CIV. ANTINEURITIC YEAST CONCENTRATES.

II. THE USE OF NORITE CHARCOAL IN THE CONCENTRATION OF TORULIN.

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(*Received June 2nd, 1927.*)

In this paper are described improved methods of concentrating torulin to an activity of 0·5–1·0 mg. per day and some of the experimental evidence upon which the better methods are based. *Torulin* is here defined as the factor in yeast curative of symptoms in pigeons of head retraction induced by feeding upon polished rice. The work forms a continuation of that previously published [Kinnersley and Peters, 1925]. Discussion of the relation of this work to that of others is postponed to the end of this paper.

Experimental.

The methods of testing have been those previously described, and the activity (curative and protective) is given throughout in mg. per pigeon per day (day-dose). Previous methods of concentrating to an activity of 1·0 mg. involved a preparation of the Osborne-Wakeman aqueous extract from baker's yeast, and the successive removal of precipitates with neutral lead acetate and acid mercuric sulphate. At this stage in the earlier experiments, barium sulphide together with barium sulphate precipitated in the solution was used to clear metals and certain colloidal matter before charcoal treatment. Subsequently this was discarded owing to the tedium of filtration. Instead, the filtrate from the mercuric sulphate precipitate was brought to p_H 5·0 approx., any precipitate which appeared being removed. Charcoal was then stirred into the solution, and the antineuritic substance together with impurities removed from the charcoal by extraction with acid 50 % alcohol. The idea which guided these experiments was based upon the belief that the earliest possible treatment with charcoal shortened stages in which large volumes of solution must be handled. Working upon the small scale and with care in the use of the mercury reagent, satisfactory concentrates can be prepared in this way. The concentrates so obtained can be further purified by alcohol fractionation. Exp. 1 gives the details of one such experiment and exemplifies the technique.

Exp. 1. 7 lb. baker's yeast (D.C.L.) were extracted twice with tap water, the combined extracts making a volume of 2700 cc. and containing 100·9 g. organic solids. 280 cc. neutral lead acetate (25 %) were added, and to the filtrate sulphuric acid until acid to Congo red and then 70 cc. of mercuric sulphate reagent (Hopkins). The filtrate was brought to p_H 5·0 with NaOH (20 %), and a small precipitate removed by filtration. It was treated successively with 30 and then 10 g. norite charcoal, allowed to stand with stirring for about 10 minutes, and the charcoal collected upon a filter and well washed with distilled water. The combined charcoals were extracted with 200 cc. and 100 cc. 55 % alcohol by volume containing 1 cc. conc. HCl %. The organic solids present in the combined alcoholic extract were 2·850 g. The solutions were concentrated to about 5 cc. *in vacuo* at about 60°, and 45 cc. 97 % alcohol were added, making an alcohol concentration of 87 %[1]. The precipitate was removed by the centrifuge and washed with 87 % alcohol. The further treatment is sketched below:

Centrifugate Test 500 + 700, 700 doses Alcohol removed, 50 cc. water added, and lead acetate (25 %) until no further precipate	87 % alcohol precipitate
Centrifugate. Passed H_2S 2 hours and washed precipitate with water containing trace of acetic acid	PbAc precipitate
Centrifugate. Boiled off H_2S and concentrated *in vacuo* to 4 cc.; added 95 cc. alcohol	Sulphide precipitate
Centrifugate Test 600 doses Concentrated with 1 cc. conc. HCl (to remove acetic) to dark brown gum. Extracted with hot 97 % alcohol. Washed the precipitate with 97 % alcohol	95 % alcohol precipitate
Centrifugate. Removed alcohol and added NaOH (20 %) to p_H 5·0. To the volume 10 cc. added 90 cc. 97 % alcohol	97 % alcohol precipitate
Centrifugate. Concentrated to about 2 cc. and added 48 cc. 97 % alcohol. The precipitate was redissolved in 3 cc. H_2O and reprecipitated with 47 cc. 97 % alcohol	88 % alcohol precipitate
Centrifugate Test 330 doses Concentrated nearly to dryness, added 45 cc. absolute[2] alcohol, washed the precipitate with 97 % alcohol	92 % alcohol precipitate
Centrifugate. Concentrated to dark gum, extracted with hot absolute alcohol, the precipitate being washed with absolute alcohol	97 % alcohol precipitate
Centrifugate. Taken to dryness, and extracted with 50 cc. ho absolute alcohol Test 700 doses. Organic solids 0·966 g.	98/99 % alcohol precipitate

This experiment is chosen out of many, because though the yield was not so good as in others or the final activity quite 1 mg., it illustrates certain

[1] By volume. Concentrations of alcohol are to be taken throughout as concentration by volume.

