Non-Enzymic Nature of the Pyridine Haemochrome-Cleaving Activity of Mammalian Tissue Extracts ('Haem α -Methenyl Oxygenase')

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1. The pyridine haemochrome-cleaving activity of extracts from mammalian liver and other tissues is shown conclusively to be entirely non-enzymic in nature and attributable to coupled oxidation with ascorbate. 2. Reduced glutathione probably contributes to the activity indirectly by continuously regenerating the ascorbate to the reduced form. 3. The cleavage shows no specificity for the α -methine bridge of pyridine haemochrome. 4. Results are presented suggesting some probable reasons for the erroneous characterization of the activity as an α -methine-specific haem-cleaving enzyme ('haem α -methenyl oxygenase') by Nakajima and co-workers (e.g. Nakajima, Takemura, Nakajima & Yamaoka, 1963; Nakajima & Gray, 1967).

The extensive studies by H. and O. Nakajima and co-workers on the pyridine haemochrome-cleaving activity of mammalian tissue extracts led them to propose a soluble, mainly hepatic, enzyme system, 'haem α -methenyl oxygenase', which required NADPH, Fe^{2+} and another, endogenous, cofactor for full activity (see, e.g., Nakajima, Takemura, Nakajima & Yamaoka, 1961*a,b,c*, 1963; H. Nakajima, 1963; O. Nakajima, 1962*a,b*, 1963, 1964, 1967; Nakajima & Gray, 1967, 1969*a,b*). This system cleaved pyridine haemochrome to a green product that was considered to have the formyl-biliverdin structure shown in Scheme 1 and that could be converted into biliverdin by mild acid hydrolysis (H. Nakajima, 1963). [The term 'haemochrome' is preferable to the older and misleading 'haemochromogen' and 'haemichromogen'. The term as used here is not necessarily meant to imply the reduced (ferrous) form, which should be indicated by italicizing the *o*, i.e. haemochrome (Lemberg & Legge, 1949; Murphy, 1968).] Haem α -methenyl oxygenase was considered to function on the main route of haem catabolism *in vivo*, operating as shown in Scheme 1, and was claimed to be very active with haemoglobin-haptoglobin complex, which was therefore proposed as the natural substrate (Nakajima *et al.* 1963; Nakajima, 1967). Kench, du Toit & Green (1963) and also R. Schmid (in a comment at the end of Kench *et al.* 1963) briefly reported difficulties in their efforts to duplicate the high yields of green product reported by Nakajima *et al.* (1961*a,b,c*). Murphy, Ó hEocha & Ó Carra (1967) and Levin (1967) showed that extracts similar to those studied by Nakajima and co-workers contained a heat-stable low-molecular-

weight component capable of promoting non-enzymic conversion of pyridine haemochrome into verdohaemochrome, a green pigment very similar in spectral properties to the product described by H. Nakajima (1963) and similarly convertible into biliverdin in acid (Scheme 1). Ascorbate and some other low-molecular-weight reductants promote a similar reaction by coupled oxidation with pyridine haemochrome (Scheme 1).

It seemed possible that the activity studied by Nakajima and co-workers was mainly composed of this non-enzymic component. However, Nakajima & Gray (1967) subsequently published results indicating that the liver extracts promoted cleavage specifically at the α -methine bridge of pyridine haemochrome. Since this same specificity is a highly characteristic property of haem catabolism *in vivo*, and since the coupled oxidation of pyridine haemochrome with ascorbate occurs non-specifically at all methine bridges (Rüdiger, 1968; Ó Carra & Colleran, 1969*a*), it seemed that the haemochrome-cleaving liver extracts must contain a significant directional enzymic component after all. Subsequent publications by Nakajima & Gray (1969*a,b*) report further studies on pyridine haemochrome cleavage by tissue extracts, but no reference is made to the possibility of a non-enzymic component. We have therefore re-examined the activity in detail and here show conclusively that the pyridine haemochrome-cleaving activity is non-enzymic and non-specific and due to coupled oxidation with ascorbate, probably backed up by other reductants such as GSH. We have already presented a summary of the main points in abstract form (Colleran & Ó Carra, 1969).

Scheme 1. Upper right-hand sequence: summary of the enzymic scheme proposed by H. Nakajima (1963) and Nakajima (1967) for the conversion of pyridine haemochrome and haemoglobin-haptoglobin complex into biliverdin. Lower left-hand sequence: summary of the non-enzymic coupled oxidation of pyridine haemochrome with ascorbate (represented by AH_2), basically according to Lemberg (1956). X and Y represent two pyridine ligands in pyridine haemochrome; in haemoglobin-haptoglobin complex X represents haptoglobin-globin and Y represents a hydroxyl ligand (P = propionic acid side chain).

