CXIII. THE ACTION OF NITROUS ACID UPON THE ANTINEURITIC SUBSTANCE IN YEAST.

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In the search for the antineuritic substance in yeast, many have abandoned the curative test upon pigeons as a guide in concentration [McCollum and Simmonds, 1918; Seidell, 1922]. In place of the curative test there have been used either (A) experiments involving weight or growth tests, or (B) experiments involving the protection of pigeons from symptoms of polyneuritis while upon a polished rice diet. Procedure (A) suffers from the objection that it assumes at once identity of the curative and growth substances. With (B), on the other hand, difficulties are raised by the variability of the times of onset of convulsions of different pigeons placed upon the same diet. If a given bird therefore upon a given diet does not get symptoms in a given time, it is not necessarily a clear proof of the activity of an experimental addition to the diet, because there is always the possibility that the bird in question might not have become polyneuritic in the time taken even upon polished rice alone. Further there is a call for comparatively large amounts of experimental material which inhibits rapid progress.

Considerations of this nature led me to adhere solely to the problem of concentration of the *curative* substance or substances in yeast extracts. An experience of over two years of the behaviour of pigeons fed upon polished rice has convinced me that curative tests can be used as a guide in concentration under stated conditions.

I have thought it worth while to enumerate these, not because they establish anything new, but because it is difficult to find such information collected.

The Curative Test.

1. *Symptoms.* Out of a given batch of pigeons fed upon polished rice, a majority show the classical symptom of head retraction. A few cases show the emprosthotonos of McCarrison [1919], while the remainder merely become progressively lame and die suddenly, usually without convulsions. It is the safest course to work with those showing the classical symptoms of head retraction, and to discard the other cases [Williams, R. R., 1917]. The cases of lameness often cannot be cured with marmite. It is a good plan to make a rule that any pigeon which has not reached the typical condition of head retraction within 30–35 days be put back upon the normal diet for a few weeks, and then returned to the polished rice.

2. *Rice feeding.* Confining ourselves to the pigeons showing the classical symptom, birds seem to get symptoms more rapidly when they are allowed to fly about outside in a large open cage. This was discovered by chance owing to the difficulty of finding accommodation for a large batch of pigeons indoors. A number of the batch were placed outside in the cool weather, the remainder being placed in cages in a warm room. To my surprise some 40 % of the pigeons outside showed symptoms in 11–30 days whereas the pigeons inside did not become polyneuritic until after this time. I have made no attempt to investigate this matter, which is possibly related to the increased metabolism. It is a curious fact that pigeons with threatening convulsions will often recover for a few days when brought into the warm room after being outside, and in conversation Col. McCarrison has told me that he has noticed the precipitating effect of cold upon the convulsions. In view of some recent remarks by Blacklock [1924], in which he states that lack of exercise is a contributory cause in the acceleration of death in polished rice pigeons, it may be that cold is the predominating factor in the acceleration of symptoms among the pigeons placed outside. As a practical point I have adopted the plan of keeping the pigeons at the beginning of their polished rice diet in an outdoor shed, in which they can fly about. When they show symptoms of weakness and are unable to fly from the ground to their perch, they are brought into cages in a warm room, where they remain until the symptoms appear. As in most cases of animals upon deficient diets, it is important in the final stages to avoid cold shock to which the birds seem to be especially liable. When treated in the above way, a good number of cases are obtained without recourse to artificial feeding and as the symptoms are reached soon, the birds have the necessary strength to weather the tests satisfactorily.

3. *The test.* The real difficulty in the use of the curative test lies in the interpretation of the effects of administration of the curative substance. The difficulties are twofold. Firstly, many substances other than the one sought for may produce temporary cures. Secondly, pigeons may rapidly get into a condition in which not even an accepted curative extract such as marmite will produce recovery. I shall deal with these two conditions in turn.

1. *Temporary improvement.* In some cases a dose of distilled water even may induce an amelioration of symptoms. The following experiment 1, illustrates this point.

Exp. 1. 11 Feb. Pigeon showing slight symptoms of head retraction and lameness.
1 p.m. 20 cc. of distilled water given by mouth.
6 p.m. Distinctly better. Polished rice given.
12 Feb. 12 noon. Symptoms worse again. Given marmite.
4 p.m. Looked normal.

