# CXXII. ANTINEURITIC YEAST CONCENTRATES. I.

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The present communication[1] deals with active curative preparations from yeast which have been obtained by the use of polyneuritic pigeons as test animals. The outstanding problems in connection with vitamin B are firstly, concentration of the vitamin, and secondly, whether the properties ascribed to vitamin B reside in one or more constituents. In order to avoid begging the question the word "torulin" is used throughout [Edie, Evans, Moore, Simpson and Webster, 1912] to mean the principle in yeast which cures symptoms of head retraction in pigeons induced by feeding upon polished rice.

## Technique.

The technique described previously has been used [Peters, 1924]. Test samples have been introduced into the crop. The pigeons have not been fed artificially. Only birds conforming to the conditions previously laid down have been used for the tests. Particular care has been taken to exclude cases of the illusory heat cures, to which allusion was made before. Since then much evidence has accumulated. Birds which happen to get convulsions in the outside aviary must be left for a clear 3 hours before treatment after bringing into the warmer laboratory air. In many cases such birds clear up completely, and do not show symptoms again for a period of 1–3 days. Three days seems to be the limit for these heat cures. We have no explanation to offer for this interesting fact which has been investigated by Roche [1925]. Excluding cases of heat cure there are still found birds which do not fall into the ordinary categories, so that for tests upon important material, it is essential to use more than one bird for standardisation.

### *Measurement of activity.*

We have standardised our results by the use of a curative and protective test, by means of the following equation,

$$\text{Torulin activity (T.A.)} = \frac{\text{Number of days' protection after cure}}{\text{Weight in mg. of the dry preparation}} \times 100.$$

[1] A preliminary account of this work appeared in *J. Soc. Chem. Ind.* (1925), 531.

Dry weight here means the weight obtained by drying at 100–105° for 1 hour, after subtraction of the weight of ash. Thus if 4 mg. of a preparation cure and protect for 4 days, the activity is considered to be 100 T.A. Use of this equation implies at least two assumptions: (1) that there are not wide variations in the amount of torulin required by different pigeons, and (2) that no antitorulin factors are present. With regard to (1) it is likely that account must be taken ultimately of both weight and temperature to which pigeons have been exposed. The average weight of the pigeons used by us at the time of convulsions is 270 ± 40 g. Young birds have not been employed. (2) is at present a pure assumption. In practice we have found that the results obtained for times of cure between 2 and 10 days are reasonably quantitative, so much so that it has been possible to follow the distribution of torulin between various fractions with ease. After 10 days the results are not reliable. An example of what is meant is given in Table I.

Table I.

| Pigeon | cc. given | Cure and protection (days) |
|---|---|---|
| 1 | 0·2 | 10 |
| 2 | 0·2 | 9 (after treatment with nitrite) |
| 3 | 0·2 | 10 |
| 4 | 0·5 | 19 |

Including the nitrite-treated preparation bird 2, 0·2 cc. of this preparation gave in days 10, 10, 9. Upon this basis 0·5 cc. should protect for 25 days, whereas protection only lasted for 19 days.

Table II deals with tests upon an active preparation. It shows what accuracy may be expected from such quantitative tests, and also certain limitations in its use.

Table II.

| Pigeon | Dose (cc.) | Cure and protection (days) | Day dose (mg.) |
|---|---|---|---|
| 5 | 0·1 | 2·5 | 0·068 |
| 6 | 0·1 | 2·0 | 0·085 |
| | 0·2 | 3·0 | 0·11 |
| 7 | 0·2 | 4·0 | 0·085 |
| 8 | 0·3 | 6·0 | 0·085 |
| 9 | 0·3 | 7·5 | 0·068* |
| 10 | 0·3 | 13·0 | 0·025 |
| 11 | 0·24 | 2·8 | 0·24 |

* This preparation was dried at 100° before administration and found to weigh 0·51 mg. The average for pigeons 5–9 is 0·084 mg. per day, 1190 T.A.

