. SO_4



of soluble phosphate from 22.22 mgs. to 92.1, 61.0, 121.2 mgs. respectively, and as might be anticipated there was a corresponding change in the P_H of the solutions. The stimulating power of manganous sulphate in biological processes has been previously established, and its influence on nitrification and ammonification studied by Brown and Minges (*Soil Sci.*, 1916, **2**, 67), Greaves (*Ibid.*, 443) and others. Montanan (*Staz. Sper. Agr. Ital.*, 1914, **47**, 441) also found that manganous salt greatly stimulated nitrification, the salts, in his opinion, either directly or indirectly furnishing oxygen to the nitrifying organisms. The influence of this compound in the oxidation of sulphur and the consequent increase in water-soluble phosphate in that series may well be similar.

Baudisch and Welo (*J. Biol. Chem.*, 1924, **61**, 261) hold the view that the assumption of variable oxidation stages in the conversion of ferrous to ferric iron does not explain the specific catalytic power of iron salts, for when once the ferrous salt is converted into ferric the re-conversion of ferric to ferrous iron requires considerable energy, so that the possibility of ferrous salt acting as a continuous catalyst appears to be out of the question. They explain the catalytic action in biochemical processes to (1) the peculiar power of divalent ferrous iron to absorb oxygen from the air and link it co-ordinatively; the auxiliary valence forces being stronger it is able to draw various organic and inorganic radicals or compounds into the inner sphere selectively; (2) the formation of ferro-ferric complexes in the presence of air, and (3) the oxidising power of the ferrous salt being enhanced by virtue of this co-ordinative linkage of oxygen, this power being governed by the concentration of each component of the mixture ferrous salt, carbon dioxide and oxygen. Under the conditions of our experiment the formation of such compounds appears quite possible, as evident from the increased solubilisation of phosphate, but further investigation is required to elucidate the nature of the stimulation.

Aluminium sulphate (Devson, *Soil Sci.*, 1922, **13**, 81) and other soluble salts of aluminium have been found to stimulate ammonification but to inhibit nitrification, for the latter process is readily checked in an acid medium. In view of the fact that the hydrolysis of the sulphate or chloride produces an acid medium it would be expected that the sulphur-oxidising organisms which readily tolerate acidity would thrive at the expense of other less adaptable organisms. Consequently it was not surprising to find that aluminium salts had a distinctly stimulating effect on the oxidation of sulphur and in consequence on the solubilisation of mineral phosphate.

The data for water-soluble sulphates produced in the various series showed clearly to what extent the sulphur was oxidised at different periods. It will be noted from Figs. II and III that there

has been a rapid increase in the amount of sulphur oxidised. It will be seen further that in the case of activated sludge to which sulphur and tricalcium phosphate were added, the oxidation of sulphur was much more rapid than in the mixture containing sulphur without phosphate, the sulphate being increased by nearly $1\frac{1}{2}$ times. The increased oxidation of sulphur in the phosphate series is probably due to two reasons, the phosphate by serving as a food supply for the oxidising organisms increasing their multiplication and also tending to maintain the reaction of the medium at a favourable P_H .

Considering the ratio of the water-soluble phosphate produced to the amount of sulphur oxidised and leaving out for the moment any insoluble sulphates which have not been estimated, it will be seen that in the pure phosphate series approximately two parts of soluble phosphoric acid (P_2O_5) have been produced by the oxidation of one part of sulphur (227 mgs. P_2O_5 to 113 mgs. sulphur). In the mineral phosphate series, however, without catalysts the phosphate required nearly four times its weight of sulphur (24 mgs. P_2O_5 to 91 mgs. sulphur); but in the presence of catalysts or salts used above, the proportion became 1:1. The influence of these salts on the oxidation process and the solubilisation of mineral phosphate is thus very clearly indicated.

SUMMARY.

1. The influence of sulphur-oxidation on the solubilisation of mineral phosphate in an activated sludge medium under various conditions is discussed.

2. In the sulphur series without a neutralising agent the P_H fell to 1.6 and there was a rapid production of titratable acidity. With the introduction of the phosphate, although there was a greater production of acid, this was utilised for the production of soluble phosphate the P_H remaining at about 4.6.

