The Alleged Presence and Role of Monoiodohistidine in Mitochondrial Oxidative Phosphorylation*

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ABSTRACT: The monoiodinated product of histidine has been prepared and is shown, on the basis of nuclear magnetic resonance (nmr) evidence, to be 5-monoiodohistidine and not, as previously supposed, 2-monoiodohistidine. A careful analysis of extracts of beef heart mitochondria using thin layer chromatography (tlc), paper chromatography, amino acid analysis, and molecular sieving indicates that the claim of L. E. Perlgut and W. W. Wainio [*Biochemistry 5*, 608 (1966)], that monoiodohistidine is present in such extracts, cannot be upheld. Monoiodohistidine had no observable effect on mitochondrial oxidative phosphorylation.

Perlgut and Wainio (1966) reported that synthetic MIH[1] increased the efficiency of ATP formation (P/O ratio) and respiratory control of isolated beef heart mitochondria. In attempting to demonstrate the presence *in vivo* of MIH, the same authors observed that crude mitochondrial extracts contained a component (or components) possessing similar R_F values to those of synthetic MIH in a number of paper chromatographic systems.

Our interest in these observations arose from the chemical relationship of the iodohistidines with those halogenated imidazoles and benzimidazoles whose effect on respiring mitochondria we had investigated (Beechey, 1966). We were disturbed, however, by the scarcity of chemical evidence presented by Perlgut and Wainio for the identity of their unknown component with MIH, and also by the apparent lack of any correlation between concentration of MIH added and the effect produced in their biochemical experiments. Accordingly, we set out to repeat and extend their work.

Materials and Methods

Preparation of MIH and DIH. MIH was prepared both by the method of Brunings (1947) and also by an adaptation of the method of Glazer and Sanger (1964). The first preparation, employing the Brunings method, followed the published procedure precisely, except that reduction in volume of the iodine-free aqueous solution was achieved by freeze drying and the final recystallization was directly from dilute HCl without addition of aniline.

In a subsequent preparation, the iodine-free, freeze-dried product was chromatographed on a silica gel column in a chloroform–methanol–ammonia (2:2:1) system and 5-ml fractions were collected. The MIH was in fractions 35–75. These fractions were neutralized with HCl and the NH_4Cl was removed by methanol extraction and, finally, by sublimation.

The alternative preparation was as follows. Histidine hydrochloride (3 g) was dissolved in 500 ml of 0.2 N NaOH, and the solution was stirred in an ice bath. Iodine monochloride (3 g) was dissolved in hexane (120 ml). Hexane (100 ml) was added to the histidine solution and then the iodine monochloride solution was added at a rate such that the color produced by each drop had disappeared before addition of the next. This addition took about 80 min. The mixture was allowed to stir for 30 min and then the two phases were separated and the water was washed three times with 100 ml of hexane. The washed aqueous phase was acidified with HCl to pH 5 and freeze dried. The freeze-dried material was leached with warm methanol. The methanol solution was reduced to dryness and the MIH was recrystallized as before.

The material purified in this way contained a trace of DIH. It could be further purified by recrystallization from glacial acetic acid or a mixture of glacial acetic acid and acetone. The analytical sample prepared in this way melted at 164–170°.[2] *Anal.* Calcd for $C_6H_8IN_3O_2 \cdot 2HCl \cdot 2H_2O$: C, 18.5; H, 3.6; Cl, 18.2; I, 32.5; H_2O, 9.3; N, 10.8. Found: C, 19.0; H, 3.7; Cl, 18.2; I, 32.4; H_2O (by loss in weight at 105°), 9.5; N, 10.8. The titration curve measured on a Radiometer TTTl automatic titrator was virtually identical with that recorded by Brunings.

DIH was prepared by the method of Brunings. The observed melting point was 117–120° (lit. (Brunings, 1947) mp 120°).

