THE ANTI-NEURITIC BASES OF VEGETABLE ORIGIN IN RELATIONSHIP TO BERI-BERI, WITH A METHOD OF ISOLATION OF TORULIN THE ANTI-NEURITIC BASE OF YEAST

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In a recent paper from these laboratories an account was given of the chief investigations into the causation of Beri-beri up to 1910; this account was largely an abstract of the masterly monograph in which Schaumann[2] described the work of other investigators, added much important work of his own, and brought evidence to suggest that other diseases might have a similar etiology to Beri-beri.

Some of our earlier results were briefly mentioned in that paper, and the account of our further researches will be prefaced by a brief summary of some of the numerous papers on this subject which have appeared in the interval.

One of the most interesting papers gives an account of the later researches of Fraser and Stanton[3] who have already contributed so largely to the knowledge of this subject.

They demonstrate that the active anti-neuritic substance of rice meal is soluble in water and in alcohol, show that it is stable in acid, unstable in alkaline solution, and that its thermolability varies with varying physical factors. They show that it is not a phytin or a fat, and that it probably is not a protein nor does it contain phosphorus.

They further confirm, however, the fact that the phosphorus content of a rice is an indicator of its safety as an article of diet, and, with a view to the prevention of Beri-beri, recommend administrative measures to prevent the sale in the Malay States of rice with less than 0·4 per cent. phosphorus pentoxide.

Eijkman[4] recapitulates his earlier work on the subject, and all must regret that he and his colleagues were prevented from continuing their work in Batavia and Java *where, in the years* 1889 *to* 1897, *they had so far advanced the study of Beri-beri and Polyneuritis on experimental lines,*

and had demonstrated that Beri-beri could be cured and its occurrence prevented by the use of hand-milled rice.

His further researches to isolate the active substance from rice meal are recorded. Cure of polyneuritis in fowls was effected by two or three doses of extracts from rice meal; less than five grams of extract containing 0·085 per cent. P_2O_5 and 0·012 per cent. N, sufficed to restore to activity birds severely lamed. He strongly opposes Schaumann's theory (1910) that the active substance is a phosphorus-containing compound, though awaiting fuller accounts of his later communications[5].

Shiga and Kusama[6] report extensive investigations disproving the bacterial and toxic theories of Beri-beri, and confirm much of Eijkman's, and Fraser and Stanton's work.

Kilbourne[7] shows that the potassium content of rice meal is of almost equal value to the phosphorus content as an indicator of its safety.

Chamberlain and Vedder[8], following Fraser and Stanton, showed that an extract of rice meal in 70 per cent. alcohol, concentrated at a low temperature till alcohol free, maintains its activity to cure polyneuritis. They further showed that the active substance is able to dialyze through parchment. A daily dose of these extracts, containing 0·16 mg. P_2O_5 and 4·06 mg. Nitrogen, cured, in a few days, fowls severely lamed.

In another contribution[9] they confirm these results, having kept fowls for 100 days on polished rice and the extract without development of neuritis. They find that the sucrose and ash in the extract are inactive, and so exclude 0·91 of the 1·34 per cent. solids in the extract.

In addition, they find that the active substance is absorbed by bone-black, and are now proceeding to attempt to isolate and analyse it.

Funk[11] has isolated from rice meal a crystalline nitrate of an organic base which is extremely active in reviving pigeons with polyneuritis from feeding on polished rice.

The necessary dose contains about 4 mgs. of Nitrogen, corresponding to 0·05 gram. of the nitrate of the base, to which he allots the provisional formula $C_{17}H_{18}O_4N(HNO_3)$.

The crystals were in the form of microscopic needles, melting at 233° C., insoluble in cold water or alcohol, soluble, with difficulty, in hot water. They were free from ash and from chlorine and sulphuric acid.

