IN VITRO CONVERSION OF PTEROYLGLUTAMIC ACID TO CITROVORUM FACTOR BY RAT LIVER ENZYMES

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SUMMARY

The enzymes in rat liver which convert pteroylglutamic acid to citrovorum factor are found to reside in the mitochondria and are dissociable into two fractions, one bringing about the initial reduction of pteroylglutamic acid to dihydropteroylglutamic acid and the other effecting the final reduction to tetrahydropteroylglutamic acid followed by formylation to the citrovorum factor-active derivatives; the former is easily released into the supernatant in isotonic saline, while the latter is bound more firmly in the mitochondrion.

The enzyme complex has been partially purified from 0.05 % sodium desoxycholate mitochondrial extract by precipitation at 30–50 % $(NH_4)_2SO_4$ saturation.

Reducing agents act as non-specific stabilizers and a nitrogen atmosphere is better for CF synthesis when compared to ascorbic acid addition. Serine is the most effective formyl donor. TPNH, ATP, Mg^{++}, homocysteine and pyridoxal phosphate are important, in that order, for optimal conversion. Their role in the conversion mechanism has been discussed.

INTRODUCTION

There have already been reports relating to the enzymic conversion of PGA to CF by bacterial[1], rat and chicken liver[2,3] preparations. Anaerobic conditions[4] as well as a source of formate[1–3] have been shown to be essential for optimal conversion. DHPGA[5,6] and THPGA[7,8] have been shown to be intermediates preceding the formylated active and labile THPGA derivatives which yield stable CF after anaerobic and non-enzymic degradation[9]. The present report relates to the CF-synthesising system from rat liver preparations.

METHODS

Animals

Albino rats (Wistar strain), 100–120 g in weight and of either sex were used. They were maintained on a laboratory stock diet which consisted of 75 % whole

Abbreviations: PGA, pteroylglutamic acid; CF, citrovorum factor; N^5, formyl tetrahydropteroylglutamic acid; DHPGA, dihydropteroylglutamic acid; THPGA, tetrahydropteroylglutamic acid; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.

wheat flour, 2 % whole milk powder, 12 % casein, 2 % dried yeast, 4 % arachis oil, 1 % vitaminised sesame oil (2000 I.U. of vitamin A and 50 I.U. of vitamin D per kg diet), 2 % sodium chloride and 2 % calcium carbonate.

Preparation of cell fractions

The animals were stunned by a blow on the head, exsanguinated and the liver removed and chilled in crushed ice. Portions of the liver were made up to 20 % homogenates in isotonic sucrose and fractionated using an International Refrigerated Centrifuge (Model PR-2) to yield the nuclear, mitochondrial and supernatant fractions according to the method of HOGEBOOM AND SCHNEIDER[10]. The supernatant was not centrifuged in order to separate the microsomes. When isotonic saline was used as the medium for homogenation, fractions were collected at 700 × *g* for 10 min (Fraction 700 × *g*) and further at 5000 × *g* for 10 min (Fraction 5000 × *g*). The particulate fractions were washed by resuspension in isotonic saline and fractionation using the same speeds and times.

Partial purification of the enzymes

For solubilizing the enzyme complex, mitochondria isolated from 5 *g* liver[10] were extracted in 25 ml of aqueous 0.05 % sodium desoxycholate solution for 10–15 min and then centrifuged. All operations were carried out at 0–5° and the centrifugations performed at 15,000 × *g* for 10–15 min.

Graded quantities of solid $(NH_4)_2SO_4$ were added to the extract with constant stirring and the precipitated protein fractions collected by centrifugation. The precipitates were dissolved in isotonic saline and their conversion abilities determined using a heated isotonic saline extract of an equivalent amount of mitochondria as the source of cofactors. Protein was determined from separate aliquots using the Folin-Ciocalteu Reagent[11].

