cipitated by the addition of 10% (w/v) sodium sulphate at pH 7·4. Two washings of the barium sulphate were sufficient to remove most of the adsorbed phosphocreatine; the third extract yielded only 22 mg. of phosphocreatine and was therefore rejected. The combined washings and mother liquor now amounted to 220 ml. Ethanol (880 ml.) was added and the mixture allowed to stand at room temperature (21°) for 12 hr. to ensure crystallization. The sodium phosphocreatine separates initially in the form of a suspension of very fine oily droplets, and it is advisable to allow the product to stand undisturbed for a considerable time in order to obtain complete crystallization. When this has occurred the mother liquor is perfectly clear and the phosphocreatine remaining in solution is recovered as the barium salt by the addition of 200 ml. of 1% (w/v) barium chloride in 80% (v/v) ethanol-water.

The first two preparations we carried out gave a product which crystallized in needles and corresponded to a tetrahydrate, but subsequently we have obtained only the hexahydrate which crystallizes in six-sided platelets. The yield of the sodium salt is consistently 3–4 g. and of the barium salt slightly over 1 g. The combined yield of sodium and barium salts is roughly equivalent to the yield of calcium salt reported by Lehninger (1945). We have not achieved the 30% yield claimed by Zeile & Fawaz (1938). The two hydrates of sodium phosphocreatine were completely free from contamination by sodium chloride and inorganic phosphate.

Sodium phosphocreatine tetrahydrate. Found: C, 14·6;* H, 5·4;* N, 12·6; P, 9·7; ash ($Na_4P_2O_7$) 40·4;* creatine 38·5; loss on drying at 70° *in vacuo* 19·2. $C_4H_8O_5N_3PNa_2.4H_2O$ requires C, 14·6; H, 4·9; N, 12·8; P, 10·0; ash, 40·5; creatine 39·9; H_2O, 22·0%.

Sodium phosphocreatine hexahydrate. Found: C, 13·9;* H, 5·4;* N, 11·4; P, 8·4; ash, 36·7;* creatine 35·3; H_2O, 27·8. $C_4H_8O_5N_3PNa_2.6H_2O$ requires C, 13·2; H, 5·5; N, 11·5; P, 8·5; ash, 36·5; creatine 36·0; H_2O, 29·7%.

SUMMARY

A method is described for the preparation of pure sodium phosphocreatine.

* Analyses by Mr F. Hall, Dyson Perrins Laboratory, Oxford.

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The Isolation of Coproporphyrin III from *Corynebacterium diphtheriae* Culture Filtrates

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It has been recognized that toxic filtrates of *Corynebacterium diphtheriae* cultures contain a red pigment, and in the routine production of diphtheria prophylactic from such filtrates using the alum precipitate method the final preparation is always coloured pink, unless preliminary steps are taken to remove the pigment. Charcoal has frequently been used as a decolorizing agent, but with culture filtrates from the modification of Mueller's (1939) hydrolyzed casein medium, used at the Wright-Fleming Institute for Microbiology for the routine production of diphtheria toxin, the use of charcoal leads to an excessive loss of antigen. Attempts were therefore made to find agents capable of removing the red pigment without serious loss of antigen. A large number of the more commonly used adsorbents were tested without success, but eventually it was found by one of us (L.B.H.) that aluminium benzoate at pH 6·0 and magnesium hydroxide at pH 9·0 were promising. Of these, the latter was found to be more selective, and in general possessed other properties more suited to the preparation of diphtheria prophylactic. The red pigment thus adsorbed on to the magnesium hydroxide proved to be porphyrin in nature, and was readily purified from the magnesium hydroxide precipitate.

The presence of a porphyrin in toxic filtrates of *C. diphtheriae* cultures was first reported by Campbell Smith (1930), and confirmed by Coulter & Stone (1931) who demonstrated the quantitative relation

between porphyrin and toxin production. Most workers (Dhéré, Glücksmann & Rapetti, 1933; Wadsworth, Crowe & Smith, 1935; Pappenheimer & Johnson, 1937) assumed that the porphyrin was a coproporphyrin, and Jakob (1939) claimed, without giving details, to have isolated coproporphyrin III from *C. diphtheriae* as well as from other organisms. However, in further work by Pappenheimer (1947) it was assumed that the diphtherial porphyrin was haematoporphyrin. It therefore appeared necessary to establish its identity on more certain grounds.