This is the first record of the isolation and analysis of an active substance, and the method by which it was obtained will be briefly described. One and a half kilograms of rice meal was extracted with four litres of acid alcohol; separation of the filtrate was completed by the hydraulic press; about three and a half litres of extract were obtained, and evaporated in vacuo at 30°, leaving a fat-like residue. This was melted and treated with water,

and filtered while warm. The aqueous part was treated with ether to remove all fatty substances; it cured pigeons in doses corresponding to 20 grams of the original polishings.

The total aqueous extracts from 54 kilos rice meal amounted to 17 litres, which was treated with sulphuric acid and phosphotungstic acid throwing down 900 grams of precipitate. The precipitate was dried, washed with 5 per cent. H_2SO_4, ground with baryta and shaken three hours with water. The precipitate was filtered off, the filtrate smelt of ammonia and methylamine. The baryta was precipitated with sulphuric acid, and the filtrate neutralised with hydrochloric acid, and evaporated in vacuo at room temperature. The residue was extracted with alcohol, and the alcoholic solution was active in doses = 40 grams of rice polishings. The solution was free from proteins, phosphorus, and carbohydrates.

The alcoholic solution gave a crystalline precipitate with mercuric chloride, which was separated, washed, and recrystallised from water; this consisted mostly of cholin, but some active substance was also present. Active substance was present in both the alcoholic and aqueous filtrates.

Aqueous filtrate. The mercury was removed, and the filtrate evaporated and taken up in alcohol was treated with platinic chloride to remove cholin. After removing the platinum from the filtrate it was treated with phosphotungstic acid, giving a crystalline precipitate which yielded an active substance when freed from phosphotungstate with baryta and carbon dioxide.

Alcoholic filtrate evaporated and dissolved in water: mercury removed by sulphuretted hydrogen; chlorine, &c., were removed by successive treatment with silver sulphate, sulphuretted hydrogen, and baryta. The alkaline solution was precipitated with silver nitrate and baryta, the precipitate decomposed with sulphuretted hydrogen and freed from silver and barium. It proved active, and, after evaporation in vacuo, crystals were with difficulty obtained from alcohol, with the composition, &c., given above.

Bio-Chemistry of Extracts of Rice Meal and Yeast

Among other points, we find that nearly twice as much of the phosphorus of rice meal goes into solution in water after denaturisation at 120° C. Of the soluble phosphorus of rice meal nearly five-sixths dialyses: of the soluble phosphorus of denaturised rice meal only two-thirds dialyses. The protective properties of the fractions separated were not tried, as the quantities were insufficient for continued feeding experiments.

More than twice as much of the phosphorus of dried yeast, after denaturisation at 120° C., appeared as so-called phosphatide phosphorus.

The pentosan content was also investigated, and it was found that:—

100 grams of rice meal yielded 3·93 grams. of phloro-glucide.

100 grams of potato scrapings yielded 0·43 grams. of phloro-glucide.

There appeared to be no pentosan in Chamberlain-Vedder extract of rice meal.

Aqueous and alcoholic extracts of rice meal have a marked reducing power after hydrolysis with dilute acids: the reducing materials were not soluble in alcohol-ether mixture.

Alcoholic (90 per cent.) extract of rice meal was found to be active (as stated by Fraser and Stanton) in protecting birds from the onset of neuritis, and in curing them, but concentration on the water bath rendered the extracts inactive.

The extracts were concentrated under a fan at room temperature to small bulk till all smell of alcohol had disappeared. These extracts preserved some activity (see Chamberlain and Vedder, loc. cit.). Four birds, which were very weak and disabled with polyneuritis, were each given the extract from 25 grams of rice meal, daily: in the first week they showed a decided improvement; became more active; three gained 5 per cent., 15 per cent. and 8 per cent. in weight, respectively, in one week (loss previous to treatment, 32 per cent., 30 per cent. and 36 per cent.). The other, though it became more active at first, lost a further 2 per cent. in 7 days, and 14 per cent. in ten days, being then 47 per cent. below its original weight. It died on the 15th day of feeding, having lost a further 7 per cent.

