The lyophilized material (total dry wt. 30 mg excluding buffer) was taken up in 2 ml water and tested for its ability to reactivate depleted enzyme preparations (Table I). The cofactor solution showed an absorption peak at 260 mμ with a ratio of absorption at 280 mμ to 260 mμ of 0.43.

A second 10-ml portion of the extract of acetone-dried cells was enclosed in Visking cellulose casing (8/32 inches). The casing was tied to a mechanical stirrer and suspended in 4 l of the above-mentioned buffer. Stirring was continued for 20 h at 5°.

The results shown in Table I indicate that the folic acid coenzyme was completely removed by the Sephadex treatment whereas the dialysed preparation still retained 54 % of the activity of the untreated extract. 92 % of the activity could be restored to the Sephadex treated extract by adding the amount of cofactor preparation (0.1 ml) derived from 0.5 ml of the untreated enzyme extract. Doubling the amount of the cofactor preparation increased the activity to 117 % that of the untreated extract. Further addition of cofactor did not result in increased methionine synthesis. The eluted cofactors also restored activity to the partially depleted dialysed extract.

This procedure for separating enzymes and soluble cofactors is more satisfactory than dialysis in that it is more rapid, cofactors are removed more completely and the cofactors are obtained in a relatively small volume.

I would like to thank Dr. E. HUFF for calling my attention to Sephadex and Mrs. M. ROMINE for performing the methionine assay.

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Received November 28th, 1959

The prosthetic group of a chromoprotein from mycobacteria

Several yellowish pigments soluble in aqueous media have been isolated from acid-fast bacteria, *e.g.*, phthiocol[1], riboflavin[2] and pterins[3,4].

During the isolation of a lactic acid oxidase from *Myco. smegmatis*[5] a yellow chromoprotein which accompanies the oxidase through several stages of purification was separated in a partly purified state.

This protein, which is not known to have enzymic activity, showed a characteristic absorption spectrum with maxima at 276 mμ and 403 mμ, the ratio $E_{403\ m\mu}/E_{276\ m\mu}$ being 1:3 for the purified preparation (Fig. 1). Acidified solutions of the protein are very pale yellow and this colour is intensified on making alkaline, the change being

accompanied by increased absorption at 403 mμ. Treatment with HCl to give a final concentration of 0.1 *N* causes precipitation of the protein and release of the bound pigment.

As only small amounts of the chromoprotein were available the pigment was isolated from intact bacteria. The bacteria were extracted with acetone in a Soxhlet apparatus and dried *in vacuo*. Portions of the acetone powder (5–10 g) were dispersed in 200 ml 5 % (*w*/*v*) trichloracetic acid and shaken mechanically for 4 h. On removal of the cells the yellow extract was treated repeatedly with ether to remove the trichloroacetic acid and evaporated to a small volume in a lyophyliser. The concentrate was run on to a column of Florisil which effectively retained a number of the contaminating compounds. With 1 % acetic acid as solvent the yellow chromophore was present in the first of several fluorescent bands eluted from the column. This fraction was concentrated and run on a starch column using 0.01 *N* HCl as solvent. Again the pigment was present in the first fluorescent band eluted. Barium acetate was added to this eluate, the pH adjusted to 8 and ethanol added to a final concentration of 80 %. A yellow flocculent precipitate was centrifuged off, washed with 80 % ethanol and dried *in vacuo*. Further purification was obtained by re-solution of the precipitate in the minimal amount of 0.1 *N* HCl, precipitation of the barium as the sulphate and re-precipitation of the pigment as the barium salt.

The pigment can also be purified by countercurrent distribution with 1 *N* HCl saturated with ammonium sulphate as the stationary phase and *n*-butanol saturated with 1 *N* HCl as the mobile phase. After 180 transfers the pigment, readily detected by its fluorescence, was obtained in the aqueous phase of the first few units.