MATERIALS AND METHODS

Haemin (twice crystallized from bovine blood) was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Meso-haemin was prepared by hydrogenating haemin in formic acid over Pd and reinserting the iron (Falk, 1964). Haemato-haemin was prepared by similar insertion of iron into haematoporphyrin (crystalline, purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.). Ascorbate oxidase was extracted from vegetable-marrow rind and purified to the second $(\text{NH}_4)_2\text{SO}_4$ fractionation step as described by Dawson & Magee (1955). NADPH, catalase and GSH were obtained from Sigma Chemical Co. All other chemicals and solvents were analytical grade.

Male Hartley-strain guinea pigs weighing 300–400 g and male Wistar rats weighing 250–350 g were stunned by a blow on the head and bled. The organs were removed (the livers first being perfused with ice-cold 0.9% NaCl) and cooled immediately by immersion in ice-cold 0.9% NaCl.

Homogenization of the organs in 0.1 M-sodium phosphate buffer, pH 7.4, and preparation of the 'crude enzyme' was carried out within 15 min. Bovine liver, spleen and kidney samples were obtained from freshly killed animals of a variety of domestic breeds, cooled immediately on ice and homogenized within 30 min. Homogenization and extraction of the tissues in 0.1 M-sodium phosphate buffer, pH 7.4, and preparation of the 'crude enzyme' were carried out exactly as described by Nakajima *et al.* (1963). The protein concentrations of the preparations from ox and guinea-pig liver were in the range 15–20 mg/ml.

Assay of the haem-cleaving activity (1 ml of 0.4 mM-haemin solution in pyridine as substrate/4 ml assay mixture), was carried out as described by Nakajima *et al.* (1963) and Murphy *et al.* (1967) in open wide-mouthed vials that were shaken continuously during the 30 min assay period. The 0.5 M-sodium veronal buffer, pH 8.3, originally used by Nakajima *et al.* (1963) is very difficult to work with since the veronal tends to come out of solution

at this molarity. In many experiments 0.5M-tris-HCl buffer, pH8.3 (used by Nakajima & Gray, 1969a) or 0.1M-sodium veronal or 0.1M-glycine-NaOH buffers, pH8.3, were used instead. Owing to the high pyridine concentration characteristic of this assay system, the final pH was generally about 9. Variation of the type and concentration of the buffer or adjustment of the final pH to values between 7 and 9 made little difference to the results reported here. The same procedure was used to assay the haemochrome-cleaving activity of ascorbate solutions. The ascorbate solution (typically 0.035%) in 0.1M-sodium phosphate buffer, pH7.4, was added to the assay mixture in place of the 'enzyme' preparation.

In studying the heat-stabilities, 1ml samples of the extracts or ascorbate solution were run into assay tubes, previously equilibrated at the appropriate temperature (e.g. 90°C), and swirled for 15s (the temperature of the 1ml sample reached that of the water bath within this period). After the appropriate period of heating, the tube was cooled in an ice-water bath and the assay was carried out as usual. In many cases precipitated protein in the heat-treated samples hindered free diffusion of oxygen during the assays. This resulted in a further spurious decrease in haem-cleaving activity that could be eliminated by stirring during the incubation.

The product of the incubation of pyridine haemochrome with tissue extracts or ascorbate was isolated both as described by H. Nakajima (1963) and by Levin (1966) and was characterized spectrophotometrically and chromatographically as described by H. Nakajima (1963), Levin (1966, 1967) and Murphy *et al.* (1967). The isomer composition was studied both by the analytical t.l.c. method of Ó Carra & Colleran (1969b, 1970a) and the degradative dichromate method of Rüdiger (1969).

RESULTS AND DISCUSSION

Jackson & Kenner (1968) have pointed out that the formylbiliverdin structure formulated by Nakajima and co-workers (Scheme 1) is unlikely from a biological point of view, and the subsequent hydrolysis step (Scheme 1) is even more unlikely from a chemical point of view, involving as it does the hydrolysis of a carbon-carbon bond. The original coining of the name 'haem α -methenyl oxygenase' (by Nakajima *et al.* 1963) seemed premature since (a) haem itself did not act as 'substrate', (b) there was no evidence at the time for α -methine specificity, and (c), although oxygen was required for the reaction, the system had not been rigorously shown to be an oxygenase. The proposal of haemoglobin-haptoglobin complex as the natural substrate seems to have been based mainly on the evidence that a crude preparation of this complex, when added to crude fractions of liver extracts, increased the oxygen uptake; the product of this system does not appear to have been characterized, apart from a 'dinitrophenylhydrazone' (O. Nakajima, 1962a,b, 1963; Nakajima *et al.* 1963).

