part by the levels of inorganic phosphate and of phosphate acceptors in the tissue. The present results with 0.05 mM-2:4-dinitrophenol show increased respiration with little change in any component measured other than creatinephosphate. Creatine may therefore be important in such control. 0.2 mM-2:4-Dinitrophenol may inhibit respiration by its great reduction in energy-rich phosphates, possibly to a level too low for phosphorylation of glucose. Inorganic phosphate, as previously concluded (McIlwain, 1952b), cannot alone control respiratory level.

SUMMARY

1. The spectrophotometric method of phosphate analysis developed by Slater (1953) has been adapted for the determination of energy-rich phosphates in mammalian cerebral tissues. It is based on the enzymic formation of dihydroxyacetonephosphate and its enzymic reduction by reduced diphosphopyridine nucleotide.

2. Assays by this method gave values for creatinephosphate which were in close agreement with those obtained by a chemical method.

3. The adenosinediphosphate and adenosinetriphosphate content of cerebral preparations determined enzymically were less than those determined by the chemical method. Only traces of adenosinediphosphate could be found in cerebral cortex *in vivo* or in slices respiring in a glucose-containing saline.

4. Creatinephosphate, adenosinepolyphosphate and inorganic phosphate have been measured in cerebral cortex slices maintained under a variety of metabolic conditions.

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On the Isolation and Properties of a Citrate Oxidation Factor from Yeast

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Recent experiments (Foulkes, 1951a, b) provided evidence for two distinct pathways of metabolism in cell-free preparations from baker's yeast. Pathway 1 is closely related to the tricarboxylic acid cycle normally found in mammalian tissues; all the citrate metabolized under suitable conditions by dialysed, unfortified preparations, can be accounted

for by the formation of α -ketoglutaric acid. The addition to such dialysed preparations of magnesium, adenosinetriphosphate (ATP) and a heat-stable organic fraction from yeast or various vertebrate tissues causes a large increase in the amount of citrate metabolized. The heat-stable factor involved in this reaction has been called the citrate oxidation factor (COF) (Foulkes, 1951b). The reaction or reactions catalysed by COF will be referred to as Pathway 2.

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The present communication deals with the concentration and partial characterization of COF. Pathway 2, an oxidative decarboxylation of citrate different from the normal tricarboxylic acid cycle, will be described in a subsequent publication.

At this stage, though considerable purification of COF has already been achieved, no final conclusion can yet be drawn on the nature of COF. This is especially so as the percentage of inactive impurities in the final product is difficult to estimate. The possibility must therefore remain that even the purest preparations contain only traces of a very active cofactor.

EXPERIMENTAL

Assay of citrate oxidation factor

In the absence of a stable reproducible assay system or of any knowledge of the chemical properties of COF, no absolute quantitative assay of the latter was possible. To obtain comparative quantitative information it was necessary to add graded dilutions of the fractions to be assayed to the same enzyme preparation. Quantitative data thus obtained could be consistently reproduced. The assumption underlying this assay was that, by analogy with other enzyme-coenzyme systems, a maximum response would be obtained for all concentrations of COF sufficient to saturate the enzyme system. That this assumption was justified can only definitely be proved when purified COF becomes available.

The interpretation of such comparative assays on less pure fractions is rendered difficult by the presence of inhibiting substances. Indeed at some of the earlier steps in the isolation, not only the activity/dry weight, but also the total activity often increases. This finding makes it impossible to attach definite values to the recovery of activity at the various stages. Approximate yields are expressed in terms of 'optimum doses', i.e. the minimum amount of a certain fraction which elicits maximum response in the assay system. It is realized that, because of differences in various enzyme preparations, the 'optimum dose' content can be no more than approximate. In view of this uncertainty no attempts have been made to estimate the final purity by determining activity/dry weight ratios.