The diphtheria porphyrin has proved to be a mixture of at least three porphyrins, but 98–99% consists of coproporphyrin III, the remainder being uroporphyrin I and a hitherto undescribed porphyrin.

EXPERIMENTAL

The lability of the crude toxin makes it preferable, when preparing the porphyrin as a by-product in the large-scale production of diphtheria prophylactic, to perform the magnesium hydroxide precipitation after conversion of the toxin to toxoid.

Preparation of the toxoid. The Park-Williams no. 8 strain of *C. diphtheriae* was grown on the casein-hydrolysate medium described by Mueller (1939), except that the concentration of nitrogen was reduced to 0·13 g./100 ml. (Holt, 1948). After 7 days' growth, the micro-organisms were filtered off and the toxin-containing supernatant liquid treated with 0·4% by volume of 40% formaldehyde solution. After standing at room temperature for 2 days, the reaction was adjusted to pH 7·5 with 10N-NaOH and the solution incubated at 32° for a further 28 days. The resulting crude toxoid solution was further treated as follows.

Preparation of magnesium hydroxide gel. 250 g. (approx. 1 g.mol.) $MgSO_4.7H_2O$ were dissolved in 5 l. of CO_2-free water, and to the resulting solution carbonate-free 0·5N-NaOH (approx. 4 l.) was added with continuous stirring until the pH was 10. After standing 7–10 days the clear supernatant liquid was decanted from the sedimented gel, the volume of which was now about 2·5 l. (i.e. the gel was approx. 0·4M).

Separation of the porphyrin. 0·4M-$Mg(OH)_2$ gel (6·5 l.) was added with stirring to 130 l. of the crude formol toxoid solution, the pH of which had been adjusted to 8·4 by the addition of about 500 ml. 5N-NH_3 solution. Spectrophotometric measurement of the original crude toxoid solution showed a content of 5·8 mg. porphyrin/l. (equivalent to a total of 755 mg.). After standing 1 hr., the $Mg(OH)_2$, now red in colour, was separated from the supernatant, which contained less than 0·1 mg. porphyrin/l., and which was subsequently employed for the preparation of diphtheria prophylactic.

Purification of the porphyrin and preparation of its ester. The red precipitate was suspended in 4 l. of distilled water, and dissolved by the addition of about 500 ml. of 10% (w/v) acetic acid, and the pH adjusted to less than 1 by the addition of 500 ml. 10N-HCl. After stirring for 10 min. the mixture was centrifuged, the purple-red supernatant liquid separated and the residue again extracted by standing overnight with 1 l. of N-HCl. The residual precipitate (now dirty-grey in colour) was centrifuged off, and the supernatant liquid added to that obtained by the first acid extraction. The united portions (6 l.) were then adjusted to pH 3·3, first by the addition of 50% (w/v) NaOH and finally with 30% (w/v) sodium acetate solution. The porphyrin separated as a brown flocculent precipitate, and after standing overnight the mixture was centrifuged. The supernatant liquid contained 0·375 mg./l. of porphyrin. The precipitate was dissolved in 4 l. 0·5N-HCl, and after standing 24 hr. the small brown residue was filtered off and rinsed with a small volume of 0·5N-HCl. The porphyrin was then reprecipitated at pH 3·3–3·4 as before. In all, the porphyrin was precipitated in this way four times at its isoelectric point. Finally, after washing with a little 2% (w/v) acetic acid, the precipitate was dried *in vacuo*. The dried porphyrin was dissolved in 150 ml. methanol previously saturated with dry hydrogen chloride. After standing overnight, the deep-red solution so obtained was poured into 2 l. of ice water in a large separating funnel, and repeatedly extracted with 150 ml. portions of chloroform until the aqueous phase was colourless. The chloroform solution (1200 ml.) was successively washed twice with 1 l. quantities of water, once with 1 l. dilute ammonia, and a further three times with 1 l. quantities of water. After filtration through a chloroform moistened paper, the chloroform was evaporated off leaving the crude ester (578 mg.).

Chromatographic purification of the crude ester. The crude ester was dissolved in 200 ml. benzene, and poured on to a column of $CaCO_3$ (4 × 34 cm.), and the chromatogram was developed with benzene. The main porphyrin fraction was adsorbed very weakly and readily passed into the eluate, leaving a brown zone and a second red porphyrin zone at the top of the column. On washing the column with chloroform in benzene (1 : 10 v/v), this second porphyrin zone split into two zones, one of which remained high in the column while the other moved fairly rapidly into the eluate. The remaining porphyrin zone and the brown zone could not be made to pass down the column even with pure chloroform. The adsorbent was allowed to dry, and on extrusion the third porphyrin zone was eluted with large quantities of chloroform containing 1% (v/v) of methanol. The top brown zone was not further examined.

