aniline phthalate. Amino acids and amino sugars were detected after hydrolysis for 16 h at 105° with 6 *N* HCl by 2-dimensional paper chromatography in pyridine–water, 4:1 followed by butan-1-ol–acetic acid–water, 6:1:2 and reaction with ninhydrin. DAP was detected[3] specifically by paper chromatography in methanol–water–10 *N* HCl–pyridine, 160:35:5:20. N-terminal amino acids were detected as their DNP-compounds after reaction with FDNB[4].

Table I gives results obtained with TCA but it was also found that cold water removed up to 10% of the weight of the dried cell walls in a form which had similar qualitative composition of amino acids, amino sugars and monosaccharides to the TCA extracts. The N-terminal amino acids in water extracts were not determined. Differences, possibly in lipid composition, between the two types of extract of the *Pseudomonas* cell walls may be inferred from the fact that removal of the TCA with ether from the TCA extract caused the latter to become turbid. The water extract was faintly opalescent.

It has been suggested that the amino acids other than the major cell-wall components of Gram-positive bacteria might be derived from cytoplasmic material which remains in close association with the cell-wall preparations[5]. To some extent, the results of extracting dried cell walls with TCA support this belief. Such treatment does not alter basic qualitative composition but chromatograms of the hydrolysed residues of some preparations of *S. faecalis* gave with ninhydrin no trace of any substance other than the two amino sugars and three main amino acids. The failure of TCA to effect complete removal of the trace amino acids in every case remains unexplained but might be due, in part, to variations in purity of the different cell-wall preparations. It is probably also true that contaminating material is removed from the *Pseudomonas* cell wall (the residue is lighter in colour than the untreated cell wall) but the extent to which this occurs is difficult to assess because of the greater number of amino acids present. Similarly it is difficult to decide whether the high proportion of N-terminal glycine in the TCA extract of the *Pseudomonas* cell wall was derived from the wall itself or from contaminating material. It is of some interest that no DAP was detected in any of the *Pseudomonas* extracts.

If the cell walls were not dried before treatment, neither water nor TCA had any appreciable effect. If, on the other hand, the washed residues were again freeze-dried, further treatment with either solvent removed more material. How many times this process can be repeated has not been ascertained. It would seem, therefore, that some of the changes occurring on drying the cell wall are irreversible, at least over relatively short periods after rewetting. It is possible that hydrogen-bond rearrangement which occurs on drying together with changes in less specific bonding forces expose to solvent action sites which were inaccessible while the cell wall was fully hydrated. Differences in drying conditions might account in part for differences in yield and apparent composition of the TCA extracts noted in Table I and it does not follow that any material extracted in this way from acetone-dried cells will necessarily be the same as that obtained from freeze-dried cell-wall preparations. Materials broadly resembling those described in Table I, however, have been found in TCA extracts of acetone-dried cells of both organisms.

*Department of Bacteriology, University of Manchester (Great Britain)* A. D. BROWN

[1] A. D. BROWN, *J. Gen. Microbiol.*, 17 (1957) 640.
[2] M. R. J. SALTON AND R. W. HORNE, *Biochim. Biophys. Acta*, 7 (1951) 177.
[3] L. E. RHULAND, E. WORK, R. F. DENMAN AND D. S. HOARE, *J. Am. Chem. Soc.*, 77 (1955) 4844.
[4] V. M. INGRAM AND M. R. J. SALTON, *Biochim. Biophys. Acta*, 24 (1957) 9.
[5] C. S. CUMMINS AND H. HARRIS, *J. Gen. Microbiol.*, 14 (1956) 583.

Received February 6th, 1958

# Preliminary Notes

## On the biosynthesis of heme and hemeproteins in liver cell

The synthesis of heme in the immature or nucleated red cell has been studied extensively by SHEMIN, RIMINGTON and other workers, with the result that the mechanism of porphyrin biosynthesis is now known. The biosynthesis of the tissue hemeproteins which play an important role in the respiration of aerobic cells, has been studied less extensively. THEORELL *et al.*[1] purified various hemeproteins from different organs after intraperitonal injection of radioactive iron into the guinea pig and studied the rate of its incorporation. DRABKIN[2] observed the incorporation of $^{14}$C-labelled glycine into cytochrome *c* by liver slice. These authors concluded that heme synthesis occurred in the individual aerobic cells.

