ON THE CHEMICAL NATURE OF THE SUBSTANCE WHICH CURES POLYNEURITIS IN BIRDS INDUCED BY A DIET OF POLISHED RICE.

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As a result of the work of a number of observers (Eykman(1), Gryns(2), and Fraser and Stanton(3)) it has been shown that the cortical layers of rice contain a substance which cures beri-beri in man and the polyneuritis which is produced in birds by feeding them on polished rice.

The present inquiry is directed to determine the chemical nature of the curative substance.

The experiments were carried out on pigeons of which a large stock was kept. Polyneuritis was induced by feeding on ordinary polished rice. The average time before the onset of symptoms was three weeks. The presence or absence of the active principle in the different fractions, obtained during the investigation of rice-polishings, was determined by administering them to pigeons already severely affected with polyneuritis and observing the result. The condition of the birds at the time of the tests was such that untreated they succumbed within 12 hours.

It may here be mentioned that the extracts, if given in too large a dose, were poisonous. This, as will be shown later, was due to the presence of choline which accompanied the active principle until the final stage of the separation. The poisonous action can be avoided by giving a dose calculated on the quantity of polishings used. With suitable doses passed into the crop by a tube, the pigeons recovered in 6–12 hours. Often, even after three hours the bird seemed quite well.

One symptom of polyneuritis in pigeons is paralysis of the crop and the birds being also generally paralysed the dose administered is very likely to run out again unless the head is supported. A further possible source of error occurs when the crop is not completely paralysed as the bird can vomit the material.

In more chronic cases of polyneuritis although recovery takes place and the animal feeds normally and appears well some amount of lameness persists due to degeneration of nerves. The pigeons which recovered after having received one dose of active substance were again fed on polished rice. Under these circumstances they showed symptoms of the disease for the second time in from 3 to 12 days according to the dose administered,

Extraction of rice-polishings. The method of extraction adopted was a modification of that used by Fraser and Stanton and gave a better yield than is obtained by their procedure. Since the amount of curative substance present in the rice-polishings is really very small, a quantity of substance amounting to 54 kilograms was used. This was exhausted in separate portions of 1½ kilograms each with 4 litres of alcohol containing gaseous hydrochloric acid to the extent of 2–5 %. The extraction was hastened by the use of the shaking machine and the liquid afterwards filtered by means of a large Buchner funnel. The residue was pressed out in a hydraulic press and the liquid obtained added to the original filtrate. The yield of alcoholic extract obtained in this way was 3½ litres for each portion treated.

Investigation of alcoholic extract. The alcoholic extract was evaporated in vacuo at 30° C. and 347 grammes of a fat-like substance obtained from each 3½ litres. This residue was melted at 50° C. and heated with 1 litre of water on the water bath; it was then filtered at a temperature of 38° C.—40° C. The filtrate obtained formed two layers which were separated by decantation, and the aqueous layer extracted three times with ether in order to remove all fatty substances. This aqueous solution contained no proteins and gave none of the known reactions for amino-acids (Millon, glyoxylic acid, bromine, xanthoproteic diazo- and diacetyl-reaction).

When tested on pigeons suffering from polyneuritis it was found to be effective in doses corresponding to about 20 grammes of the original polishings.

Treatment of fraction soluble in water with phosphotungstic acid. The total aqueous extracts were now combined and amounted to 17 litres. After adding sufficient H_2SO_4 to give a 5 % solution the acid liquid was treated with a 5 % phosphotungstic acid solution and left to stand overnight. The precipitate obtained weighed when dry 900 grammes. The filtrate freed from phosphotungstic acid by means of $Ba(OH)_2$ was tested and found ineffective; it still contained traces of nitrogen. The phosphotungstic acid precipitate was washed with 5 % H_2SO_4, then ground up with $Ba(OH)_2$ in a mortar and after the addition of H_2O shaken for three hours. The barium phosphotungstate precipitate

was filtered off and washed thoroughly with water. Air was passed through the alkaline filtrate to get rid of ammonia and the excess of $Ba(OH)_2$ carefully precipitated by means of H_2SO_4 and separated. The filtrate which was still very alkaline with a strong methylamine smell, was quickly neutralised with HCl and evaporated in vacuo at ordinary temperature. On extracting the residue with absolute alcohol a considerable portion remained behind; this was found to consist mostly of inorganic chlorides and amounted to 21 grms. The solution thus obtained was freed from alcohol and was tested on six fowls and found to be effective in doses corresponding to about 40 grms. of the original rice-polishings. The solution was free from proteins, phosphorus and carbohydrates.

