in no case shown the presence of free di-iodotyrosine, but since the analysis was performed on only 50 μl. of plasma, any di-iodotyrosine present may have been too low in amount for detection, but might have been revealed by extracting larger quantities of material.

Di-iodotyrosine could appear in urine without being in the plasma by the breakdown of thyroxine or thyroglobulin in the kidney tissue with the elimination of the di-iodotyrosine thus formed in the urine. There is, however, no evidence on this point and elucidation must await the results of future work. At the moment it would appear probable that the di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid of the urine are produced by the breakdown of thyroglobulin.

SUMMARY

1. The urine of patients receiving large doses of radioactive iodine for treatment of thyroid carcinoma has been investigated.

2. The amount of the iodine present in organic combination has been found to be proportional to the known uptake of radioactive iodine by the thyroid or neoplastic tissue.

3. The organic fraction has been studied by paper chromatography.

4. Two main compounds in the organic fraction have been shown to be chromatographically identical with 3:5-di-iodotyrosine and 3:5-di-iodo-4-hydroxyphenyl-lactic acid.

5. Several compounds of unknown structure have also been shown to be present.

6. The origin of the urinary di-iodotyrosine is discussed. It is considered probable that this is derived from circulating thyroglobulin.

REFERENCES

Albert, A. & Keating, F. R. (1949). *J. clin. Endocrin.* **9**, 1406.

Albert, A., Rall, J. E., Keating, F. R., Power, M. H. & Williams, M. M. D. (1949). *J. clin. Endocrin.* **9**, 1392.

Benua, R. S., Albert, A. & Keating, F. R. (1952). *J. clin. Endocrin.* **12**, 1461.

Brown, F. & Jackson, H. (1954). *Biochem. J.* **56**, 399.

Brown, F. & Jackson, H. (1956). *Biochem. J.* **62**, 295.

Fletcher, K. (1957). *Biochem. J.* **67**, 136.

Flock, E. V. & Bollman, J. L. (1954). *Fed. Proc.* **13**, 209.

Foster, G. L. & Gutman, A. B. (1930). *J. biol. Chem.* **87**, 289.

Gross, J. & Leblond, C. P. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 686.

Gross, J. & Pitt-Rivers, R. (1953). *Biochem. J.* **53**, 645, 652.

Harington, C. R. (1927). *Biochem. J.* **21**, 169.

Johnson, H. W. & Albert, A. (1951). *Endocrinology*, **48**, 669.

Kalant, H., Lee, R. & Sellers, E. A. (1955). *Endocrinology*, **56**, 127.

Pitt-Rivers, R., Stanbury, J. B. & Rapp, B. (1955). *J. clin. Endocrin.* **15**, 616.

Querido, A., Stanbury, J. B., Kassenaar, A. A. H. & Meijer, J. W. A. (1956). *J. clin. Endocrin.* **16**, 1096.

Roche, J., Jutisz, M., Lissitsky, S. & Michel, R. (1951). *Biochim. biophys. Acta*, **7**, 257.

Roche, J., Michel, R. & Tata, J. (1952). *C.R. Soc. Biol., Paris*, **146**, 1003.

Roche, J., Michel, R. & Tata, J. (1954*a*). *C.R. Soc. Biol., Paris*, **148**, 1036.

Roche, J., Michel, R. & Tata, J. (1954*b*). *C.R. Soc. Biol., Paris*, **148**, 642.

Saul, J. A. & Trikojus, V. M. (1948). *Biochem. J.* **42**, 80.

Stanbury, J. B., Kassenaar, A. A. H., Meijer, J. W. A. & Terpstra, J. (1956*a*). *J. clin. Endocrin.* **16**, 735.

Stanbury, J. B., Meijer, J. W. A. & Kassenaar, A. A. H. (1956*b*). *J. clin. Endocrin.* **16**, 848.

Taurog, A. (1955). *The Thyroid, Brookhaven Symposia in Biology*, no. 7, p. 111. Upton, New York: Brookhaven National Laboratory.

Taurog, A., Briggs, F. N. & Chaikoff, I. L. (1952). *J. biol. Chem.* **194**, 655.

Taurog, A., Tong, W. & Chaikoff, I. L. (1950). *J. biol. Chem.* **184**, 83.

Thibault, O. & Pitt-Rivers, R. (1955). *Lancet*, **268**, 285.

Tishkoff, G. H., Bennett, R., Bennett, V. & Miller, L. L. (1949). *Science*, **110**, 452.

Tong, W., Taurog, A. & Chaikoff, I. L. (1951). *J. biol. Chem.* **191**, 665.

Tong, W., Taurog, A. & Chaikoff, I. L. (1954). *J. biol. Chem.* **207**, 59.

Preparation and Properties of Highly Purified Diaphorase

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In 1937 an enzyme catalysing the reaction between reduced coenzyme I (Co I-H_2) and methylene blue (or other suitable carriers) was found to be present in the plant and animal kingdoms and was named at the time 'coenzyme factor' or diaphorase by two groups of workers who independently discovered it (Green, Dewan & Leloir, 1937; Dewan & Green, 1937; Adler, Euler & Hellström, 1937; Adler, Euler & Günther, 1938). It was subsequently found widely distributed in animal tissues (Green, Needham & Dewan, 1937; Dewan & Green, 1938; Green & Dewan, 1937; Euler & Hasse, 1938; Euler & Günther, 1938*a*); in yeast (Euler & Günther, 1938*b*; Dewan & Green, 1938); and in plant tissues (Lockhart, 1939).

Straub (1939) reported the isolation of a soluble flavoprotein from heart muscle and showed that the prosthetic group was flavinadenine dinucleotide (FAD). Straub, Corran & Green (1939), on testing the catalytic properties of the enzyme, concluded that this flavoprotein was identical with the 'coenzyme factor'; this was confirmed later by Adler, Euler & Günther (1939). A detailed account of the catalytic properties of the enzyme was subsequently published by Corran, Green & Straub (1939). But no criteria of purity such as electrophoresis or sedimentation were applied and, in fact, surprisingly little is known about the physicochemical properties of the enzyme. Straub's best preparation contained $0{\cdot}54 \pm 0{\cdot}02$ % of riboflavin, a figure on which his molecular weight of 70 000 is based, assuming 1 mole of flavin/mole of protein.

It may be thought strange that a classical flavoprotein such as Straub's diaphorase has been so little investigated. Yet it is a somewhat controversial enzyme. Thus Lockhart & Potter (1941) and Potter (1950) suggested that diaphorase might represent a transformation artifact derived from Co I–cytochrome *c* reductase. Edelhoch, Hayaishi & Teply (1952) and Mahler, Sarkar, Vernon & Alberty (1952) have again brought up this possibility. Slater (1950) proposed that in a heart-muscle preparation diaphorase is associated with an unknown factor to form the so-called Co I–cytochrome *c*-reductase system.