Preparation of Mitochondria. Rat heart and beef

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[1] Abbreviations used in this work: 5-monoiodohistidine, MIH; 2,5-diiodohistidine, DIH; adenosine triphosphate, ATP; adenosine diphosphate, ADP; ferric ferricyanide–arsenious acid, FFCA.

[2] Brunings (1947) gives 204–206° as the melting point of the monohydrochloride.

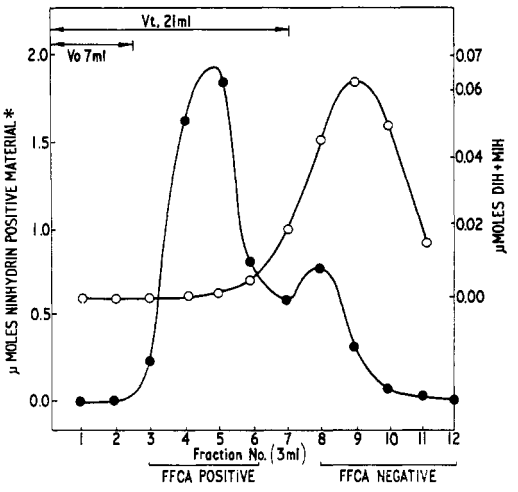


FIGURE 1: Chromatography of mitochondrial extract on a Bio-Gel P-10 column. O, elution pattern of MIH and DIH; ●, elution pattern of mitochondrial extract from 132 mg of heavy beef heart mitochondria; *, assayed using histidine as standard

heart mitochondria were prepared essentially by the method of Chance and Hagihara (1961). Rat liver mitochondria were prepared by disrupting rat liver in 0.25 M sucrose and centrifuging according to the method of Aldridge (1957).

Assay of Protein Concentration. Protein was measured by the biuret method (Gornall *et al.*, 1949).

Preparation of Mitochondrial Extracts. Extract A was prepared as follows. Heavy beef heart mitochondria (4 g of protein), suspended in 0.25 M sucrose, were freeze dried. The freeze-dried material was extracted by shaking for 30 min with 1 l. of 95% ethanol (adjusted with 1 N KOH to an indicated pH of 13 measured with a glass electrode) and centrifuged at 500*g* for 10 min. The supernatant was evaporated at 50° to a volume of approximately 30 ml, acidified with 1 N HCl to an indicated pH 8, and further evaporated. It was necessary to use 60 ml of water to dissolve the sediment, giving an opalescent solution. This extract was used routinely.

Extract B was prepared as follows. A suspension of heavy beef heart mitochondria (20 ml, 4 mg of protein/ml) in 0.25 M sucrose plus 0.002 M EDTA, pH 7.4, was added to 40 ml of a solution containing 0.01 M α-ketoglutarate, 25 μM ATP, and 0.005 M Tris chloride buffer, pH 7.4. The mixture was incubated for 30 min with occasional shaking at 25°. The mitochondria were removed by centrifugation and extracted by shaking for 30 min with 20 ml of 95% ethanol at an indicated pH of 12.7. After centrifugation the volume of the extract was reduced to 1 ml at 50°. The extract was acidified with 0.05 ml of concentrated HCl.

Extract C was prepared as follows. Rat heart mitochondria (122 mg of protein), suspended in 1.35 ml of 0.25 M sucrose, were added to 30.5 ml of ethanol at an indicated pH 12.7. This mixture was shaken for 30 min on a mechanical vibrator. The mitochondria were removed by centrifugation in an MSE bench centrifuge. The supernatant fluid was taken to dryness in a rotary evaporator at 50° and the residue was suspended in 2 ml of water, giving an opalescent solution.

