Biochemical Pharmacology, 1966, Vol. 15, pp. 927–935. Pergamon Press Ltd., Printed in Great Britain.

CHANGES IN THE LIVER CONCENTRATIONS OF THE NICOTINAMIDE ADENINE DINUCLEOTIDE COENZYMES AND IN THE ACTIVITIES OF OXIDOREDUCTASE ENZYMES FOLLOWING TREATMENT OF THE RAT WITH ETHYL CHLOROPHENOXYISOBUTYRATE (ATROMID-S*)

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(*Received* 1 *November* 1965; *accepted* 12 *January* 1966)

Abstract—The effect of short periods of treatment (up to 2 months) with ethyl chlorophenoxyisobutyrate (CPIB) on the concentrations of nicotinamide adenine dinucleotide (pyridine nucleotide) coenzymes and on the activities of several oxidoreductase enzymes in rat liver has been described. The results were obtained in the equilibrium phase of the response to CPIB and a reproducible pattern of changes was found. The concentrations of both oxidized and reduced pyridine nucleotide coenzymes, particularly NAD, were raised, as were the activities of several dependent dehydrogenases. The activities of the reduced pyridine nucleotide:cytochrome-c dehydrogenases were either unaffected or reduced, as was the activity of tryptophan pyrrolase. The relationship of these results to the mode of action of CPIB is discussed.

INTRODUCTION

EXTENSIVE study has been made of the effect of ethyl α-(4-chlorophenoxy)-α-methylpropionate (CPIB, clofibrate, "Atromid-S") on rat liver during investigations into the mode of action of this compound. CPIB binds to a particular site of serum albumen,[1] that is also occupied by several endogenous factors such as thyroxine, tryptophan, pyridoxal phosphate and androsterone sulphate.[2] The mechanism of action of CPIB depends on the redistribution of these factors, principally between the serum and liver,[3–5] and studies were undertaken to measure the biochemical changes in rat liver. It has been shown previously,[3] that the rat liver responds to CPIB treatment in distinct stages; firstly an initial stimulation of protein synthesis, an increase in liver weight and a decrease in glycogen content, followed by an equilibrium phase, which is maintained throughout drug treatment and where liver weight and liver protein concentration are maintained at high levels and glycogen at low levels compared to the controls.

The establishment of a "pattern of response" to various factors is often of use in characterising mechanisms of action,[6–8] and the results of observations made in the equilibrium phase of CPIB treatment are reported in this paper. Nicotinamide adenine dinucleotide coenzyme (pyridine nucleotide) concentrations, the activities of several

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dependent dehydrogenases and reduced pyridine nucleotide: cytochrome-c dehydrogenases together with tryptophan pyrrolase activities (both in the early and in the equilibrium phases) were measured and the significance of the results is discussed with reference to the proposed mode of action of CPIB.

METHODS

Experimental animals

Male rats of a specific pathogen-free Wistar-derived Alderley Park strain were used throughout, ranging in weight from 150 to 400 g. In any one experiment, equivalent rats in terms of body weight were always included to enable direct comparisons to be made.

Diet

Rats were fed a powdered diet (Scottish Agricultural Industries) containing 0·25% CPIB by weight. No restriction was placed on the quantities eaten, nor on the availability of water. The period of dosing ranged from 1 to 60 days.

Assay procedures

Liver concentrations of NAD, $NADH_2$, NADP and $NADPH_2$ were determined by the method of Lindall and Lazarow,[10] this being a modification of the well-established method of Bassham *et al.*[11]

At the termination of an experiment, the animals were killed by stunning with a blow to the head and exsanguinating from a severed throat. Livers were rapidly removed to an ice-cooled container, the organ weight recorded and samples homogenised at 0° with a Potter–Elvehjem Perspex–Fluon homogeniser (clearance 0·5 mm) driven by an air-motor. The homogenising procedure was the same in all cases and homogenates of 10% strength were prepared in either 0·25 M sucrose or 0·15 M KCl containing 0·8% (v/v) 0·02 M $KHCO_3$ at pH 7·0. The sucrose homogenates were centrifuged at 4° at 600 *g* for 5 min to give a supernatant fraction containing mitochondria, lysosomes, microsomal material and cytoplasm. The KCl-homogenates were centrifuged at 4° at 15,000 *g* for 15 min to give a supernatant containing principally microsomal material and cytoplasm; an MSE-17 refrigerated centrifuge was used throughout. To avoid excessive freezing and thawing, the homogenates were subdivided into several portions and stored at −20° until used. Further dilution of homogenates was made with the respective homogenising medium. Significant changes in the activities of the enzymes measured were not apparent in preparations stored up to three weeks in this way.