It is true that starting from the same material (pig heart) a true diaphorase (without cytochrome *c*-reductase activity) may be isolated by a different procedure from that used for the preparation of the Co I–cytochrome *c*-reductase. But the relationship between the two enzymes still remains obscure. The recent work of Strittmatter & Velick (1956) and Velick & Strittmatter (1956) on the microsomal cytochrome from liver has brought to light a new feature in the Co I–cytochrome *c*-reductase system. They have shown that the large fraction of Co I–cytochrome *c*-reductase activity of the liver which is contained in the microsome fraction can in part be ascribed to a Co I-H_2-specific dehydrogenase which is completely inactive with cytochrome *c* but catalyses the reduction by Co I-H_2 of the microsomal cytochrome b_5. The latter cytochrome reacts directly with cytochrome *c* thereby completing a Co I-H_2–cytochrome *c*-reductase system.

The work described in this paper was undertaken with the object of obtaining more precise information on the purity and properties of diaphorase. The isolation procedure, described in detail, has been slightly modified from that originally developed by Straub (1939) and also from that published by Mahler (1955).

MATERIALS AND METHODS

Reduced coenzyme I (*Co* I-H_2). The disodium salt from yeast was obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). The stock solution (1 mg./ml.) was prepared in 0·002 M-phosphate buffer, pH 7·2. The solution was stored at 4° but deteriorated fairly rapidly and was not kept longer than a fortnight. Concentration was estimated from the molecular-extinction coefficient $\epsilon = 6{\cdot}22 \times 10^3$ l. mole^{-1} cm.$^{-1}$ (Horecker & Kornberg, 1948).

2:6-*Dichlorophenolindophenol.* A sample from British Drug Houses Ltd. was purified by dissolving it in 0·001 M-phosphate buffer, pH 7·5, and extracting the acid form with ether. The ether extract was washed with water, extracted with 0·001 M-Na_2CO_3 solution and the dye was precipitated from the slightly warm (40°) carbonate solution by adding NaCl solution and allowing to cool. The precipitate was filtered off and washed with dilute NaCl solution. The dye was standardized by titration of the iodine liberated from acidified KI (Rosin, 1954), which gave a purity of 86 %. The optical density at 600 mμ of a sample of 2:6-dichlorophenolindophenol was determined after suitable dilution with phosphate buffer, pH 8, and calculated on the basis of 86 % purity, gave $\epsilon = 21 \times 10^3$ l. mole^{-1} cm.$^{-1}$. A stock solution of the dye (10^{-4} M) was prepared in 0·002 M-phosphate buffer, pH 7·2. The solution deteriorated slowly and was freshly prepared once a fortnight.

Other materials. Alumina C_γ was prepared according to the method of Willstätter & Kraut (1923, 1924).

Apoenzyme of D-amino acid oxidase was prepared from sheep kidney according to the method of Negelein & Brömel (1938–39).

Measurements of enzyme activity. The method developed by Edelhoch *et al.* (1952) and Mahler *et al.* (1952), which makes use of the decrease in optical density at 600 mμ when 2:6-dichlorophenolindophenol is reduced, was used for assaying diaphorase activity, but the amount of reagents used differed slightly. Thus 1·5 ml. of 0·2 M-phosphate buffer, pH 7·2, 0·2 ml. of stock Co I-H_2 (see above), 1 ml. of stock dye solution and 0·2 ml. of water were placed in quartz cells (1 cm. path). The optical density at 600 mμ was determined and the enzyme solution (0·1 ml. appropriately diluted) was added at zero time. The change in optical density was followed at 30 sec. intervals for 3 min. Enzymic activity was determined from the initial velocity, corrected for the blank rate without any enzyme. Unless otherwise stated, all determinations were carried out at room temp. (18 ± 2°). The reaction was usually carried out in evacuated Thunberg tubes with attached optical cells (obtainable from the Thermal Syndicate, Ltd., Wallsend, Northumberland), for it was found that aerobically when all the indophenol had been reduced, slow reoxidation occurred. However, the initial velocity of the enzyme reaction, with a large amount of dye, was found to be the same whether carried out aerobically or anaerobically.

Enzyme unit. One enzyme unit is defined as that amount which causes an initial rate of change of optical density at 600 mμ of 0·01/min. under the above conditions. Protein was estimated by the optical density at 280 mμ according to the method of Warburg & Christian (1939–40). An optical density of 1·0 at this wavelength is found to be approximately equivalent to a protein concentration of 1 mg./ml.

Electrophoresis. This was carried out in the Tiselius electrophoresis apparatus (Perkin–Elmer Corpn., Conn.,

U.S.A.; model 38) at 4°. For most of the preparative runs the 6·6 ml. cell was used, though the 2·2 ml. cell was used in the expt. described in Table 2. Photographs were recorded on rapid process P 200 panchromatic plates (Kodak, Ltd.). Before electrophoresis the protein was dialysed at 0° against buffer for 16–19 hr.

Sedimentation. Sedimentation runs were made in the model E Analytical Spinco Ultracentrifuge at room temp. and at top speed (59 780 rev./min.). The protein was dialysed overnight at room temp. against 0·05 M-phosphate buffer, pH 7·2, containing 0·05 M-NaCl.

Diffusion. The Perkin–Elmer electrophoresis apparatus, with the modified Longsworth scanning technique, was used for the diffusion measurement. The boundaries were brought into view by a clockwork mechanism devised by Dr J. Keilin and Mr B. R. Slater. The experiment was done at 17° on the sample of dialysed diaphorase used in the sedimentation experiments. Photographs were taken at intervals over a period of 3 days. The diffusion coefficient was calculated by the height–area method from enlarged projections of the photographs on graph paper.

Spectrophotometric measurements. With the exception of the measurement of the absorption spectrum of diaphorase (Fig. 5), for which the Unicam photoelectric spectrophotometer was used, all measurements were made in the Beckman Model DU spectrophotometer.

