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THE CHEMISTRY OF THE VITAMINES [1]

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Since the appearance of the last article in which I collaborated with Doctor Vedder,[2] I have made more than forty attempts to obtain pure vitamine from large quantities of rice polishings. While other methods were tried, the prime endeavor was to perfect the method described by Funk,[3] as it appeared to be preferable to that of Edie et al.[4] or Suzuki [5] and his coworkers, with whose methods I have experimented. The modification of Wellman, Eustin, and Scott [6] was also tested. It failed to facilitate the final purification of the vitamine and apparently resulted in greater loss of curative power in the vitamine fraction. On the other hand, it possesses the great advantage of effecting an enormous saving in the quantities of phosphotungstic acid and barium hydroxide used.

In addition to these methods, many other precipitants have been tried, such as mercuric acetate and zinc and cuprous salts, but of these none produced promising results and may be dismissed. Other attempts were directed to finding a better method of decomposing the phosphotungstates produced by Funk's method. For this purpose amyl alcohol was used as

[1] Received for publication April 26, 1915.

[2] *This Journal, Sec. B* (1913), 8, 175.

[3] *Journ. Physiol.* (1911), 43, 395; (1912), 45, 75; *Ergeb. d. Physiol.* (1913), 13, 125.

[4] *Bio-chem. Journ.* (1912), 6, 234.

[5] *Bio-chem. Zeitschr.* (1912), 43, 89.

[6] *Am. Journ. Prev. Med.* (1914), 1, 295.

suggested by Jacobs.[7] This, however, afforded no particular advantage, as the vitamine apparently distributed itself more or less equally between the amyl alcohol and water layers. It was also discovered that the phosphotungstate of the vitamine dissolved fairly readily in an excess of phosphotungstic acid, thus enabling it to be separated from the bulk of the precipitated phosphotungstates. But since barium hydroxide was the only reagent found which could be used effectively for decomposing the separated phosphotungstates, this method was also abandoned.

When it was desired to isolate considerable quantities of vitamine in a fairly pure or at least highly concentrated condition, it was necessary to fall back on the method of Funk in practically its original form as nearly as I now understand it. This was the method used for the treatment of the human cases recently reported by Doctor Saleeby and me.[8]

Every resource has been exhausted to avoid mixed deposits from the final liquor and to increase the yield of needles melting at 233°, but without avail. Every step in the process has been modified in turn, but without materially improving the final result. Among such modifications may be mentioned the following:

1. Varying degrees of expression of the original polishings.
2. The use of neutral alcohol for extraction and subsequent hydrolysis of the aqueous extract with dilute sulphuric acid.
3. The use of a glass still and high vacuum for concentrating the extract.
4. More or less prolonged maceration of the phosphotungstates with barium hydroxide.
5. Removal of ammonia from the resulting solution with a stream of air.
6. Varying the strength of reagents and solvents at many points in the process.

I was, therefore, at a loss for an adequate explanation of the discrepancies between my results and those of Funk. Recent work[9] now shows that Funk was in error in reporting the isolation of the curative substance as a pure chemical individual and that the substance melting at 233° consisted almost entirely of nicotinic acid. A number of other differences exist between Funk's results and my own, which are not as yet wholly accounted for. I have noted that the quality of the rice

[7] *Journ. Biol. Chem.* (1912), 12, 429.
[8] *This Journal, Sec. B* (1915), 10, 99.
[9] Drummond and Funk, *Biochem. Journ.* (1914), 8, 598.

polishings appears to affect the yield of curative substances considerably and have selected the raw material accordingly. Perhaps more suggestive is the idea which has but recently occurred to me that rice polishings, like yeast,[10] may undergo an autolysis which splits the mother substances of the vitamines in a different way than does acid hydrolysis. Funk obtained his entire supply of rice polishings from the Orient,[11] and the bran was well aged before being submitted to chemical manipulation. Here comparatively fresh material has been used uniformly on account of the difficulty of protecting it during storage from the attacks of insects which multiply in it with tremendous rapidity. The presence of large quantities of enzymes in rice polishings is readily demonstrable, as there is rapid production of carbon dioxide from its suspensions in water in the presence of sufficient formaldehyde or phenol to prevent bacterial growth.

In as much as Funk's descriptions of his chemical methods have been so brief and incomplete as to puzzle me greatly, the method as used by myself is recorded in detail, with notes on the apparent discrepancies in results.