[2] No precautions to dry the absolute alcohol have been taken.

points, in particular the best method of fractionating with alcohol. It appears to represent the simplest possible method of reaching this activity from the charcoal concentrates previously described. The principle involved is the removal of some interfering substance with lead acetate, removal of metals in acetic acid solution, followed by gradual removal of acetic acid and material insoluble in alcohol in acid solution. This is followed by a repetition of the same steps in rather more alkaline solution. The steps are fairly expeditious. Experience has convinced us that it is dangerous to venture from the path described. For instance, too rapid use of alcohol does not take to pieces the adsorption complexes efficiently, and there is much loss of activity upon the alcohol precipitates. Omission of the HCl after treatment with H_2S leads to the throwing out of torulin upon the alcohol precipitates. Improper removal of alcohol before bringing to p_H 5·0 is apt to cause destruction when soda is used, etc. It is abundantly clear that torulin of this activity is soluble in absolute ethyl alcohol containing mere traces of water.

The method exemplified in Exp. 1 can be relied upon to give some 700–800 doses from 7 lb. D.C.L. baker's yeast of an activity of about 5·0 mg. a day at the charcoal stage and about 1 mg. per day after alcohol fractionation. A trial of brewer's yeast did not give more activity, and the material was much more coloured.

When these methods were applied to large scale batches of yeast (56 lb.), it became evident that other factors intervened, complicating the subsequent fractionation. These and the slow filtration of the earlier stages led to a return to a more careful study of the pre-charcoal steps. It was found that by the removal of gum and subsequent adsorption of the torulin upon norite charcoal at p_H 7·0, the yield of active substance may be doubled, and the activity at the charcoal stage improved to between 0·5–1·5 mg. per day. Moreover, the speed of preparation is so much increased that it is possible for two persons to work up 14–28 lb. yeast in 2–3 days to give a yield of 3000–6000 day-doses of this activity.

It is proposed to present certain of the experimental evidence bearing upon the method of extraction used.

(1) *The use of baryta to clear gum and other colloidal matter.*

It has been found that treatment with cold saturated baryta after removal of the neutral lead acetate precipitates under certain circumstances rapidly flocculates the gum and clears the solution. Immediately following this step, sufficient sulphuric acid can be added to make acid and throw down the barium as sulphate which rapidly settles. This treatment much accelerates filtration in the subsequent stages. Certainty as to the exact mechanism of clearance has not yet been reached. It appears to be essentially a colloidal flocculation. It is suggested as an explanation that in the presence of traces of lead, baryta removes the gum as a coarse flocculent precipitate which in its turn adsorbs other colloidal substances. It is certainly not simple because the optimum

conditions vary slightly according to the condition of the yeast. Provided that the amount of lead is kept low, the losses at this stage are small. This is not so if the solution is cleared by the addition of baryta and basic lead acetate. In one case in which this was tried, a magnificent clearance was effected, but at the expense of large losses of active material by adsorption upon the precipitate. Table I gives some results.

Table I.

Exp.	Condition of material	Dose given	Bird test (days)	Total doses
2	Pb filtrate before baryta	1/3000	4·0	12,000
	Same after baryta	1/2200	5·0	11,000
3	Aqueous yeast extract before lead acetate	1/1000	3·5	3,500
	Same after lead and baryta stage	1/1000	6·0	6,000

In Exp. 3 there is an apparent increase in activity. As this has been noticed more than once it is probably not due to experimental error and is analogous to the increase in growth-promoting activity for yeast observed by Funk and Dubin [1920] after treatment of yeast extracts with lead acetate and mercury. It suggests that any estimates of actual torulin content obtained by the feeding of a natural food-stuff are apt to be erroneous. The inhibitory effect of impurities may be due either to an actual toxic antagonism or merely to a failure of absorption in the gut.