Measurement of the flavin content. The flavin content of the protein was determined on a sample of the enzyme solution used for ultracentrifugal analysis. This had previously been exhaustively dialysed against distilled water and made up to 5 ml. The FAD content was determined on a boiled portion of the solution by means of the D-amino acid oxidase system (Warburg & Christian, 1938). The apoenzyme of the D-amino acid oxidase was calibrated against a standard FAD solution. The sample of FAD used in this calibration had been purified on a cellulose column according to the method of Whitby (1953), and was free from interfering adenylic compounds (see Burton, 1951). Chromatography on paper with the solvent system butanol–acetic acid–water (Crammer, 1948), and with a 5% aq. soln. of $Na_2HPO_4, 12H_2O$ (ascending chromatogram; Whitby, 1950), showed that FAD was the only fluorescent flavin nucleotide present in the sample. An independent estimate of the FAD content was made by determination of the optical density at 451 mμ, a value of $\epsilon = 1{\cdot}04 \times 10^4$ l. mole.$^{-1}$ cm.$^{-1}$ being used. In both estimations the FAD content was calculated from the dry wt. of the solution after freeze-drying.

Purification procedure

The modified method described below, up to step VIII, is one developed by Drs M. Dixon and G. Weber in this Department. The procedure for further purification is original.

Step I. Mincing and washing. Twelve pig hearts were freed from fat and connective tissue and passed through an electric meat mincer. The mince was repeatedly washed with 20 vol. of tap water until the washings were no longer red (usually eight washings). The mince was stirred vigorously with a mechanical stirrer for 20 min./wash. The mince was then squeezed through calico cloth.

Step II. Preparation of homogenate. The mince was homogenized with 0·02 M-Na_2HPO_4 soln. in a Waring Blendor (200 g. of mince plus 300 ml. of phosphate) for 4·5–5 min. To the homogenate a volume of water equal to the volume of phosphate used was added and the extract spun in an International serum centrifuge for 30 min. at 2000 rev./min. The precipitate was discarded.

Step III. Acidification to pH 4·6. To each l. of supernatant 25 ml. of M-acetate buffer, pH 4·6, was added and the solution left overnight in the cold room. The precipitate was centrifuged (International serum centrifuge for 20 min.) and the supernatant discarded.

Step IV. First heating. The precipitate was suspended in water (1 l./kg. of original washed mince used) and the suspension made as fine as possible by mechanical stirring. Ammonium sulphate to 1% was added. The solution was now quickly heated to 38–40°, kept at this temp. for 5 min., and then rapidly cooled and spun in a refrigerated centrifuge for 30 min. (2000 rev./min.). The precipitate was discarded.

Step V. Treatment with alumina C_γ. The yellow fluorescent supernatant was adjusted, if necessary, to pH 6–6·5 with N-acetic acid (usually the pH needed no adjustment). Alumina solution (65 ml.; dry wt. 18–19 mg./ml.), was added. After 30 min. in the cold the solution was centrifuged. The supernatant was treated with a further quantity of gel to adsorb any remaining diaphorase and the centrifuging repeated. The two lots of alumina were combined and the adsorbed enzyme was eluted with successive lots of 50 ml. each of 0·2 M-Na_2HPO_4 soln., until the eluate ceased to be yellow (usually 4 or 5 lots). The combined eluates were dialysed overnight against two changes of distilled water (15 l. each).

Step VI. Second heating and ammonium sulphate fractionation. The dialysed solution (usually 500 ml.) was made 1% with respect to $(NH_4)_2SO_4$. The solution was heated to 60° by a water bath and kept at this temp. for 5 min. The solution was cooled, centrifuged and the precipitate discarded. The supernatant was then brought up to 0·45 saturation with $(NH_4)_2SO_4$, allowed to stand in the cold for 1 hr. and spun in the Serval High-Speed centrifuge. The precipitate was discarded. The supernatant was then brought up to 0·8 saturation with $(NH_4)_2SO_4$, allowed to stand for 1 hr. in the cold, and again centrifuged. The precipitate was dissolved in about 20 ml. of water.

Step VII. Dialysis. The solution was then dialysed against distilled water until a precipitate appeared. This was spun off and the dialysis continued until further precipitation occurred.

Step VIII. Third heating and second ammonium sulphate fractionation. The precipitate was dissolved by the slow addition of aq. 0·1% (w/v) NH_3 soln. (pH 7–7·4). The solution was now heated to 62° for 5 min. After cooling, the solution was brought up to 0·55 saturation with $(NH_4)_2SO_4$. The precipitate was discarded after centrifuging. The supernatant was then brought up to 0·65 saturation. The diaphorase appeared as a yellow precipitate. It can be stored at this stage in the refrigerator. Alternatively, the $(NH_4)_2SO_4$ precipitate may be centrifuged off, dissolved in dilute phosphate buffer, pH 7–7·5, and stored at −15°. Step VIII corresponds essentially to the last stages of Straub's method of preparation. The yield of enzyme obtained is about 50–90 mg./kg. of original washed mince.

The instability of the enzyme during the initial stages presents a problem, especially during the phosphate extraction just before acidification. When step IV has been started it is advisable to work as rapidly as possible to step VII. Enzyme activity was determined at each stage of the preparation. Table 1 is representative of a typical purification procedure.

Table 1. *Summary of diaphorase purification*

Procedure	Volume (ml.)	Activity (units/ml.)	Protein (mg./ml.)	Purity (units/mg. of protein)	Purification factor	10^{-3} × Total units	Yield (%)
Washed pig-heart mince (2000 g.) extracted with 0·02 M-phosphate	4000	600	44	13·6	—	2400	100
Ppt. obtained after acidification resuspended in water	2000	1100	91	12·0	0·8	2200	91
Suspension heated to 38–40° for 5 min. Supernatant after centrifuging	1600	600	6·2	97·0	7	960	40
Alumina eluate after dialysis	700	680	4·5	151	11	476	20
Ppt. from 0·8 sat. $(NH_4)_2SO_4$, redissolved in water	35	7000	13·6	514	37	245	10
Final 0·65 sat. $(NH_4)_2SO_4$ ppt., redissolved in water	7·8	8100	9·5	852	63	63	2·6

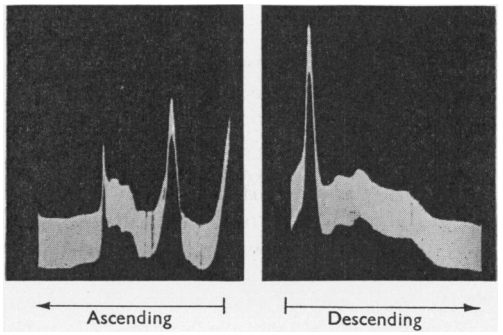

Fig. 1. Electrophoretic diagrams of ascending and descending boundaries of a solution of diaphorase at step VIII in 0·05 M-tris buffer, pH 8·5, 95 min. after beginning of migration.