This is a case of temporary improvement for 24 hours in which the pigeon has been able to right its physiological instability for a short time with the help of the water. There are many recorded cases of temporary improvement

following the injection or giving by the mouth of diverse substances, a case in point being the series of purine compounds tested by Funk [1913]. I have come to the general conclusion that a curative experiment cannot be considered decisive unless (*a*) the symptoms ameliorate considerably within 3–6 hours, and (*b*) the cure persists for over 48 hours, while the bird is still upon a polished rice diet. It is further more satisfactory if symptoms can be cleared up more than once, though this has certain limitations. Upon a second or third appearance of the convulsions the condition seem to be more rapidly progressive than upon the first appearance. This imposes the limitation that the curative substance be administered within an hour or so of the second onset of symptoms. Where this has been done, it has been found possible in good cases to clear up symptoms as many as four times. Exp. 2 illustrates this.

Exp. 2. 21 April. Head retraction.
7.20 p.m. Curative dose. Rather better in 3 hours. Cured next morning.
23 April, 12 noon. Symptoms again, treated within an hour with more curative extract. Improved in 6 hours and well next day.
28 April, 9.15 a.m. Symptoms again. More extract. Well next day.
6 May, 12 noon. Symptoms again. More curative extract. Well next day.
8 May. Placed upon normal diet again.

2. *Intractable cases*, in which even marmite will not produce a cure. Comparatively often I have been unable to cure pigeons by feeding them upon marmite. Among a series of over 200 cases I find that I have tried to clear up some 59 with marmite at various stages after the onset of symptoms, and usually after attempts to cure with other substances. An analysis of these cases shows the results in Table I.

Table I.

	Number of hours after the onset of symptoms	
	24	48
Not cured	11	10
Cured	31	7

Out of 42 pigeons, therefore, attempts to clear up within 24 hours gave 31 cures, some 75 %, whereas the proportion is much less (about 40 %) for birds allowed to continue longer. Not much stress is laid upon these figures, but they show the order of things which can be expected. The practical conclusion to be drawn is that a failure to produce cure by a test substance administered after long continuance of the symptoms should not be accepted as proof of the absence of the curative substance from the extract. We may summarise the conditions for the curative test in somewhat the following way:

1. Only those pigeons should be used for the test which show well-marked head retraction, in which the symptoms have appeared within 30–35 days, and which appear strong enough to withstand treatment.

2. If the symptoms clear up completely upon administration of the extract within a period of 6–12 hours, recovery must be a persistent one for two or better three days, in order to establish the activity of an extract.

3. If the symptoms do not clear up, it is not proof of the non-activity of an extract unless (*a*) the bird is strong, and (*b*) the extract is administered within 12 hours from the start of the convulsions if it is the first attack, and within three hours if it is the second.

Extracts have been administered by passing directly into the crop by the use of a pipette fitted with a small piece of bicycle valve tubing. Care must be taken to see that the bird does not drown itself when there is spasm of the throat, leading to passage of the administered solution into the trachea, and so to the lungs.

Histamine not the curative substance.

The asserted presence of histamine in yeast extracts [Seidell, 1921], together with the claim made by Danysz, Michel and Koskowski [1922] and by Koskowski [1922] that pigeons could be protected from convulsions upon a polished rice diet by injections of histamine, led me early in the work to test the curative effect of this substance. The results of the experiments confirm in the main the conclusions of Abderhalden [1923] that definite cures can be produced, but that they are quantitatively and usually qualitatively different from those produced by yeast extracts.

I have made curative experiments both by feeding and injection, and protective feeding experiments, using a B.D.H. specimen of histamine phosphate.

Curative experiments.

Exp. 3. *Injection experiments.* In three experiments in which varying amounts of histamine were injected no effect was observed. This confirms the results of Danysz, Michel and Koskowski [1922].