Nature of the activity. We have repeated the published procedures of Nakajima *et al.* (1963) and

of Murphy *et al.* (1967) and Levin (1967) on numerous occasions and in general our results were in agreement with the latter authors, and indicated at least a very considerable non-enzymic component in the pyridine haemochrome-cleaving activity that was diffusible through dialysis membranes and that emerged with the low-molecular-weight fraction (mol.wt. <200) from Sephadex G-10 columns. No protein fractions could be found that enhanced the activity of this low-molecular-weight fraction.

The haemochrome-cleaving activity was consistently found in the low-molecular-weight fraction of bovine and guinea-pig liver extracts and similar results were obtained with all other tissue extracts displaying pyridine haemochrome-cleaving activity (e.g. rat liver and bovine, guinea-pig and rat spleen and kidney). This is consistent with the idea that coupled oxidation with ascorbate might be the basis of the activity, but Murphy *et al.* (1967) considered that liver extracts were more than twice as active as could be accounted for by their ascorbate contents. Some of this discrepancy seems to have been due to a failure to make adequate allowance for the non-linear relationship between activity and ascorbate concentration discussed below. Eliminating this source of error, we still observe a smaller discrepancy (about 50%) between the activity of ascorbate solutions in the range 0.01–0.035% and liver extracts of the same ascorbate concentrations. This could be accounted for by GSH and/or other reductants continually regenerating the ascorbate to the reduced form and thereby indirectly contributing to the activity. Such an effect can readily be demonstrated in a protein-free system: for example, at pH7.5 the activity of a solution containing 0.02% ascorbate and 7mM-GSH was double that of 0.02% ascorbate alone, although the GSH on its own showed only slight activity.

The stimulation reported by Nakajima *et al.* (1963) with NADPH and Fe^{2+} may have been due to a similar reinforcing effect and the unidentified endogenous cofactor, whose properties and partial purification were also described by Nakajima *et al.* (1963), may well have been GSH. It also seems possible that ascorbate and/or dehydroascorbate, loosely bound to protein, could have come through the steps described by Nakajima *et al.* (1963) for the preparation of their 'enzyme' fraction and could have been activated by such a cofactor preparation.

The fact that catalase failed to inhibit the activity of liver extracts significantly has been cited by Nakajima *et al.* (1963) as proof that a peroxide-generating system could not be involved and, by implication, that coupled oxidation with ascorbate was ruled out (since the mechanism of the coupled oxidation has been supposed to involve peroxidation of the haemochrome by peroxide formed on reduction of molecular oxygen by the ascorbate). We have

confirmed the lack of inhibition reported by Nakajima *et al.* (1963), but we have also found the coupled oxidation of pyridine haemochrome with ascorbate is not significantly inhibited by a similar concentration of catalase (3000 Sigma units/4ml incubation mixture). The breakdown of pyridine haemochrome by hydrogen peroxide is completely inhibited by such concentrations of catalase under the same conditions. Higher concentrations of catalase (10000 Sigma units) caused partial (about 25%) inhibition of the activity of the ascorbate system, but had the same effect on incubations of pyridine haemochrome with liver extracts. It thus appears that free peroxide is not an essential intermediate in either system and so any arguments based on such an assumption are not valid.

When portions of guinea-pig liver extracts were preincubated with ascorbate oxidase (150 units at pH 5.6 at 30°C) 80% of the pyridine haemochrome-cleaving activity was abolished within 5 min, proving that ascorbate is an essential ingredient of the system. (The residual activity may be due to other endogenous reductants such as cysteine and GSH.)