The absorption spectra in 0.1 *N* HCl and in 0.1 *N* NaOH are given in Fig. 2. The spectrum shows a marked bathochromic shift on change from acid to alkali, with absorption maxima at 265 mμ and 375 mμ in 0.1 *N* HCl and at 252 mμ, 268 mμ and 418 mμ in 0.1 *N* NaOH. The pigment dissociated from the chromoprotein appears

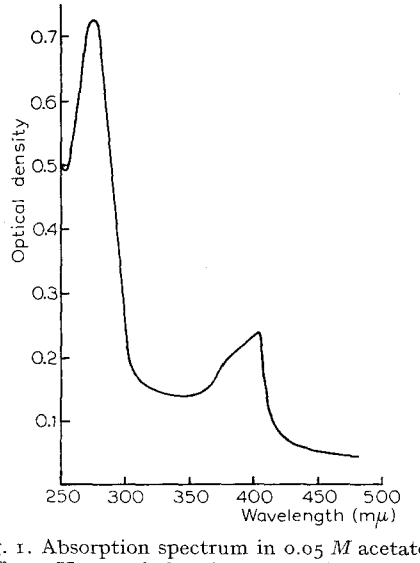

Fig. 1. Absorption spectrum in 0.05 *M* acetate buffer, pH 4.5, of the chromoprotein isolated from *Myco. smegmatis*.

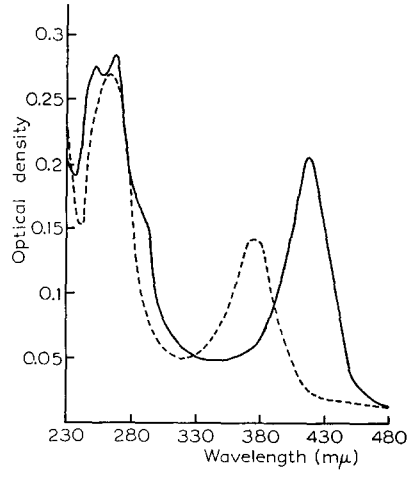

Fig. 2. Absorption spectrum of the yellow pigment isolated from acetone-treated cells of *Myco. smegmatis*. – – –, 0.1 *N* HCl; ———, 0.1 *N* NaOH.

to be identical with the pigment isolated from the intact bacteria as judged by similarity in absorption spectra, changes in fluorescence and behaviour on paper chromatograms with 5% Na_2HPO_4 (R_F, 0.64) and butanol–acetic acid–water (4:1:5) (R_F, 0.06) as solvents.

The pigment is strongly yellow coloured in neutral or alkaline solutions but the colour decreases on acidification or addition of $Na_2S_2O_4$. Under u.v. light solutions of the pigment show an intense yellow-green fluorescence below pH 2 and a blue fluorescence at higher pH values. This strong fluorescence and the colour change in fluorescence induced by alteration of pH afford a ready means of following the isolation of the pigment.

The pigment was insoluble in diethyl ether, light petroleum, chloroform, ethyl acetate, and *n*-butanol, sparingly soluble in cold water and ethanol, but readily soluble in dilute acids, salt solutions and liquid phenol, particularly on warming. The pigment showed remarkable resistance to acid hydrolysis (refluxing in 5 *N* HCl for 18 h) and towards mild oxidation with alkaline $KMnO_4$. Both treatments give rise to a number of fluorescent compounds detected by paper chromatography but the principal compound present showed the properties of the original pigment (R_F, colour change, fluorescence). After hydrolysis with $HClO_4$, purines and pyrimidines could not be detected by paper chromatography with isopropanol–2 *N* HCl as solvent[6].

An insoluble compound could be formed from the pigment by treatment of solutions with 0.01 *M* $FeCl_3$ at pH 5 and a barium salt of the pigment was precipitated at pH 8 by 80% ethanol in the presence of barium acetate. Although insufficient material was available for full elementary analysis, estimation of nitrogen (micro-Kjeldahl) on a sample of the free pigment showed the presence of 18% N and estimation of phosphorus on a sample of the Ba salt gave 5.2% P. A pigment with identical properties to those described was isolated by the same procedure from *Myco. Karlinski* and *Myco. phlei*.

The pigment described has certain properties (solubilities, absorption spectrum, bathochromic shift, fluorescence and acid stability) which are consistent with those of a pteridine derivative. Features of the spectrum and its bathochromic shift are similar to those of a pteridine isolated from the eye pigments of *Drosophila melanogaster*[7] but there are marked differences in stability and other properties. The possibility that pteridines have coenzymic functions other than those associated with tetrahydrofolic acid has been suggested[8]. It is conceivable that the mycobacterial pigment, which contains phosphate and is firmly bound to protein, could possess a nucleotide structure and could function as a coenzyme.

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Received December 30th, 1959