Detection of Compounds on Thin Layer Chromatography (tlc) Plates and Paper Chromatograms. The ferric ferricyanide–ferric chloride–sodium arsenite [FFCA] spray was prepared by the method of Postmes (1963). After development, the paper or thin layer chromatography plates were air dried, exposed to unshielded ultraviolet light from a Hanovia-type mercury lamp for 30 min, and then sprayed. The plates were allowed to develop in the dark for 20–30 min; sensitivity 3×10^{-11} mole of MIH. The detection of compounds by ninhydrin and the Pauly reagent followed standard procedures (Dawson *et al.*, 1959).

Gel Filtration of the Mitochondrial Extract A. The extract was partially purified by passing it through a Bio-Gel P-10 column. The gel (20 g) was allowed to swell for 2 days in distilled water and then poured into a glass column fitted with a "Perspex" plug covered with nylon netting. The void volume of the column was determined using Sephadex Blue Dextran; this volume was 7 ml (see Figure 1). The internal volume of the gel was assumed to be not greater than 30% of the total column volume; the latter was 21 ml. The elution volumes of MIH and DIH were determined by passing them through the column and assaying for ninhydrin-positive material. The crude extract was placed on the Bio-Gel P-10 column and was eluted with distilled water. All fractions were tested with ninhydrin using histidine as a standard.

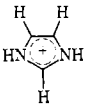
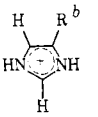
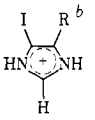
Hydrolysis of the Mitochondrial Extracts. A 0.5-ml aliquot of the crude extract (A or B) was hydrolyzed by adding 0.2 ml of 10% ethanolic KOH. The tube was stoppered with a glass bead and placed in a boiling water bath for 20 min. The extract was cooled to 0° and neutralized with 0.04 ml of 60% perchloric acid. The precipitate of $KClO_4$ was removed by centrifugation.

Amino Acid Analysis. An EEL amino acid analyzer was calibrated with standard amino acids and synthetic MIH and DIH, using a 15-cm column of 8% cross-linked sulfonated polystyrene resin and elution with 0.14 M sodium citrate buffer, pH 4.37. Retention times of 85.6 ± 2.7 min for MIH and 113.5 ± 2 min for DIH were obtained at 50°, flow rate 0.5 ml/min. The elution sequence of the basic amino acids was MIH, tryptophan, DIH, lysine, and histidine.

Results

Constitution of MIH. This was achieved by nuclear magnetic resonance (nmr) spectroscopy. The chemical shifts of the ring protons in imidazole hydrochloride, histidine hydrochloride, and monoiodohistidine hydrochloride (in parts per million relative to water) are shown in Table I. The assignments of the absorption bands to the 2 or 5 positions of the imidazole ring are obtained from the weights of the two peaks for the case of imidazole hydrochloride. It is clear that on

TABLE 1: Nmr Spectra of Imidazole Hydrochloride, Histidine Hydrochloride, and Iodohistidine Hydrochloride in D_2O.[a]

Compound	Structure	2 Position	4(5) Position
Imidazole hydrochloride		−3.70 (1 H)	−2.47 (2 H)
Histidine hydrochloride		−4.07 (1 H)	−2.70 (1 H)
Iodohistidine hydrochloride		−4.17 (1 H)	No absorption

[a] Figures quoted are in parts per million relative to water. [b] R = $CH_2CH(NH_2)CO_2H$.

introduction of the iodine atom, absorption due to the 5 proton is removed, while that of the 2 proton survives and is moved downfield by 0.1 ppm. Monoiodohistidine is thus correctly described as 5-monoiodohistidine.

When Brunings first synthesized MIH in 1947, he suggested that the most likely constitution for the compound was that in which the iodine occupies position 2, in analogy with the constitution of monoiodoimidazole. This constitution has been widely accepted and quoted, although Brunings himself recognized clearly the lack of proof for his formulation. In fact, the analogy with monoiodoimidazole is not strictly valid, since this compound is prepared by deiodination of triiodoimidazole, while MIH is prepared by stepwise iodination of histidine.