RESULTS

Purity of preparation

Different diaphorase preparations (step VIII) were examined in the Tiselius electrophoresis apparatus. Electrophoresis was carried out in 0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 8·5. (In later experiments the ionic strength of the buffer was increased by the addition of 0·05 M-NaCl.) In all cases a typical pattern was obtained. A similar pattern was obtained in one case with a preparation made exactly according to the procedure of Straub. Thus within 10 min. of commencing the electrophoresis there was a rapid splitting into several components (see Fig. 1). It will be seen that in addition to a major component there are at least five minor components. The flavin content (as riboflavin phosphate) of diaphorase is given by Straub (1939) as 0·66 % (0·54 ± 0·02 % riboflavin). If one assumes this value to be that of pure diaphorase then on the basis of the flavin content, at the step VIII, different diaphorase preparations were 50–90 % pure.

Further purification of diaphorase

It was observed that if the electrophoresis of the impure diaphorase (Fig. 1) was continued for a long time, the aggregate of faster-moving components migrated out of the field of view leaving behind the major component, which had a much lower mobility at this pH. Isolation of the slow component, by removal of the solution from the electrophoresis cell with a syringe and determination of the specific activity, showed that this component represented the diaphorase. Use was then made of this observation for the final purification of diaphorase.

Electrophoresis. The $(NH_4)_2SO_4$ precipitate obtained in step VIII was centrifuged and dissolved in the minimum of water. The protein solution was then dialysed in the cold against distilled water until a precipitate appeared (this precipitate was colourless and was spun off and discarded). The solution was diluted so that the protein concentration was approx. 1–2 %. Dialysis was then carried out against 0·05 M-tris buffer, pH 8·5, containing 0·05 M-NaCl for 16–24 hr. in the cold. Electrophoresis was then carried out in the Tiselius apparatus, with a potential of 100 v and a current of 11 mA. The duration of the electrophoresis varied from run to run but the usual time was 8–9 hr. After each run the descending limb was isolated from the remainder of the cell and the contents were removed. The contents from several runs were combined.

Ammonium sulphate fractionation. The combined fractions, still in 0·05 M-tris, pH 8·5, were treated with $(NH_4)_2SO_4$ up to 0·3 saturation. Some inert protein was precipitated and was removed by centrifuging. The diaphorase was precipitated by bringing the supernatant up to 0·65 saturation with $(NH_4)_2SO_4$.

Table 2 is a summary of the purification procedure. The recovery of diaphorase and its purity (more especially with the purer fractions) can be

Table 2. *Summary of further purification of diaphorase*

For details see Materials and Methods.

Procedure	Volume (ml.)	10^{-3}Activity (units/ml.)	Protein (mg./ml.)	Purity (units/mg. of protein)	Purification factor	10^{-3} × Total units	Yield (%)
Solution before electrophoresis	2·2	20·25	14·7	1730	—	44·5	100
Descending-limb fraction after electrophoresis for 8 hr.	0·8	23·0	6·3	3600	2	18·0	40
Fraction remaining after isolation of descending limb	1·5	16·0	7·7	2616	—	24·0	—
Descending-limb fraction after $(NH_4)_2SO_4$ fractionation and dialysis	2·7	3·5	0·69	5000	2·8	10·0	22

Table 3. *Decrease in $E_{280\ m\mu}/E_{451\ m\mu}$ with purification of diaphorase*

Values of E refer to 1 cm. thickness of solutions.

Fraction	$E_{280\ m\mu}$	$E_{451\ m\mu}$	Ratio $E_{280\ m\mu}/E_{451\ m\mu}$
Solution after step VIII, before electrophoresis	9·79	0·95	10·3
Descending limb after electrophoresis	6·3	0·84	7·5
Remainder of solution after electrophoresis	7·7	0·85	9·0
Descending limb after $(NH_4)_2SO_4$ fractionation	6·9	1·15	6·0

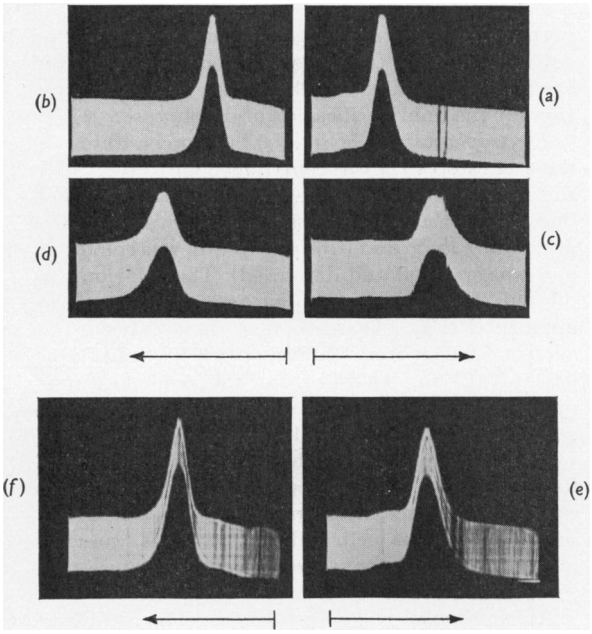

Fig. 2. Electrophoretic diagrams of ascending and descending boundaries of a solution of diaphorase (about 1%) after further purification. (*a*) and (*b*), Descending and ascending limbs respectively, 221 min. after migration in 0·05M-phosphate–0·05M-NaCl, pH 6·5; (*c*) and (*d*), descending and ascending limbs respectively of the same solution after 405 min. migration; (*e*) and (*f*), descending and ascending limbs respectively after 420 min. migration in 0·05M-phosphate–0·05M-NaCl, pH 7·2.

conveniently followed during isolation by determination of the ratio of the optical density at 280 mμ to that at 450 mμ. Table 3 is an example of the method applied to a preparation from the electrophoresis stage. The purest fraction obtained was subjected to physical tests.

Physicochemical properties of the purified enzyme

Electrophoresis. To date there is no published work on the electrophoresis of diaphorase. The fraction obtained by electrophoresis followed by $(NH_4)_2SO_4$ fractionation was examined in the Tiselius apparatus at different pH values.

At pH 7·2 (0·05M-phosphate–0·05M-NaCl) the protein appeared to be homogeneous after electrophoresis for up to 3 hr. When the process was continued for a longer time, however, a small amount of slower-moving component could be detected. At this pH the main component had a mobility of $\mu = 2{\cdot}43 \times 10^{-5}$ cm.^{2} sec.$^{-1}$v^{-1} (see Fig. 2*e*, *f*).