The preparation of the cell-free enzyme from yeast has been previously described (Foulkes, 1951*a*). It is dialysed for 4-4.5 hr. in the refrigerator in a rocking dialyser against 0.1 M-phosphate, pH 7.1. The period of dialysis is rather critical: fairly complete removal of COF is not achieved under the present conditions in less than 3-4 hr., whilst irreversible inactivation of the enzyme sets in if dialysis is prolonged much over 5 hr.

To 1 ml. of this dialysed enzyme are added 10 μ moles citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$, British Drug Houses Ltd., A.R.), 0.1 ml. of 0.85% (w/v) $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 mg. ATP (Boots Pure Drug Co., Nottingham) as the Na salt and the fraction to be assayed, or water; final vol., 2.2 ml. Incubation and analysis are carried out as previously described (Foulkes, 1951*a*). Assays on duplicate flasks should not differ by more than $\pm 5\%$.

The fraction to be assayed must be neutral and free of organic solvents. These may be removed either by distillation *in vacuo* or by exhaustive extraction with ether.

Properties of citrate oxidation factor

In the course of prolonged efforts to isolate COF, over seventy preparations were carried out involving a total of 0.5 ton of yeast. Of the many different purification procedures tested, the one finally recommended is given in the next section; here are outlined some chemical properties of COF as deduced from its behaviour during a variety of methods of isolation.

Stability of citrate oxidation factor. At room temperature COF is stable in a pH range 2-11 for several hours. Normal acid or alkali at higher temperatures caused rapid and complete inactivation. In the frozen state neutral solutions of purified COF have been kept for several months without great loss of activity, although COF appears generally less stable in the purified state than when crude.

Reaction with heavy metals. COF is not precipitated by basic lead acetate, Lohmann's reagent ($\text{Hg}(\text{NO}_3)_2$ in HNO_3), or Ba^{2+} (in the presence of 90% ethanol at pH 8.4).

Reaction with base precipitants. COF is completely precipitated at pH 1.5-2 by phosphotungstic acid. The recovery of activity from the precipitate is difficult. There is no precipitate formation on addition of picric or picrolonic acid to even relatively concentrated solutions of COF.

Solubility in organic solvents. COF is insoluble in ether. It is precipitated from crude solutions on the addition of 3 vol. acetone. Acetone precipitation from purified solutions leads to considerable inactivation. It is soluble at all pH values in 90% ethanol. In the higher alcohols (*n*-propanol, *n*-butanol) it is more soluble at alkaline than at neutral or acid pH, an observation which has been made use of for chromatographic separation on cellulose columns. From aqueous, salt-saturated solution COF is extracted quantitatively by phenol. Over 90% of the activity is recovered in the combined aqueous phase on washing the phenol layer three times with an equal volume of water.

Use of adsorbents. At pH 3.5, COF is adsorbed by fuller's earth and acid-washed Norite, but not by cellulose (Whatman no. 4 filter paper). Elution is conveniently carried out with dilute ammonia (0.1%).

Behaviour on ion-exchange resins. (a) *Basic resins.* COF is not taken up from purified solution by the strongly basic Amberlite resin IR 400. To avoid an undue rise in pH the carbonate form of this resin is to be preferred. There is no interaction between COF and the weakly basic Amberlite resin IR 4B. (b) *Acidic resins.* COF is irreversibly inactivated by the weakly acidic Amberlite resin IRC 50. At pH 3.5, COF reacts with the strongly acidic Amberlite resin IR 100. It can be eluted slowly at pH 3.5, more rapidly at higher pH values.

Chromatography on Amberlite IR 100 has been included in the final purification procedure (step E), details of which are given in the next section. Fig. 1 illustrates in detail a typical experiment.