3. In the absence of catalysts, even though there was increased sulphur oxidation, as indicated by the sulphate figures, there was not a corresponding increase in the production of water-soluble phosphate; but with sulphates of manganese, iron and aluminium the solubility rapidly increased.

4. With the rapid oxidation of elemental sulphur there was an accumulation of water-soluble sulphate. Generally the amount of sulphur oxidised in relation to the water-soluble phosphate produced was found to be in the ratio of 4 of sulphur to 1 of mineral phosphate. The introduction of the catalysts reduced the ratio to 1:1.

Department of Bio-Chemistry,
Indian Institute of Science,
Bangalore.

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II.—STUDIES IN THE PROTEINS OF INDIAN FOODSTUFFS

PART I.—THE PROTEINS OF RAGI (ELEUSINE CORACANA): ELEUSININ, THE ALCOHOL-SOLUBLE PROTEIN.

By Nuggchalli Narayana and Roland V. Norris.

Ragi (*Eleusine coracana*), the staple cereal crop of the Mysore State, forms the greater part of the diet of the lower classes in that province. Its food-value is popularly supposed to equal that of wheat and to be much higher than that of rice. The proteins of this grain, hitherto uninvestigated, therefore appeared to merit study. Ragi contains from 1.1 to 1.7 per cent. of nitrogen or 6 to 11 per cent. crude protein ($N \times 6.25$), typical analyses of the dry grain being given below expressed as percentages.

TABLE I.

Analysis of Ragi: dried at 100°.

...	Ash	Ether extractives	Crude protein* ($N \times 6.25$)	Crude fibre	Carbohydrates (by difference)
I	2.78	1.82	6.90	3.27	85.23
II	2.52	1.61	9.83	3.54	82.50

In common with other cereal grains a considerable proportion of the protein consists of a prolamine, or alcohol-soluble protein which may be given the name eleusin. In the present paper a preliminary account is given of an examination of this substance.

Separation of Eleusin.

The grain was finely ground and passed through a 30 mesh sieve. The flour was then treated with three times its weight of 70 per cent. alcohol and vigorously stirred by mechanical means for six to ten hours. The extract was then filtered, yielding a clear, yellow liquor.

* True protein, 6.78 and 9.75.

In the earlier experiments the prolamine was separated by concentrating the extract at a temperature not exceeding 45° under reduced pressure. When the volume is reduced by about one-third precipitation of the protein begins and is complete when about half the liquid has been evaporated. Separated in this way, however, the protein is tough and leathery; after being washed, dried, powdered and extracted with ether to remove fatty material it was found to contain only 13.9 per cent. of nitrogen, and was therefore obviously impure. Moreover, probably owing to denaturation, it is no longer soluble in 70 per cent. alcohol and its further purification becomes a matter of difficulty.

A more convenient method of separation is to concentrate the alcoholic extract under reduced pressure until the first signs of precipitation are evident. The liquid is then filtered and poured slowly with constant stirring into ten times its volume of distilled water. A milky fluid results from which, on addition of a small quantity of an electrolyte such as sodium chloride, the protein is readily precipitated in flakes and being then easily soluble in 70 per cent. alcohol can be purified by repeated solution and reprecipitation. The precipitated protein is finally washed on a filter-funnel with large quantities of water to remove the sodium chloride and dried in a desiccator over sulphuric acid. The dry powder is then extracted in a Soxhlet with ether to remove traces of fat and again dried, when the protein is obtained as a greyish white powder containing on an average 15.9 per cent. of nitrogen. After treatment in the above manner it is no longer soluble in dilute alcohol. It gives positive reactions with the usual protein tests and contains both tyrosine and tryptophan. It contains no phosphorus however, and cystine is probably absent; but this point is receiving further investigation.

It is advisable not to prolong the preliminary extraction with 70 per cent. alcohol for more than ten hours: Otherwise denaturation takes place and the protein is always precipitated in a leather-like mass insoluble in dilute alcohol and therefore incapable of further purification. For comparative purposes slight variations were made from time to time in the method of preparation, e.g.,

(a) Direct extraction of the flour with cold 70 per cent. alcohol.

(b) Extraction at 50–60°.