Of the other three birds, two fell in weight (5 per cent. and 2 per cent.) between the 7th and 10th days, the other one gained a further 2 per cent: two fell again in weight, one losing a further 5 per cent., the other falling 5 per cent. more to nearly its weight at the commencement of treatment. The last just maintained its increase. All were now extremely weak again and could scarcely survive more than a day: they were now put on yeast, and rapidly improved; walking and flying well in a few days, and gaining, respectively, 9·6 and 6 per cent. in the week.

Attempts were made to precipitate the active principle from these extracts by the lead acetate method, which will be described in more detail in dealing with yeast.

Neither the normal or basic lead acetate precipitates proved active: the filtrate, however, was active: the precipitate from this by phosphotungstic acid did not, however, prove active.

Funk's method was now tried directly on the original extract of the meal. A strong odour of ammonia and methylamine was noticed in the treatment with baryta. In spite of continued treatment with this extract, however, the birds died in a few days.

We considered that other foodstuffs might give more favourable results, and so, for the present, abandoned the investigation of rice meal.

On account of the resemblance of the active substance in solubility, etc., to some of the peculiar lecithins and bases described by Winterstein in wheat meal, we tried the lead acetate precipitable portions of Katjang beans, but found them inactive. Feeding with fresh brain also failed to preserve birds from death when incapacitated with neuritis.

An attempt to isolate the active substance from Katjang beans proved

unsuccessful; neither the lead acetate filtrate or precipitate proving active. Further experiments will be made.

Natural yeast had been previously found to possess marked preventive and curative properties, and extracts from yeast were next investigated.

Investigation of Anti-neuritic Powers of Extracts of Yeast

I.—*Cold Alcohol.* Extract of yeast with 90 per cent. alcohol in the cold takes out the active substances.

The residue had lost its power to cure neuritis.

The extract rapidly revived birds suffering from neuritis, both of the convulsive and lame types. The weights improved markedly under treatment, but occasionally relapsed on continued treatment.

Bird *a*2.—Loss of weight 25 per cent., and marked lameness: 1 c.c. extract (6 grams yeast) given daily. Bird able to walk and fly next day. Improved in weight 5 per cent. in 7 days, but then began to lose again. Lived 4 weeks on this dose of extract, and re-developed lameness slowly on cessation of the extract.

Bird *d*5.—Lameness, loss 32 per cent. Improved 6 per cent. in weight in 3 days; 11 per cent. in 21 days on 1 c.c. extract daily.

Bird *e*1.—Lameness, moribund, loss 23 per cent. Improved 4 per cent. in weight in 3 days; 14 per cent. in 14 days on 1 c.c. extract daily.

Bird *f*2.—33 per cent. loss, severe convulsions. Active in 24 hours. Gained 3 per cent. in 3 days.

The first extracts were made by standing the yeast under alcohol, shaking at intervals. The later extracts were made by passing the alcohol successively over fresh portions of yeast. Finally, the principle of the 'Gegenstrom' was adopted: the alcohol first passing over partly exhausted yeast and then over successive fractions, and finally over fresh yeast. These extracts proved much more potent than the earlier ones.

The alcohol was removed under the fan at room temperature before use.

All extracts were thus made alcohol free before further precipitations or feeding experiments.

II.—*Hot Alcohol.* Hot alcoholic extracts of yeast, or extracts concentrated on the water bath, proved inactive.

III.—*Acetone.* The precipitate thrown down by acetone at 0°C. from cold alcoholic extract proved inactive.

The filtrate after removal of the acetone proved active.

Bird *e*3.—Loss 40 per cent., lameness very marked, moribund. 2 c.c. daily acetone filtrate. Flying and walking well in 24 hours. Gain in weight 14 per cent. in 3 days; 18 per cent. in 10 days. Lived on this extract for 15 days, though losing a little weight in the last 4 days, and was then put on another extract.