A fraction (25 ml.) purified up to step D (see below) was acidified to pH 3.5 with HCl, and applied to columns (int. diam. 1.5 cm., length 35 cm.) containing the resin ground and sieved to 200-500 mesh size. The column had been equilibrated with 0.05 M-sodium citrate, pH 3.5, in 0.05 M-NaCl. Elution was carried out with the same solution. Rate of passage of eluent was 4 ml./hr. It will be seen that the major part of the ultraviolet-absorbing material is eluted before the appearance of COF. This material has been shown to contain uracil and pentose but no phosphate, and it is

hoped to describe it fully elsewhere. In some experiments, due presumably to the effect of uncontrolled variables such as temperature, or to differences in concentration, there may be a greater overlap between the uracil-containing compound and COF. The second lower absorption peak bears no obvious relation to COF and it is not apparent in all experiments.

Reaction with HNO_2 . The activity and basic properties of COF are not affected by prolonged treatment with NaNO_2 and acetic acid under the conditions employed by Schlenk, Hellström & v. Euler (1938) for the deamination of diphosphopyridine nucleotide (DPN).

Fig. 1. Behaviour of citrate oxidation factor on Amberlite IR 100. For experimental details, see text. The curve represents extinction at 265 $\text{m}\mu$. The superimposed histogram gives the COF activity in arbitrary units.

Properties of aqueous solutions of citrate oxidation factor purified to stage F (see below, p. 327). Such solutions are colourless. There is no specific absorption in the ultraviolet. The ninhydrin test with the sensitive reagent of Moore & Stein (1948) is positive, but can be rendered very weak or absent by treatment with HNO_2 without affecting the activity of COF. It contains no organic or inorganic phosphate, as estimated by the method of Berenblum & Chain (1938) after digestion and hydrolysis in the usual way. There are no reducing sugars before or after 10 min. boiling in 0.3N-HCl, as estimated by the arsenomolybdate method of Nelson (1944). The reaction with conc. HNO_3 (xanthoproteic reaction) is very weak or absent; the Sakaguchi reaction and biuret test are negative. Only traces of pentose are present as estimated by the orcinol reaction after preliminary bromination to weaken base-sugar bonds (Massart & Hoste, 1947). A fluorescent compound is formed under the conditions of the acetone and alkali reaction for quaternary nicotinamides (Huff & Perlzweig, 1946). However, no evidence could be obtained for the presence of a quaternary nicotinamide by the spectrophotometric cyanide method of Colowick, Kaplan & Ciotti (1951). Drying solutions of purified COF *in vacuo* leads to complete and irreversible inactivation. This occurs also on drying in the presence of dilute NH_3 , acetic acid, 2:3-dimercaptopropanol or cysteine. The distillate collected in the liquid air-cooled trap has no activity. There is thus no evidence to indicate that COF is volatile, especially as crude extracts can safely be taken to dryness.

Concentration of citrate oxidation factor

The procedure finally adopted is outlined in Fig. 2 and has yielded consistently good results on a scale ranging from 1 lb. to several cwt. of yeast. The source material is commercial baker's yeast purchased from the Distillers Company Ltd. The values given refer to the preparation from 1 cwt. (51 kg.).

That the overall yield of activity represents only a small part of the COF originally present in the yeast can be deduced from the fact that boiled yeast extract, freshly prepared on a small scale, contains, as far as can be judged, of the order of one 'optimum dose' $\frac{1}{2}$ g. yeast. Each single isolation step recommended below gives satisfactory yields of activity under suitable conditions. Since in large-scale preparations the major losses appear to occur during the initial extraction, this step should be completed as rapidly as possible.

The purification procedure is as follows.

(A) One cwt. (51 kg.) of fresh crumbled yeast is extracted with 50 l. tap water. The water is heated to 85° and the yeast added at such a rate that the temperature remains between 85 and 88°. Efficient stirring is essential. When addition is completed, the suspension is heated to 90–92°, then cooled as rapidly as possible below 70°. The faster the original rate of heating, the shorter the time during which COF is exposed to high temperatures, and thus presumably the higher the yield. The cooled suspension is filtered or centrifuged. The final volume is of the order of 55–60 l. No attempt has been made to subject the residue to a second extraction.