The methods of enzyme determination employed were either direct applications of established methods or were minor modifications of the methods indicated. In all experiments adequate controls were included, enabling the results to be expressed as percentages of the control animal values. Where enzyme activity was measured by direct spectrophotometry, a Bausch & Lomb "Spectronic-505" dual-beam recording spectrophotometer was employed, the activities being derived from the initial linear sections of the recordings.

The dehydrogenase enzymes were measured spectrophotometrically at 25° and 340 mμ, by following either the rate of reduction of oxidised pyridine nucleotide

coenzyme in the case of glutamate dehydrogenase* [EC 1.4.1.2, L-glutamate : NAD oxidoreductase (deaminating)],[12] isocitrate dehydrogenase* [EC.1.1.1.42, threo-DS*i*-isocitrate : NADP oxidoreductase (decarboxylating)],[13] glucose-6-phosphate dehydrogenase* [EC.1.1.1.49, D-glucose-6-phosphate : NADP oxidoreductase][14], phosphogluconate dehydrogenase* [EC.1.1.1.44, 6-phospho-D-gluconate : NADP oxidoreductase (decarboxylating)],[14] and lactate dehydrogenase* [EC.1.1.1.27, L-lactate : NAD oxidoreductase],[15] or by following the rate of oxidation of reduced pyridine nucleotide coenzyme in the case of malate dehydrogenase* [EC.1.1.1.37, L-malate : NAD oxidoreductase].[16] The respective coenzyme and homogenate were included in both test and control cuvettes, the specific substrate being present only in the test cell. Phosphogluconate dehydrogenase was assayed at pH 7·6.[14] Homogenates in KCl were employed for the assay of the soluble enzymes, LDH, MDH, G6PDH, and PGDH and sucrose homogenates for determination of the mitochondrial enzyme, GDH, and for the soluble ICDH activity.

The reduced pyridine nucleotide coenzyme : cytochrome-c dehydrogenases were assayed spectrophotometrically in the sucrose preparations at 550 mμ and 25° by following the rate of reduction of cytochrome-c. The method of Mahler[17] was used to determine the $NADH_2$-dehydrogenase [EC.1.6.99.3, $NADH_2$: cytochrome-c oxido-reductase] but a modification of several methods[18–20] was used for the $NADPH_2$-dehydrogenase [EC.1.6.99.1, $NADPH_2$: cytochrome-c oxido-reductase].

Tryptophan pyrrolase, [EC.1.13.1.12, L-tryptophan : oxygen oxido-reductase, or trivially, tryptophan oxygenase] was measured by the method of Knox and Auerbach[21] in 600 *g* sucrose supernatants. All assays of this enzyme were made within 6 hr of sacrifice.

RESULTS

Pyridine nucleotide coenzyme concentrations

The concentrations of NAD, $NADH_2$, NADP and $NADPH_2$ were measured during the "equilibrium-phase" of the response to CPIB treatment,[3] i.e. after 14 or more days of continuous treatment, with the results shown in Table 1. The increase in the liver weight of the treated animals is of the same order as reported previously.[3] The extent of the increase in concentration of NADP and $NADPH_2$ is similar, with the result that no change occurs in the NADP : $NADPH_2$ ratio following CPIB administration. The increase in NAD concentration, however, is considerably greater than that of $NADH_2$, giving, as a result, a raised NAD : $NADH_2$ ratio.

Liver dehydrogenase activities

The results shown in Table 2 are those from representative experiments and are not average results from several experiments. The pattern of response was obtained during the "equilibrium-phase" of CPIB treatment and is reproducible. The initial weight of the rat, in the range 150–400 g, does not significantly influence the response to 0·25% CPIB. The effects of CPIB are not confined to either NAD or NADP-dependent dehydrogenases although a considerable variation in response is seen within each

* Abbreviations for convenience:glutamate dehydrogenase, GDH; isocitrate dehydrogenase, ICDH; glucose-6-phosphate dehydrogenase, G6PDH; phosphogluconate dehydrogenase, PGDH; lactate dehydrogenase, LDH; and malate dehydrogenase, MDH.

group. LDH and G6PDH consistently show the largest responses to CPIB treatment, whereas GDH and PGDH show no effect. The extent to which ICDH and MDH respond is comparable to the change in overall liver protein concentration previously reported.[3]

TABLE 1. RAT LIVER PYRIDINE NUCLEOTIDE COENZYME CONCENTRATIONS FOLLOWING TREATMENT WITH 0·25% CPIB IN THE DIET FOR 35 DAYS

