Preparation of Uricase

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Following the pioneer work of Battelli & Stern (1909), research on the uric-acid-oxidizing enzyme, uricase, present in the liver and kidneys of most mammals, was much advanced by the studies of Keilin & Hartree (1936) who demonstrated the specificity of the enzyme, the reversibility of the cyanide poisoning, and the formation of hydrogen peroxide as reaction product; the enzyme preparation used by Keilin & Hartree (1936) was an acetone powder from pig liver. A little later Davidson (1938) succeeded in obtaining a purified preparation which was 550 times more active than the original acetone powder, and shortly afterwards Holmberg (1939) improved the purification of uricase still further. More recently, Altman, Smull & Barron (1949) described another method of uricase purification.

Recently, uricase has been introduced as an analytical tool for the determination of uric acid in biological material. Conventional colorimetric procedures using Folin's phosphotungstic acid or Benedict's arsenophosphotungstic acid reagents, have been shown to be unspecific; reducing substances other than uric acid, such as ergothioneine, glutathione, cysteine, ascorbic acid and certain sugars, also produce positive colour reactions. Therefore, methods which make use of uricase in combination with colorimetric or other procedures (e.g. spectrophotometric, titrimetric) have been recommended for the determination of 'true uric acid' in blood and urine (Blauch & Koch, 1939; Bulger & Johns, 1941; Schaffer, 1944; Silverman & Gubernick, 1947; Leone, 1947; Kalekar, 1947; Block & Geib, 1947; Praetorius, 1949).

The object of the present study has been to work out a simplified and reproducible method which would make it possible to prepare uricase in sufficient quantities, and in a sufficiently stable and pure form, to be used in routine determinations of uric acid in biological material (preliminary communication, Leone, 1952).

EXPERIMENTAL

Determination of enzyme activity

Uricase activity was determined manometrically, in Barcroft differential manometers. A borate buffer, 0.1M, pH 9, was used. Amounts of enzyme were chosen so as not

to exceed an uptake of 60–65 μ l. O_2 during the first 15 min. A dangling cup, with 2 mg. uric acid, was tipped into the flask at zero time. KOH papers were placed in the centre wells. The activity of uricase was expressed in terms of Q_{O_2} , defined as the amount of O_2 taken up in μ l./hr./mg. dry weight of enzyme, and calculated from the actual O_2 uptake during the first 15 min. of incubation, at 39° and in an atmosphere of air. The extent of purification has been assessed on the basis of (i) activity of enzyme = Q_{O_2} under standard conditions (see above); (ii) concentration of enzyme = O_2 uptake, in μ l./ml. of enzyme extract; (iii) yield of enzyme = O_2 uptake, in μ l., calculated for the total amount of the enzyme preparation.

Method of preparation

The purification of uricase consists of the following stages.

Stage 1. Borate-butanol extraction of ox kidneys. Ox kidneys, which can be conveniently stored for several weeks in the frozen state without much loss of activity, were cut into small pieces, freed from fat, blood vessels and connective tissue, and passed through a mechanical meat mincer. The mince was then homogenized for 5 min. in a Waring Blendor with 0.1M-borate buffer, pH 10, and *n*-butanol, in the proportion of 300 ml. borate buffer and 10 ml. *n*-butanol to every 100 g. of mince. The borate was heated to about 40° before homogenization, and the suspension was brought to pH 10 by the addition of 0.1N-NaOH. The whole suspension was incubated at 37° for 16–18 hr. At the end of the incubation it was cooled down and centrifuged for 30 min. at 3000 rev./min.; a yellow, cloudy supernatant was obtained, which had a volume corresponding to about one-third that of the whole suspension. The supernatant was decanted, and more 0.1M-borate buffer, pH 10, was added to the residue to bring the whole mixture to the original volume; the mixture was well stirred, and again centrifuged for 30 min. at 3000 rev./min. This was repeated twice; the four supernatants together represent the 'borate-butanol extract'. The Q_{O_2} value at this stage was 40; about 90% of the total uricase content originally present in the whole suspension was recovered in the extract.

Stage 2. Calcium phosphate gel treatment (alkaline). To the borate-butanol extract, about one-fifth of its volume of calcium phosphate gel was added; the gel had a dry-weight content of 25 mg./ml., and was prepared according to Keilin & Hartree (1951). The mixture was centrifuged after 10–15 min. The supernatant was preserved, while the residual calcium phosphate cake was thrice extracted with small volumes of a one-fifth-saturated solution of $(NH_4)_2SO_4$. The three $(NH_4)_2SO_4$ extracts were added to the supernatant from the first centrifugation.