Exp. 4. *Feeding by the mouth.* The administration of histamine in doses of approximately 0·2 mg. up to a maximum of 0·6–1·0 mg. gave the following results. Out of 18 birds treated, not improved 6, improved for periods up to 24 hours 4, and up to 48 hours 3. The remaining 5 were cured for 3, 5, 6, 8, 12 days respectively. Accepting the criteria laid down in the earlier part of this paper, 28 % would be regarded as cured. It is clear, however, that we are not dealing with the certainty of a yeast extract with which, under comparatively unfavourable conditions, a 75 % cure was obtained. The difference of the histamine effect from that of the curative substance in yeast extracts is also made clear by Exp. 5, in which daily feeding of histamine in 0·4 mg. doses did not influence the onset of convulsions.

Exp. 5. Two batches of 10 pigeons were kept under the same experimental conditions outside in an outdoor cage, merely being separated by a wire partition. One batch received 0·4 mg. of histamine by the mouth each day after the second. Up to the thirty-first day the following were the days after starting the feeding upon which the pigeons showed polyneuritis.

Day upon which convulsions appeared:

Histamine fed	12, 15, 21, 25, 25, 31, 31.
No histamine	19, 23, 26, 26, 26.

Exp. 5 shows that there was nothing to choose between the times of onset of convulsions for the two sets of birds. Moreover, none of these cases could be cleared up by giving more histamine. Presence of vitamine contamination in the preparation would seem to be excluded by experiment, so that we must attribute the occasional curative effect of histamine by the mouth to some other obscure cause, possibly a family relation to the actual curative substance.

Certain other substances.

A variety of other substances were tried in an attempt to see whether the effect of histamine was related in any way to its basic character. Table II shows that all the substances tried were definitely less effective than histamine, a result which has also been reported by Abderhalden for amines excluding tyramine. Out of the 22 pigeons treated with various substances, only one case could be ranked as a cure, a different picture from histamine. The failure of effect of sodium bicarbonate shows that the onset of symptoms is not due to an upset of the alkalinity of the blood owing to discharge of acid into the blood. This was suggested by the ease with which exercise precipitates the convulsive condition in these birds.

Table II.

Substance	Dose	No. of birds	Effect
Na bicarbonate	0·5 g.	5	Nil in three cases. Temporary improvement for 10 hours and 30 hours in two cases
Hexylamine	20 mg. (approx.)	6	Nil in two cases. Temporary improvement for 24 hours in two cases; 2 days in two cases
Guanidine	7 mg.	1	No effect
Dimethyl urea	9 mg.	1	Improvement for 24 hours
Urea	0·05 g.	2	Nil in one case. Slight improvement for 4 hours in other case
Olive oil	4–7 cc.	5	Nil in one case. Improved for 12 hours, 30 hours, 45 hours (three cases). One case cured for 6 days

That the curative effect of a yeast extract is not due to primary amines is, I think, definitely shown by the experiments with nitrous acid described later in the paper. Before turning to these, however, a convenient method of obtaining concentrated extracts of yeast will be described in which use is made of a charcoal method of extraction.

Preparation of concentrates of the curative substance in yeast.

Yeast in 7 lb. samples is kept at room temperature for two days. It is then boiled up with tap water twice, approximately 1500 cc. being used for each of the boilings [Osborne and Wakeman, 1919]. To the combined filtrates, 25 % neutral lead acetate is added in an amount just short of that required to produce the maximum precipitate. This is usually slightly more than 300 cc. The filtrate from this is made definitely acid with sulphuric acid and treated with the acid mercuric sulphate reagent (Hopkins), until the maximum

precipitation is obtained. It is allowed to stand overnight and then filtered. The clear filtrate is allowed to stand for a day in the course of which time it usually deposits a faint cloud. This should be removed by filtration. To the clear filtrate some 10 g. of BaS is added, and the mixture is filtered clear. If this should prove difficult at this stage, a cautious addition of $Ba(OH)_2$ will usually make it possible to reach a stage at which a clear filtrate is obtained. The fluid, however, should not be allowed to get alkaline, and is usually of a high acidity when the filtering point is reached. The fluid cleared of the sulphides and $BaSO_4$ is boiled to expel H_2S and cooled. It is then exactly neutralised with NaOH to litmus, and made just acid with acetic acid, any precipitate which forms being removed by filtration. 30 g. of "norite" charcoal is then stirred into the fluid, and allowed to settle for 30 minutes. The charcoal is separated upon a Buchner funnel, and washed thoroughly with distilled water. If the filtrate from the charcoal is still coloured it is treated with 20 g. more charcoal, and this is then combined with the original charcoal fraction.