(B) The filtrate is reduced *in vacuo* to about 6 l. The temperature should not exceed 45°. The pH of the resulting brown syrup is about 5. By the careful addition of 40% KOH the pH is brought to 8.5–9, i.e. just alkaline to phenolphthalein. 600 g. $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ are added and the suspension stirred vigorously. The amount of BaCl_2 used probably represents an excess, but because of the copious precipitate formed it is difficult to see whether any BaCl_2 remains undissolved. The pH is again adjusted to 8.5. With constant stirring 4 vol. ethanol are added. This concentration in particular is not critical so that the ethanol used may be 95 or 99%. The precipitate is allowed to settle at 0° overnight. There will then be a very heavy, gummy precipitate, and a fairly clear, yellow supernatant. This is poured off and filtered. The most convenient way of recovering the large amount of solution imprisoned in the solid residue is to knead by hand in a few changes of 80% ethanol. Two treatments with 2 l. each should be sufficient. Each time the yellow solution is poured off and filtered. The combined filtrates are then evaporated *in vacuo* to 2 l. As the next step involves extraction of COF into phenol, it is essential at this stage to remove the last traces of ethanol. For this purpose, about 0.5 l. distilled water is added before finally reducing the volume to about 1.5 l. (The Ba precipitate has been used as a convenient source of coenzyme A.)

(C) The ethanol-free concentrate is brown in colour, and syrupy in consistency. It contains BaCl_2 which would render difficult a determination of the point where sufficient $(\text{NH}_4)_2\text{SO}_4$ has been added to saturate the solution. An excess of $(\text{NH}_4)_2\text{SO}_4$ (70 g./100 ml.) is therefore added to the solution at room temperature. The saturated salt solution and sludge is now extracted with repeated amounts of 50–100 g. phenol (A.R.). The extraction is continued till most of the colour has been recovered in the combined

phenolic layers. If the phenolic layer becomes too concentrated at the early stage of the extraction, it will not separate cleanly from the aqueous solution and has to be diluted by the addition of further phenol. It is essential to use a separating funnel with a sufficiently wide bore tap to allow the excess $(\text{NH}_4)_2\text{SO}_4$ to be withdrawn. In a typical preparation about 400 g. of phenol were used, giving a dark brown phenolic solution of 600 ml. To remove as much of the suspended aqueous layer as possible, the phenol is clarified by spinning and carefully poured off from the salt and salt solution.

$(\text{NH}_4)_2\text{SO}_4$ carried through the phenol extraction into the final aqueous solution by repeating the $(\text{NH}_4)_2\text{SO}_4$ -phenol-water treatment. The final volume of light brown, aqueous concentrate should be of the order of 200 ml./cwt. yeast.

(E) The 200 ml. concentrate is carefully acidified with conc. HCl to pH 3.5. It is then divided into two portions and applied separately to ion-exchange columns. Increasing to any large extent the total material applied to each column leads to overloading and consequent leakage of activity. The columns (int. diam. 2.3 cm.) are tapered at one end and fitted with a coarse sintered-glass filter and tap to

Fig. 2. Purification of citrate oxidation factor.

(D) The phenol is extracted three times with 1 vol. each of water, allowing sufficient time for the layers to separate on each occasion. The combined aqueous extracts are freed from phenol by extraction with ether, and evaporated *in vacuo* to about 200 ml. Especially in large-scale preparations it may be desirable to reduce the high concentration of

control the rate of flow. Amberlite IR 100 resin, 200-500 mesh/in., is suspended in dilute HCl and poured into the column to a final height of solid of 48 cm., after removal of excessively fine particles by flotation.

