The Formation of Verdohaemochrome from Pyridine Protohaemichrome by Extracts of Red Algae and of Liver

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Nakajima, Takemura, Nakajima & Yamaoka (1963) claimed that the conversion of haem into a precursor of biliverdin by ox-liver homogenates was catalysed by an enzyme (haem α -methenyl oxygenase) that required a cofactor extracted from the liver by boiling water. In their assay procedure pyridine protohaemichrome was a better substrate than haemoglobin-haptoglobin complex, the best natural substrate encountered. The product of the reaction was reported by Nakajima (1963) to be different from verdohaemochrome, which is produced by coupled oxidation of pyridine protohaemichrome with ascorbate (Lemberg, Cortis-Jones & Norrie, 1938a, b; Lemberg, 1956). The results of the Japanese workers appeared to indicate that such coupled oxidations with low-molecular-weight reductants played no part in the activity of liver extracts.

In the present investigation we sought similar porphyrin-cleaving activity in red algae, in which bile pigment-protein complexes (biliproteins) participate in the light reactions of photosynthesis (see Ó hEiocha, 1965). Such activity was indeed found in extracts of acetone-dried powders of *Porphyra umbilicalis* (formerly *P. laciniata*) and other red algae that were collected on the shores of Galway Bay. However, our results indicate that this activity, and the activity from ox liver, is entirely non-enzymic, at least in the assay system of Nakajima *et al.* (1963).

Liver extracts corresponding to Nakajima's 'crude enzyme' solution were prepared by homogenizing washed liver macerates in cold 0.1M-phosphate buffer, pH 7.4 (3 vol.), at 4° for 4 min. and keeping the homogenates at 4° for 15 min. Acetone-dried powders of *P. umbilicalis* (prepared at -20°) were extracted with the same buffer (10 ml./g. of powder) at 4° for 48 hr. Extracts were clarified by centrifugation at about 30 000g for two periods of 15 min. each, followed by filtration at 4°. These extracts are referred to below as the cold-water extracts. Boiled-cell extracts were prepared from fresh minced tissue in 0.1M-phosphate buffer, pH 7.4 (3 ml./g. of liver; 4 ml./g. of *P. umbilicalis*), at

90–100° for 15 min., followed by filtration of the extracts. 'Purified cofactor' was prepared according to the method of Nakajima *et al.* (1963).

Reaction mixtures (4 ml.) consisted of 0.1M-veronal buffer, pH 8.3 (1 ml.), pyridine protohaemichrome solution (0.4M-haemin in pyridine) (1 ml.) and samples of tissue extracts and/or the various preparations and fractions obtained from algae and liver. When necessary, an appropriate volume of distilled water was added before these samples to give a final volume of 4 ml. Incubation was at 37° for 30 min. and reaction was terminated by the addition of 10% (w/v) trichloroacetic acid (1.5 ml.). Insoluble material was removed by centrifugation and green product estimated spectrophotometrically at 650 m μ . Assays of preparations containing protein displayed some variability, which was found to be due to adsorption of some substrate and product on precipitated protein. This could be avoided by adding urea (2.5 g.) instead of trichloroacetic acid after incubation, both to denature and to redissolve the protein, all substrate and product thus being kept in solution.

The activity in extracts of *P. umbilicalis* was qualitatively similar to that of liver extracts. Similar activity was found in the red algae *Ceramium rubrum* and *Polysiphonia elongata*. Except where otherwise stated, the following observations apply equally to the systems from both liver and *Porphyra umbilicalis*. The cold-water extracts retained most of their activity when deproteinized with trichloroacetic acid followed by readjustment to pH 7, and also when heated on a boiling-water bath for 15 min. followed by removal of precipitated protein. The boiled-cell extracts and the 'purified cofactor' preparations were active alone and showed no synergistic effect with the cold-water extracts. The activity in all preparations diffused rapidly and completely through dialysis membranes and could be recovered by concentration of the diffusate in a rotary evaporator. When active preparations were filtered through columns of Sephadex G-10 and G-15 only the lowest-molecular-weight fractions, which emerged at the same elution volume as NaCl, were active. All high-molecular-weight fractions prepared from cold-water extracts, whether by dialysis, gel filtration or (NH₄)₂SO₄

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fractionation, were inactive and their addition did not increase the activity of low-molecular-weight active fractions, boiled-cell extracts or 'purified cofactor' preparations.