Conversion of PGA to CF

The optimal conditions reported for the conversion with rat liver[2,12] were maintained. The reactions were carried out in conventional Warburg flasks. Each vessel contained 200 μg PGA, 10 mg serine, and 3 ml of 20 % liver homogenate or the corresponding cellular fraction dissolved in 0.066 *M* phosphate buffer pH 6.4, final volume 5 ml. The reaction was allowed to proceed under N_2 at 37° for 100 min after which it was arrested by steaming the stoppered flasks for 5 min. CF was assayed using *Leuconostoc citrovorum* (American Type Cultures Collection No. 8081) as the test organism in a basal medium of SAUBERLICH AND BAUMANN[13]. Conversion values, after correction for endogenous CF, are expressed as μg CF formed by 1 g liver or the cellular fractions corresponding to 1 g liver.

DHPGA synthesis: This was studied by incubating PGA (200 μg) under N_2 for 1 h at 37° in 0.06 *M* phosphate buffer, pH 6.4, with isotonic saline liver supernatant (3.0 mg nitrogen) in a total volume of 3.0 ml. The product was then acidified, aerated and read at 420 mμ on a Beckman DU spectrophotometer against an enzyme blank in a reference cuvette[5]. For the zero blank, the reactants were mixed just before acidification.

Succinoxidase: Determinations of succinoxidase were carried out according to the method of SCHNEIDER AND POTTER[14].

Radioactivities: These were determined in infinitely thin measured portions evaporated to dryness on 25 mm diameter pyrex glass planchettes, using a helium-filled SL-16 flow gas counter in conjunction with a SC-51 Autoscaler (Tracer Lab. Inc., Waltham, Mass., U.S.A.) operating at 40–45 % efficiency.

RESULTS

Experiments using fractions from liver homogenates in isotonic saline

The conversion of PGA to CF did not proceed satisfactorily when isotonic sucrose was used as the medium for homogenation. This effect was not further investigated. However, conversion values for homogenates in isotonic saline were comparable to those obtained when aqueous phosphate buffer[12] was used for homogenation. The CF synthesized from added PGA was in a free and assayable form since chemical and enzymic liberation procedures only served to increase the endogenous levels (unpublished data).

No single cellular fraction of the isotonic saline homogenate was found to effect CF synthesis. A combination of the supernatant ($> 5000 \times g$) and the fraction $700 \times g$ accounted for the entire conversion of the whole liver homogenate (Table I). By heat denaturation of either fraction or by substitution of the co-factors[3] (ATP, TPN and Mg^{++}) for the supernatant, CF synthesis did not proceed, indicating an enzymic contribution by each fraction.

The conversion was then shown to proceed in two subsequent stages, each separately terminable by heating the reaction system. The first stage is brought about by the supernatant enzymes, while the fraction $700 \times g$ undergoes conversion in the presence of a formyl donor (Table I). The conversion did not proceed when this sequence was reversed. Aminopterin addition inhibited CF synthesis even when

TABLE I

CONVERSION OF PGA TO CF BY ISOTONIC SALINE HOMOGENATE FRACTIONS OF RAT LIVER

Conversion values were obtained as described in the METHODS section. Results are expressed as mean ± S.E.M.

Expt. No.	*Enzyme source*	*CF formed (μg/g liver)*
1	Total homogenate	1.52 ± 0.15
2	Fraction 700 × *g* and supernatant	1.50 ± 0.16
3	Fraction 5000 × *g* and supernatant	0.08 ± 0.02
4	Supernatant (1st step), then fraction 700 × *g* (2nd step), stepwise*	1.35 ± 0.18
5	Fraction 700 × *g*, then supernatant, stepwise*	0.16 ± 0.04
6	1st step aerobic, 2nd step anaerobic*	1.28 ± 0.25
7	1st step anaerobic, 2nd step aerobic*	0.37 ± 0.21
8	Aminopterin added in 2nd step*	0.04 ± 0.02

* The reaction was carried out stepwise. In the 1st step PGA was reacted with a cellular fraction for 50 min and the reaction stopped by steaming. The 2nd cellular fraction was then allowed to react for a further 50 min, before termination of the experiment. Unless otherwise specified, all incubations were anaerobic. Attention is drawn to Expt. No. 4 where serine addition was found necessary in the 2nd step. In Expt. Nos. 6 and 7 the steps are as in Expt. No. 4. Aminopterin, where added, amounted to 10 μg level per flask.

the substance was added in the latter stage where anaerobic conditions were most crucial for optimal conversion (Table I).