The charcoal is treated with 300 cc. of 50 % alcohol containing 1 cc. of strong HCl per 100 cc. The mixture is heated for half an hour upon the water bath and filtered. The charcoal is again extracted with two successive 150 cc. amounts of alcohol of the same strength and acidity. The alcoholic extract is evaporated *in vacuo* at a temperature not exceeding 60°, to a small bulk of about 20 cc.

Several extracts of yeast have been made in this way and have all proved highly curative. An amount of the order of 0·05 cc. will cure the usual pigeon of symptoms within 6 hours and keep it from convulsions upon a diet of polished rice for a matter of $3\frac{1}{2}$ days. Such an amount after drying at 100° will contain about 10·5 mg. of solid of which 1·2 mg. is ash, so that under 3 mg. is needed as a daily dose. This compares favourably with other concentrates. Provided that the HCl present in these concentrates is not removed they will keep for a period of several months at room temperature. This amply confirms the remarks upon stability in the presence of acid made by other observers (Seidell).

The action of nitrous acid.

So far as I know there is no experiment in the literature upon the action of nitrous acid upon the curative effect of yeast extracts. McCollum and Simmonds [1918] showed that the growth substance for rats was not much affected by treatment for four hours with nitrous acid gas. In my experiments (Table III), treatment with an acid solution of sodium nitrite did not destroy the curative action of the yeast concentrates prepared with charcoal. Exps. 6, 7, 8 (Table III) shows that it is possible to get roughly quantitative results with these extracts in terms of the time after cure for which protection lasts upon the polished rice diet. Exps. 9, 10, 11, 12 (Table III) represent the effects of part of the same yeast concentrate treated with nitrite before administra-

tion. The nitrite treatment has been varied in different experiments without effect upon the results obtained. The usual procedure has been to add saturated sodium nitrite drop by drop to the acid yeast concentrate either at room temperature or in ice water, allowing the action to proceed for 12 hours or more. The mixture is then warmed for a short time and finally boiled, cooled and neutralised before administration to the pigeon. In all cases there has been no appreciable destruction of the curative substance so far as can be judged from the rough quantitative results obtainable.

Table III.

Exp.	Amount given	Improvement in	Protection for over
	Yeast concentrate before nitrite treatment.		
6	1 cc.	5 hours	16 days*
7	$\frac{1}{6}$,,	4 ,,	9 ,,
8	$\frac{1}{12}$,,	4 ,,	4 ,,
	Yeast concentrate after nitrite treatment.		
9	$\frac{1}{12}$ cc.	5 hours	5 days
10	$\frac{1}{12}$,,	7 ,,	6 ,,
11	$\frac{1}{12}$,,	4 ,,	10 ,,
12	$\frac{1}{12}$,,	6 ,,	7 ,,

* Bird then placed on normal diet.

The curative activity of the concentrates after treatment with nitrous acid eliminates not only histamine but probably all primary amines as being the curative substance. A large number of secondary amines are also excluded, though certain of the more complicated ring structures containing the :NH grouping will probably survive the treatment mentioned. In the case of certain nitrosamines, reconversion to the secondary amine by heating with HCl is known to be readily brought about.

There is still left the puzzle as to the action of histamine in certain cases. Upon the whole I feel tempted to view the symptoms as an expression of some complex local vascular disturbance and to attribute the effect of histamine to some temporary vascular action, but any such view only adds one more to the speculations which have already been advanced in connection with the antineuritic vitamin.

Conclusions.

1. The curative test upon the polyneuritic pigeon can be used as a guide in concentration experiments under stated conditions.

2. In confirmation of Abderhalden, the administration of histamine as a curative agent sometimes produces cures in pigeons. Daily doses of histamine do not delay the onset of symptoms.

3. Treatment of acid extracts of yeast with nitrite does not destroy the curative property of such extracts. This excludes primary amines and many secondary amines as responsible for antineuritic action.

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(For references to the vast literature see the Report of the Medical Research Committee on the present state of knowledge of accessory food factors, 2nd ed. 1924, *Med. Res. Council Special Rep. Series,* No. 38 (revised).)

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