IV.—*Platinic Chloride.* Cold alcoholic yeast extract throws down a golden yellow precipitate with platinum chloride.

Precipitate (freed from platinum) dissolved in very dilute hydrochloric acid. Proved inactive.

Filtrate (freed from platinum) seemed largely inactivated.

V.—*Phosphotungstic Acid.* A bulky greyish white precipitate was obtained by treating cold alcoholic yeast extract free from alcohol with phosphotungstic acid. This was separated and allowed to stand with excess of baryta at 57° C., being frequently shaken. The colour changed to bright yellow. On filtering, a bright yellow liquid was obtained, from which the baryta was precipitated with carbon dioxide. Tremethylamine and ammonia are liberated by the baryta.

The filtrate from the phosphotungstic acid was similarly treated.

The active substance is separated in the phosphotungstic precipitate. The filtrate is inactive.

The solution of the phosphotungstic precipitate, prepared as above described, was fed to several birds with neuritis.

The convulsive form of neuritis is rapidly cured by it, but the improvement in lameness is not so marked, and the lameness sometimes progresses in spite of the treatment being continued.

Bird *p*4.—Loss 17 per cent., severe convulsions, lame. Bird flying in 3 days, but lost 10 per cent. more weight in a week.

Bird *o*3.—Loss 17 per cent., very severe convulsions. Convulsions cured. Bird became markedly lame, and lost 3 per cent. more weight in 3 days.

Bird *o*1.—Loss 25 per cent., convulsions, lame. Convulsions cured. Lameness improved in 2 days. Gain 5 per cent. in 3 days.

The solution of the precipitate freed from baryta was concentrated to a syrup under the fan: crystals separated from the syrup. A solution of these crystals was active.

V.—The active filtrate after lead acetate precipitation was similarly treated with phosphotungstic acid: the baryta was added in the solid form (thoroughly ground into the precipitate) to avoid great dilution. A large amount of amine is liberated.

The active substance was precipitated by phosphotungstic acid from the lead acetate filtrate.

Bird *o*3.—20 per cent. down, very lame. Continuous treatment with extract. Lameness cured in 7 days, but bird still very thin and weak. Gained 3 per cent. in 10 days.

VI.—Benzoylation of lead acetate filtrate.

No satisfactory benzoyl compound isolated.

VII.—Precipitation of lead acetate soluble, phosphotungstic precipitable, fraction.

Active substance was removed by silver nitrate and baryta, but the amount obtained was too small to be tested.

VIII.—*Ammonia* throws down a scanty fine white precipitate from yeast extract: the precipitate dissolves in dilute acid; it is inactive.

The filtrate (neutralised) remains active.

Bird 12.—Loss 33 per cent., very lame, severe convulsions; 2 c.c. ammonia filtrate daily. Running about, no convulsions, 24 hours. Quite lively, slightly lame, 48 hours.

IX.—*Lead Acetate* (normal and basic).

Cold alcoholic yeast extract was precipitated with slight excess of normal lead acetate, giving a fine white precipitate.

(i) Normal lead acetate. Precipitate was freed from lead by sulphuretted hydrogen, and from the latter under the fan and concentrated.

The solution of the precipitate proved inactive.

(ii) Normal lead acetate. The filtrate remained active when freed from lead and sulphuretted hydrogen.

This was now treated with basic lead acetate in slight excess.

A yellow precipitate in small amount was obtained.

Basic lead acetate precipitate (freed from lead and sulphuretted hydrogen) inactive.

Basic lead acetate filtrate (freed from lead and sulphuretted hydrogen) active.

X.—*Yeast extract—portion non-precipitable by lead acetates.*

A small portion of the filtrate, after removal of lead and sulphuretted hydrogen, was concentrated in vacuo to a thick syrup.