An observed increase in the optical density at 420 mμ, following acidification and exposure to air of the products of the supernatant reaction (Table II) was interpreted as indicating the formation of DHPGA, which is known to give a yellow degradation product[5,15].

The cellular fraction sedimenting at 700 × *g* from isotonic saline homogenates accounted for 90 % of the total liver succinoxidase activity (Table III). When mitochondria labelled *in vivo*, by sedimentation from isotonic sucrose liver homogenates of rat killed 2 h after intraperitoneal injection of an aqueous solution of 1 mg [3-^{14}C]-serine (2.5 × 10^5 counts/min per 100 g body wt.), were added to a liver homogenate in isotonic saline and re-sedimented, it was observed (Table IV) that 70 % of the mitochondrial radioactivity was recoverable in fraction 700 × *g*. Fraction 5000 × *g* accounted for only 5 % of the activity, while 20 % was recovered in the supernatant (> 5000 × *g*).

Experiments using fractions from liver homogenates in isotonic sucrose

CF synthesis was demonstrable with purified nuclei and mitochondria prepared from isotonic sucrose homogenates and suspended in isotonic saline (Table III). The mitochondria were responsible for most of the conversion ability of the particulate fraction. The small conversion by the nuclear fraction was attributed to contaminating

TABLE II

CONVERSION OF PGA TO CF BY SUPERNATANT OF RAT LIVER ISOTONIC SALINE HOMOGENATES

See METHODS section for details.

No.	*Optical density at 420 mμ*	
	0 h	*1 h*
1	0.12	0.41
2	0.08	0.33
3	0.09	0.47

TABLE III

COMPARATIVE STUDIES WITH CELLULAR FRACTIONS FROM ISOTONIC SALINE AND ISOTONIC SUCROSE HOMOGENATES OF RAT LIVER

For details of procedure see METHODS section. Results are expressed as mean ± S.E.M.

Medium of homogenisation (isotonic)	*Source of enzyme*	*CF formed (μg/g liver)*	*Succinoxidase activity (μl O_2/h/g liver)*
Saline	Whole homogenate	1.52 ± 0.15	3868 ± 122
Sucrose	Whole homogenate	0.68 ± 0.14	3917 ± 145
Saline	Fraction 700 × *g* and supernatant	1.50 ± 0.16	3595 ± 155
Sucrose	Nuclei*	0.28 ± 0.03	495 ± 36
Sucrose	Mitochondria*	1.19 ± 0.12	2908 ± 55
Sucrose	Nuclei and mitochondria*	1.49 ± 0.18	3508 ± 88

* Prepared from isotonic sucrose homogenates and suspended in isotonic saline.

mitochondria, since this fraction was not completely devoid of succinoxidase activity (Table III).

The protein fraction, separating at between 30–50% $(NH_4)_2SO_4$ saturation of the 0.05% aqueous sodium desoxycholate mitochondrial extract, accounted for 90% of activity of the mitochondria (Table V). Fractions separating at between

TABLE IV

DISTRIBUTION OF MITOCHONDRIA IN CELLULAR FRACTIONS FROM ISOTONIC SALINE LIVER HOMOGENATES

Labelled mitochondria were obtained by fractionation of isotonic sucrose liver homogenates of rats killed 2 h after intraperitoneal injection of an aqueous solution containing 1 mg [3-^{14}C]serine (2.5×10^5 counts/min/100 g body weight). These were added to a liver homogenate in isotonic saline, re-sedimented and washed at $700 \times g$ and $5000 \times g$ for 10 min, each. Radioactivities of the particulate and supernatant ($> 5000 \times g$) fractions were determined as described in the METHODS section. Results are expressed as mean ± S.E.M.

Fraction	*Activity (counts/min)*	*% of total activity*
Total homogenate	420 ± 32	100
Fraction $700 \times g$	295 ± 20	70
Fraction $5000 \times g$	30 ± 4	7
Supernatant ($> 5000 \times g$)	88 ± 6	21

TABLE V

PARTIAL PURIFICATION OF THE CF-FORMING ENZYMES OF RAT LIVER MITOCHONDRIA

For details of assay see the METHODS section. Specific activities are expressed as mμg CF formed/mg protein.