It will be seen that there are three groups: (*a*) the majority of birds 5–9, which behave well to a standard, (*b*) bird 10 which required much less than the standard, and (*c*) bird 11 which required much more. Bird 10 has been omitted, and represents an abnormality in response, which appears occasionally. It is possible that such birds still have sufficient torulin left in their system, and that the small dose given is enough to set free this store. Bird 11 has also been omitted, for the reason that it was not in good condition when the dose was given, and was also a bird which had been subjected to the tests

over a period of 5 months in the laboratory. It must be admitted that at present it is not possible to eliminate the element of judgment in the selection of suitable cases. Use of the equation is however justified by the fact that by its aid we have obtained torulin concentrates some 200 times as concentrated as in the charcoal concentrates. During the progress of the research we have been increasingly impressed by the quantitative response of the majority of test birds to the fractions given.

## Experimental.

It is not proposed to give at length the numerous curative experiments made in the course of fractionation. In each case, before proceeding to a further step, results have been checked by the use of birds. A few general statements may be made as to procedures which have not been helpful, when applied to the charcoal concentrates. With phosphotungstic acid it was not found possible by a varied extraction to recover the active principle in reasonable amount. At this stage also adsorption upon kaolin, or upon specimens of fuller's earth procurable by us proved valueless, as did also precipitation with lead acetate in alkaline solution. In the latter case all the torulin present was recovered from the filtrate. We have not been successful in preparing from these concentrates active picrates similar to those described by Seidell [1924].

The following method we have found successful, when working upon a scale of some 7–21 lb., using D.C.L. baker's yeast.

Up to the stage involving the use of acid alcohol to extract torulin from the charcoal, the technique has been the same as previously described [Peters, 1924], with the exception that in later preparations the treatment with BaS has been omitted. Instead charcoal adsorption has been applied direct to the acid fluid after removal of the mercury precipitate. After removal of the first amount of charcoal, the fluid has been neutralised to methyl red and again treated with charcoal in similar amount. The charcoal residues are combined after washing with distilled water, and concentrated. Further treatment can be then carried out as described below. Unless otherwise stated, all precipitates have been removed by the centrifuge and washed with appropriate solution. Concentration has been carried out *in vacuo* under the water pump with a temperature not exceeding 60° in the apparatus described [Peters, 1924].

*Stage* 1. The charcoal concentrate was diluted with a convenient amount of water and alcohol added to a concentration of 60 %, the precipitate removed and discarded, and the centrifugate (cf.) concentrated to remove alcohol.

*Stage* 2. The concentrate from stage 1 was diluted with water and 25 % lead acetate added until precipitation was complete, the precipitate removed, the centrifugate and washings combined, treated with HCl to remove excess of lead and concentrated. Any precipitate appearing during concentration can be discarded.

*Stage* 3. The acid concentrate from stage 2 was treated with methyl alcohol to a concentration of at least 90 % (more does not matter), the

precipitate removed, and the centrifugate concentrated to remove methyl alcohol.

*Stage* 4. The solution from stage 3 was made up to a convenient volume with water and treated with "dialysed iron" 10 %, about 7 cc. being required for a 7 lb. sample. The precipitate was removed by the centrifuge, and washed. This washing was found to be important. Normal NaOH, about 10 cc., was then added until a precipitate ceased to come down, at which stage the fluid is very alkaline. Care must be taken to carry this stage through quickly, and not to allow the solutions to rise above room temperature. We have found in confirmation of the work of others that torulin is inactivated by heating with NaOH. The heavy precipitate is removed and washed with dilute caustic soda. The centrifugate was made acid to litmus as soon as possible and concentrated.

*Stage* 5. Ethyl alcohol was then added to a concentration of 85 %, the precipitate removed and washed with 85 % alcohol, and the centrifugate concentrated. Alcohol was then added to 95 % and the process repeated. It is best to re-dissolve the gummy precipitates obtained in a minimum amount of water and throw out again with alcohol at this stage, at which there is liable to be loss.