A comparison of the paper chromatographic R_F values obtained by us for MIH with those quoted by Perlgut and Wainio (1966) for the compound which they prepared, but did not characterize, taken together with their method of synthesis, makes it likely that the compound which they used as a reference in their work was indeed, as they supposed, MIH.

Is MIH Present in Mitochondrial Extracts? Paper chromatography of the mitochondrial extract A confirmed the observation of Perlgut and Wainio that an FFCA-positive material with an R_F similar to that of MIH was present in the extract. Using the solvent system *n*-butyl alcohol–acetic acid–water (78:5:17) (system I), the R_F for MIH was 0.08 and that for KI was 0.19; our extract contained materials with R_F values of 0.08 and 0.18. Using the solvent system chloroform–methanol–17% ammonia (2:2:1), the R_F for MIH was 0.63; the extract contained materials with R_F values of 0.62 and 0.24. However, there were indications of the presence of other FFCA-positive materials in the extract.

The extracts A and C were also analyzed using tlc on silica gel G and GF plates and the solvent system I. After ultraviolet irradiation and spraying with the FFCA reagent, we could observe materials with R_F values which were similar to both MIH and DIH (see Table II). The presence of other FFCA-positive material in the extract was also observed, and we could separate the extract into seven discrete spots. Many of these spots were positive to ninhydrin, and at least three gave positive reactions to the Pauly reagent. All spots were positive to Rhodamine 6G. In all the solvent systems used, two bands which gave positive reactions with the FFCA, ninhydrin, and Pauly reagents ran with R_F values almost identical with those of MIH and DIH.

With this apparent confirmation and extension of the work of Perlgut and Wainio, we attempted to isolate MIH and DIH from mitochondria. The experimental plan was to separate MIH and DIH from mitochondrial extracts using an amino acid analyzer fitted with a split-stream device. The retention times of MIH and DIH showed that they could be separated from the basic amino acids. The isolated iodinated amino acids were then to be analyzed by mass spectrometry. As the mitochondrial extracts were opalescent, we attempted to clarify them by centrifugation at 140,000*g* for 2 hr, but were unable to do so. Gel filtration, using a polyacrylamide gel (Bio-Gel P-10), was attempted. As can be seen in Figure 1, synthetic MIH and DIH are slightly retained by the gel (their elution volumes were greater than the total column volume). When the mitochondrial extract was eluted from the column, two

TABLE II: Diagrammatic Representation of the Migration of Mitochondrial Extracts on Thin Layer Chromatography on Silica Gel G.[a]

Rel Position of Spots			Reaction of Spots with Reagents			
Std		Extract	FFCA	Pauly	Rhodamine 6G	Ninhydrin
			Solvent Front (10 cm)			
		0	−	+	−	−
		0	+	+	+	+
		0	+	−	+	+
DIH	0	0	+	+	+	+
		0	+	+	+	+
MIH	0	0	+	+	+	+
Histidine	0	0	+	+	+	+
		0	+	−	+	+
			Origin			

[a] Solvent system: butanol–acetic acid–water (78:5:17). +, positive reaction with reagent. −, no reaction with reagent. 0, relative position of spots after development of chromatogram.

distinct peaks of ninhydrin-positive material were observed. The first contained the opalescent material and was eluted without retention, and the second peak had approximately the same elution volume as MIH and DIH. The fractions from each peak were pooled and freeze dried. An aliquot of each freeze-dried fraction was chromatographed on tlc silica gel G plates using system I. After ultraviolet irradiation, FFCA-positive material with R_F values similar to MIH and DIH was present in the first peak (see Figure 1). Aliquots of the second peak did not yield any FFCA-positive material regardless of the quantity used.

A large volume of the ethanolic extract A (equivalent to 2.0 g of protein) was fractionated on the P-10 column and fractions with the same elution volume as MIH and DIH were concentrated and subjected to analysis in the amino acid analyzer. No ninhydrin-positive material was present in the elution position of MIH. A small amount of some component was eluted at the DIH position, but the absorption characteristics of the color produced with ninhydrin were different from those of DIH.