A fundamental discrepancy between the results of Nakajima and co-workers (e.g. Nakajima *et al.* 1963) and those of Murphy *et al.* (1967) and Levin (1967) concerns the heat-lability of hepatic pyridine haemochrome-cleaving activity. Whereas the first group reported complete abolition of the activity by

heating at 80–100°C, the last two found the activity to be stable towards this treatment. We found some extracts to be heat-stable, but most were heat-labile to various degrees, and some gave results superficially very suggestive of enzymic activity. The differences in stability were related to the initial activity. Highly active or concentrated extracts appeared to be stable, whereas dilute extracts, with a relatively low activity, were heat-labile. However, even in dilute extracts the activity survived short periods of heating, and very little activity was lost by holding the extracts at 100°C for 2 min. Ascorbate solutions with the same pyridine haemochrome-cleaving activity displayed a similar heat-lability under the same conditions. A typical result is illustrated in Fig. 1. It is clear from this that the heat-lability of the activity of the liver extracts is not characteristic of an enzyme system but is very similar to that of ascorbate.

The relationship between the pyridine haemochrome-cleaving activity and the concentration of ascorbate was found to be sigmoidal rather than linear, as illustrated in Fig. 2. This relationship seems to be due to a critical balance of ascorbate and oxygen concentrations and it varies with oxygen partial pressure. This same characteristic relationship was found also with liver extracts (Fig. 2). Levin (1967) and Murphy *et al.* (1967) had indicated that the relationship between concentration and activity of liver extracts was not linear, and some results published by Nakajima and co-workers

Fig. 1. Heat-stability at 90°C (in 25 mM-sodium phosphate buffer, pH 7.4) of the pyridine haemochrome-cleaving activity of 0.035% ascorbate (●) and of ox liver extract (○). Portions (1 ml) were incubated at 90°C for various periods, cooled and then assayed as usual (final volume of assay mixture 4 ml).

Fig. 2. Relationship between pyridine haemochrome-cleaving activity and concentration of ascorbate (●) and ox liver extract (○). Units of haemochrome-cleaving activity are as defined by Nakajima *et al.* (1963); concentrations are in terms of no. of ml of 0.033% ascorbate or ox liver extract added/4 ml assay mixture. (The ascorbate concentration was chosen to match the activity of the liver extract.)

(e.g. Fig. 6 of Nakajima *et al.* 1963) show clearly that their preparations gave a relationship similar to that reported here as characteristic of ascorbate. This relationship explains the reason for the apparently different heat-stabilities of extracts of high and low activity. At high concentrations corresponding to the horizontal portion of the curve in Fig. 2 limited destruction of ascorbate does not lead to a decrease in activity, but at concentrations corresponding to the sloping portion of the curve activity decreases rapidly with destruction of ascorbate.

Detailed analysis of these results revealed no evidence of an enzymic component in the activity of the liver extracts. Further, when incubation mixtures were made up in 8M-urea, no decrease in activity was observed. Also, the high concentration of pyridine (25%) characteristic of the assay system used by Nakajima and co-workers was found to be necessary for the activity. Lowering of the pyridine concentration to 10% inhibited the process almost completely whereas raising it to 40% did not depress the activity. This dependence on such a high pyridine concentration is again a very unlikely characteristic for an enzymic system. However, just this same dependence is characteristic of the ascorbate system, where it seems to be a solvent effect, most of the pyridine being replaceable by some other organic solvent, e.g. ethanol. The initial step in the coupled oxidation (hydroxylation of a methine bridge of the haemochrome to yield a green oxyhaemin derivative) is much accelerated in a non-aqueous medium, e.g. 95% ethanol containing only 5% pyridine (Ó Carra & Colleran, 1970b). When portions of the liver extracts were added to solutions of pyridine haemochrome in ethanol (in the ratio 1:3) the same accelerated formation of the green hydroxylated derivative was observed.

Nature of the product and specificity of the reaction. In agreement with the reports of Murphy *et al.* (1967) and Levin (1967), the product of the aerobic action of liver or other tissue extracts on pyridine haemochrome was in all cases spectrally and chromatographically identical with verdohaemochrome obtained by coupled oxidation with ascorbate, and no evidence whatever was obtained in favour of the 'formylbiliverdin' structure. We have verified Murphy's (1968) contention that the green crystalline material prepared from veronal-buffered incubation mixtures as described by H. Nakajima (1963) and Nakajima & Gray (1967) is mainly composed of crystals of veronal with adsorbed or occluded verdohaemochrome. It seems possible that the results on which the formylbiliverdin structure was based by H. Nakajima (1963) were obtained with such contaminated material. When veronal buffer is replaced by tris or glycine buffer no crystals are obtained, although the green

product is formed at the same rate. It is significant that in two published experiments in which veronal buffer does not seem to have been used O. Nakajima (1963, 1964) reported failure to crystallize the green product.