*Stage* 6. The 90 % alcoholic solution was treated with half a volume of ether, the precipitate removed, taken up in a minimum of water, made up to 90 % alcohol with absolute alcohol, and re-precipitated with ether. The combined centrifugate from the two precipitations was concentrated as far as possible, the extract dissolved in a minimum of water, and made up to a strength of 95 % alcohol with absolute alcohol, any precipitate appearing at this stage being removed.

*Stage* 7. The fraction obtained was then gradually worked up to higher concentrations of alcohol, the object being to obtain the fraction soluble practically in absolute alcohol. When the fraction soluble in about 99 % alcohol is obtained it is well to take it to dryness, and then treat with absolute alcohol, and allow to stand out of contact with air overnight. About 40 cc. of absolute alcohol were used at this stage for a 7 lb. preparation. The precipitate can then be re-extracted with fresh alcohol. The torulin will be found in the absolute alcohol solution. The final stages require care, and it is well to check the absence of torulin from the final precipitates. The material at this stage is apt to vary. In the case of four preparations which have been worked up to this stage, the activity of the final product has varied between 50–100 T.A. A yield of some 2800 day doses may be expected from 21 lb. of yeast.

Owing to the variability of material, we do not claim that the method described is more than empirical. We have found in working upon the larger scale that it is necessary to repeat stages in order to get these final fractions soluble in alcohol and free from gum. The advantage of separation from solvents lies in the freedom from ash and gum of the final fraction, and the ease with which a false step may be retrieved.

*Further treatment of the concentrates.*

By fractionation of a product of 63 T.A. in mixtures of ether and alcohol in which the amount of water was reduced to a minimum three fractions were obtained: (*a*) a gummy fraction thrown out of the alcohol by the addition of mere traces of ether, (*b*) an intermediate fraction thrown out by ether up to four volumes of ether to one of alcohol, and (*c*) a fraction soluble in ether-alcohol. The latter contained an oil, comparatively insoluble in water. Fractions (*a*) and (*c*) were relatively inactive. The intermediate fraction when treated with silver sulphate in acid solution threw down an inactive precipitate. From the centrifugate after treatment with silver nitrite in the presence of HCl, ammoniacal silver hydroxide threw down a precipitate, which was extracted with HCl and alcohol, and yielded the preparation of 1190 T.A., tests upon which are described in Table II. This contained ammonium chloride as an impurity, and some evidence has already been obtained that the activity may be pushed to 3000 T.A. This being so, it does not seem unlikely that the activity of torulin itself may prove to lie between 10,000 and 100,000 T.A. or in the region of 0·001 mg. per day for the normal pigeon. This would only bring it into line with substances like pituitrin.

*Weight maintenance activity.*

It is difficult to know how to relate our preparations to those described by Seidell [1921, 1924] and Emmett and Peacock [1925], both of whom have employed weight maintenance tests. The latter observers describe a preparation, from a source unmentioned, capable of maintaining the weight of pigeons fed upon polished rice in doses of 0·1 mg. per day and of causing gain of weight in doses of 0·15 mg. per day. For the sake of some comparison with the weight maintenance tests, we have carried out the following experiments 1 and 2, of which the details are recorded in Figs. 1 and 2. Exp. 1 was designed to constitute a severe test for protection against polyneuritis, and Exp. 2 to place the bird under a condition in which it should easily show a gain in weight.

*Exp.* 1 (Fig. 1). Two pigeons upon a polished rice diet were kept outside in the winter weather. For the first 16 days the diet was polished rice alone; following this each bird received every second day 0·17 mg. of the preparation used in Table II. This should constitute a border-line amount to protect against polyneuritis at laboratory temperature. On the 32nd day, as one day was accidentally missed, four doses were given. The weight of the birds steadily fell. Bird 12 died of general weakness on the 44th day without showing symptoms of head retraction. It was found to have undigested rice in its crop. Under the conditions of the experiment the birds might be expected to get symptoms by the 30th day at latest, so that this bird may be considered to have been protected for at least 14 days. Bird 13 lasted until the 50th day, when it developed symptoms of head retraction thereby showing that

protection had been afforded previously by the preparation, but that the amount given was rather low. An extra dose of the preparation on this day cured the symptoms, but the bird died of general weakness on the next day, and was found to have no food in the crop. Doubtless not much stress can be laid upon evidence drawn from prolonged feeding upon polished rice at a stage when deficiencies other than the one under study may be present, but it seems reasonable to conclude that protection against polyneuritis may be afforded for many days under severe conditions without weight maintenance.