To check that the MIH was not complexed with lipid, the extracts were hydrolyzed in ethanolic KOH. Control solutions of 0.5 μmole of MIH plus extract A or B, 0.5 μmole of DIH plus extract A or B, and 0.5 μmole of MIH and 0.5 μmole of DIH alone were treated similarly. Various aliquots of the neutralized hydrolysate were analyzed by tlc with the accompanying controls using system I. All FFCA-positive material from the mitochondrial extract had been destroyed by this treatment, but the controls showed that added MIH and DIH were unaffected. There was no detectable iodide spot after tlc of the control hydrolysates.

The above evidence suggested that the FFCA-positive material might well be associated with lipid and not MIH. Using silica gel G in the solvent system chloroform–methanol–water (65:25:4), the FFCA-positive material could be separated from MIH and DIH. Furthermore, extraction of lipid from heavy beef heart mitochondria by the method of Folch *et al.* (1956) and tlc on silica gel G as above yielded four FFCA-positive regions corresponding in R_F values to phosphatidylcholine, phosphatidyllecithin, neutral lipids, and cardiolipin. All of these phospholipids gave strong FFCA-positive reactions using the equivalent of 0.1 mg of mitochondrial protein.

The Effect of MIH on P/O and ADP/O Ratios. Table III lists the results of experiments with rat liver and beef heart mitochondria. It can be seen that MIH had no significant effect on the P/O ratios when measured by the orthophosphate uptake method using the conditions of Perlgut and Wainio (1966). The presence of MIH in the reaction medium gave no significant change in the P/O, ADP/O, and respiratory control ratios (rat heart or liver mitochondria) when assayed polarographically. MIH had no effect on the ADP/O ratios and respiratory control ratios of beef heart mitochondria which had been stored at −20° for 3 weeks. The mother liquors from which the MIH had been crystallized were also without effect on ADP/O ratios and respiratory control ratios of rat heart mitochondria. The addition of extracts A and C to the reaction medium during the assay of ADP/O ratios caused a slight decrease in the efficiency of ATP synthesis.

Discussion

Perlgut and Wainio's (1964, 1966) results may be summarized briefly thus. They obtained, on paper chromatography in five solvent systems of a crude mitochondrial extract, an unknown compound which, in these systems, had R_F values similar to those of MIH. The compound also gave a blue color with the

TABLE III: The Effect of MIH on P/O Ratios.[a]

Type of Mitochondria	P/O Ratios and Additions: O	10^{-7} M MIH	5×10^{-7} M MIH
Rat liver mitochondria	2.2	2.0	2.3
	2.2	2.2	2.2
Heavy beef heart mitochondria	2.5	2.5	2.6
	2.5	2.6	2.5
Heavy beef heart mitochondria	2.9	3.0	3.0
	2.9	3.1	3.0
Heavy beef heart mitochondria[b]	3.0	3.0	2.8
	2.7	2.6	2.9
		2.8	2.7

[a] The mitochondria were preincubated with MIH as described by Perlgut and Wainio (1964). The mitochondria were then suspended in a medium containing 5 mM dipotassium glutamate, 5 mM dipotassium malate, 5 mM magnesium chloride, 1 mM tripotassium adenosine triphosphate, 10 mM potassium orthophosphate, pH 7.5, and 0.19 M sucrose, giving a final protein concentration of 13–16 mg/ml. This suspension (0.9 ml) was added to the main compartment of a 7-ml manometer flask. KOH (0.1 ml of 40%) was placed in the center well and 0.1 ml of 1 M glucose solution, containing 2 mg of crystalline hexokinase, in the side arm. After a 10-min equilibration period at 25°, the flask contents were mixed. The reaction was stopped by the addition of 0.1 ml of 50% trichloroacetic acid. The orthophosphate uptake was measured as the difference between the orthophosphate contents of the experimental flasks and zero-time controls. [b] MIH in the P/O assay medium.