At pH 6·5 (0·05M-phosphate–0·05M-NaCl) the same picture was obtained, as shown in Fig. 2*a–d*. The mobility of the main component was

$$\mu = 2{\cdot}2 \times 10^{-5}\ \text{cm.}^2\ \text{sec.}^{-1}\text{v}^{-1}.$$

At both pH values the direction of migration was anodic. In another experiment, carried out in 0·05M-acetate (containing 0·05M-NaCl), pH 5·5, no movement of the boundary was observed after electrophoresis for 5 hr. In acetate, pH 4·89, there appeared to be a very slight movement towards the cathode after electrophoresis for the same period of time. A large amount of precipitation occurred at this pH and the experiment was abandoned. No

electrophoresis experiments were carried out at lower pH values owing to the fact that some irreversible splitting off of the flavin occurred. At pH 8·5, with the same buffer used in the preparative procedure, there was a single component in the ascending boundary but the descending boundary showed the presence of a slower component and the major component itself was somewhat asymmetrical. The mobility was not determined at this pH. With this buffer system distinct boundary anomalies were apparent; these were not observed in veronal buffer, pH 8·5.

Sedimentation. The sedimentation coefficient $S_{20,w}$ was determined at different protein concentrations. $S_{20,w}$ was found to vary slightly with concentration (Fig. 3). A value

$$S_{20,w} = 5{\cdot}38 \times 10^{-13} \text{ cm.}^2\text{/sec.}$$

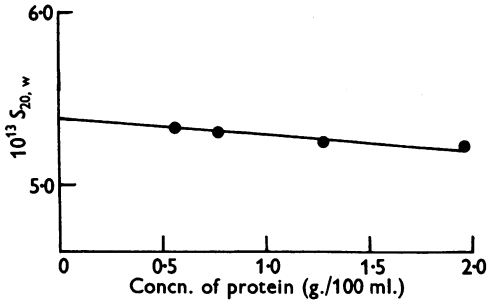

Fig. 3. Relationship between concentration of diaphorase and its sedimentation coefficient in 0·05 M-phosphate–0·05 M-NaCl, pH 7·2.

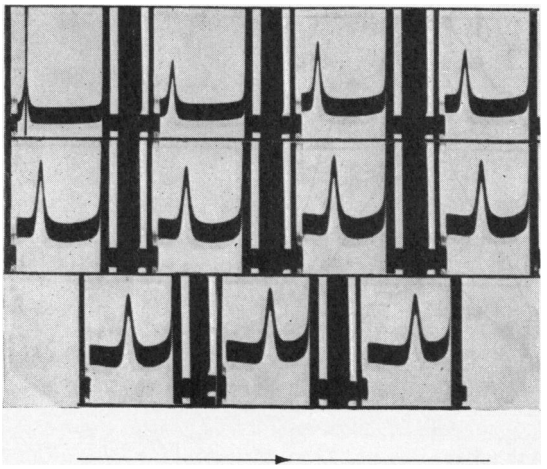

Fig. 4. Sedimentation-boundary diagrams of a 1·2% solution of diaphorase in 0·05 M-phosphate–0·05 M-NaCl, pH 7·2. Speed, 59 780 rev./min. Mean temp. of rotor, 19°. First exposure 16 min. after reaching top speed. Interval between exposures is 8 min.

was obtained by extrapolation to zero protein concentration. In all cases the sedimentation constant was calculated assuming a partial specific volume for the solute of 0·73. The diaphorase in all these experiments showed one component only, and the peak remained symmetrical throughout (see Fig. 4).

Diffusion constant. One diffusion measurement was carried out on a 0·5% solution of diaphorase which had previously dialysed overnight at room temperature against the same solvent system as was used in the sedimentation experiments. A corrected value of $D_{20,w} = 6{\cdot}08 \times 10^{-7}$ cm.²/sec. was obtained.

Molecular weight. The molecular weight has been calculated by using the Svedberg equation (see Svedberg & Pedersen, 1940)

$$M = RTs/D(1-V_\rho).$$

Combining the extrapolated value for $S_{20,w}$ of $5{\cdot}38 \times 10^{-13}$ cm.²/sec. with the diffusion coefficient $D = 6{\cdot}08 \times 10^{-7}$ cm.²/sec. and an assumed partial specific volume of $V = 0{\cdot}73$, a molecular weight of 81 000 is obtained. Allowing for the assumptions made in this calculation, there is fairly good agreement with the value of 70 000 found by Straub (1939), on the basis of the flavin content and assuming one flavin group per molecule of protein, and that of 72 000 ± 4900 found by Weber (1951), using the polarization of fluorescence technique. The sedimentation experiments now show that there is only one molecule of flavin per mole of protein, a fact that has hitherto been assumed.

Flavin content. The solution of diaphorase of highest purity, containing 0·859 mg./ml., was found to contain 0·01006 mg. of FAD/ml. by the D-amino acid oxidase test, and 0·00998 mg./ml. spectrophotometrically. This corresponds to a FAD content of 1·17 and 1·16% respectively (0·56% riboflavin). The minimum molecular weight, based upon the flavin content, is $786 \times 100/1{\cdot}165 = 67\,000$. On the basis of this flavin content, Straub's preparation would have been about 98% pure.

Absorption spectrum. The absorption spectrum of the purest preparation is similar to that published by Straub (1939), in that there are maxima at 274, 359 and 451 mμ, and minima at 397 and 315 mμ (see Fig. 5). But the ratios of the light absorption at the maxima are somewhat different. The ratio of light absorption at 274 mμ to that at 451 mμ obtained in the present study was 6·72. Comparison with the figure of 7·33 observed for this ratio by Straub indicates that some colourless impurities absorbing in the region 270–280 mμ in Straub's original preparation have been removed in the present preparation. The ratio of absorption at 359 mμ to that at 451 mμ was 0·97 (Straub found 0·91). Fig. 6 shows the absorption spectra of diaphorase in the oxidized and reduced forms in the

visible region. Curve 1 is the oxidized form of diaphorase. Curve 2 was obtained by treating the same solution of diaphorase with excess of reduced Co I. The large amount of absorption by the reducing agent below 400 mμ precludes any accurate measurement in this region. Addition of Co I-H_2 eliminates the fluorescence of the enzyme, but it will be seen from Fig. 6, curve 2 that a large amount of residual absorption still remains in the region of the flavin peak at 450 mμ. If dithionite is added to the enzyme solution reduced by Co I-H_2 there is a further decrease in absorption in the region 450–500 mμ (see Fig. 6, curve 3). Dithionite alone produces a slightly greater decrease in absorption in the visible region than either Co I-H_2 or Co I-H_2 plus dithionite, as shown in Fig. 6, curve 6.