From this a deposit of fine feathery crystals takes place (also a few other crystals). One decigram of the crystals was separated as well as possible, dissolved in water, and given to a bird lame with neuritis. The bird recovered and was able to walk and fly in forty-eight hours. It lived for a week without further treatment, and then again become lame, and died fourteen days after the one dose.

Method of Isolation Finally Adopted

Twenty pounds of commercial fresh pressed yeast were extracted in the cold with successive quantities of methylated spirits, using in all, about twenty litres of spirit; the yeast was filtered through thick calico, and the alcoholic filtrate was freed from alcohol at room temperature by means of an electric fan. There remained about 7 litres of watery fluid, dark yellow in colour, smelling strongly of beer, and with an intense bitter taste.

This water extract was mixed with sufficient plaster of Paris to make it 'set.' The plaster matrix, after standing overnight, was ground to a fine powder, and extracted in the shaking machine with successive small quantities of methylated spirits made faintly acid with hydrochloric acid. These extracts were freed from alcohol, as before, and the watery fluid obtained, amounting to 3-4 litres, was precipitated with excess of basic lead acetate. The lead precipitate, having previously been found to be inactive, was discarded. The filtrate was freed from lead with sulphuretted hydrogen, and then concentrated to a syrup in vacuo at 38° C. This syrup was treated with absolute alcohol, and the sticky hygroscopic yellow precipitate (creatinin, etc.) was filtered off. The alcoholic filtrate was again freed from alcohol and then precipitated with baryta and silver nitrate. This precipitate was decomposed with sulphuretted hydrogen, filtered, excess of sulphuretted hydrogen removed by the fan, and then taken to dryness in vacuo at 38° C. A small quantity of a brown, sticky, hygroscopic mass was obtained in this way, easily soluble in cold water, and intensely active.

A dose of 0·006 gram administered to a bird with severe convulsions and lameness, improved the convulsions in four hours: the bird was flying strongly in twenty hours, and the lameness disappeared in forty-eight hours. Two further doses of 0·003 gram were given on the third and eighth day; the bird appeared normal, and gained weight on polished rice diet, but died on the 15th day without return of lameness or convulsions. Other results were equally favourable, the dose (3 mgs.) corresponds to 15 grams of yeast.

The substance was further purified by treatment with alcohol: it was insoluble in ether and acetone, and on standing yielded feathery crystals identical with those found in Experiment X.

The ash consisted principally of barium nitrate and a small amount of phosphate.

Pending further investigations into the exact nature of the ash and its relationship to the organic compound, it is assumed to consist entirely of impurity, and the composition of the residue is approximately:—

C =	40·5
H =	8·07
N =	13·32
O =	38·11
	100·00

This corresponds to the formula $C_7H_{17}N_2O_5$ or $C_7H_{16}NO_2(HNO_3)$.

As the action of baryta splits off trimethylamine we may assume further the presence of this group, and write the formula as:—

$$N(CH_3)_3 \cdot C_4H_7O_2 \,.\, (HNO_3).$$

The substance isolated we propose to call Torulin, and we hope to prepare a larger amount with a view to further investigations into its exact composition and relationships, and also into its physiological action; among other questions to be determined may be mentioned:—

(i) Whether pigeons, etc., can fully maintain their weight and activity on a diet of polished rice with the addition of small doses of Torulin; or whether it will only prevent the onset of convulsions or nervous changes without being able to maintain full nutrition.

(ii) Whether it is active in itself or only serves as an 'activator' for some other substance (cp. Schaumann).

(iii) In what state does it exist in the food stuffs? Has each food stuff a special base of this class, these bases being interchangeable in animal metabolism?

(iv) What is the cause of the convulsive form of polyneuritis? Is it an early neuritis of the labyrinth or of the cerebral or cerebellar cortex? How does Torulin cure it so rapidly?

(v) Why does the phosphorus content of a food stuff serve as an indicator of its richness in antineuritic bases? Was the phosphate in our purified product merely an accidental contamination?

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