	Control† mean	Treated† mean	Treated % control	P*
Initial body wt. (g)	165 ± 5·0(10)	171 ± 5·6(10)	104	>0·2
Terminal body wt. (g)	259 ± 8·5(10)	263 ± 10·0(10)	102	>0·2
Liver weight (g/100 g body wt.)	4·25 ± 0·09(10)	6·18 ± 0·23(10)	145	<0·001
NAD (mμmole/g fresh liver)	384 ± 36(8)	973 ± 112(7)	254	<0·001
$NADH_2$ (mμmole/g fresh liver)	298 ± 13(8)	435 ± 20(7)	146	<0·001
NAD : $NADH_2$	1·29 ± 0·12(8)	2·24 ± 0·26(7)	174	<0·01
NADP (mμmole/g fresh liver)	19·0 ± 2·8(6)	29·3 ± 2·6(8)	154	0·02
$NADPH_2$ (mμmole/g fresh liver)	191 ± 8·4(7)	324 ± 31·6(6)	169	0·001
NADP : $NADPH_2$	0·100 ± 0·011(6)	0·091 ± 0·014(6)	91	>0·20

* Treated vs. control group.
† Mean ± S.E.M. (number of animals).

TABLE 2. RAT LIVER DEHYDROGENASE ACTIVITIES AFTER TREATMENT WITH 0·25% CPIB IN THE DIET FOR 14–30 DAYS

Enzyme	Coenzyme requirement	Enzyme activity* Control (Mean†)	Treated (Mean†)	Treated (% controls)	P
G6PDH	NADP	3·62 ± 0·50(10)	5·50 ± 0·50(10)	152	<0·02
PGDH	NADP	4·94 ± 0·25(10)	5·22 ± 0·25(10)	105	>0·20
ICDH	NADP	52·5 ± 2·23(8)	64·3 ± 2·39(7)	122	0·005
GDH	NAD or NADP	4·86 ± 0·22(8)	4·82 ± 0·24(7)	101	>0·20
LDH	NAD	344 ± 23(8)	535 ± 24(7)	155	<0·001
MDH	NAD	1290 ± 32(7)	1540 ± 32(7)	119	<0·001

For identity of enzymes, see Methods section.
* Activity expressed as units/mg. fresh liver, where 1 unit = change in extinction at 340 mμ of 0·001/min.
† Mean ± S.E.M. (number of animals).

Liver reduced pyridine nucleotide:cytochrome c dehydrogenase activities

The results shown in Table 3 were again obtained during the "equilibrium-phase" of the response to CPIB treatment and show that the $NADH_2$- and $NADPH_2$:cytochrome-c dehydrogenases react differently, the former being actively reduced, the latter showing levels identical to the controls.

Liver tryptophan pyrrolase activity

The results of CPIB treatment for varying periods are shown in Table 4. In experiment A, a progressive decline of measurable activity in response to CPIB administration is observed. The extent of reduction may vary from one experiment to the next,

as shown by experiments A and B. The evidence obtained from several observations on this and other parameters suggests that the level is reduced to a minimum point and is then maintained as long as drug is given, thus corresponding to the equilibrium-phase response to CPIB previously mentioned. Normal levels are re-attained about 14 days after cessation of treatment.

TABLE 3. RAT LIVER REDUCED PYRIDINE NUCLEOTIDE:CYTOCHROME-C DEHYDROGENASE ACTIVITIES AFTER TREATMENT WITH 0·25% CPIB IN THE DIET FOR 48 DAYS

	Enzyme activity*			
	Control group Activity†	Treated group Activity†	% controls	P‡
$NADH_2$-dehydrogenase	465 ± 22(10)	279 ± 26(10)	60	<0·001
$NADPH_2$-dehydrogenase	23·1 ± 1·2(10)	22·0 ± 0·9(10)	95	>0·2

* Activity expressed as units × 10^{-3}/mg fresh liver, where 1 unit is a change in extinction at 550 mμ of 0·001/min, due to the reduction of cytochrome c.

† Mean ± S.E.M. (number of animals).

‡ Treated vs. control group.