About 100 ml. of the acidified concentrate are applied at room temperature to the column previously equilibrated

with citrate buffer pH 3.5 (10.5 g. citric acid monohydrate, 50 ml. *N*-HCl, 100 ml. *N*-NaOH, made up to 1 l.), and elution is carried out with this buffer. A constant head of liquid is maintained over the resin. Collection of fractions is begun when the peak of ultraviolet-absorbing material has appeared in the column effluent. The total volume collected from each column is of the order of 1.5 l. For assay purposes, it is sufficient to neutralize a suitable portion of the citrate buffer and add Mg, ATP and enzyme.

The total time required for this step is about 1 week. The rate of passage of liquid cannot be increased if a good separation is to be achieved. Although contamination of the top of the column by fungi has been observed at the later stages of the operation, no visible growth has ever been found to extend into the column, and the separation of COF is not affected.

(F) The combined active fractions (about 3 l.) are neutralized to pH 7 with 40% NaOH. The volume is then reduced *in vacuo* to 300 ml. A high concentration of citrate interferes with the phenol extraction. To repeat the ethanol-Ba fractionation for removal of citrate, alkali (40% NaOH) to pH 8.5, BaCl₂ · 2H₂O (12.5 g./100 ml.) and 4 vol. ethanol are added as before. It is sufficient to allow the solution to stand at room temperature for 1 hr. before filtering off the barium citrate. This is washed with a few small volumes of 80% ethanol and the combined filtrates are evaporated *in vacuo* to 150 ml., taking the same precautions as previously to remove the last traces of ethanol. The faintly yellow, clear solution is saturated with (NH₄)₂SO₄ (75 g./100 ml.) and extracted three times with about 10 g. phenol. The combined phenol layers are clarified by spinning and extracted three times with an equal volume of water. Traces of phenol are removed from the pooled aqueous layer by ether extraction. This leaves 80–100 ml. of a clear, colourless, highly active solution, of which 0.02–0.05 ml. is required for an assay.

(G) Further purification can conveniently be achieved by treating this solution with HNO₃, as described above, and rechromatographing it on a small column of Amberlite IR 100 at pH 3.5 as before. The yield of organic material/lb. fresh yeast, averages about 0.2 mg., of which 5–10 μg. are required to elicit an optimum response under the present conditions.

DISCUSSION

In spite of considerable information gained on the chemical properties of COF it is not yet possible to identify it with any compound, or assign to it any particular structure. The following substances were tested for COF-like activity before the information provided by purified preparations narrowed the range of possible structures to weakly basic, phosphate-free compounds: nicotinamide, nicotinamide riboside (given by Dr T. P. Singer), diphosphopyridine nucleotide (40% pure), triphosphopyridine nucleotide (crude), coenzyme III (Singer & Kearney, 1952), biotin (Ashe Laboratories, Leatherhead, Surrey), spermine (given by Prof. Peters), thiamine, cytosine, adenine, cytidine (this and the following five substances were given by Dr D. D. Woods and Dr R. H. Nimmo-Smith), uridine, adenosine, guanosine, cytidylic acid and adenosine-5'-phosphate, and coenzyme A from liver prepara-

tions. None of these compounds possesses any activity in the yeast assay system with the exception of the preparation of coenzyme III. This was slightly active, but purified COF has no activity in the coenzyme III-catalysed oxidation of cysteine sulphinic acid (Kearney & Singer, private communication).

The identity of many cofactors with COF can be excluded *a priori* on known chemical data. Among these factors are phosphate-containing compounds such as the cogalactowaldenase of Caputto, Leloir, Cardini & Paladini (1950) or adenosine-5'-phosphate, shown by Kornberg (1951) to be required by a DPN specific isocitric dehydrogenase from yeast. Another compound, the chemical properties of which differ greatly from those of COF, is the pyruvate oxidation factor (Gunsalus, Struglia & O'Kane, 1952).

COF has further been tested with negative results for coenzyme A activity in the liver acetylase system of Kaplan & Lipmann (1948). COF has no significant vitamin B activity when assayed after autoclaving with *Streptococcus faecalis* R as test organism (Dr J. Lascelles, private communication). Excluding the identity of various growth factors with COF does not, of course, exclude the possibility that COF is a compound allied to a known factor (conjugated form, breakdown product or precursor).