Treatment with mercuric chloride. In the next stage of purification mercuric chloride was used. The alcoholic solution described above was treated with an alcoholic solution of mercuric chloride and after standing in the cold for some time the *crystalline* precipitate obtained was filtered off and washed with cold alcohol. The weight of this precipitate was about 45 grms. and by partially evaporating the alcoholic solution 5 grms. more were obtained. The 50 grms. of mercury salt was recrystallised from water containing some mercuric chloride and 42 grms. of needle-like crystals obtained. Three portions composed of: (1) Crystalline substance, (2) Aqueous filtrate, (3) Alcoholic filtrate, were therefore obtained in treating with mercuric chloride as indicated in the following table:

Alcoholic solution of extract treated with mercuric chloride = alcoholic filtrate (3); ppt. of crystals which on recrystallising from H_2O gave = H_2O filtrate (2); crystalline substance (1)

These three portions were now investigated separately.

(1) *Treatment of the crystalline substance.* This substance was suspended in H_2O and decomposed with H_2S. The Hg-free liquid proved effective in curing pigeons when administered in doses equivalent to about 100 grms. of the original polishings.

As some preliminary experiments showed that the bulk of the above crystals consisted of choline, an aqueous solution was made and treated with $AgNO_3$ in the presence of $Ba(OH)_2$. This reagent does not precipitate choline but a small precipitate was obtained which was decomposed by H_2S and tested on one pigeon with positive result. The filtrate of the $AgNO_3$ and $Ba(OH)_2$ precipitate was proved to be without effect.

(2) *Treatment of mercuric chloride aqueous filtrate.* This filtrate was decomposed by H_2S, the precipitate filtered off and the liquid

evaporated in vacuo. The residue was dissolved in alcohol and fractionated by consecutive additions of an alcoholic platinic chloride solution. In this way eight fractions of a platinum chloride salt were obtained. All these were devoid of curative properties and were found on analysis to consist of choline.

·1718 grm. substance gave ·0544 Pt.

·2310 grm. „ required 7·5 c.c. N/10 H_2SO_4 (Kjeldahl),

i.e. 31·66 % Pt and 4·54 % N.

$(C_5H_4NOCl)_2 . PtCl_4$ requires 31·65 % Pt and 4·54 % N.

The alcoholic filtrate from the last platinum chloride precipitate was evaporated in vacuo, the residue dissolved in H_2O and the platinum eliminated by H_2S. This solution was given to two pigeons and found effective in doses corresponding to 40 grms. of polishings.

As the platinic chloride salt of choline is known to be very insoluble and the choline itself was proved to be ineffective, it was thought that another base must be present in the filtrate. Mercuric chloride was again added but in this case no insoluble salt was formed; it would seem that the active substance is but partially precipitated by mercuric chloride and then only in the presence of some other substances such as choline. An attempt to form a picrate was equally unsuccessful. These points suggested that the curative substance was probably present in all the mercuric chloride fractions and curing experiments proved this surmise to be correct.

Since it was difficult to find a suitable precipitant for the active substance phosphotungstic acid was again used. By this means 1·4 grms. of a crystalline phosphotungstate were obtained. This salt, which was entirely insoluble in H_2O, was recrystallised from dilute alcohol; in this way 1·1 grms. of a crystalline substance composed of five and six sided prisms were obtained. This substance gets black when heated to 200° C. and does not melt at 300° C. It contains 1·59 % of nitrogen after drying in vacuo at 110° C. Half a gram of this substance was decomposed with baryta and the excess of barium removed by CO_2.

On administering the filtrate to two pigeons suffering from polyneuritis they quickly recovered. Since each dose contained only 4 mgrs. of nitrogen it is evident that a very small amount of the substance is effective.

As the amount of this substance obtained was insufficient for further analyses it is at present impossible to say anything with regard to its chemical nature.