(c) A preliminary extraction with 4 per cent. sodium chloride for 12 hours followed by extraction with cold 70 per cent. alcohol.

(d) As in (c) but with the alcoholic extraction carried out at 50–60°.

All four methods have given a final product with much the same degree of purity, the figures obtained by elementary analysis being as follows:—

TABLE II.

Composition of Eleusinin prepared by Different Methods.

Method of Preparation	a	b	c	d	Mean Result
C ...	52.83	53.87	53.56	52.92	53.29
H ...	7.14	7.60	7.73	7.34	7.35
N ...	16.10	15.98	15.91	15.65	15.91
O ...	23.93	22.53	22.79	24.09	23.34

The nitrogen distribution was next determined in a mixed sample by the Van Slyke method (*J. Biol. Chem.*, 1911, 10, 15) as modified by Plimmer (*J. Biol. Chem.*, 1925, 10, 1004). Hydrolysis was effected by boiling 3–4 grams of the protein with 100 c.c. of 20 per cent. HCl under a reflux condenser for 30 to 36 hours. Even after a prolonged digestion an insoluble residue is invariably left containing nitrogen equivalent to about 0.8–0.9 per cent. of the total nitrogen.

TABLE III.

Nitrogen Distribution in Eleusinin.

(Results expressed as percentages of total nitrogen).

Sample	I	II	III	Average
Humin				
Insoluble ...	0.85	0.88	0.89	0.87
Soluble ...	0.25	0.29	0.31	0.28
Amide ...	19.72	20.97	20.87	20.52
Basic, comprising	5.71	6.23	5.87	5.93
Arginine ...	2.37	2.90	2.54	2.60
Histidine ...	2.79	2.52	2.76	2.69
Lysine ...	0.55	0.81	0.57	0.64
Non-basic, comprising	71.86	70.52	71.11	71.16
Amino ...	69.31	68.93	68.85	69.03
Non-amino ...	2.55	1.59	2.26	2.13
Total ...	98.39	98.89	99.05	98.76

TABLE IV.

*Comparison of Eleusinin with other Prolamines.**

(Results expressed as percentages of total nitrogen).

	Gliadin from <i>Triticum</i> <i>vulgare</i>	Secalin from <i>Secale</i> <i>cereale</i>	Sativin from <i>Avena</i> <i>sativa</i>	Hordein from <i>Hordeum</i> <i>vulgare</i>	Zein from <i>Zea mays</i>	Kafrin from <i>Andropogon</i> <i>sorghum</i>	Sorghumin from <i>Sorghum</i> <i>vulgare</i>	Teozein from <i>Euchlaena</i> <i>Mexicana</i>	Eleusinin from <i>Eleusine</i> <i>coracana</i>
Amide ...	25.52	22.18	22.20	23.38	18.06	20.76	18.96	18.99	20.52
Humin ...	0.87	1.17	1.25	1.44	1.11	1.35	3.08	1.19	1.17
Basic, comprising									
Arginine ...	6.38	6.80	8.93	6.22	3.92	3.92	4.83	3.90	2.60
Histidine ...	5.41	7.21	2.31	10.36	2.45	1.71	1.19	3.58	2.69
Cystine ...	1.68	1.44	1.74	1.38	0.98	1.23	0.92	0.80	?
Lysine ...	0.57	0.42	1.20	3.02	0.89	2.48	3.45	2.57	0.64
Non-basic, comprising									
Amino ...	53.49	50.14	54.60	50.41	66.08	68.85	60.01	64.44	69.03
Non-amino ...	6.14	9.77	5.86	3.65	5.90	0.32	5.33	3.62	2.13
Total ...	99.77	99.13	98.09	100.26	99.40	100.62	97.77	97.09	98.76

* Cf. Hoffman and Gortner, Colloid Symposium Monograph, Vol. II, Chemical Catalog Co., New York, 1925.

The essential difference in nitrogen distribution between eleusinin and most of the other prolamines examined is, therefore, the lower content of basic nitrogen including the possible absence of cystine and a correspondingly high percentage of amino-nitrogen. The work is being extended and a complete estimation of the constituent amino-acids is being attempted.

Department of Bio-Chemistry,
Indian Institute of Science,
Bangalore.

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