In the present work, the incorporation of $^{59}Fe$ into heme by homogenates of rat liver, as well as of other organs, such as heart muscle, kidney, brain, spleen and voluntary muscle, was studied. The homogenate was incubated with $^{59}Fe$ (0.1–0.5 $\mu$C, 0.1–0.5 $\mu$g as $FeCl_3$) at 37°. Heme was extracted by HCl–acetone and purified by chromatography on Hyflo Super-Cel[3]. The peak of the radioactivity coincided well with that of protoheme. In some experiments the hemin was crystallized after addition of carrier.

The radioactivity of heme was high in the mitochondrial fraction after incubation of liver homogenate with radioactive iron (Table IA). The mitochondrial preparation separated from the homogenate by differential centrifugation also incorporated radioactive iron into heme (Table IB). The cholate extract from liver mitochondria also had this activity. The optimum range of pH lay between 6 and 8 and the activity was abolished completely by heating for 30 sec in a boiling water bath. The activity was augmented about two-fold by adding a reducing agent, such as cysteine. These properties were similar to those of the cholate extract of chicken erythrocyte studied in our laboratory[4].

TABLE I

INCORPORATION OF RADIOACTIVE IRON INTO HEME BY THE PARTICULATE FRACTIONS OF RAT LIVER

A. After incubation of the liver homogenate with $^{59}Fe$ ($3 \cdot 10^5$ counts/min) at 37° for 1 h, the homogenate was fractionated by differential centrifugation. Heme was extracted by HCl–acetone and purified chromatographically[3]. The radioactivity was measured by well-type scintillation counter. B. The liver homogenate was fractionated by differential centrifugation. Each fraction was incubated with $^{59}Fe$ ($1.5 \cdot 10^5$ counts/min) at 37° for 1 h, and the heme isolated and counted as in A. The values given are counts/min in the isolated heme.

| | *Nucleus* | *Mitochondria* | *Microsome* | *Supernatant* |
|---|---|---|---|---|
| A. | 4,520 | 14,900 | 4,720 | |
| B. | 1,250 | 7,080 | 2,380 | 2,260 |

The cholate extract (1 % sodium cholate in phosphate buffer, pH 7.4) was purified by ammonium sulfate fractionation. The activity was found in the fraction obtained between 0.50 and 0.75 ammonium sulfate saturation (Table IIA). This fraction could incorporate $^{59}Fe$ into heme without addition of protoporphyrin even after dialysis. To examine the nature of hemeprotein synthesized, the mitochondrial fraction after incubation with $^{59}Fe$ was extracted by cholate and fractionated with ammonium sulfate. Most of the radioactivity in heme was found in the 0.35–0.50 fraction (Table IIB). Cytochrome *b* has been found in the 0.35–0.50 fraction of the cholate extract by CLARK *et al.*[5].

The present findings support the view of THEORELL[1] and DRABKIN[2] that all aerobic cells possess independent biosynthetic capability for the manufacture of hemeproteins and show that this activity is retained in the isolated particles of cell. The possibility that the 0.50–0.75 fraction of the cholate extract contains a protoporphyrin–protein complex and that the hemeprotein is synthesized by the direct incorporation of iron into the complex is suggested by the fact that this fraction could incorporate radioactive iron into heme without the addition of protoporphyrin. This view is in accord with that of ERIKSEN that "bound" porphyrin is the direct precursor of hemoglobin[6]. He suggested that the protein which forms a complex with protoporphyrin may be globin. The protein in our experiment, however, seems to differ from the protein moiety of any

TABLE II

INCORPORATION OF RADIOACTIVE IRON INTO HEME BY THE CHOLATE EXTRACT (A) AND DISTRIBUTION OF RADIOACTIVE HEMIN IN THE CHOLATE EXTRACT AFTER INCUBATION OF MITOCHONDRIA (B)

A. The cholate extract of mitochondria was fractionated with ammonium sulfate. Each fraction was incubated with $^{59}Fe$ ($5 \cdot 10^5$ counts/min) at 37° for 1 h. The procedure for the purification of heme and measurement of the radioactivity was the same as in Table I. B. After incubation of the mitochondrial fraction with $^{59}Fe$ ($5 \cdot 10^5$ counts/min) at 37° for 1 h, the mitochondria were extracted with cholate and fractionated with ammonium sulfate. The values given are counts/min in the isolated heme.

| | *Ammonium sulfate saturation* | | |
|---|---|---|---|
| | *0–35 %* | *35–50 %* | *50–75 %* |
| A. | 1,820 | 1,750 | 19,100 |
| B. | 2,900 | 10,500 | 1,230 |

known hemeprotein. The possibility may be suggested that in mitochondria, the incorporation of iron into the porphyrin–protein complex occurs by way of a hemeprotein intermediate which transfers the synthesized heme to form the hemeprotein found in the 0.35–0.50 fraction of the cholate extract. The hypothesis of heme transfer has been repeatedly proposed by several authors.