(2) *Specificity of adsorption by charcoal.*

Charcoal as an adsorbent for the antineuritic factor from rice polishings was first employed by Chamberlain and Vedder [1911, 1912]. These authors were not able to recover the active principle from the charcoal by extraction with ether, absolute alcohol or water. Cooper [1913] confirmed the extraction by animal charcoal from horse-flesh extract, but stated that water and alcohol would recover the factor. Eddy, Heft, Stevenson and Johnson [1921] used charcoal (norite) for the adsorption from alfalfa extract of the growth-promoting substance for yeast, part of which they attributed to vitamin B. They recovered the principle with glacial acetic acid. Funk and Dubin [1921] have employed norite for fractionating vitamin B and the yeast growth-promoting factor. Mueller [1922] used it for separating streptococcal growth-factors.

Earlier in this work the active principle was recovered from norite charcoal by the use of 50 % alcohol by volume containing 1 cc. HCl %. More lately this treatment has been varied, and also the norite charcoal itself purified as described by Merrill [1921] by boiling with strong HCl, washing until free from chlorides and drying. Such purified norite does not seem to be changed in adsorptive properties so far as this work is concerned. In the attempt to improve the use of norite for concentrating torulin, it became of importance to settle how far adsorption upon charcoal was a property of torulin itself. After treatment with mercuric sulphate, adsorption at p_H 5·0–7·0 is a property of the torulin in solution. Exp. 4 shows that it is not necessarily a property of torulin at a more active stage, viz. 1·0 mg. day-dose.

Exp. 4. Preparation from baker's yeast, made by the older technique and worked up to solubility in 99 % alcohol. Organic solids 0·525 g. Bird tests: 7 Aug. 1/100, 5 days; 26 Oct. 1/100, 4 days; activity 1·2 mg. Alcohol removed *in vacuo*, taken up in 50 cc. H_2O and brought to p_H 5·0 with NaOH, a small insoluble precipitate being removed by the centrifuge. Treated with 1 g. of washed norite charcoal, which largely decolorised the preparation. Filtrate from charcoal gave organic solids 0·425 g. Bird tests: 1/50, 8 days; 8 days, or 400 doses; activity 1·05 mg.

Hence at p_H 5·5 at a later stage charcoal does not extract torulin from solution when 10 doses to the cc. are present, although at an earlier stage it extracts it when not more than one dose is present to 1 cc. It is still possible to obtain some adsorption of torulin at p_H 7·0 and thereby a concentration of the active principle from a solution of activity 1·0 mg., provided that the torulin concentration is 50–200 doses per 10 cc., but a second treatment of such a solution adsorbs no more torulin, so that even here torulin is not itself being adsorbed.

It may be concluded therefore that adsorption of torulin by norite charcoal is not a property of torulin itself, but that it depends upon the presence of some co-adsorbent. The problem in the early stages therefore narrows down to the issue of removing the maximum of interfering substances without removing the co-adsorbent.

(3) *The optimum conditions for adsorption.*

This conclusion leads to the following three questions. What is the optimum p_H for adsorption? Does the optimum p_H differ for material that has undergone previous treatment with lead and baryta only, as distinct from that which has also been treated with mercuric sulphate? Is treatment with mercuric sulphate necessary? Exps. 5–9 were designed to settle these questions, all except the last being performed upon an extract from 56 lb. of baker's yeast, kindly made by Messrs The British Drug Houses, Ltd., so that the basic extract should be homogeneous. In Exp. 5 adsorptions at various p_H were made after mercuric sulphate treatment. In Exp. 6 the effect of a re-extraction at p_H 5·0 after one at p_H 1·0 was tried. In Exp. 7 adsorption at various p_H in absence of mercuric sulphate was tried, this being reinvestigated in Exp. 8.

Exp. 5. Preliminary treatment—lead acetate, baryta, and mercuric sulphate. The total filtrate from these precipitates, equivalent to some 2800 day-doses, was divided into four equal portions, which were brought by addition of NaOH to p_H about 1·0, 4·0, 6·0 and 7·0. Each portion was then treated with 15 g. followed by 5 g. of washed norite, each lot of charcoal being then treated with acid 50 % alcohol. Table II shows the tests for the 15 g. charcoal extracts.

Second charcoal extracts in the case of p_H 6·0 and 7·0 yielded 180 and 72 doses of activity 3·4 mg. and 9 mg.

Table II.

Exp.	p_H	Organic solids (g.)	Pigeon tests (doses)	Activity (mg.)	% extraction
5	1·0 approx.	1·248	186	6·7	27
	4·0	0·990	164, 82 } 120	8·2	17
	6·0	1·064	456, 760 } 603	1·8	86
	7·0	1·275	850, 680 } 760	1·7	108
6	5·0 (after 1·0)	0·976	120, 270 } 200	4·8	—

The most efficient extraction was at p_H 7·0, where in this case within the limits of error practically all the torulin was removed by one charcoal treatment, to yield material of an activity below 2 mg. The result at p_H 6·0 is near to this. The experiment shows how profoundly the charcoal adsorption is influenced by p_H.