In its weakly basic properties, acid and alkali lability and resistance to nitrous acid, COF closely resembles nicotinamide derivatives (Schlenk *et al.* 1938). The presence of *N*-substituted nicotinamide is further suggested by a positive Huff & Perlzweig (1946) reaction, but as pointed out above, no spectrophotometric evidence could be obtained to support the view that COF is a nicotinamide derivative.

Whatever structure is finally assigned to COF, it will have to explain the instability of purified fractions under various conditions. It cannot be decided at the moment whether this property is due to presence of damaging impurities (e.g. compounds originating from the resin) or whether it is caused by some intramolecular reaction such as a lactone formation. Further work along these lines is in progress.

SUMMARY

1. A method is described for the partial purification of the citrate oxidation factor (COF). This is a compound required by cell-free yeast extracts for the metabolism of citrate by a pathway apparently outside of, and additive to, the normal tricarboxylic acid cycle.

2. COF is a weakly basic compound, free from phosphorus. Its possible structure is discussed in the light of this and other data.

This work was carried out during the tenure of an Australian National University Scholarship. It was made possible by the constant guidance and encouragement of Prof. Sir Rudolph Peters, F.R.S., to whom I wish to express my sincerest thanks. Dr D. D. Woods, F.R.S., Dr L. A. Stocken, Dr P. W. Kent, Mr R. W. Wakelin and many others greatly assisted by their continuous helpful discussions and

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The Effect on Mouse-liver Catalase Activity and Blood-haemoglobin Level of a Milk Diet Deficient in Iron, Copper and Manganese

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Recent work has shown that tumours contain some agent capable of rapidly and markedly depressing liver catalase activity in mice (Nakahara & Fukuoka, 1949, 1950; Fukuoka & Nakahara, 1951; Adams, 1950*a*, 1951*a*; Greenfield & Meister, 1951). Further, the importance of adrenal and testicular hormones in the maintenance of mouse-liver catalase activity was demonstrated by Adams (1950*b*, 1952) and may be briefly summarized as follows. There is a sex difference in enzyme activity, males having the higher level, and castration results in a fall in activity to slightly below the normal female level. Testosterone injected into castrated males or normal females produces a rise in catalase activity to approximately that of normal males. A marked fall in enzyme activity follows adrenalectomy in both sexes, and the level is restored to normal by the injection of cortisone. Evidence was then obtained that the tumour agent acts by blocking the normal hormonal stimulation of the liver (Adams, 1951*b*).

In order to gain more information about the normal mechanisms involved in the maintenance of mouse-liver catalase activity it was decided to examine the role of trace metals. Since catalase is an iron-containing enzyme, it is not unreasonable to suppose that trace metals play an important part

in its synthesis, and in fact Schultz & Kuiken (1941) found that rat-liver catalase activity was depressed when the animals were maintained on a diet deficient in iron or copper. Addition of the missing metal restored the activity to normal. Fukuoka & Nakahara (1951) stated that feeding or injecting ferric chloride stimulated liver catalase activity in the tumour-bearing animal. Little or no other work on the influence of trace metals appears to have been done, apart from a report by Day & Skidmore (1947) that catalase activity is depressed in zinc deficiency.

The present work began with an attempt to repeat in mice the effects of iron and copper deficiency noted in rats by Schultz & Kuiken (1941). However, the results with a trace-metal-deficient diet differed so widely from those obtained by these authors that it was found necessary to carry out a more detailed investigation. Accordingly, the effects of deficiencies of iron, copper and manganese, on liver catalase activity have been studied. The degree of iron and copper deficiency was checked by corresponding determinations of haemoglobin level. An attempt was also made to find a connexion between the observed effects and the action of testosterone on catalase level.