The Q_{O_2} value of the combined extracts was about 50, and not more than 8–10% of the enzyme present in the

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borate-butanol extract remained adsorbed to the calcium phosphate gel precipitate.

Stage 3. $(\text{NH}_4)_2\text{SO}_4$ precipitation and further purification by dialysis. The concentration of $(\text{NH}_4)_2\text{SO}_4$ in the combined extracts from stage 2 was increased to 50% saturation by the addition of the required amount of the solid salt. The mixture was left for some time in the cold and then centrifuged. The precipitate, which settled on the bottom of the centrifuge tubes, was easily disturbed by attempts at decantation, therefore the bulk of the supernatant fluid was siphoned off and preserved in the cold; if necessary, the fluid collected in this way was again centrifuged to recover all the precipitate.

The $(\text{NH}_4)_2\text{SO}_4$ precipitate was then suspended in distilled water, made up to a volume corresponding to about one-fifth of the original extract from stage 2, and dialysed for 12–20 hr. at room temperature against running tap water, until a definite brown precipitate settled in the cellophan bag, while the rest of the fluid became quite clear. The dialysed preparation was then centrifuged at about 5000 rev./min. in a Sorvall centrifuge (model SS1); this produced a complete separation of the precipitated protein fraction from the clear yellow supernatant, which was found to be inactive. The precipitate was ground in a pre-cooled mortar with ice-cold 0.1 M-phosphate buffer, pH 7.3, and then centrifuged in the cold at about 10000 rev./min. in the Sorvall centrifuge. The clear, almost colourless phosphate extract was separated by decantation, the protein residue ground with 0.1 M-borate buffer, pH 10, and centrifuged at high speed, at room temperature, for about 10 min. The resulting supernatant, slightly opalescent but almost colourless, was preserved, and the borate extraction was repeated another three times. From 45 to 80% of the enzyme present in the extract from stage 2 was usually recovered in these borate extracts, while relatively little activity was found in the phosphate extract, which contained a considerable amount of inactive protein material. The volume of the combined borate extracts was about one-third of the extract from stage 2, and the enzyme had a Q_{O_2} value of about 300.

Stage 4. Calcium phosphate gel treatment (acid). The slightly opalescent borate extract was cooled in an ice bath and, with continuous stirring, enough 0.5 N-acetic acid was added to give a faintly acid reaction (methyl red just pink). Next, calcium phosphate gel, 0.1 vol., was added and the pH adjusted again. After 10–15 min. the mixture was briefly centrifuged. The supernatant was decanted and the calcium phosphate precipitate with the adsorbed enzyme was washed with distilled water, care being taken to maintain a weakly acid reaction. After centrifugation and washing, the precipitate was ground in a mortar with a one-fifth-saturated solution of $(\text{NH}_4)_2\text{SO}_4$, at a neutral to slightly alkaline pH. Centrifugation was again carried out, and the $(\text{NH}_4)_2\text{SO}_4$ extraction of the calcium phosphate gel was repeated two or three times; the water-clear and almost colourless supernatants were combined. The volume at this stage was equal to that of the extract from stage 3. The activity corresponded to a Q_{O_2} value of about 700, and accounted for approximately 60% of that present in the preceding stage.

Stage 5. $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis. The extracts from stage 4 were brought to half saturation with $(\text{NH}_4)_2\text{SO}_4$, the resulting precipitate centrifuged down and washed in the centrifuge tubes with a half-saturated solution of $(\text{NH}_4)_2\text{SO}_4$. The final precipitate was dissolved in

0.1 M-borate buffer, pH 10, corresponding to no more than one-tenth of the volume of the extract from stage 4, and dialysed in the cold against three changes of 0.01 M-borate buffer, pH 10. After dialysing for 36 hr., a small amount of brownish precipitate had collected in the cellophan bag and was removed by centrifugation. The clear, slightly yellowish uricase solution obtained in this way usually showed a Q_{O_2} of 750–800, and contained 95–100% of the enzyme from stage 4. Its volume corresponded to about one-tenth of the enzyme extract from stage 4.