Enzyme preparation	*CF formed (μg/g liver)*	*Apparent yield (%)*	*Protein content (mg/g liver)*	*Specific activity*
Homogenate	1.52	100	220.0	6.7
Mitochondria	1.21	79	44.2	27.5
0–60% $(NH_4)_2SO_4$ fraction*	0.40	26	28.4	14.5
30–50% $(NH_4)_2SO_4$ fraction*	0.38	25	7.9	48.9
30–50% $(NH_4)_2SO_4$ fraction with cofactors**	1.32	88	7.9	167.1

* PGA to CF conversion abilities were studied with protein fractions precipitating at different $(NH_4)_2SO_4$ saturations of the 0.05% desoxycholate extract of mitochondria, in the presence of a heated isotonic saline extract of an equivalent amount of mitochondria to provide the cofactors.

** In this case the source of cofactors was a mixture containing 5 mg $MgCl_2$, 1 mg TPNH, 1 mg ATP, 3 mg homocysteine and 1 mg pyridoxal phosphate, in 5 ml of the reaction system.

TABLE VI

FORMYL DONORS AND THE CONVERSION OF PGA TO CF BY RAT LIVER MITOCHONDRIA

Formyl donors were added at 0.1 mmole level per flask and the conversion values obtained as described in the METHODS section. Results are expressed as mean ± S.E.M.

Formyl donor	*CF formed (μg/g liver)*
Serine	1.19 ± 0.12
Formate	0.52 ± 0.07
Formaldehyde	0.07 ± 0.01

0–30 % and 50–60 % $(NH_4)_2SO_4$ saturation showed negligible activities on analysis.

Formyl donors: In the next experiment the effect of different formyl donors on the CF synthesis by the mitochondria was studied (Table VI). Serine, formate and formaldehyde are effective, in that order, as formyl donors.

Cofactors and other requirements: The heated extract of mitochondria which supplied cofactors for the conversion effected by the partially purified enzyme precipitate (Table V) could be completely replaced by a mixture containing TPNH, ATP, $MgCl_2$, homocysteine and pyridoxal phosphate, with vastly improved yields (Table VII). The lower yields with the former were probably due to the absence of a TPNH regenerating system in the heated mitochondrial extract (unpublished data). By optional omission of each component of the mixture, it was found (Table VII) that the TPNH and ATP additions were most crucial for optimal conversion, followed by Mg^{++}, homocysteine and pyridoxal phosphate.

Reducing conditions: Table VIII would indicate that reducing conditions are essential for conversion and act non-specifically. A nitrogen atmosphere favoured conversion better than ascorbic acid addition. Further, in a N_2 atmosphere, ascorbic acid addition was without effect.

TABLE VII

EFFECT OF VARIOUS ADDITIONS ON THE CONVERSION OF PGA TO CF

The partially purified enzyme precipitate obtained at 30–50 % $(NH_4)_2SO_4$ saturation of the 0.05 % deoxycholate extract of mitochondria was used as the enzyme source. The additions were as described in Table V and the conversion values, representing the average of 4 different experiments, were determined omitting each of the components in turn.

Omission	*CF formed (µg/g liver)*
Nil	1.32
TPNH*	0.13
ATP	0.40
$MgCl_2$	0.51
Homocysteine	0.84
Pyridoxal phosphate	1.13

* TPN showed an effect only in the presence of isocitrate and isocitric dehydrogenase (supernatant).

TABLE VIII

REDUCING CONDITIONS FOR THE CONVERSION OF PGA TO CF BY RAT LIVER MITOCHONDRIA

Freshly neutralised ascorbic acid was added in 10 mg amounts per flask. Conversion values were obtained as described in the METHODS section. Results are expressed as mean ± S.E.M.

Ascorbic acid addition	*Under N_2*	*CF formed (µg/g liver)*
+	—	0.66 ± 0.04
—	+	1.19 ± 0.12
+	+	1.10 ± 0.12
—	—	0.02 ± 0.01

DISCUSSION

Experiments with cellular fractions from liver homogenates in isotonic saline indicated that the conversion proceeded in two subsequent stages, the first brought about by the supernatant ($> 5000 \times g$) enzymes and the second by the particulate enzymes of fraction $700 \times g$.