The nature of the 0.50–0.75 fraction and the product of $^{59}Fe$ incorporation is now being investigated.

*Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Tokyo (Japan)*

SHIGEKI MINAKAMI
YOSHIMASA YONEYAMA
HARUHISA YOSHIKAWA

1 H. THEORELL, M. BÉZNAK, R. BONNICHSEN, K. G. PAUL AND Å. ÅKESON, *Acta Chem. Scand.*, 5 (1951) 445.
2 J. B. MARSH AND D. L. DRABKIN, *J. Biol. Chem.*, 224 (1957) 909.
3 J. E. RICHMOND, K. I. ALTMAN AND D. SALOMON, *J. Biol. Chem.*, 211 (1954) 981.
4 Y. KAGAWA, S. MINAKAMI, Y. YONEYAMA AND G. URATA, *J. Jap. Biochem. Soc.*, 29 (1957) 611 (in Japanese).
5 H. W. CLARK, H. A. NEUFELD, AND E. STOTZ, *J. Biol. Chem.*, 210 (1954) 851.
6 L. ERIKSEN, in *Porphyrin Biosynthesis and Metabolism*, J. and A. Churchill, London, 1955.

Received February 10th, 1958

## Further studies on enzymic adrenal 11-β-hydroxylation

11-β-Hydroxylation of the steroid nucleus, occurring in adrenal mitochondria, has been under study for a considerable period in several laboratories[1–4], during which it has become apparent that molecular oxygen provides the hydroxyl oxygen and that reduced triphosphopyridine nucleotide (TPNH) is a necessary reactant.

More recently[5] we have found that two heat-labile enzyme fractions, in addition to TPNH, were required to catalyse the reaction. One of these was extracted from acetone powder of adrenal mitochondria with an aqueous digitonin solution. The second could be prepared from other tissues as well as the adrenal, *e.g.*, liver, spleen, plasma.

In the experiments reported here, deoxycorticosterone, the substrate, was 11-hydroxylated to form corticosterone. The extent of the reaction was assayed fluorimetrically[5].

Because the digitonin used in our earlier adrenal preparation rendered its further purification difficult, the adrenal mitochondrial acetone powder was extracted instead with 10 vol. distilled water ($H_2O$ extract) and the extracted residue was extracted a second time, using 10 vol. 0.15 *M* KCl (KCl extract). The results, some of which differed from those reported earlier[5], are shown in Table I.

Both adrenal extracts (*i.e.*, $H_2O$ and KCl) were apparently necessary in order for the hydroxylation to proceed. Further, it was found that the rate was greatly enhanced if an aqueous extract of rabbit-liver acetone powder were added to the two adrenal fractions. This liver extract could be heated to 100° for several minutes without losing its ability to stimulate the reaction. Several adrenal enzyme preparations were obtained which were completely inactive without the addition of a boiled liver extract. A heat-stable hydroxylation cofactor could also be obtained from acetone powders of human placenta and bovine testis, and from crude adrenal homogenates. It thus appeared that not only were two enzymes and TPNH needed for 11-hydroxylation, but that

TABLE I

COFACTOR REQUIREMENT FOR 11-β-HYDROXYLATION

The reaction mixtures contained 0.04 μmole TPNH, 0.029 μmole deoxycorticosterone, 10 μmoles tris(hydroxymethyl)aminomethane buffer, pH 7.4, enzymes, and other additions in a vol. of 0.57 ml. Incubated 20 min at 37° in air.

| | *Corticosterone found (mμmoles)* |
|---|---|
| $H_2O$ extract | 1.33 |
| KCl extract | 0 |
| $H_2O$ extract + liver extract | 1.66 |
| KCl extract + liver extract | 0.42 |
| $H_2O$ extract + KCl extract | 0.84 |
| $H_2O$ extract + KCl extract + liver extract | 6.72 |
| $H_2O$ extract + KCl extract + boiled liver extract | 5.72 |