The main benzene eluate was evaporated to dryness, and crystallized twice from chloroform and methanol. 429 mg. of coproporphyrin III methyl ester (m.p. 155–157° with a remelt at 181–182°) were obtained. This material in chloroform showed absorption bands at 622·1, 572·0, 533·7 and 502·5 mμ., intensity IV > III > II > I (Hartridge reversion spectroscope). A further 50 mg. were obtained by working up the mother liquors. After saponification in conc. HCl and dilution to 0·15N in respect of HCl, the spectral absorption curve over the range 400–750 mμ. was identical with that of coproporphyrin I obtained from the faeces of a patient with congenital porphyria. The Cu complex of the ester was prepared by Fischer's (1937) method, and after five crystallizations gave crystals melting at 218° with absorption maxima at 563 and 526 mμ.

Aronoff (1947) has pointed out that the relative intensities of the absorption bands of diphtherial porphyrin in ether, as given in our preliminary communication (Gray & Holt, 1947), are not typical of a porphyrin. We have re-examined the absorption spectrum of our material in ether solution and, although inspection through a hand spectroscope at first sight seemed to confirm our finding, when the spectral absorption curve was plotted with a spectrophotometer, there was no doubt that the relative intensities were in the order IV > III > II > I. It was obvious that with a hand

spectroscope the narrow width of the absorption band in the red led to an overestimate of its intensity. Others have also been misled by the narrowness of the first absorption band; thus Grinstein, Schwartz & Watson (1945) describe the relative intensities of the absorption bands of coproporphyrin I as IV, III, I and II in decreasing order.

The eluate containing the second porphyrin zone was evaporated to dryness, and after recrystallization from chloroform and methanol gave 7·1 mg. of the typical hair-like crystals of uroporphyrin I octamethyl ester (m.p. 288°). In chloroform, this ester showed absorption bands at 625·7, 576·7, 536·5 and 503·6 mμ., intensity IV>III>II>I (Hartridge reversion spectroscope). The third porphyrin zone has not yet been identified.

DISCUSSION

The isolation of coproporphyrin III ester in relatively enormous quantities in the large-scale production of purified diphtheria toxoid provides an abundant and hitherto unexplored source of coproporphyrin III, which will be of the greatest value in the investigation of the metabolic relationships of this material in the intact animal. The production of small quantities of uroporphyrin I provides yet another example of the so-called dualism of the porphyrins, attention to which was first drawn by Fischer (1937). In the light of present knowledge it is not possible to speculate on the significance of this dualism to the economy of the organism. The implications suggested by Pappenheimer (1947) regarding the relationship of the production of porphyrin to toxin formation will be the subject of a separate paper to be published elsewhere.

SUMMARY

Coproporphyrin III tetramethyl ester in large yield and uroporphyrin I octamethyl ester in very small yield have been prepared from toxic culture filtrates of *Corynebacterium diphtheriae*.

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Crystalline Bacterial Catalase*

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In recent years, the trend of enzyme research has shifted from the study of the reactions catalyzed to a study of the chemical nature of the enzymes themselves. This has led in the last fifteen years to the isolation of some forty enzymes in a crystalline or highly purified state, and in many cases to the identification of their prosthetic groups, resulting in a completely new outlook on enzyme chemistry.

Little progress, however, has been made in the study of bacterial enzymes from this standpoint, and up to the present there has not been recorded the isolation of a single bacterial enzyme in a pure state. This is the more regrettable since many interesting enzymes exist in bacteria which have not been found elsewhere, and some bacterial enzymes at least (e.g. lactic dehydrogenase, cytochromes a_1 and a_2) are known to differ greatly from their counterparts in animal tissues. This relative neglect of bacterial enzymes is in part due to purely technical reasons, namely, the difficulties involved in growing the large quantities of bacteria required, and the problem of liberating endo-enzymes from the bacterial cell. The first of these problems is well on its way to solution, but the second is more difficult. Most of the techniques hitherto used for destroying the cell wall and liberating intracellular enzymes (for example, autolysis, vacuum- or acetone-drying followed by extraction, shaking with glass beads (Curran & Evans,

* A preliminary account of part of this work appeared in *Nature, Lond.*, **160**, 125 (1947).