FFCA spray reagent. We have been able to reproduce these findings, but we cannot agree that they constitute evidence for the presence of MIH in these extracts.

The color reaction is the sole evidence that the observed compound(s) of R_F values similar to MIH contain iodine. Only two papers, other than the contribution of Perlgut and Wainio, describing the use of this reagent are known to us (Postmes, 1963; Gmelin and Virtanen, 1959). In the first (Postmes, 1963), the FFCA spray was used to identify iodinated tyrosine derivatives from a thyroid extract which had been treated so as to separate such derivatives from other contaminating material. In the second (Gmelin and Virtanen, 1959), only reference compounds were examined in a preliminary evaluation of the reagent. Neither paper described a systematic attempt to discover the range of interfering substances.

Any compound capable of reducing Fe(III) to Fe(II) will produce a blue color with the FFCA reagent. The greater sensitivity of iodide depends upon its ability to catalyze the reduction of Fe(III) by As(III). That iodide does indeed possess this ability was first established by Iwasaki *et al.* (1953) in a paper which was apparently not known to the other users of the FFCA spray reagent. Gmelin and Virtanen (1959) and Postmes (1963) have observed that the reagent is also sensitive to iodinated phenols. This probably, though the fact is not established, depends on oxidation of the phenol by iron with liberation of iodide, in a way similar to that observed by Bowden *et al.* (1955) for the case of the ceric sulfate–arsenious acid reagent. There is no evidence whatsoever that the FFCA or, for that matter, the ceric sulfate–arsenious acid reagents are similarly affected by organic iodine compounds *per se*. In both cases there is evidence that iodide (or iodine) must first be liberated (Bowden *et al.*, 1955; Bush, quoted in Bowden *et al.*, 1955, p 97). It is perfectly possible that the irradiative treatment used on paper chromatograms by Perlgut and Wainio may liberate iodine in some form or other, but they have offered no independent evidence that this is so. In our hands, histidine itself reacts with the FFCA spray on paper after irradiation,[3] and tlc of the (crude) mitochondrial extracts used by Perlgut and Wainio, and by ourselves, has given up to seven distinct fractions which are positive to the FFCA spray. This need occasion no surprise; such an extract might reasonably be expected to contain a number of compounds possessing reducing properties. To distinguish such compounds from iodide (if produced *in situ*) would require considerably more independent evidence of the nature and quantity of the constituents of the extract than was offered by Perlgut and Wainio.

In view of these facts, quantitative estimation on paper chromatograms of an unknown compound, not independently identified, by densitometric means using the color produced by the FFCA spray must be even more suspect. Such suspicion becomes intensified when it is realized that the quantitative estimation of iodide (not, we repeat, iodinated compounds) by the FFCA or related reagents depends upon its effect on the rate of reduction of Fe(III). Such an effect can be measured accurately only by kinetic techniques such as those generally used for iodide determination with the ceric sulfate–arsenious acid reagent (Zak *et al.*, 1952; Barker *et al.*, 1951; Pileggi *et al.*, 1961; Zak, 1958). Finally, Perlgut and Wainio (1966) and Postmes (1963) appear to have been unaware of the marked temperature sensitivity of the iodide-catalyzed reduction of Fe(III) by As(III) (Iwasaki *et al.*, 1953).

We have confirmed that ethanolic extracts of mitochondria contain FFCA-positive compound(s) with R_F values similar to MIH using the paper chromatographic systems of Perlgut and Wainio and using some, but not all, of the thin layer chromatographic systems described here. However, since there are at least seven such compounds in the extract, we must emphasize that we have no evidence that the compound(s) running as MIH in the different chromatographic systems is the same compound(s).

[3] For an indication of the possible breakdown products involved see Pfinnigsdorf and Schriber (1961).