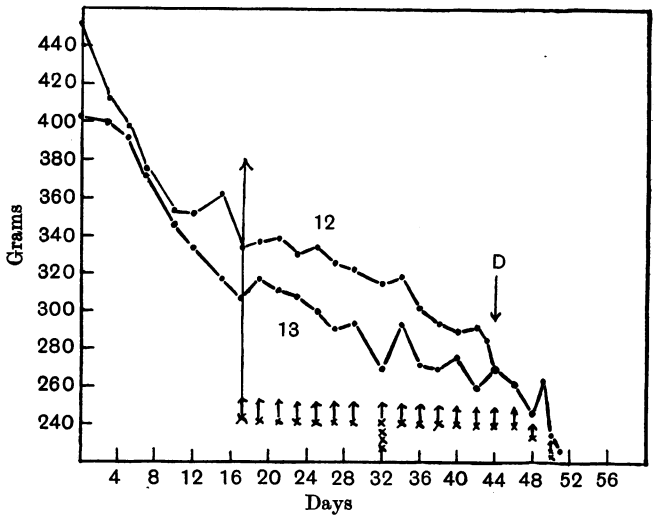


Fig. 1. Exp. 1. Pigeons 12, 13. Ordinates g. weight. Abscissae days.
D = death of pigeon 12. × = 2-day dose ≡ 0·17 mg. T.A. 1190.

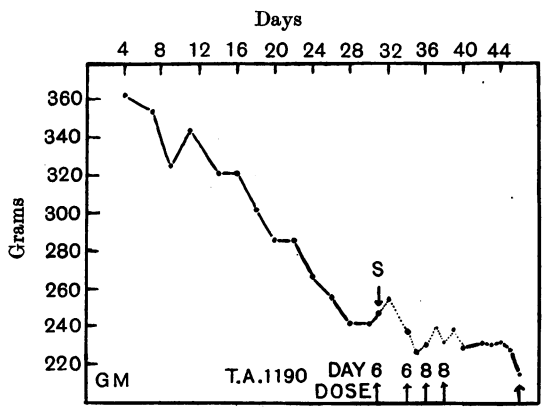


Fig. 2. Exp. 2. Pigeon 14. S = symptoms of head retraction. Diet, polished rice.

*Exp.* 2 (Fig. 2). During the hot summer weather, bird 14 was kept indoors until it showed symptoms of head retraction on polished rice. After this it was given several doses of the same preparation as in Exp. 1. The time at which

symptoms appear in these cases is usually later than under the conditions of Exp. 1. In this case it was the 29th day. The pigeon did not maintain weight even with 3–4 times the day dose, when conditions as regards the general rate of metabolism must have been particularly favourable.

It is clearly necessary to carry out extended tests with other preparations of this nature, but we feel that it is justifiable to conclude that highly active antineuritic preparations can be obtained which have no effect upon the weight curves in doses above the amount necessary to cure symptoms of head retraction. The evidence in this communication therefore supports the view that vitamin B consists of two factors.

## Summary.

1. Heat cures must be avoided in working with pigeons suffering from symptoms of head retraction induced by feeding upon polished rice.

2. Using the word "torulin" to mean the principle in yeast curative of these symptoms, the following equation gives quantitative results in most cases, namely,

$$\text{Torulin activity (T.A.)} = \frac{\text{Number of days' protection after cure}}{\text{Weight in mg. of the dry preparation}} \times 100.$$

3. A yeast concentrate has been obtained of 1190 T.A. which cures and protects in doses of 0·084 mg. per day.

4. Doses of this concentrate which protected against polyneuritis for many days did not cause increase in weight of pigeons after cure of the polyneuritic symptoms.

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