acid present may well have resulted from isomeric 1-oleodistearin present as impurity in the original 2-oleodistearin. Hence the positional specificity of this preparation is very high, if not indeed absolute. The advantage of this modification lies in the elimination of high-speed centrifugation, and this makes it possible to prepare eight times as much material in a single batch as the amount prepared by the original method.

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Human-erythrocyte reduced triphosphopyridine nucleotide oxidase

TPNH oxidase which is present in the mature human erythrocyte has been purified by KIESE *et al.*[1,2] and HUENNEKENS *et al.*[3,4]. The enzyme studied by HUENNEKENS *et al.*[3,4] was found to be devoid of flavin at all stages of its purification, could utilize as terminal acceptors either oxygen, methemoglobin, or cytochrome *c*, and by spectrophotometric examination contained a rather large Soret peak at 406 mμ. As a result of their investigations HUENNEKENS *et al.* proposed that an iron-porphyrin moiety served as a prosthetic group for the enzyme.

As part of a study of the metabolism of the mature human erythrocyte, we have had occasion to purify this enzyme according to the method of HUENNEKENS *et al.*[3]. Starting from an acetone-powder extract prepared from 200 ml of packed washed human erythrocytes, the enzyme was carried through an ethanol chloroform fractionation, lyophilization, fractionation at pH 5.4 and ammonium sulfate fractionation. In accordance with the findings of HUENNEKENS *et al.* a 180-fold purification was achieved. Further purification was attained by use of DEAE-cellulose chromatography. The ammonium sulfate fraction with the highest specific activity, which in our hands precipitated between 70–80% saturation, was dialyzed against 0.005 M potassium phosphate buffer at pH 7.0 and then placed on a DEAE-cellulose column which had been equilibrated against the same buffer. One active fraction was eluted with 0.005 M potassium phosphate buffer at pH 7.0, while another more active fraction was eluted with 0.02 M potassium phosphate at pH 7.0. The fraction eluted with the higher concentration of potassium phosphate buffer showed as 5–10-fold increase in specific activity over that of the previous step and resulted in an

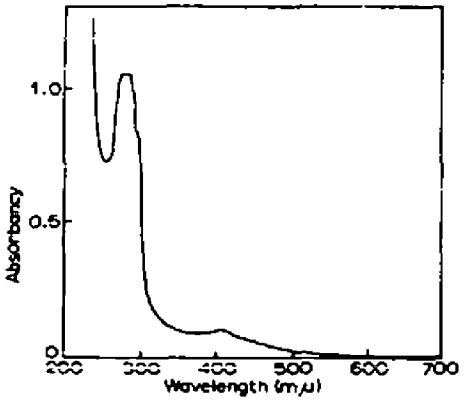

Fig. 1. Absorption spectrum of purified enzyme. The enzyme was carried to the ammonium sulfate fraction step. The spectrum was determined by means of a Cary Recording Spectrophotometer Model 11. Specific activity of the enzyme, 90. The protein concentration was 0.85 mg/ml.

overall purification of the enzyme of 900–1800-fold. Experiments reported in Fig. 2 of this communication were performed with this fraction.

Spectral analysis of various enzyme preparations after precipitation of the hemoglobin with the ethanol–chloroform mixture showed a peak at 406 mμ of various heights. It was found that by extracting the acetone powder 1 to 10 (w/v) in 0.15 M NaCl and precipitating the hemoglobin in the extract by consecutive addition of $^1/_3$ vol. of ethanol and $^1/_5$ vol. of chloroform that the 406-mμ peak was virtually absent on spectral examination of this and subsequent fractions. A typical example

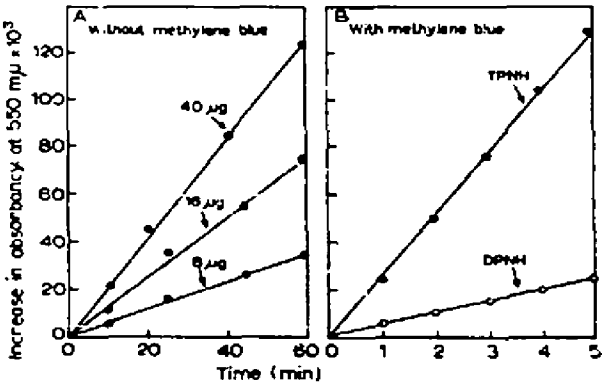

Fig. 2. A. TPNH–cytochrome *c* oxidoreductase activity without addition of methylene blue. The assay system contained 100 μmoles of Tris–HCl buffer (pH 7.5), 0.2 μmole TPNH, 0.2 μmole cytochrome *c*, indicated amounts of enzyme eluted from a DEAE-cellulose column with 0.02 M potassium phosphate buffer (pH 7.0), and water to 3 ml. Changes in absorbancy were determined at 550 mμ with a light path of 1 cm using a Beckman DU Spectrophotometer. Absorbancy changes were determined in an appropriate blank containing all components except enzyme and used to correct absorbancy changes obtained with enzyme present. B. TPNH–cytochrome *c* oxidoreductase activity with addition of methylene blue. Conditions were identical to those stated in A except that 0.027 μmole methylene blue was added to the reaction mixture, the amount of enzyme was held constant at 8 μg, and in one of the reaction mixtures 0.2 μmole DPNH was added instead of TPNH as indicated.

of the enzyme spectrum is presented in Fig. 1. The specific activity* of various enzyme preparations was found to be independent of the absolute magnitude of the 406-mμ peak or ratio of the protein peak at 278 mμ to that at 406 mμ. These data may be taken as evidence that the component responsible for light absorption at 406 mμ plays no role in the functioning of the enzyme. The findings stand in sharp contrast to those of HUENNEKENS *et al.*[3,4]. It is noteworthy that efficient removal of the peak at 406 mμ could not be accomplished by ethanol–chloroform treatment of hemolysates. On the other hand, ethanol–chloroform treatment of dilute acetone-powder extracts was consistently quite effective in removing most of the component absorbing at 406 mμ.

Using cytochrome *c*, which has been shown to give the highest activity as a terminal electron acceptor[3], and relatively large amounts of the purified enzyme, the activity was compared with and without addition of catalytic amounts of methylene blue. As shown in Fig. 2, enzymic activity was present without methylene blue, but was only a fraction of that obtained on addition of this dye. In this regard it is of interest that HUENNEKENS *et al.*[3] observed that the enzyme as isolated from human erythrocytes manifested an absolute requirement for methylene blue, or other autoxidizable dyes, as an electron carrier. The data in Fig. 2, B also illustrate the comparative activities of TPNH and DPNH when assayed under identical conditions with cytochrome *c* as the terminal electron acceptor. Inclusion of *p*-chloromercuribenzoate at a concentration of $3 \cdot 10^{-5}$ M resulted in an inhibition of 60% of the enzyme's activity when assayed under the conditions outlined in Fig. 2, B with TPNH as the electron donor. These results are similar to those noted previously[3]. It should be noted that SCOTT AND MCGRAW[6] have recently reported the purification of a diaphorase from human erythrocytes which is relatively specific for DPNH and appears to be distinct from the enzyme described here.

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* Specific activity is defined as units of enzyme per mg of protein. 1 unit of enzyme is defined as that amount which causes a decrease of absorbancy at 340 mμ of 0.010 under the conditions outlined by HUENNEKENS *et al.*[3].

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