Despite these chromatographic similarities, we believe that MIH is not present in the mitochondrial extracts for the following reasons. The FFCA-positive compounds present in the mitochondrial extracts may be separated from authentic MIH on tlc run with chloroform–methanol–water and also by gel filtration.

All the FFCA-positive materials in the extract were alkali labile. In contrast, authentic MIH treated under similar conditions was stable.

We have not been able to detect a trace of MIH in extracts from 2 g of beef heart mitochondria when these extracts were fractionated on the calibrated ion-exchange resin of an amino acid analyzer. Perlgut and Wainio (1966) admitted to a similar lack of success.

The mitochondrial extraction procedure used by Perlgut and Wainio to remove FFCA-positive substances from heavy beef heart mitochondria was obviously not specific for MIH and would dissolve many other mitochondrial components, including phospholipids. Our results show that the phospholipid present in 100 μg of mitochondria gave, after tlc, four well-defined areas of FFCA-positive compounds corresponding to known phospholipids. Mitochondria (100 μg) would contain a maximum of 7×10^{-11} mole of MIH (based on Perlgut and Wainio's highest value for mitochondrial MIH content). This amount of MIH is very close to the limits of detection; hence the presence of interfering substances and the lack of specificity of the FFCA reagent render this method useless for the identification of MIH.

The possibility that MIH is an intermediate in oxidative phosphorylation in rat liver mitochondria may be further discounted by studies of the iodine content of mitochondria. The concentration of cytochrome *a* in rat liver mitochondria is 0.2 mμmole/mg of protein (Chance and Hess, 1959). It would be expected that an intermediate of oxidative phosphorylation would be present in mitochondria at similar concentrations. However, Carr and Riggs (1953) state that the concentration of protein-bound iodine in rat liver mitochondria is 0.003 mμmole/mg of protein and F. L. Hoch (personal communication, 1966) found a total iodine content of 0.0033 mμmole of iodine/mg of protein. Furthermore, Hoch (1966) found that the iodine level in mitochondria isolated from hypothyroid rats was only 21% that of the normal, and yet these mitochondria synthesize ATP with an efficiency equal to that of mitochondria isolated from normal rats.

L. E. Perlgut and W. W. Wainio (private communication) have informed us that they can only detect MIH after preincubating mitochondria with substrate, etc. Our extract C was made following their conditions as closely as possible. However, all the FFCA-positive substances in this extract were alkli labile in contrast to authentic MIH.

Using Perlgut and Wainio's conditions, freshly made beef heart and rat liver mitochondria were preincubated with MIH; this treatment had no effect on the P/O, ADP/O, and the respiratory control ratios when assayed both manometrically and polarographically. Addition of MIH directly to the assay medium was also without effect. Partially uncoupled heavy beef heart mitochondria were also treated with MIH and the ADP/O and respiratory control ratios were then assayed; no difference between the control and MIH-treated samples was detected. Thus, in our hands MIH has no effect on mitochondrial oxidative phosphorylation.

The results presented here eliminate 5-MIH from consideration as an intermediate in oxidative phosphorylation. The possibility that 2-MIH, present as an impurity in Perlgut and Wainio's preparation, was responsible for any effect they observed on oxidative phosphorylation and corresponds to the supposed intermediate present in mitochondria can also be eliminated: (1) by the lability to alkali of the FFCA-positive mitochondrial material; (2) by the lack of any unknown peak on amino acid analysis; and (3) by the separation of all FFCA-positive mitochondrial material by a molecular sieving process which would be unable to differentiate between 2- and 5-MIH. Further addition of crude material from our 5-MIH preparations had no effect on oxidative phosphorylation using Perlgut and Wainio's conditions.

Acknowledgment

We thank Mr. I. G. Blackwell of Woodstock Agricultural Research Center for performing the chemical analysis of MIH.