Reduction of PGA to THPGA precedes formylation and aminopterin addition inhibits conversion by blocking the reduction of PGA[16,17]. In a bacterial system WRIGHT AND ANDERSON[5] indicated that aminopterin did not inhibit the initial reduction of PGA to DHPGA. In our studies aminopterin inhibited CF synthesis when added in the final stage (fraction $700 \times g$) reaction where the reducing conditions were most crucial and the addition of a formyl donor was found necessary (Table I). This would suggest that reduction of DHPGA to THPGA and the subsequent formylation were mediated by enzymes of the fraction $700 \times g$. Reduction of PGA to DHPGA by the supernatant enzymes is implied in Table II. To confirm this, CF synthesis from synthesized DHPGA[18] corresponding to 1.50 μg/g liver (average of 4 different incubations) was demonstrable under optimal conditions in the presence of fraction $700 \times g$ together with the heated supernatant and serine.

Succinoxidase determinations (Table III) indicated the presence of mitochondria in the particulate fraction sedimenting at $700 \times g$. Experiments with purified cellular fractions showed that the mitochondrion was the active site of conversion (Table III). Further labelled work (Table IV) suggested agglutination and disintegration of the mitochondria in isotonic saline, followed by release of intra-mitochondrial contents. HOGEBOOM, SCHNEIDER AND PALLADE[19] have reported similar observations on mitochondria prepared in isotonic saline. Thus it would seem that the enzyme systems in the mitochondria could be divided into the one which brings about the initial reduction of PGA to DHPGA and the other which effect the final reduction to THPGA followed by formylation to CF active derivatives; the former was easily released into the supernatant in isotonic saline, while the latter was more firmly bound in the mitochondrion.

The distribution of CF-synthesising enzymes in rat liver differs from that in chicken liver, where they are apparently more readily soluble and reside in the supernatant[17]. Extraction of the mitochondria by 0.05 % aqueous sodium desoxycholate solubilized the enzyme complex and 90 % of the activity was recovered in the protein fraction precipitating at between 30 and 50 % $(NH_4)_2SO_4$ saturation (Table V). These results are in accord with those of DEODHAR, SAKAMI AND STEVENS[20] who have reported a solubilized preparation from rat liver particles that accumulated a CF-active product when THPGA was activated in the glycine–serine interconversion. NADKARNI AND SREENIVASAN[21] attributed 70 % of the liver activity for the reverse reaction involving serine synthesis with CF utilisation to the mitochondria.

A need for pyridoxal phosphate (Table VII) in the overall serine hydroxymethylase reaction has constantly been observed[22]. DOCTOR *et al.*[3] have also reported stimulatory effects of Mg^{++} and homocysteine. The latter probably enhance the utilisation of 1-C fragments in CF synthesis by forming a homocysteine-C_1 common precursor in 1-C transfers[23].

The multiplicity of the formyl donors, essential for the conversion, lends uncertainty to the exact nature of the active intermediates formed. In the present

study serine was the most effective (Table VI). For rat liver enzymes, ELWYN *et al.*[24] have also reported serine to be the most effective precursor ot the methyl group of choline, a process in which the C:H bond of the β-C of serine remained unaltered. This would suggest that the N^{10}-hydroxymethyl derivative of THPGA[25] was formed in an ATP dependent reaction[26] without the obligatory formation of the formyl derivative. The two derivatives are known to exist in a redox equilibrium with a TPN-linked dehydrogenase which has been isolated in a purified form[27]. This reaction could be compared with the known TPNH-linked reduction of PGA to DHPGA[5,6] and THPGA[18,28]. Under anaerobic conditions labile THPGA and its N^{10}-formylated derivatives undergo non-enzymic degradation to CF, the stable N^5-formyl derivative[9]. As observed by DONALDSON AND KERESZTESY[29], reducing agents act only as non-specific stabilizers (Table VIII). Under a nitrogen atmosphere the addition of ascorbic acid had no effect (Table VIII). The latter is known to inhibit specifically the aerobic cleavage of CF[30].

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