Absence of catalase in uricase preparations

No catalase appeared to be present in the borate-butanol extract at stage 1 of the purification procedure. This was indicated by the final O_2 uptake of the extract, which corresponded to the reaction leading to H_2O_2 formation; furthermore, no appreciable O_2 evolution occurred in manometric experiments in which the enzyme extract was incubated with suitable amounts of H_2O_2 . A microspectroscopic examination of a sample of the extract, treated with pyridine and sodium dithionite, usually showed a distinct but faint band of a haematin compound in the visible region, when observed in a layer of about 2 cm. thickness. However, no such band could be seen in samples at the more advanced stages of purification.

Value of the borate-butanol treatment

The addition of small amounts of *n*-butanol to the borate suspension of ox kidneys, prior to incubation, has been found particularly useful; it has been established that the concentration of *n*-butanol must never exceed its solubility in water at room temperature; denaturation and loss of enzyme occurred as soon as separation of two (aqueous + alcoholic) or three (aqueous + alcoholic + precipitate) phases took place. On the other hand, the use of *n*-butanol alone was insufficient for the preparation of an active uricase extract; alkaline borate incubation was also important, as shown by the figures in Table 1. It will be seen that the extraction of the enzyme was greater in the butanol suspension after incubation (380 as against 268 $\mu\text{l. O}_2/\text{ml.}/\text{hr.}$); the accompanying increase of the Q_{O_2} value was considerable (88 as against 13 $\mu\text{l. O}_2/\text{mg.}/\text{hr.}$); thus the addition of *n*-butanol had a twofold effect; it enhanced extraction of the enzyme, and in addition, caused denaturation and precipitation of inactive proteins.

As to the relative amounts of borate buffer and of *n*-butanol, the figures in Table 2 show that higher Q_{O_2} values were obtained with increasing amounts of the alcohol; however, there was a corresponding difficulty in the $(\text{NH}_4)_2\text{SO}_4$ precipitation of the extract after incubation and centrifugation, so that it was necessary to dialyse the borate-butanol extract before precipitation with $(\text{NH}_4)_2\text{SO}_4$. The proportion of 300 ml. borate buffer and 10 ml. *n*-butanol/100 g. of kidney mince was optimal, as it resulted in satisfactory Q_{O_2} values and made it unnecessary to dialyse the extract before the first $(\text{NH}_4)_2\text{SO}_4$ fractionation. However, when small amounts (500–1000 ml.) of an enzyme preparation, with a Q_{O_2} value about 70–100, are required for analytical purposes, one can conveniently increase the butanol concentration in the suspension up to 22–25 ml./100 g. mince and 260 ml. borate buffer. In this way after incubation and centrifugation, an extract is obtained which either can be used as such or can be dialysed against running

Table 1. *Uricase concentration ($\mu\text{l. O}_2/\text{ml.}/\text{hr.}$) and activity ($\mu\text{l. O}_2/\text{mg.}/\text{hr.}$) in centrifuged borate extracts from beef kidney suspensions, before and after incubation, with and without *n*-butanol*

	Concentration		Activity (Q_{O_2})	
	Before incubation	After incubation	Before incubation	After incubation
Without <i>n</i> -butanol	162	144	5.6	5.0
With <i>n</i> -butanol	268	380	13.2	88.0

Table 2. *Effect of varying concentrations of butanol on uricase preparations*

No.	Ratio mince/borate buffer/ <i>n</i> -butanol (g./ml./ml.)	Enzyme concentration ($\mu\text{l. O}_2/\text{ml.}/\text{hr.}$)	Enzyme activity Q_{O_2}
1	100:260:22	380	88
2	100:260:22	220	70
3	100:290:22	204	66
4	100:290:14	248	45
5	100:300:15	300	56
6	100:300:15	248	43
7	100:260:9.5	236	24
8	100:290:10	248	24
9	100:290:10	136	14
10	100:300:10	295	64
11	100:300:10	296	38
12	100:300:10	198	26
13	100:300:10	240	24
14	100:320:10	192	33
15	100:330:10	196	31
16	100:360:9	188	19
17	100:500:16	168	24

tap water for 12–16 hr. at room temperature, if further treatment such as freeze-drying is intended.

Reproducibility and yields

The method showed satisfactory reproducibility. Consistently good activity and concentration of enzyme were obtained in several preparations, so far as the first step of purification is concerned. Variable losses were encountered during the precipitation by dialysis in stage 3, and in the acid calcium phosphate gel treatment in stage 4. In both instances, it appeared that the losses were due not so much to enzyme denaturation as to a partial, but irreversible, adsorption to the precipitated protein, or to the calcium phosphate cake. Precipitation by dialysis caused losses from 4 to 55%, and absorption on acid calcium phosphate gel from 5 to 40%. In general, as much as 30% of the original uricase present in the ox-kidney mince is recovered by the new method, so that it is possible to obtain from 1 kg. ox kidneys about 620 mg. of a uricase preparation with a Q_{O_2} value at least 700.