We may next consider the effect of treating the filtrate from p_H 1·0 with fresh charcoal at p_H 5·0. This is seen in Exp. 6 (Table II). Assuming that some 100 doses were extracted by the second charcoal treatment at p_H 1·0 (this test was accidentally omitted), which is certainly an outside figure, there should be at least 414 doses remaining. Of these not more than half can be extracted at p_H 5·0 following the first charcoal treatments. It will be noticed that irrespective of p_H about 1·0 g. of organic solids is adsorbed by 15 g. of charcoal. After adsorption 48 g. of organic solids remained in the filtrate.

It may be asked now what is the effect of omitting mercuric sulphate, a step that is undesirable from several points of view. Exp. 7 (Table III) deals with this point.

Exp. 7. A volume of solution which had not been treated with mercuric sulphate, equivalent to some 2100 doses, was divided into three portions and treated as before with 15 g. washed norite charcoal, after being brought respectively to p_H 5·0, 6·0 and 7·0 with NaOH.

Table III. (*Exp.* 7.)

p_H	Organic solids (g.)	Pigeon test (doses)	Activity (mg.)	% extraction
5·0	1·300		3·4	55
6·0	1·036	200, 400 } 300 av.	3·4	43
7·0	1·170	304	3·9	43

In this experiment adsorption has been relatively inefficient, The presence of the substances in solution precipitable by mercuric sulphate has therefore depressed the adsorption at p_H 6·0 and 7·0 as compared with Exp. 5. This result was curious but has received later confirmation. In Exp. 8 the remainder of the batch was treated at p_H 6·0 and 7·0.

Exp. 8. The remaining material from the last experiments, as in Exp. 7, was treated at p_H 6·0 and 7·0 with 60 and then 20 g. of washed norite charcoal.

The method of extraction was varied in this case. Successive extractions with $N/10$ HCl, and then 50 % acid alcohol were employed for the first charcoal, and with 50 % acid alcohol alone for the second. Table IV gives the results.

Table IV. (*Exp.* 8.)

p_H	Charcoal (g.)	Extract by	Organic solids (g.)	Doses		Activity (mg.)	% extraction
6·0	60	$N/10$ HCl	1·127	840	1589	1·35	—
		50 % alc.	1·926	749		2·6	50
	20	50 % alc.	1·430	440		3·2	14
7·0	60	$N/10$ HCl	1·044	725	1165	1·44	—
		50 % alc.	2·354	440		5·4	38
	20	50 % alc.	1·44	?840*		—	—

* Probably an irregular bird.

Exp. 9 is a further variation of the same type. The extract from 14 lb. baker's yeast after treatment with lead acetate and baryta, and removal of baryta with sulphuric acid yielded a filtrate of 10,000 cc., which was divided into two equal portions *A* and *B*. To *A* 50 cc. mercuric sulphate reagent were added and the whole filtered immediately. To the filtrate from *A* and to *B* (untreated with mercury), sufficient NaOH was added to bring the p_H to 7·0. Each was then treated successively with 30 and 10 g. washed norite. Subsequently the charcoals were extracted with $N/10$ HCl and 50 % acid alcohol. Table V gives the results for the first charcoal (30 g.) extracts.

Table V. (*Exp.* 9.)

	Extract by	Organic solids (g.)	Doses	Activity (mg.)
A.	$N/10$ HCl	0·440	900	0·49
			900+	
	50 % alc.	1·400	1800+	0·78
B.	$N/10$ HCl	0·560	600+	0·93
	50 % alc.	1·592	Nil	6·6 ?
			240	

Exps. 8 and 9 seem to confirm the fact that treatment with mercuric sulphate facilitates adsorption of torulin. It is possible to remove the substance precipitated by mercury in large part by treatment with charcoal at p_H 2·0, and to follow this with the usual adsorption at p_H 7·0. Experiments upon the yield of material show that it is as good for the $N/10$ HCl extract both as regards amount and activity, but the yield extracted by 50 % acid alcohol is not so good as following the mercury treatment. A detailed account of this method of treatment is given later, as it is likely to prove of value to those who require these concentrates for feeding experiments, owing to the complete freedom from mercury. Enough evidence has been adduced to show how variable is the character of an adsorption of this kind, and how dangerous it might be to draw conclusions about the adsorption of torulin from one class of mixture such as yeast extract and apply this to another. Such experience may be regarded as familiar to the enzyme investigator, as shown by the work of Willstätter and his colleagues upon invertase.