Hope of isolating large quantities of an individual vitamine from rice polishings having been abandoned, it was decided to attempt the synthesis of curative substances, which might throw light on the nature of the vitamines. As a discontinuance of the work for the present is now necessary, the very incomplete results are reported in the experimental part.

In choosing the direction for a beginning in this work, the facts adduced by Funk and others pointed to the pyridine ring as the most promising nucleus. The most important facts in favor of this choice are the occurrence of nicotinic acid with the vitamine in several natural substances and the resistance of the vitamine to the Kjeldahl method of digestion for the determination of nitrogen. That this property of the vitamine is shared to the greatest degree by bodies containing the pyridine ring has been shown by the as yet incompleted work of Doctor Brill and Mr. Agcaoili of the Bureau of Science who have kindly communicated their results to me. This resistance is well exemplified by the method of Weidel and Murmann [12] for preparing pyridine sulphonic acids.

[10] Cooper, *Biochem. Journ.* (1914), 8, 250; Abstract in *Zentralbl. Biochem. u. Biophysik.* (1914), 17, 481.
[11] *Journ. Physiol.* (1911-1912), 43, 400.
[12] *Monatsh. f. Chem.* (1895), 16, 749.

The earlier surmise of Funk that the vitamines are purine or pyrimidine derivatives, on the other hand, does not appear to agree with the known facts above cited. It is supported by the semicurative properties of allantoin and other purine and pyrimidine bodies, which is, however, largely offset by the similar though less-marked property of nicotinic acid. No doubt the conception of the vitamines as necessary foodstuffs for the elaboration of certain tissue constituents had weight with Funk in this surmise. Experience with human beriberi, however, throws much doubt on the truth of this conception, the evidence against which is presented elsewhere.[13]

EXPERIMENTAL PART

METHOD OF SEPARATION OF THE VITAMINE OF RICE POLISHINGS

Twenty-five kilograms of selected, finely ground, light-colored rice polishings are allowed to macerate with occasional stirring for from twelve to twenty-four hours in a mixture of 75 liters of 95 per cent alcohol and 5 liters of concentrated hydrochloric acid. The mass is then pressed out at a pressure of about 1,000 pounds to the square inch. The collected liquid amounts to from 60 to 65 liters. It is evaporated in a copper still at a pressure of from 10 to 12 centimeters of mercury to a volume of about 3 liters. The concentration requires about seven hours. The temperature should not rise above 50°. The concentrated extract separates into layers of fat and aqueous liquid. The aqueous extract is separated from the fat while still warm and is filtered from a copious black sediment, which after being washed is discarded with the fat. The filtered extract and washings are further concentrated in glass under a good vacuum to 2 liters.

Phosphotungstic acid is added gradually till permanent precipitation is complete. An excess must be avoided. Some samples of phosphotungstic acid were encountered which produced temporary precipitates which later redissolved when concentrated solutions were added to an extract already completely precipitated. Therefore care should be taken that the further precipitation is permanent. After standing overnight, the precipitated phosphotungstates are collected and thoroughly washed with 5 per cent sulphuric acid till the washings show only traces of chlorides. When thoroughly air-dried the phosphotungstates so produced weigh from 2,200 to 2,500 grams, com-

[13] Williams and Johnston, *This Journal, Sec. B* (1915), 10, 337.

pared with about 450 grams obtained by Funk[14] from the same quantity of polishings. This large yield is somewhat, but not greatly, reduced if the original extract is made with neutral alcohol and the concentrated extract hydrolyzed by boiling with 10 per cent sulphuric acid under a reflux condenser for five hours. However, the maceration in acid alcohol produces an extract which cures neuritic fowls in doses corresponding to 50 grams, whereas extracts made by the latter method must be given in doses corresponding to 100 grams to produce the same result.

The mass of dry phosphotungstates is ground intimately with two and a half times its weight of barium hydroxide. During this process the mixture becomes pasty and develops an evanescent green. The odor evolved is somewhat distinctive and resembles that of methyl amine. The pasty mass is collected in a large bottle and is shaken with 3 liters of water for five hours and filtered. The residue is again shaken twice with water for an hour each time. The combined filtrates, from which in the meantime the excess of barium hydroxide has been completely removed with sulphuric acid without the least unnecessary delay, are now carefully tested for excess of sulphuric acid or barium. Any excess of either having been carefully removed, the solution is neutralized with nitric acid, using litmus paper as indicator, and is concentrated in vacuo to a volume of about 1 liter. At this point the toxic properties of the liquid are so great that it is impossible to demonstrate its curative power.