These experiments show that the haem methenyl oxygenase activity of ox liver and of *P. umbilicalis*, as measured by the assay procedure of Nakajima *et al.* (1963), does not contain any detectable enzymic component.

The low-molecular-weight active factor was most suitably obtained by extraction of minced liver or acetone-dried powders of *P. umbilicalis* for 15 min. at 100° with constant stirring, followed by centrifugation, and had the same solubility properties as the 'cofactor' described by Nakajima *et al.* (1963). All active preparations had reducing power, as measured by titration against 2,6-dichlorophenol-indophenol. When oxygen was passed through the preparations, activity and reducing power decreased concomitantly. The activity was restored by reduction with ascorbate, which was removed before assay by precipitation with a slight excess of HgCl_2 . The active factor therefore appears to be a reductant.

The conversion of pyridine protohaemichrome into the green product required oxygen; 80% degradation of haemichrome was the highest value observed. Spectral analysis indicated that protohaemichrome was initially reduced to protohaemochrome. The relationship between activity and concentration of the active factor was not linear, particularly at extremes of concentrations. Similar relationships were observed in the coupled oxidation of pyridine protohaemichrome with ascorbate or cysteine.

The products of the action of liver and *P. umbilicalis* extracts on pyridine protohaemichrome and of its coupled oxidation with ascorbate were isolated from scaled-up reaction mixtures by following the procedures of Nakajima (1963) and Levin (1966) and were compared by thin-layer chromatography on silica gel G in the solvent systems chloroform-pyridine-water (6:4:1, by vol.) (Levin, 1966) and chloroform-acetic acid (10:5 and 10:8.5, v/v). In each case there was a major green component and a number of minor green and brown components. Apart from some slight variation in the relative amounts of the minor components, the chromatographic patterns of the products from all three reaction mixtures were the same.

On elution from the thin-layer plates and careful rechromatography, the major green components from the three reactions were identical and can be identified as verdohaemochrome, as this is the major product in the coupled oxidation of pyridine protohaemichrome with ascorbate (Levin, 1966). Moreover, the purified major component in each case was spectrally identical with purified verdohaemo-

chrome (Levin, 1966), and was shown in the following way to have retained the iron atom of the protohaemichrome (as does verdohaemochrome). Thin-layer chromatograms of the green products were bleached by spraying lightly with 30% (w/v) H_2O_2 and heating at 100°. They were then sprayed with $\text{K}_3\text{Fe}(\text{CN})_6$ solution and kept overnight or until the iron was located by the appearance of blue spots. These spots coincided exactly with the original green pigment. On heating in conc. HCl at 70° for 15 min. the purified green pigment was converted in about 95% yield into a mixture of biliverdin and bilipurpurin, identified by thin-layer chromatography and spectrophotometry, consistent with the proposed structure of verdohaemochrome (Lemberg & Legge, 1949, pp. 464-468).

It therefore seems that the degradation of pyridine protohaemichrome by the liver and algal extracts is a coupled oxidation with low-molecular-weight reductant(s), very similar to the coupled oxidation of haemichrome with ascorbate. However, the active factors from both liver and *P. umbilicalis* were more than twice as active in verdohaemochrome formation as ascorbate solutions of the same reducing power and were far more active than cysteine. Although NADPH on its own did not degrade pyridine haemichrome, it enhanced the effect of active factor, presumably by regenerating it by reduction during the coupled oxidation.

The level of activity in *P. umbilicalis* showed seasonal variation. Activity was highest in early spring whereas biliprotein content was highest in late spring.