The experiments in this section permit us to draw the following conclusions in answer to the questions. The optimum p_H varies according to the mixture from which adsorption takes place. For material not treated with mercuric sulphate the optimum is p_H 5·0–6·0, and this adsorption is less efficient than adsorption at p_H 7·0 after treatment with mercuric sulphate. The mercuric sulphate stage or its equivalent cannot be omitted if the maximum yield is to be obtained.

(4) *The optimum conditions for extraction.*

It will have been observed in Exps. 8 and 9 that two extractions of the charcoal were made, namely, with $N/10$ HCl followed by 50 % acid alcohol. Further, the material extracted by $N/10$ HCl in this way tends to have a high activity, 0·4 mg. having been observed at this stage. In Exp. 9 the activity was 0·5 mg. This was rather unexpected in view of earlier experiments and suggested that the gum had modified conditions in previous work. It further appeared possible that the use of stronger alcohol as a first step might produce a differential extraction, and give even higher activity in the extract. Exp. 10 illustrates the effect of extracting first with strong alcohol.

Exp. 10. 14 lb. baker's yeast. Extraction of the charcoal was carried out first with 300 cc. 97 % alcohol containing 1 cc. conc. HCl %; allowing for water retained by the charcoal this means extraction with 85–90 % alcohol by volume. Subsequent extractions were with $N/10$ HCl and 60 % acid alcohol. Table VI gives the results obtained for the first and second charcoals.

Table VI.

Extract by	Organic solids (g.)	Doses	Activity (mg.)
1st charcoal:			
acid 97 % alcohol	2·450	1250 | 1250	2·0
$N/10$ HCl	0·560	500 | 150	1·7
acid 60 % alcohol	1·080	300 | 300	3·5
2nd charcoal:			
acid 97 % alcohol	0·852	360 | 360	2·5
$N/10$ HCl	0·130	100 | 200	0·9
acid 60 % alcohol	0·231	under 50	? 4·5

Total doses 1875 + 560 = 2435.

The material obtained in this way is not so good as that from the initial $N/10$ extraction. Nothing is therefore to be gained by the insertion of the strong alcohol step. It is clearly ineffective as a means of removing impurities, owing to the large number of doses extracted by the strong alcohol.

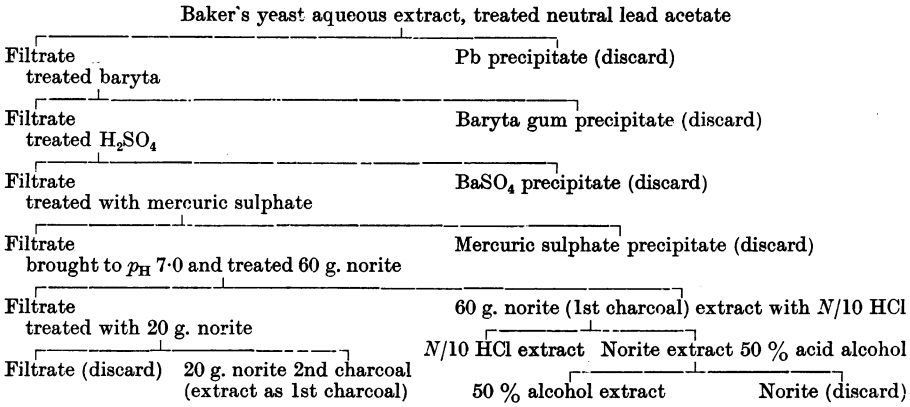
It may be further enquired whether anything is to be gained by an extraction with stronger alcohol following 50 %. This was tried in one case giving no appreciable yield of torulin although organic substances were still

extracted from the charcoal. In view of the above results, as a routine, two extractions of the charcoal are now made, one with $N/10$ HCl to which sufficient HCl is added to make the p_H definitely acid to Congo red if necessary. A further extraction is then made with 50 % acid alcohol by volume.

Optimum amount of charcoal. Experiment has shown that for the aqueous extract from 14 lb. of yeast, treatment with 60 g. of charcoal followed by a subsequent 20 g. cannot be improved.