(3) *Treatment of mercuric chloride alcoholic filtrate.* This filtrate was evaporated in vacuo and the residue extracted with water. On removing the Hg by means of H_2S a fluid was obtained which was

effective in curing pigeons. Chlorine was removed from the liquid by Ag_2SO_4, the Ag eliminated by H_2S and the H_2SO_4 by means of $Ba(OH)_2$.

To the alkaline solution was added $AgNO_3$ and $Ba(OH)_2$ as long as a precipitate continued to form. The precipitate, which was at first white but on standing became brown, was washed with water and decomposed by H_2S. A very dilute solution of H_2SO_4 was used to remove the last traces of barium and the filtrate was then tested on eight pigeons and found effective. The $AgNO_3 + Ba(OH)_2$ filtrate was freed from silver by hydrochloric acid and from barium by sulphuric acid; on testing it was found inactive.

The solution of the decomposed Ag salt was evaporated in vacuo and an endeavour made to crystallise the residue from dilute alcohol, but no crystals formed at first though some were obtained after the solution had been kept in a vacuum desiccator for some time. These crystals which consisted of long microscopical needles proved by their reactions with brucine and diphenylamine to be the nitrate of a base. They gave no reaction for substances such as Arginine, Histidine, Carnosine, and Creatinine which from their chemical behaviour might be present in this fraction. They left no residue on burning and melted at 233° C. This high melting point does not correspond with any of the nitrates of the known bases which might be present in this fraction. The product of the first crystallisation weighed 0·25 grm. and the second 0·15 grm. The crystals contained nitrogen and were free from Ba, Ag, Cl, and H_2SO_4. They were insoluble in cold water and in alcohol but dissolved with difficulty in hot water. They gave the following results on analysis[1] after being dried at 110° in vacuo.

·1481 grm. substance gave ·3021 grm. CO_2 and ·0706 grm. H_2O.
·0878 grm. „ „ 5·9 c.c. N (moist) at 749 m.m. and 16°.

Found 55·63 % C.
„ 5·29 % H.
„ 7·68 % N.

These figures correspond best to the formula $C_{17}H_{18}O_4N$ (HNO_3) for which following figures are required:

56·16 % C.
5·28 % H.
7·71 % N.

[1] By the time my work had reached this stage my material was nearly exhausted and only just enough for one analysis of carbon and nitrogen was available. I therefore asked my friend Dr Nierenstein, Lecturer in Biochemistry in Bristol, who at the present time is in constant practice in organic analyses, to undertake the determinations for me. Dr Nierenstein has been good enough to do this and I beg to express here my gratitude for his kindness.

Although the active substance has been definitely identified with the Ag salt obtained by precipitating with $AgNO_3$ in presence of baryta, the proof of activity of the nitrate obtained from this Ag salt and which was the salt submitted to analysis is less conclusive, as by this time the exhaustion of the material permitted of but four experiments, three of which were successful and one failed to cure the bird. For the same reason it was not possible to investigate further the chemical reaction of the substance but attempts to obtain by extraction of more raw material the active substance from other food-stuffs are in progress.

In conclusion I wish to express my thanks to Dr Leonard Braddon of Seremban (F.M.S.) for kindly supplying the necessary rice-polishings.

SUMMARY.

(1) Polyneuritis of birds as shown by Eykman, Gryns, Fraser and Stanton, is due to the lack of an essential substance in the diet. The substance is only present in minute amount, probably not more than ·1 grm. per kilo of rice.

(2) The substance which is absent in polished rice and is contained in rice-polishings is an organic base which is completely precipitated by phosphotungstic acid and by silver nitrate and baryta. It is partially precipitated by mercury chloride in alcoholic solution in the presence of choline and is not precipitated by platinum chloride in alcoholic solution.

Reasons for provisionally regarding the active substance as a body giving a crystalline nitrate which has the percentage composition of 55·63 % C, 5·29 % H and 7·68 % N are adduced, but as by the time the search had approached the final stages the material became exhausted; duplicate analyses could not be made and but few animal experiments performed.

The chemical nature of the curative substance could not be further investigated immediately but larger quantities of raw material are being worked up.

(3) The curative dose of the active substance is small; a quantity of substance which contains 4 mgr. of nitrogen cured pigeons.

REFERENCES.

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3. Fraser and Stanton. Studies from the Institute for Medical Research. Federated Malay States, No. 12. The Etiology of Beri-beri, 1911.