Stability of enzyme

Even at the most advanced stage of purification, uricase activity was unchanged for many weeks in alkaline extracts. The activity was particularly stable in the first borate-butanol extract, where it remained unchanged sometimes for months. Altman *et al.* (1949) have reported the stabilization of uricase by freeze-drying their active extract. This

has been confirmed for uricase prepared by the present method; a freeze-dried powder preparation preserved its activity for 5 months at 4°.

DISCUSSION

The method of uricase purification described above possesses several advantages. First, there is a good yield of enzyme throughout the whole procedure. Whereas in the older methods great losses were encountered at an early stage, with the present method 90% of the original amount of enzyme could be recovered at the first stage of purification. The addition of *n*-butanol to the suspension prior to incubation is of special importance. Askonas (1950) has shown the usefulness of some alcohols in enzyme purification; and recently Morton (1950) has reported on the use of *n*-butanol as a means of bringing enzymes into solution. The action of *n*-butanol has been explained as a solubilization of the lipoprotein complexes in which form enzymes presumably occur in the cells. While the procedure described here differs in several ways from that of Morton, it is quite conceivable that the same mechanism is concerned in both instances.

The satisfactory yield and good reproducibility of the present method make it particularly well suited for the purpose of quantitative determination of uric acid. The use of crude preparations of uricase is inadvisable. It has been correctly observed by Schaffer (1944) that the accuracy of uricase methods for uric acid analysis depends on the purity of the enzyme used. In most methods, crude uricase preparations have been used in the form of acetone powders either as such (Blauch & Koch, 1939; Bulger & Johns, 1941; Schaffer, 1944; Silverman & Gubernick, 1947) or after extraction and partial purification (Block & Geib, 1947; Leone, 1947). The procedure just described yields at a very early stage a uricase preparation which is catalase-free, has a sufficiently high activity (Q_{O_2} 50–100) for analytical purposes, and can be conveniently stabilized in powder form by freeze-drying.

SUMMARY

1. A new procedure for the purification of uricase is described. Essential features of it are: the preparation of an extract from ox-kidney mince incu-

bated for 16–18 hr. with borate buffer, pH 10, in the presence of *n*-butanol; purification by calcium phosphate gel at alkaline and acid pH; repeated precipitations of the enzyme with half-saturated ammonium sulphate; and separation from inactive proteins by dialysis.

2. From 1 kg. ox kidneys it is possible to obtain 620 mg. of a preparation with a $Q_{0.5}$ value not less than 700.

3. Uricase prepared according to the present

method was free from catalase at an early stage of purification.

4. The suitability of this enzyme preparation for the purpose of uric acid determination is discussed.

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A Method for Measuring the Deposition of ^{32}P in Phosphatidylethanolamine and its Application to Rat-brain Tissue

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There is at present no method for determining the radioactivity of phosphatidylethanolamine in small samples of animal tissues labelled with radioactive phosphorus (^{32}P). Such a determination requires the isolation of a pure sample of phosphatidylethanolamine.

It was shown by Folch (1942) that phosphatidylethanolamine is appreciably soluble in ethanol, so that the serine- and inositol-containing phospholipins are selectively precipitated on adding ethanol to an ethereal solution of the phospholipins. Therefore, former methods for determining the specific activity of 'kephalin' phosphorus which were based on ethanolic precipitation do not give a true indication of the phosphorus turnover in phosphatidylethanolamine. Solvent fractionation methods for the isolation of phosphatidylethanolamine require large quantities of tissue and careful analytical

control. Moreover, for the fractionation to be successful, it is necessary to avoid the preliminary treatment of the tissue with trichloroacetic acid solution. Precipitation of the phospholipins with trichloroacetic acid helps to free them from troublesome contamination with acid-soluble phosphorus of high specific activity.

The method to be described for determining the specific activity of phosphatidylethanolamine phosphorus is based on the finding that the phospholipin can be catalytically hydrolysed by dilute mercuric chloride solution to a water-soluble derivative of ethanolamine whose behaviour on chromatography and hydrolysis corresponds to glycerylphosphorylethanolamine (GPE). This compound can be separated by paper chromatography and its radioactivity measured. The specific activity of the phosphatidylethanolamine phosphorus can then be calculated.

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