Improved technique for concentrating torulin.

Based upon the foregoing experiments, the following technique has been elaborated.



Preparation of norite concentrate.

The description applies to 14 lb. of baker's yeast (D.C.L.). The yeast is allowed to remain 3 days at laboratory temperature, 15–20°, in the 7 lb. bags. Less yield seems to be obtained if this preliminary autolysis is omitted. An aqueous extract is then made by stirring the yeast gradually in small quantities into 3500 cc. of boiling tap water until all the yeast is added. (We cannot find any difference in results if acetic acid be added.) The mixture is not allowed to boil for more than 5 minutes. It is then filtered upon Büchner funnels with constant renewal of papers. This process takes about 2 hours. A second extraction with 2800 cc. of water is made. The whole operation of extraction takes about 4 hours and gives a volume of 7000 cc. The combined filtrates are treated with about 400 cc. of 25 % neutral lead acetate solution. This amount is more than the amount necessary to bring down the main bulky precipitate, but is short of complete precipitation. It seems to be the optimum for the next stage. The lead precipitate is removed by filtration overnight through folded filter papers in ordinary funnels. The speed of this filtration varies, most preparations filtering in a few hours. No attempt is made to wash the precipitates. The filtrate often clouds upon standing, but

need not be filtered again on this account. The filtrate from the lead precipitate measuring some 5500 cc. is treated with cold saturated baryta solution containing a little precipitated baryta in suspension. About 3000–4000 cc. are usually required. Immediate flocculation of the gum should take place which should then settle rapidly to the bottom of the vessel, clearing the solution to leave a crystal clear lemon yellow supernatant fluid.

It is well to test this step upon a small scale (50–100 cc.). If flocculation is not obtained by the addition of baryta, a small amount of the lead acetate solution, 2 cc. per litre, can be added, *before* adding the baryta, which will usually settle matters (see Section (1) of this paper). After flocculation of the gum, filtration is rapidly effected through folded papers, and the filtrate treated as soon as possible with sufficient sulphuric acid to make acid to Congo red. The barium sulphate, etc., are removed by decantation and filtration. At this stage the volume of the solution may measure about 10 litres.

Occasionally we have met with a yeast extract which will not filter overnight at the lead acetate stage. If this be so, the only course is to treat directly with baryta in the presence of the lead precipitate. The yield from such a preparation is not so good, the precipitates adsorbing torulin.

The filtrate from the barium sulphate, which need not be absolutely clear, is brought to p_H 2·5 (approx.) with addition of either sulphuric acid or of NaOH, and is then treated with mercuric sulphate in sulphuric acid, 80–100 cc. being used. It is then filtered through folded papers without delay. After filtration a faint cloud often appears in the solution upon standing, which is not removed. Care must be taken not to have excess of mercury present at this stage. The point to which we work is reached when 10 cc. of the solution taken into a test-tube ceases to give an immediate flocculent precipitate with addition of one drop of the reagent. At this point it gives a cloud.

After removal of the mercury precipitate, NaOH is added to p_H 7·0 (at which point the faint cloud if present redissolves) and the solution is treated with 60 g. dry purified norite charcoal. The charcoal is allowed to stand in contact with the solution with stirring for about 10 minutes, and is then collected upon a Büchner funnel. Filtration is usually rapid at this stage. The filtrate from the first charcoal is treated with 20 g. more of charcoal, this second charcoal being kept separate from the first. The charcoal precipitates are thoroughly washed with distilled water upon the funnel. The first and second charcoals are each treated separately as follows. Two extractions are made with $N/10$ HCl, *ca.* 200 cc., upon the hot water-bath, the papers and charcoal being placed with the HCl for this purpose in a beaker. If the extracting solution during the boiling becomes alkaline to Congo red, strong HCl is added drop by drop until the whole is acid to Congo red paper. After heating for 1 hour, the charcoal is filtered off while hot. A second extraction of the charcoal is then made in the same manner. These two almost colourless extracts are combined, dilute $BaCl_2$ is added to remove the last traces of sulphuric acid and the extracts are concentrated *in vacuo* at a temperature

not exceeding 60° to a volume of 50 cc. If a cold store is not available, sufficient alcohol is added to make a volume concentration of about 15 %. This acts as a preservative; in its absence we have found moulds growing in such solutions.

After extraction of the charcoal with $N/10$ HCl, a further yield of less active material can be obtained by extraction with 50 % alcohol (by volume) containing 1 cc. strong HCl %; two extractions are made, each with about 150 cc. acid alcohol.