To the concentrated liquid a 10 per cent solution of silver nitrate is added till no further precipitation occurs. The bulky yellow precipitates of purine bases are separated by filtration, and the filtrate is reserved for further fractionation. The purine bases are discarded. I am yet uncertain whether or not Funk carried out this separation of the purine bases in the case of rice polishings, although he reports having done so with yeast. At all events the purine bases so separated, although considerable in amount, do not when freed from silver possess curative properties to a high degree. Moreover it would be quite hopeless under my conditions of work to try to insolate any substance in a pure state from the vitamine fraction if the purines were included with it.

A small aliquot part of the filtrate from the purine bases is titrated with a 5 per cent silver nitrate solution till a drop of

[14] *Journ. Physiol.* (1912), 43, 396.

the clear titration liquid placed on a test plate gives with a saturated solution of barium hydroxide a distinctly brown rather than white or pale yellow precipitate. To the main portion of the filtrate from the purine bases is added an amount of silver nitrate slightly less than that calculated from the above titration.

A small aliquot part of the resulting liquid is now titrated with a saturated solution of barium hydroxide. A white precipitate forms, which at first redissolves on stirring. Later the precipitate becomes permanent and gradually darkens. As long as insufficient barium hydroxide is present the precipitate will settle on standing a few minutes, leaving the supernatant liquid bright and clear. Upon the addition of an excess of barium hydroxide the supernatant liquid becomes cloudy or muddy in proportion to the excess of barium hydroxide. Having thus determined the quantity of barium hydroxide necessary, the aliquot part is combined with the main portion and the calculated quantity of barium hydroxide solution is added. A slight deficiency is preferable to an excess.

The method suggested by Funk of testing with ammoniacal silver nitrate to determine the necessary quantity of barium hydroxide was of very little use to me, as this reagent at no stage produces more than a faint precipitate with the filtrate from the vitamine fraction.

When the precipitation with barium hydroxide is completed, the precipitate is allowed to settle and after decantation of the clear liquid is collected on a Büchner funnel and is thoroughly washed with water. The precipitate, which has by this time assumed a dark green, is suspended in 100 cubic centimeters of water, into which is led a stream of hydrogen sulphide. The suspension is shaken continuously during the passage of the hydrogen sulphide, which continues for from thirty to forty minutes. The solution is filtered, and the precipitate is repeatedly macerated and washed with small quantities of water containing hydrogen sulphide. The combined washings and filtrate from the silver sulphide are carefully treated with very dilute sulphuric acid until all traces of barium are just removed. After filtering from barium sulphate, the solution is evaporated at room temperature to a volume of 50 cubic centimeters. One hundred cubic centimeters of 70 per cent alcohol are added. A rather copious, brown, flocculent precipitate results, which is separated by filtration and washed with 70 per cent alcohol. This precipitate has rather slight curative powers, a dose equivalent to 15 or 20 kilograms of the original polishings being necessary to cure a fowl. It gives the uric acid reaction.[15] It is fairly freely soluble in water, but is very slightly soluble in alcohol. From it considerable nicotinic acid can be crystallized from hot water.

The alcoholic filtrate and washings, which are now curative for fowls in doses equivalent to 3 kilograms of the original polishings as compared with 100 grams in the case of the original extract,[16] are collected and evaporated in a dessicator over sulphuric acid at ordinary pressure. A vacuum dessicator produces too rapid an evaporation for the best results. This solution is now distinctly acid to litmus, but not to methyl orange. As evaporation proceeds, the acidity diminishes. Occasionally a faint alkalinity is developed, resulting in considerable loss of curative power on further standing. In the course of a week's standing in the dessicator, a few needle crystals, 2 to 3 millimeters in length, appear. These show a marked tendency to float on the surface of the mother liquor. At the same time a deposit collects in the bottom of the vessel, consisting of mixed forms of crystals and much amorphous matter. The floating crystals when removed and dried melt at 233° and produce cures in fowls in doses of from 10 to 20 milligrams. The yield, however, amounts to only from 30 to 40 milligrams. If the crystals are allowed to remain in the mother liquor, there is no further accumulation. The amorphous material, however, gradually increases in amount. If separated when the volume has diminished to 10 or 12 cubic centimeters, it shows curative power in fowls in doses of from 20 to 30 milligrams. The yield from 25 kilograms of polishings amounts to 0.2 to 0.25 gram. It is difficultly soluble in water and alcohol and when recrystallized from dilute alcohol is very little altered in appearance or character. This is the product which was used in treating the human cases recently reported.[17]

On further evaporation of the mother liquor from this product either with or without the addition of alcohol, a syrup results which eventually dries to a hard clear film, weighing from 0.2 to 0.3 gram. It possesses curative properties in mod-

[15] Folin and Macallum, jr., *Journ. Biol. Chem.* (1912), 11, 265; (1912–13), 13, 363.