Lemberg suggested that coupled oxidation with ascorbate might reflect the mechanism of the breakdown of haemoglobin to choleglobin *in vivo* (Lemberg, Lockwood & Legge, 1941; Lemberg & Legge, 1949, pp. 475-477; Lemberg, 1956). This seemed to have been discounted by the results of Nakajima *et al.* (1963), but now again must be considered the most substantiated theory for the formation of bile pigments *in vivo*, unless and until assay systems are found that reveal hitherto undetected enzymic mechanisms.

While this paper was in preparation, Levin (1967) published findings on liver homogenates that are in agreement with ours.

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The Relationship between Neurophysin and the Soluble Proteins of Pituitary Neurosecretory Granules

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The polypeptide hormones, oxytocin and vasopressin, have been isolated from pituitary posterior lobes as a hormone-protein complex by several workers. van Dyke and his associates isolated from fresh tissue an apparently homogeneous protein with mol. wt. 30000. It possessed oxytocic, pressor and antidiuretic activities in the same ratios as they occur in the gland (van Dyke, Chow, Greep & Rothen, 1942). Later Acher and his co-workers isolated similar material from acetone-desiccated tissue (Acher, Manoussos & Olivry, 1955; Acher, Chauvet & Olivry, 1956). The hormones were dissociated from the complex by precipitation of the protein constituent, neurophysin, with trichloroacetic acid. The hormone-protein complex dissociates in dilute acid, and this property has been used to isolate neurophysin entirely free from biological activity by gel filtration in 0.1N-formic acid on Sephadex G-25 (Hope, Schacter & Frankland, 1964; Frankland, Hollenberg, Hope & Schacter, 1966). The neurophysin, isolated from acetone-desiccated glands, consisted of several constituents separable by electrophoresis. Seven protein fractions have now been isolated by column-chromatographic procedures (Hope & Hollenberg, 1966; Hollenberg & Hope, 1967); all but one was found to bind the polypeptide hormones. The amino acid analyses of the fractions with binding capacity were similar to one another and to the analysis reported by Block & van Dyke (1952) for the hormone-protein complex isolated from fresh tissue (van Dyke *et al.* 1942).

Surprisingly, neurosecretory granules isolated from the posterior lobes of bovine pituitary glands contain only two soluble proteins (Dean & Hope,

1966). Since the components absent from neurosecretory granules could not be detected in any other subcellular fraction, it was evident that the composition of neurophysin changed during the isolation procedure (Dean & Hope, 1967).

The possibility that the two soluble proteins of neurosecretory granules are partially degraded by the activity of a proteolytic enzyme(s) has now been studied. Since extracts of bovine pituitary posterior lobes have proteolytic activity (Adams & Smith, 1951; Ramachandran & Winnick, 1957; La Bella, Reiffenstein & Beaulieu, 1963; Dean & Hope, 1967), we examined extracts of pituitary posterior lobes in media of different pH values for proteolytic activity and for the protein constituents by starch-gel electrophoresis. A procedure suitable for the isolation of the soluble proteins of the neurosecretory granules from acetone-desiccated pituitary tissue is described.

Methods and materials. Acetone-desiccated powder of bovine pituitary posterior lobes (1.8 i.u. of oxytocic activity/mg.) was extracted by suspending the powder (2.5 g./100 ml.) in a suitable medium. After 18 hr. at 4° the insoluble material was removed by centrifugation. The supernatant was dialysed against distilled water and freeze-dried.

Zone electrophoresis was done in horizontal starch gels according to the method of Smithies (1955) in the buffer system described by Ferguson & Wallace (1961) with a starch concentration of 15 g./100 ml. of buffer. Protein samples (50 µl. of 5 mg./ml.) were dissolved in the same buffer used to prepare the gel. Protein was stained with 0.05% Nigrosine.

Proteolytic activity was assayed by a modification of the method described by Anson (1938). The