TABLE 4. RAT LIVER TRYPTOPHAN PYRROLASE ACTIVITY AFTER TREATMENT WITH CPIB IN THE DIET FOR 1–22 DAYS

Experiment	Dose* of CPIB (%)	Duration of dosing (days)	Tryptophan pyrrolase activity (μmole kynurenine formed/g fresh liver/hr) Control Group Mean†	Treated Group Mean†	% controls	P‡
A	0·25	1		2·27 ± 0·30(3)	104	>0·2
		2		2·16 ± 0·30(3)	99	>0·2
		3		1·79 ± 0·30(3)	81	>0·1
		4	2·19 ± 0·11(23)	1·50 ± 0·30(3)	69	<0·05
		5		1·54 ± 0·30(3)	71	0·05
		8		0·95 ± 0·36(2)	44	0·005
		10		0·93 ± 0·30(3)	43	<0·001
		Recovery day 14		1·83 ± 0·23(5)	84	>0·1
B	0·25	22	1·87 ± 0·12(10)	1·29 ± 0·12(10)	69	0·005

* CPIB was incorporated in the diet (w/w) at the levels shown.

† Mean ± S.E.M. (number of animals).

‡ Treated vs. respective control group.

A diagrammatic representation of the pattern of response to treatment with 0·25% CPIB in the diet is shown in Fig. 1, the significance of which is discussed later.

DISCUSSION

The mechanism of action of CPIB has been discussed previously.[3] In brief, the available evidence indicates that CPIB causes a redistribution of several plasma-protein bound compounds such as thyroxine and tryptophan into the liver. The result of this is a disturbance in the metabolic equilibrium of the cell, which changes until a new balance is attained; this balance is then maintained throughout drug treatment.[3]

The results reported in this paper were obtained when the new equilibrium had been established following CPIB administration. The pattern of response is reproducible and characteristic for CPIB treatment.

The levels of NAD and $NADPH_2$ found in the control SPF-Wistar rats are in the low normal range of published values[10, 11, 22–30] whereas the level of $NADH_2$ is

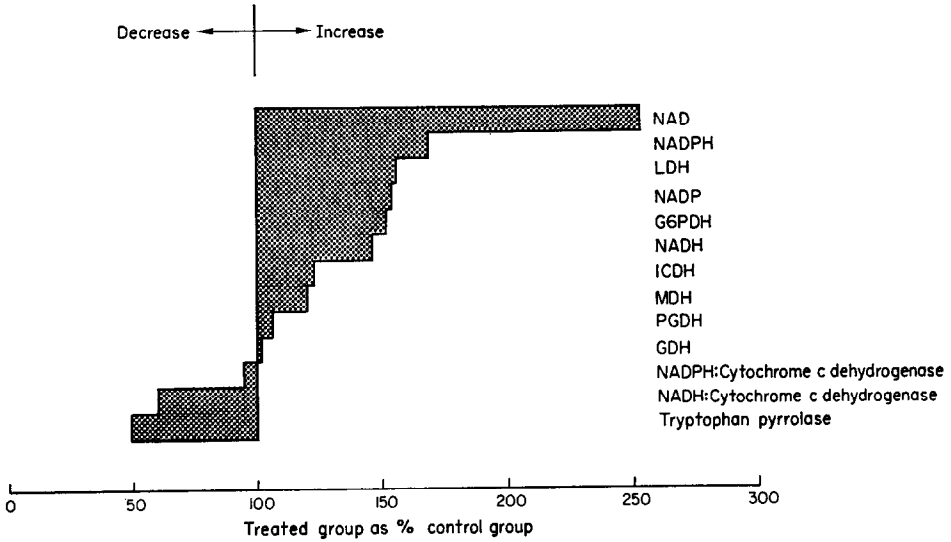

FIG. 1. The pattern of response to CPIB (0·25% in the diet) during the equilibrium-phase. The abbreviations used are indicated in the Methods section.

marginally higher. The NADP concentration found is of the same order as most of the published data and corresponds to the so-called "acid-stable" NADP described by Burch *et al.*[31] Apart from the controversy over true liver NADP levels, it is felt that the variation in the concentrations of pyridine nucleotide coenzymes is explained by strain difference.

The effects of several treatments on rat liver pyridine nucleotides have been examined, for example, diabetes[10, 23, 27], starvation,[10, 23, 27] growth hormone,[23] administration of thyroxine[23] or tryptophan,[33] carbon tetrachloride poisoning[29] and ethanol intoxication,[30] but none except tryptophan administration produces a pattern of response similar to that shown by CPIB. It is noteworthy that Garcia-Bunuel *et al.*[28] demonstrated a general decline in the concentrations of all the pyridine nucleotides, but particularly of NAD, during periods of niacin deficiency in rats, an effect almost exactly opposite to that seen after CPIB. The possible inference to be drawn is that CPIB in some way affects the rate or the extent of synthesis of the pyridine nucleotides, in particular NAD, this being the precursor of the other pyridine nucleotides. NAD is generally believed to be synthesised principally from niacin but tryptophan is now an accepted precursor, the conversion of quinolinic acid to nicotinic acid mononucleotide (an intermediate also in the pathway from niacin to NAD) having been recently demonstrated in rat liver.[32]