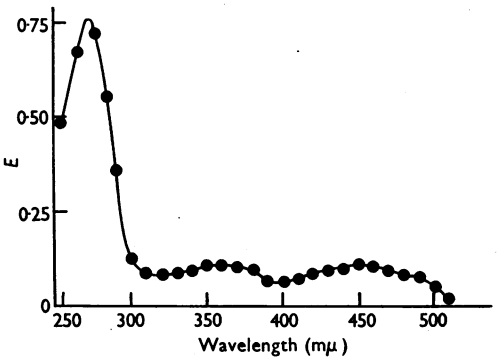

Fig. 5. Absorption spectrum of a 0·07% solution of diaphorase in 0·01 M-phosphate buffer, pH 7·2.

A characteristic feature of the absorption spectrum of diaphorase is the presence of a shoulder at 480 mμ. Straub (1939) reported that examination of a solution of diaphorase under the low-dispersion spectroscope indicated the presence of two bands, one at 451 mμ and another at 480–490 mμ. The difference spectrum, obtained by subtracting curve 2 from curve 1, and plotted in curve 4, clearly indicates the presence of a two-banded spectrum. This phenomenon is not so apparent in the difference spectrum obtained by subtracting curve 3 (Co I-H_2 plus dithionite) from curve 1, as shown in curve 5, which resembles more closely that of the oxidized form (curve 1). Another feature of the absorption curves obtained by reduction of diaphorase with Co I-H_2 or with Co I-H_2 plus dithionite is the increase in general absorption in the region 510–600 mμ. This effect is not observed to the same extent in a solution of the enzyme reduced with dithionite, as shown in curve 6. When a pure solution of FAD was reduced with excess of Co I-H_2 a typical flavin-difference spectrum was obtained, indicating that the anomalies in the absorption spectrum of diaphorase when it is reduced with Co I-H_2 are not apparently an inherent property of the free prosthetic group. Reduction of FAD by Co I-H_2 was brought about by allowing solutions of the two substances to react until no further change in optical density was observed.

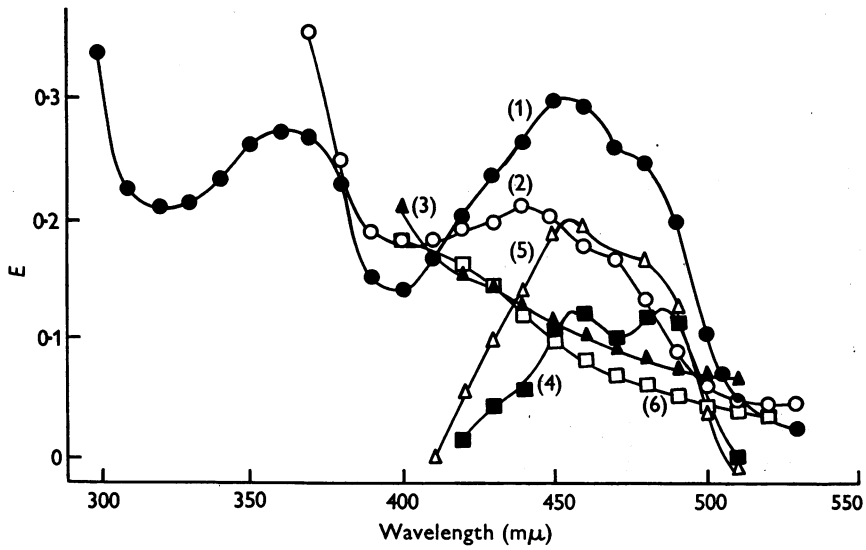

Fig. 6. Absorption spectra of the oxidized and reduced forms of a 0·2% solution of diaphorase. Curve 1 (●), oxidized form; curve 2 (○), diaphorase reduced by excess of Co I-H_2; curve 3 (▲), after reduction with Co I-H_2 plus dithionite; curve 4 (■), difference spectrum obtained by subtracting curve 2 from curve 1; curve 5 (△), difference spectrum between that of oxidized diaphorase and diaphorase reduced with Co I-H_2 plus dithionite; curve 6 (□), enzyme reduced with dithionite alone.

FAD was also reduced with dithionite. The results are shown in Fig. 7. Curve 1 is oxidized FAD. After treatment of the oxidized solution for 120 hr. with excess of Co I-H_2, curve 2 was obtained. Curve 3 represents the difference spectrum between oxidized and reduced forms. Curve 4 is FAD reduced by dithionite, curve 5 the difference spectrum. From these curves it is clear that with both Co I-H_2 and dithionite reduction, typical flavin-difference spectra are obtained. Nor does reduction of FAD by Co I-H_2 give rise to an appreciable increase in general absorption above 500 mμ. Beinert (1956*a*) has studied the spectra of flavins over the wavelength range 230–1300 mμ. By recording families of curves during progressive oxidation of the reduced to the oxidized forms at various conditions of pH and concentration, he has obtained evidence for the existence of semiquinoid intermediates in both their monomeric and dimeric forms. In the spectra of FAD shown in Fig. 5 the conditions are such that these intermediates cannot be detected.

Combination of FAD with the apoenzyme to form diaphorase has resulted in a 15 mμ shift toward shorter wavelengths of the characteristic flavin peak at 375 mμ, whereas the peak at 450 mμ is unaffected. The ratios of the light absorption at the two maxima to the minimum at 397 mμ in the visible region with diaphorase varies quite markedly from the corresponding values for FAD. The ratio $E_{375\ m\mu}:E_{405\ m\mu}$ for FAD is about 1·5; the value of $E_{359\ m\mu}:E_{397\ m\mu}$ for diaphorase is 2·0. Diaphorase gives a value of 2·2 for $E_{451\ m\mu}:E_{397\ m\mu}$ (FAD gives a ratio $E_{450\ m\mu}:E_{405\ m\mu}$ of 1·8). It is doubtful whether these differences can be explained entirely on the basis of the flavin to protein linkage. The possibility of the presence of a trace of a haem-protein impurity cannot be entirely excluded. Haem proteins have a Soret band in the region 400–430 mμ, with very high extinction coefficients in both the oxidized and reduced forms. Contamination of diaphorase with a haem protein, in an amount not detectable electrophoretically, could possibly be observed spectrophotometrically. The fact that when FAD is reduced by dithionite more than half the absorption is abolished in the region 400–430 mμ (see Fig. 7, curve 4), whereas reduction of diaphorase by dithionite (see Fig. 6, curve 6) has resulted in an increase in absorption from 400 mμ to about 410 mμ, and considerable absorption still remains in the region from 410 mμ to about 435 mμ, might be explained on the basis of the presence of a haem impurity.