[16] Funk's statement that the vitamine fraction retains nearly the whole of the curative substance does not appear to be borne out by his own experimental results.

[17] *This Journal*, Sec. B (1915), 10, 99.

erate doses and gives a blue color with phosphotungstic acid and alkali.[18]

SYNTHETIC PRODUCTS

The following derivatives of nicotinic acid were prepared and their curative properties tested by feeding to neuritic fowls per os:

TABLE I.—*Derivatives of nicotinic acid fed to neuritic fowls.*

Substance.	Dose.	No. of fowl.	Effect.
	Grams.		
Nicotinic acid	0.05 to 0.2	6	Little improvement, but some prolongation of life.
Trigonellin	0.05 to 0.2	3	Do.
Hydrochloride of methyl ester of nicotinic acid.	0.1 to 0.2	2	Marked, but temporary improvement.
P-oxynicotinic acid.	0.1 to 0.3	4	Approximately the same as nicotinic acid.

The nicotinic acid was prepared by oxidizing nicotine with nitric acid;[19] trigonellin, by the method of Hantzsch;[20] the methyl ester, by allowing nicotinic acid chloride to react at ordinary temperature with methyl alcohol; and the *p*-oxynicotinic acid, from methyl coumalat.[21] Oxynicotinic acid gives an intense blue reaction with phosphotungstic acid and alkali.[22] This color reaction given by the vitamine fraction of rice polishings may well be due to oxypyridine derivatives.

The methods used by Fischer were tried for the purpose of condensing nicotinic with *p*-oxynicotinic acid and the latter with itself. *P*-oxynicotinic acid was dissolved in aqueous acetone and the theoretical amount of dimethylaniline. To this solution was added finely ground hydrochloride of nicotinic acid chloride, for which no suitable solvent could be found. It decomposed to nicotinic and hydrochloric acids, while the oxynicotinic acid was largely recovered unchanged. As the residue from the reaction mixture did not possess marked curative properties, it was not further investigated.

An attempt to make the carbomethoxy derivative of *p*-oxyni-

[18] Folin and Macallum, jr., *Journ. Biol. Chem.* (1912), **11**, 265; (1912–13), **13**, 363.

[19] *Chem. Centralbl.* (1898), **1**, 677.

[20] *Ber. d. deutsch. chem. Ges.* (1886), **19**, 31.

[21] Pechmann, *Welsh Ber.*, **17**, 2390.

[22] Folin and Macallum, jr., *Journ. Biol. Chem.* (1912), **11**, 265; (1912–13), **13**, 363.

cotinic acid likewise failed to produce satisfactory yields. A small percentage of a substance which from its solubilities appeared to be carbomethoxy derivative was obtained. The body, however, was so unstable as to make its isolation in a pure state difficult. As the yield was very unpromising, this method was abandoned. If, however, carbomethoxy derivatives could be obtained the acid chlorides of which are soluble in some neutral solvent, they would possess a great advantage. The greatest difficulty encountered in working with the pyridine carboxy acids and their derivatives is their slight solubility in all ordinary solvents.

An attempt to condense *p*-oxynicotinic acid with itself by the use of fuming sulphuric acid resulted in a mixture of what appeared to be sulphonated bodies which possessed no marked curative properties. Phosphorus pentoxide gave better results.

Oxynicotinic acid was intimately ground with an excess of phosphorus pentoxide. The mass was quickly transferred to a dry flask fitted with a calcium chloride tube and heated in an oil bath to 150° for twelve hours. The mass gradually softened and was converted into a brown gum, which was taken up in a small quantity of warm water. On cooling, some crystals of oxynicotinic acid separated out together with a quantity of an amorphous, rather flocculent precipitate. This was separated and tested on three neuritic fowls, using a dose of 0.1 gram per os. Great improvement resulted in two cases and an apparently complete cure in the third.

In another experiment acetic anhydride was used as a condensing agent. Oxynicotinic acid was boiled with acetic anhydride under a reflux condenser for thirty minutes. On cooling, a mass separated very similar to that obtained in the previous experiment. Three neuritic fowls were each given by mouth 0.1 gram of this substance. Considerable improvement followed in all cases, but the effect seemed less promising than that of the previous preparation.