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The 11β-Hydroxylation of Progesterone and Deoxycorticosterone by Rat Adrenal Mitochondria*

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ABSTRACT: A study was undertaken to evaluate the *in vitro* 11β-hydroxylation of progesterone and 11-deoxycorticosterone (DOC) by rat adrenal mitochondria. Incubations of DOC, progesterone, or both steroids were carried out with mitochondria in the presence of reduced triphosphopyridine nucleotide (TPNH) with or without calcium ions (Ca^{2+}). The effect of increasing Ca^{2+} concentration on the conversion of DOC into corticosterone was a biphasic one; slight stimulation of the reaction occurred with Ca^{2+} up to 55 μM and maximum 11β-hydroxylation was achieved between 1 and 22 mM. In the absence of Ca^{2+}, progesterone appeared to be somewhat more readily 11β-hydroxylated than DOC. At concentrations above 55 μM, the effect of Ca^{2+} on progesterone 11β-hydroxylation was similar to that for DOC. In the mixture of the two steroids, the presence of DOC markedly inhibited the 11β-hydroxylation of progesterone. At the higher concentrations of Ca^{2+}, progesterone slightly reduced the 11β-hydroxylation of DOC; however, at low Ca^{2+} or in the absence of Ca^{2+}, progesterone markedly enhanced the formation of corticosterone from DOC. Radioisotope studies indicated that the extra corticosterone formed from incubations of progesterone plus DOC did not arise from progesterone. Moreover, studies on mitochondrial swelling suggested that the slight additional swelling of the particles in the presence of progesterone plus DOC would not account for the enhanced corticosterone formation.

Incubation of 10–120 μg of DOC with 60 μg of progesterone revealed that corticosterone formation was suppressed only at the higher steroid concentrations. The reduction of DOC 11β-hydroxylation by progesterone may be related to total steroid concentration rather than to a specific effect of progesterone. Incubation of 20–60 μg of progesterone with 30 μg of DOC revealed a progressive inhibition of 11β-hydroxyprogesterone formation by DOC. Thus, while progesterone is 11β-hydroxylated, the normal steroid substrate, DOC, is more readily 11β-hydroxylated by rat adrenal mitochondria incubated with both steroids in the presence of Ca^{2+} and TPNH.

In some studies of steroid 11β-hydroxylation by rat adrenal mitochondria, incubations of the particulate fraction with progesterone have been undertaken to show the quality of the preparation. A lack of microsomal contamination in the mitochondrial preparation was shown by the finding of 11β-hydroxyprogesterone[1] rather than 11-deoxycorticosterone (DOC) following incubations of the particulate fraction with progesterone (Péron *et al.*, 1964a,b, 1965a,b; Roberts *et al.*, 1964, 1965). It has been established that the 11β-hydroxylation of DOC by rat adrenal mitochondria in the presence of reduced triphosphopyridine nucleotide (TPNH, NAD PH) requires the presence of calcium ion (Ca^{2+}) (Péron *et al.*, 1965a,b, 1966).

* From the Department of Biology, Southern Methodist University, Dallas, Texas, and the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts. *Received February 24, 1966.* Supported in part by grants from the National Institutes of Health, U. S. Public Health Service AM-05744, AMO-4899, and T4-CA-5001.

† Recipient of Research Career Award AM-K3-19-190 National Institute of Arthritis and Metabolic Diseases.

[1] The systematic chemical names of substances for which trivial names are used on this report are the following: 11-deoxycorticosterone (DOC), 21-hydroxy-4-pregnen-3,20-dione; 18-hydroxy-11-deoxycorticosterone (18-OHDOC), 18,21-dihydroxy-4-pregnen-3,20-dione; progesterone, 4-pregnen-3,20-dione; 11β-hydroxyprogesterone, 11β-hydroxy-4-pregnen-3,20α-dione; corticosterone, 11β-21-dihydroxy-4-pregnen-3,20-dione; TPNH, reduced triphosphopyridine nucleotide; ACTH, adrenocorticotrophin.