DISCUSSION

Direct comparison of diaphorase obtained in the present study with that originally isolated by Straub in 1939 is difficult as he has not published sufficient physicochemical information. Methods for determining the homogeneity of proteins at that time were relatively undeveloped and the degree of purity of diaphorase remained an open question. The present work has shown that Straub's method of preparing diaphorase gives a product which, when examined by electrophoresis and in the ultracentrifuge, is not pure. A method for further purification of diaphorase has been developed and

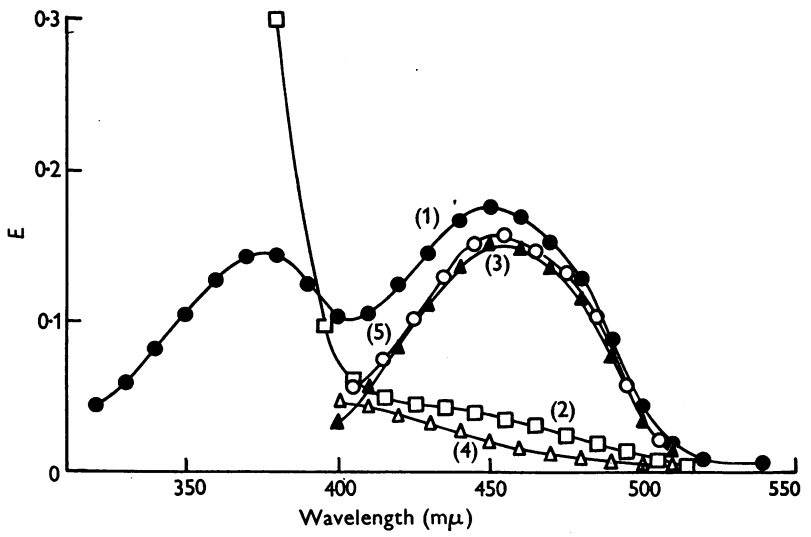

Fig. 7. Absorption spectrum of a $1{\cdot}5 \times 10^{-5}$M-solution of FAD. Curve 1 (●), oxidized form; curve 2 (□), reduced with excess of Co I-H_2, after 120 hr.; curve 3 (▲), difference spectrum, i.e. curve 2 subtracted from curve 1; curve 4 (△), FAD reduced with dithionite; curve 5 (○), difference spectrum, i.e. curve 4 subtracted from curve 1.

the enzyme has been obtained at least 95 % pure, as judged by electrophoresis in the Tiselius apparatus and sedimentation in the ultracentrifuge.

From the flavin content of the enzyme Straub (1939) determined the molecular weight of diaphorase and found a value of 70 000, assuming 1 mole of flavin/mole of protein. This assumption is not justified in the absence of experimental evidence; it has been shown that some flavoproteins contain more than one flavin group/molecule. Keilin & Hartree (1948, 1952) have shown that glucose oxidase has two prosthetic groups per molecule of 149 000, while more recently Theorell & Åkeson (1956) have found that the 'old yellow enzyme' has a minimum molecular weight of 52 000 on the basis of the flavin mononucleotide content, but a mol.wt. of 100 000–105 000 as determined by sedimentation. There are thus two flavin mononucleotide groups/molecule of protein.

The absorption spectrum of diaphorase is unusual in that there is an absorption band at about 485 mμ in addition to the usual flavin peak at 450 mμ. A similar band at 490 mμ is observed in the absorption spectrum of L-amino acid oxidase of snake venom, purified by Singer & Kearney (1950). It may be of significance that when Co I-H_2 is added to a solution of diaphorase there is not only a decrease in absorption in the flavin-peak region at 450 mμ but a significant decrease in absorption in the region of the other band at about 485 mμ, as shown by Fig. 6, curve 4, which is the difference spectrum between oxidized diaphorase and diaphorase reduced with Co I-H_2. The significance of this band at 485 mμ is unknown. For lack of any other satisfactory explanation the following may be mentioned. Examination of the absorption spectrum of FAD reveals a slight but definite asymmetry toward the longer wavelengths in the flavin peak at 450 mμ, which is manifest as a broad band from about 440–500 mμ when a solution of FAD is examined with a low-dispersion spectroscope. A solution of diaphorase, on the other hand, when examined in this way shows two distinct bands, one at 450 mμ and another at about 485–490 mμ. Although it has not been possible to show the existence of two separate bands in FAD in the spectrophotometer, the broad nature of the absorption band from 450 to 500 mμ might be due to the presence of two bands. Combination of FAD with the protein to form diaphorase may be such that a more distinct separation of the two bands occurs.

The observation that if Co I-H_2 is added to a solution of diaphorase only about half the absorption in the region at 450 mμ is abolished, and there is an increase in absorption in the region 510–600 mμ, is of interest although it is a phenomenon not entirely new to flavoproteins when reduced by their substrates. Thus Dolin (1956) observed a similar phenomenon in the absorption spectrum of Co I-H_2 peroxidase (a flavoprotein peroxidase) when reduced with Co I-H_2 and dithionite. Dolin interpreted this as a combination of Co I-H_2 with the enzyme to form an enzyme–substrate complex. Beinert & Crane (1956) report the same phenomenon with the three fatty acyl coenzyme A dehydrogenases. The same effect is not observed with a solution of diaphorase reduced by dithionite and it is tempting to interpret this phenomenon as a combination of Co I-H_2 with the enzyme to form an enzyme–substrate complex, as suggested by Dolin (1956) and Beinert & Crane (1956). Beinert (1956*b*), with another flavoprotein, has now shown that this is not the case. By means of a rapid-scanning spectrophotometer and by using a solution of that fatty acyl coenzyme A dehydrogenase which is most active with the C_8–C_{12} derivatives, he showed that this phenomenon is due to the formation of an intermediate oxidation stage. The intermediate is unstable when produced by addition of dithionite in the absence of substrate. The results obtained when diaphorase is reduced by substrate or by dithionite would seem to support Beinert's view.

The present work has not clarified the relationship between diaphorase and cytochrome *c* reductase of pig heart, but it is hoped that this study of some of the properties of diaphorase may be useful in the elucidation of the problem at a future date. Highly purified diaphorase, as now obtained, is completely inactive with cytochrome *c* as electron acceptor, nor has it been possible to convert diaphorase into a cytochrome *c* reductase by the addition of either Fe^{2+} or Fe^{3+} ions. In the absence of any added factors diaphorase is also found to be completely inactive with the cytochrome b_5 isolated by Strittmatter & Velick (1956).

SUMMARY

1. A modified method for the preparation of Straub's diaphorase from pig heart is described.

2. Sedimentation and diffusion measurements and electrophoretic examination have established the homogeneity of the enzyme preparation.