O. Nakajima (personal communication) has claimed that 'haem α -methenyl oxygenase' displays a specificity for the haemochrome of protohaemin and does not promote the breakdown of other haemochromes, including mesohaemochrome. We have verified that the activity is much greater with the protohaemochrome, but green products are also formed slowly from the meso and haemato compounds. The ascorbate system, however, displays exactly the same preference for the protohaemochrome, and it is clear that this effect is dependent on differences in the intrinsic reactivities of the various haemochromes rather than an enzymically determined specificity.

Nakajima & Gray (1967) concluded that guinea-pig liver extracts promote a specific cleavage at the α -methine bridge of pyridine haemochrome, a specificity not characteristic of the ascorbate system (Rüdiger, 1968; Ó Carra & Colleran, 1969a). Their conclusions were based on the application of the permanganate degradation method of Nicolaus (1960) to the crystalline green product isolated from veronal-buffered incubation mixtures and discussed above. The chromatographic micro-method of isomer analysis of biliverdins developed by Ó Carra & Colleran (1969a, 1970a) is a much more sensitive and reliable method, and we have applied this to the product obtained after incubation of pyridine haemochrome with various tissue extracts, avoiding contamination with veronal by using tris or glycine buffers. Table 1 lists some typical results, and also the isomer composition of the product obtained with ascorbate. The compositions are almost identical and indicate non-specific cleavage at all four methine bridges. Changing the pH of

Table 1. *Isomer composition of the products of pyridine haemochrome cleavage by various tissue extracts and by ascorbate*

Product of cleavage by	Isomer composition (%)			
	IX α	IX β	IX γ	IX δ
Ascorbate	32	25	23	20
Ox liver	28	27	25	20
Guinea-pig liver	31	25	24	20
Rat liver	29	28	24	19
Guinea-pig spleen	31	25	24	20
Sheep spleen	28	27	23	22

The products were analysed by the t.l.c. method of Ó Carra & Colleran (1969a, 1970a). Other experimental details are given in the Materials and Methods section.

the incubation mixture or the concentration of pyridine did not significantly change the isomer composition obtained with guinea-pig or ox liver extracts; nor did the addition of catalase to the incubation mixtures have any effect (eliminating the possibility that the formation of free peroxide might be causing the non-specific cleavage). These results were carefully checked by using the highly sensitive dichromate degradation technique of isomer analysis developed by Rüdiger (1968, 1969) and the results fully confirmed the non-specific nature of the hepatic pyridine haemochrome-cleaving activity. W. Rüdiger (personal communication) has independently confirmed this for guinea-pig liver extracts. On the other hand, Nichol & Morell (1969) concluded that guinea-pig liver extracts probably cleaved pyridine haemochrome specifically at the β - or the δ -methine bridge. We have considered their rather complex technique of isomer analysis elsewhere (Ó Carra & Colleran, 1970a) and have shown that it is open to serious misinterpretation.

These results, taken as a whole, indicate that the pyridine haemochrome-cleaving activity of liver extracts is entirely non-enzymic and attributable primarily to ascorbate. The non-specific nature of the cleavage renders its biological significance extremely doubtful. Some other reported haem-cleaving systems (Tenhunen, Marver & Schmid, 1968, 1969; Ó Carra & Colleran, 1969a,b) are probably of biological significance, but it should be pointed out that these systems act on haemin, not pyridine haemochrome, and are inactivated by the high pyridine concentrations used by Nakajima and co-workers. Tenhunen *et al.* (1968, 1969) have demonstrated haem-cleaving activity associated with spleen microsomes, and we have shown (Ó Carra & Colleran, 1969a,b) that the apoproteins of a number of haemoproteins are capable of acting as haem-cleaving oxygenases with various degrees of specificity for the α -methine bridge with ascorbate, or in one case NADPH, as cofactor. This specificity is absolute with myoglobin, and seemingly also with a microsomal haemoprotein (possibly cytochrome P-450) that may be responsible both for the activity described by Tenhunen *et al.* (1968, 1969) and also for the rapidly turning-over fraction of microsomal haem revealed by the results of Garner & McLean (1969) and Levin & Kuntzman (1969).

Nakajima *et al.* (1963) and Nakajima (1967) have proposed another enzyme, 'haem α -methenyl formylase', which is supposed to catalyse the conversion of formylbiliverdin into biliverdin (see Scheme 1). This 'enzyme' has been referred to in a number of review articles (With, 1968; Ó hEocha, 1968), but its original proposal appears to have been based mainly on reasoning *a priori*, assuming the existence of a formylbiliverdin substrate, and the

rationale behind this reasoning now seems untenable.

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