The second charcoal extracts are worked up similarly; lately the extraction with 50 % acid alcohol has been omitted. The above operations take approximately 3 days, the last day being occupied in the extraction of the charcoals. We have been able upon occasion to work up twice the quantity in this time in the laboratory.

Yields. We have now worked up some nine 14 lb. preparations and eight 28 lb. preparations by variants of this technique, and have given above the one which we consider to be most satisfactory. The $N/10$ HCl extract for 14 lb. contains between 1200–2000 doses of activity 0·4–1·0 mg. The alcoholic extracts which are coloured give a yield of some 1500 more doses of activity 1·0–1·5 mg. The second charcoal $N/10$ HCl extract gives about 600 further doses of between 1·0 and 2·0 mg. activity. The 50 % extract of the second charcoal is relatively inactive.

The highest yield which we have obtained from 14 lb. yeast has been 4400 doses. This is only some 70 % of the total doses present in the gum-free aqueous extract, which has given us a figure corresponding to 6000 doses in the 14 lb. This latter figure corresponding to one dose in 1 g. of pressed yeast is much higher than we had originally supposed, and than the figure given by Cooper [1912] of 2·5 g.

Though the yields are doubled as compared with our previous methods, and are more active at the same stage, it appears that there is still some considerable loss in the processes connected with the charcoal stage. Tests upon the filtrate from the charcoal have not given sufficiently reliable results to enable us to draw conclusions as to the amount of torulin present after the charcoal adsorption.

The presence of traces of metals and of a relatively large amount of acid is an objectionable feature to those who wish to employ concentrates prepared in this way for prolonged feeding experiments. These can be eliminated by removal of alcohol *in vacuo*, bringing to p_H 4·5 and then treating with H_2S. After this, the fluid is warmed upon the water-bath until flocculation of any colloidal sulphide takes place, filtered, and then boiled over the naked flame until free from H_2S. It is then evaporated to a small volume after addition of a small amount of HCl to drive off any acetic acid, and then subjected to the alcohol fractionation as described in the introduction to this paper (Exp. 1), but leaving out the lead acetate stage. In cases in which this technique has been applied the material has concentrated to an activity of 0·15–0·3 mg. a day, giving material of N content varying from 15–25 %.

The following alternative technique is suggested for use in feeding experiments, charcoal being used instead of mercury.

Alternative technique avoiding the use of mercury.

After removal of the gum with baryta, the barium is removed with sulphuric acid so as to leave only a minimal excess of sulphuric acid in solution. HCl is then added to p_H about 2·5 and barium sulphate removed as usual. 60 g. of norite charcoal is then stirred into the solution. This removes the bulk of the substances precipitable with mercuric sulphate leaving the torulin in solution at this p_H. After filtration, the solution is brought to p_H 7·0, and the usual charcoal extractions made. Upon the four occasions when this technique has been followed there has been a good yield in the $N/10$ HCl extracts of a high activity 0·3–0·5 mg., but the extra amount obtained in the 50 % acid alcohol extracts is poor. The method has much to recommend it to those who do not wish to obtain the highest possible yield of active material, but are satisfied to get a reasonable yield with the least trouble.

Discussion.

It is hoped that the methods of obtaining reasonably active preparations of the curative substance with comparative ease may be of use to workers in many fields of nutrition. Now that such preparations can be obtained, there would seem to be no longer excuse for working with such impure mixtures as yeast autolysates as a source of this factor. It is not known yet what amount of torulin is needed in the nutrition of the rat, a matter which it is hoped to investigate shortly. This opens the necessity of discussing the place of torulin among the water-soluble vitamins, as also the excuse for the retention of this word. When this work was commenced, some years ago now, it was decided to adhere to the older curative test in spite of its many deficiencies because it seemed to be more likely to be testing for one factor than any test which involved the maintenance of weight or growth. Recent work upon the durability of vitamin B has tended to strengthen the view that this is so [see Harege and Carrick, 1926]. More especially Goldberger, Wheeler, Lillie and Rogers [1926] have shown that two factors are needed for the growth of rats, one of which is thermostable and present in autoclaved yeast, believed to be the pellagra-preventing factor, and both of which are of course present in fresh yeast. By the use of charcoal concentrates prepared by the early methods described by one of us [Peters, 1924], Chick and Roscoe [1927; Roscoe, 1927] have shown that the thermolabile factor for rat growth is present in the concentrates of the curative substance for pigeons (torulin concentrates).