3. The molecular weight of diaphorase calculated from sedimentation and diffusion measurements is about 80 000. From the flavin content the minimum molecular weight is 67 000, which shows that the molecule of enzyme contains one prosthetic group.

4. The addition of reduced coenzyme I to a solution of diaphorase under anaerobic conditions only partly abolishes the absorption at 450 mμ and there is an increase in absorption above 510 mμ. The difference spectrum shows the presence of a further peak with maximum absorption at 485 mμ, in addition to the usual flavin peak at 450 mμ.

I wish to thank Dr M. Dixon, F.R.S., for suggesting the problem and for many valuable suggestions throughout the course of the work. I am grateful to Dr E. Webb for his help and advice. I am indebted to Mr B. Boon for carrying out the ultracentrifuge determinations and to Mr B. R. Slater for his help and co-operation with the electrophoresis experiments and for carrying out the diffusion measurement. I also wish to thank Mr D. Gardiner for much assistance during the preparation of diaphorase. This paper is published with the permission of the South African Council for Scientific and Industrial Research, to whom I am grateful for the award of a Research Bursary.

REFERENCES

Adler, E., Euler, H. von & Günther, G. (1938). *Ark. Kemi. Min. Geol.* **12**, No. 54.
Adler, E., Euler, H. von & Günther, G. (1939). *Nature, Lond.*, **143**, 641.
Adler, E., Euler, H. von & Hellström, H. (1937). *Ark. Kemi. Min. Geol.* **12**, No. 38.
Beinert, H. (1956*a*). *J. Amer. chem. Soc.* **78**, 5323.
Beinert, H. (1956*b*). *Biochim. biophys. Acta*, **20**, 588.
Beinert, H. & Crane, F. L. (1956). *Inorganic nitrogen Metabolism.* Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Burton, K. (1951). *Biochem. J.* **48**, 458.
Corran, H. S., Green, D. E. & Straub, F. B. (1939). *Biochem. J.* **33**, 793.
Crammer, J. L. (1948). *Nature, Lond.*, **161**, 349.
Dewan, J. G. & Green, D. E. (1937). *Nature, Lond.*, **140**, 1097.
Dewan, J. G. & Green, D. E. (1938). *Biochem. J.* **32**, 626, 1200.
Dolin, M. I. (1956). *Arch. Biochem. Biophys.* **60**, 499.
Edelhoch, H., Hayaishi, O. & Teply, L. J. (1952). *J. biol. Chem.* **197**, 97.
Euler, H. von & Günther, G. (1938*a*). *Naturwissenschaften*, **26**, 677.
Euler, H. von & Günther, G. (1938*b*). *Hoppe-Seyl. Z.* **256**, 229.
Euler, H. von & Hasse, K. (1938). *Naturwissenschaften*, **26**, 187.
Green, D. E. & Dewan, J. G. (1937). *Biochem. J.* **31**, 1069.
Green, D. E., Dewan, J. G. & Leloir, L. (1937). *Biochem. J.* **31**, 934.
Green, D. E., Needham, D. M. & Dewan, J. G. (1937). *Biochem. J.* **31**, 2327.
Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
Keilin, D. & Hartree, E. F. (1948). *Biochem. J.* **42**, 221.
Keilin, D. & Hartree, E. F. (1952). *Biochem. J.* **50**, 331.
Lockhart, E. E. (1939). *Biochem. J.* **33**, 613.
Lockhart, E. E. & Potter, V. R. (1941). *J. biol. Chem.* **137**, 1.
Mahler, H. R. (1955). *Meth. Enzymol.* **2**, 707.
Mahler, H. R., Sarkar, N. K., Vernon, L. P. & Alberty, R. A. (1952). *J. biol. Chem.* **199**, 585.
Negelein, E. & Brömel, H. (1938–39). *Biochem. Z.* **300**, 225.
Potter, V. R. (1950). *Annu. Rev. Biochem.* **19**, 9.
Rosin, J. (1954). *Recent Chemicals and Standards*, 3rd ed., p. 162. New York: D. van Nostrand Co. Inc.
Singer, T. P. & Kearney, E. B. (1950). *Arch. Biochem.* **27**, 348.
Slater, E. C. (1950). *Biochem. J.* **46**, 499.
Straub, F. B. (1939). *Biochem. J.* **33**, 787.
Straub, F. B., Corran, H. S. & Green, D. E. (1939). *Nature, Lond.*, **143**, 119, 334.
Strittmatter, P. & Velick, S. F. (1956). *J. biol. Chem.* **221**, 253.
Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge.* Oxford University Press.
Theorell, H. & Åkeson, Å. (1956). *Arch. Biochem. Biophys.* **65**, 439.
Velick, S. F. & Strittmatter, P. (1956). *J. biol. Chem.* **221**, 265.
Warburg, O. & Christian, W. (1938). *Biochem. Z.* **298**, 150.
Warburg, O. & Christian, W. (1939–40). *Biochem. Z.* **303**, 62.
Weber, G. (1951). Ph.D. Thesis: University of Cambridge.
Whitby, L. G. (1950). *Nature, Lond.*, **166**, 479.
Whitby, L. G. (1953). *Biochem. J.* **54**, 437.
Willstätter, R. & Kraut, H. (1923). *Ber. dtsh. chem. Ges.* **56**, 1117.
Willstätter, R. & Kraut, H. (1924). *Ber. dtsh. chem. Ges.* **57**, 1088.

Studies in the Biochemistry of Micro-Organisms

102. QUADRILINEATIN (1:2-DIFORMYL-5-HYDROXY-3-METHOXY-4-METHYLBENZENE), A METABOLIC PRODUCT OF *ASPERGILLUS QUADRILINEATUS* THOM & RAPER*

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Aspergillus quadrilineatus Thom & Raper, a species in the *A. nidulans* group, has been shown by Howard & Raistrick (1955) to produce in the perithecia a crystalline polyhydroxyanthraquinone pigment which has been named asperthecin. By degradative methods Neelakantan, Pocker & Raistrick (1957) have demonstrated that asperthecin is either 3:4:5:6:7-pentahydroxy-2-hydroxymethylanthraquinone or 3:4:5:7:8-pentahydroxy-2-hydroxymethylanthraquinone.

Howard & Raistrick (1955) noted the almost immediate production of a copious, heavy, dark-orange precipitate on the addition of Brady's reagent (2:4-dinitrophenylhydrazine in dilute hydrochloric acid) to the culture solution from strain S.M. 297 of the same organism. They recrystallized

* Part 101: Neelakantan, Pocker & Raistrick (1957).