Plasma protein-bound tryptophan and thyroxine are displaced by CPIB[1, 2, 4, 5] into the liver. The primary influence on the liver pyridine nucleotide concentrations

will depend upon the interplay of these displaced compounds and the relative importance of each at different times; this is not understood at present, although an increase in the liver concentration of tryptophan would be expected to lead to a raised level of pyridine nucleotides.[33] Tryptophan pyrrolase constitutes the first step in the pathway of conversion of tryptophan to quinolinic acid and NAD, and Wagner[34] has demonstrated feed-back inhibition of tryptophan pyrrolase *in vitro* by NAD. It is possible therefore, that the raised levels of pyridine nucleotides following CPIB treatment leads to a similar inhibition to account for the low enzyme levels recorded. Glock and McLean[23] published data on the effects of thyroxine administration (0·5 mg sub. cut.) on rat liver pyridine nucleotide concentrations, but the results were unlike those seen after CPIB in this study where only physiological amounts of thyroxine were redistributed.[4, 5] The reported antagonism between thyroxine and tryptophan[35, 36] may have some influence on the position of the final equilibrium, although an accurate assessment of this interplay is not possible *in vivo*.

Measurement of $NADPH_2$:cytochrome-c dehydrogenase was made for comparison with the known effects of thyroxine administration, which specifically stimulates the activity of this enzyme in liver tissue;[37] a similar effect has been observed in hyperthyroidism.[38] Thyroxine at physiological levels reduces the activity of microsomal $NADH_2$:cytochrome-c dehydrogenase,[39] similarly to the effect of CPIB treatment. The failure of $NADPH_2$: cytochrome-c dehydrogenase to show a change in activity following CPIB administration cannot be explained at present. In view of the raised concentrations of the pyridine nucleotides and of the increased activities of the dehydrogenase enzymes after CPIB treatment, it is surprising that the activity of the reduced pyridine nucleotide:cytochrome-c dehydrogenases is either reduced or unchanged, since it is generally held that these enzymes provide the link to the respiratory chain and parallel the changes in the activities of the dehydrogenases.[38]

The reproducible pattern of response of the various dehydrogenase enzymes examined has several possible explanations; for example, the activity of a particular dehydrogenase may be regulated by the level of available pyridine nucleotide coenzyme. Garcia-Bunel *et al.*[28] have speculated that in niacin-deficiency, where the coenzyme levels are generally depleted, the enzymes with the highest dissociation constants for NAD or NADP show the greatest fall in activity; the opposite may hold true when the levels are raised, some enzymes showing greater responses than others, as observed after CPIB administration. Secondly, the enzyme activity may reflect the increase in total protein that occurs with CPIB treatment and may, therefore, represent secondary hepatic effects of thyroxine displacement.[3] The fact that some enzyme activities are increased and others decreased favours the explanation that the increase in liver protein following CPIB dosing is not a general increase but a selective increase, as postulated for the mode of action of thyroxine by Moury and Crane.[39] Thirdly, the effect may reflect the relative importance of these enzymes in their respective metabolic cycles, those exerting a rate-limitation showing the larger changes in response to the altered metabolism of the liver cell. This is consistent with current concepts of hormonal activity,[6–8] where regulation of specific enzyme synthesis is implicated.

The results of the present study do not contradict the proposed mode of action of CPIB,[3–5] i.e. the displacement of endogenous hormones and cofactors from serum to liver. The observed pattern of response reflects the interplay of some or all of these factors, and until the relative importance of each factor is understood, it is not possible

to say which predominates. The effects on rat liver weight, protein and glycogen concentrations and on the ribosomal incorporation of amino-acid following CPIB treatment indicate that thyroxine may be of primary importance in causing the change in the metabolic balance of the rat liver,[3] but comparison with the reported effects of thyroxine administration is difficult, since considerable discrepancies have occurred in the reported results due to the widespread use of pharmacological doses of this hormone. Pitt-Rivers and Tata[9] have reviewed this field and have emphasised the inadequacy of assessing thyroxine function following pharmacological doses of this hormone. It is possible, therefore, the CPIB may constitute a useful tool in assessing the primary effects of thyroxine in rat liver, since it is possible to displace physiological amounts from the serum to the liver and at the same time to prevent the extrahepatic secondary effects of conventional thyroxine administration.

Acknowledgement—The technical assistance of Miss J. Hamer is most gratefully acknowledged.

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