It is therefore tempting to identify torulin with the antiberiberi vitamin, but this can at present be done only with reserve. It does not seem quite clear that symptoms of head retraction in the pigeon are certainly identical with beriberi [Eijkman, 1913], and it must be further remembered that

preparations of an activity of even 0·1 mg. per day for the pigeon are still sufficiently impure to admit of the presence of more than one factor. Some evidence that more than one factor may still be present has been obtained already in collaboration with Miss Reader, in work which is now proceeding. The various workers who have been recently directing their attention to the problem of isolating the antineuritic vitamin would appear to be investigating different problems. Levene and van de Hoeven [1926] using the growth of rats for their tests have obtained preparations of which 0·08 mg. daily are required for the rat. Seidell [1926] uses the maintenance of weight in pigeons as a standard test. This test has also been employed by Funk and Paton [1922]. Both of these tests would seem likely to test for one factor, only so long as all the others are supplied in the diet or from stores accumulated by the animal.

In view of the uncertainty and differences of opinion as to the tests to be employed for the so-called antineuritic factor, it is probably best for the present to use the term torulin, as defined in the introduction to this paper.

Quite recently Jansen and Donath [1926, 1927] have described the isolation of a crystalline substance from rice polishings, making use of a protective test in birds. The substance described is protective to *Munia maja* (a small tropical bird) in doses of 0·002 mg. a day, and to the pigeon in doses of 0·01 mg. per day. This would be eight times more active than the preparation previously described by the authors [Kinnersley and Peters, 1925]. No mention, however, is made as to whether this substance is also curative. In connection with preventive tests, it will be remembered that Funk [1913] showed that even synthetic allantoin and hydantoin would lead to diminished loss of weight by pigeons upon polished rice, and also tend to delay symptoms of polyneuritis.

The interesting results of the Dutch workers led us to apply their methods to some of the 0·4 mg. a day material obtained in the course of this work. We are obliged, however, to record our disappointment, having been unable to concentrate the torulin present by the use of their technique, starting in one case at the silver stage, and in another at the platinum stage. It would appear therefore that their methods are not applicable to the concentration of torulin in these fractions. Nor can we obtain evidence that the Pauly reaction fractionates with the activity, material of activity 0·1 mg. giving a much less intense Koessler and Hanke [1919] reaction than the less active material from which it had been derived, when equi-dose amounts were compared. An amount equivalent to two torulin doses of activity 0·1–0·2 mg. per day gave a yellow reaction of less colour intensity than the reddish reaction given by 0·01 mg. histidine hydrochloride.

We lay no stress upon our failure to concentrate torulin in our fractions by a method applicable to the protective (? antiberiberi factor) in rice polishings. Doubtless it lies in some peculiarity of the yeast extracts[1]. It is, however, possible that the antiberiberi factor is distinct from torulin. The question at

[1] Assuming that the curative substances in yeast and rice polishings are identical.

issue seems to be whether torulin is distinct from the antiberiberi factor, and what relation these properties of extracts bear to the protective factor in the sense of Jansen and Donath. It is indeed possible that the train of symptoms cured by torulin can only arise when absence of some other factor has accentuated a special phase of metabolism. This would explain certain abnormal responses to test doses, and would also justify the feeling that curative tests upon pigeons are not a reliable guide where human beriberi is concerned [Eijkman, 1913; Jansen and Donath, 1927].

SUMMARY.

Improvements in methods of concentrating torulin are described, torulin being defined as the factor in yeast curative of symptoms of head retraction in pigeons induced by a diet of polished rice. By a removal of gum with baryta, subsequent procedures are simplified. Adsorption upon norite charcoal is not a property of torulin of activity 1·0 mg. per day, and is therefore due to a co-adsorbent in the earlier stages. The optimum p_H for adsorption upon norite charcoal is 7·0, provided that the extract has undergone a treatment with mercuric sulphate, but this is not true if this step be omitted. An improved technique for obtaining torulin concentrates of an activity of 0·2–1·0 mg. per day is described, based upon the above observations.

We are indebted to W. Wakelin for assistance both with the pigeons and the preparations. We are also grateful to Mr Carr of the British Drug Houses for his kind co-operation. One of us (H. W. K.) is indebted to the Medical Research Council for a personal grant, and we have to thank the Medical Research Council and The Christopher Welch Trustees for a grant for materials.

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