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Effect of α-*p*-Chlorophenoxyisobutyrate on the Metabolism of Isoprenoid Compounds in the Rat

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1. Feeding of α-*p*-chlorophenoxyisobutyrate (CPIB) to rats increased ubiquinone concentration in the liver but not in other tissues. The increase was progressive with the time of feeding and related to the concentration of CPIB in the diet. 2. Incorporation of [1-^{14}C]acetate, but not of [2-^{14}C]mevalonate, into sterols in the liver *in vivo* or by liver slices *in vitro* was decreased on feeding the rats with CPIB. However, incorporation of mevalonate into ubiquinone increased. 3. CPIB, when added in low concentrations to liver slices, had no effect on isoprene synthesis from acetate; higher concentrations, however, were inhibitory. 4. No activation of ubiquinone synthesis from mevalonate was observed when CPIB was added to the liver slices synthesizing ubiquinone. 5. The increase in ubiquinone in CPIB-fed animals appears to be due to increased synthesis in the initial stages and to decreased catabolism in the later stages. 6. An inverse relationship was found between the concentration of ubiquinone in the liver and the serum sterol concentration in CPIB-fed rats.

Several inhibitors of cholesterol biosynthesis have been used to decrease serum concentrations of triglycerides and cholesterol in hypercholesterolaemic patients. One such inhibitor is CPIB* ethyl ester, also known as Atromid-S or Clofibrate. Thorp & Waring (1962) first reported that this compound was able to lower the serum cholesterol concentration in the rat. Since then, a large number of publications have appeared describing clinical trials and the metabolic effects of the drug, but its mode of action remains unknown (Oliver, 1967).

Earlier work in which rats were fed with CPIB (Avoy, Swyryd & Gould, 1965) or ubiquinone (Krishnaiah, Joshi & Ramasarma, 1967*a*) showed remarkably similar effects of inhibition of hepatic synthesis of cholesterol and lowering of serum sterol concentrations. This was considered to be due to end-product inhibition by excess of ubiquinone in the liver of a common step in the synthesis of both cholesterol and ubiquinone. It was then discovered in this laboratory that CPIB increased ubiquinone concentration in the liver to the same extent as did feeding with exogenous ubiquinone (Krishnaiah, Inamdar & Ramasarma, 1967*b*). This was confirmed independently by Phillips, Lakshmanan & Brien (1968), who also found that the catabolism of ubiquinone was lowered under these conditions (Lakshmanan, Phillips & Brien, 1968). In the present paper the details of our study on the effect of feeding with CPIB on the synthesis of isoprenoid compounds are given. The results show that increased synthesis in the initial stages and decreased catabolism during the later stages of feeding with CPIB are responsible for the increase in ubiquinone concentration in the liver.

* Abbreviation: CPIB, α-*p*-chlorophenoxyisobutyrate.

EXPERIMENTAL

CPIB was obtained as a gift from Dr J. M. Thorp of Imperial Chemical Industries Ltd., Pharmaceuticals Division, Macclesfield, Cheshire, U.K.

[^{14}C]Ubiquinone-9 was obtained as a gift from Professor O. Wiss of Hoffman–La Roche and Co., Basle, Switzerland. Sodium [1-^{14}C]acetate (specific radioactivity 6.71mCi/mmol) was purchased from Bhabha Atomic Research Centre, Trombay, India. DL-[2-^{14}C]Mevalonic acid lactone (specific radioactivity 2.73mCi/mmol) was bought from The Radiochemical Centre, Amersham, Bucks., U.K. Samples of the lactone were delactonized before use by incubation with 0.05M-NaOH for 15min at 37°C followed by neutralization.

Adult male albino rats from this Institute's colony, weighing 150–180g, were used. The rats were fed on a synthetic casein diet with vitamins (Joshi, Jayaraman & Ramasarma, 1963). Water was given *ad libitum*.

The animals were generally killed under ether anaesthesia. For studies on liver slices the rats were killed by stunning and decapitation. When the blood was drawn, the rats were kept under mild ether anaesthesia and the blood was obtained by heart puncture.

The methods of administration of the tracers, processing of the livers, preparation and incubation of liver slices, saponification of the samples, fractionation of the unsaponifiable lipids, purification of the ubiquinone and sterol samples and their determination, and the measurement of radioactivity were the same as described by Krishnaiah *et al.* (1967*a*). The values reported are means of independent determinations on three rats in each group with less than 10% variation. In each case, the results are compared with those from corresponding normal animals.

Preparation of CPIB acid from the ethyl ester. This was done by the method of Avoy *et al.* (1965). The ester was hydrolysed with alkali and acidified, and the CPIB acid was extracted into diethyl ether. The solvent was evaporated and the acid was titrated with 1M-KOH to pH7.4.

RESULTS

Ubiquinone concentrations in tissues of CPIB-fed rats. It was earlier observed that the liver ubiquinone concentration increased in CPIB-fed rats (Krishnaiah *et al.* 1967*b*). The results in Table 1 indicate that the liver showed a considerable increase in ubiquinone content but other tissues did not. In kidney it increased to a slight extent whereas intestine and spleen showed no change.

Table 1. *Ubiquinone content of various tissues of CPIB-fed rats*

The rats were given CPIB (0.5% in the diet) for 10 days. The various tissues were removed after the animals were killed and the ubiquinone contents were determined. Only the change in liver ubiquinone is significant ($P < 0.01$). Six animals were used in each group; means ± S.D. are given.

	Ubiquinone (nmol/g of tissue)	
Tissue	Normal	CPIB-fed
Liver	98±7	218±20
Kidney	54±15	87±25
Intestine	24±7	26±8
Spleen	15±2	19±3

Incorporation of radioactive tracers in CPIB-fed rats. Feeding the rats with CPIB inhibited the incorporation of [1-^{14}C]acetate into total non-saponifiable lipids and sterols at the two time-intervals tested (Krishnaiah *et al.* 1967*b*) (Table 2). There was no quantitative difference in the extent of inhibition of sterol synthesis obtained when feeding with CPIB was continued for a longer time (25 days), although there was a further increase in liver weight. An increase in the incorporation into 'fatty acids' was observed in CPIB-fed rats. These results are in agreement with the results of Avoy *et al.* (1965).

The experiments with slices of the livers of the rats fed with CPIB showed results (Table 3) exactly similar to those obtained with intact animals: increase in ubiquinone content with increase in time, inhibition of sterol synthesis and no change in fatty acid synthesis.

The specificity of the tissue with respect to its susceptibility to the inhibition by CPIB was investigated. The results in Table 4 show that the drug produced inhibition of sterol synthesis only in the liver and not in the kidney or the intestine, effects similar to those obtained by dietary cholesterol and ubiquinone administration (Krishnaiah *et al.* 1967*a*). The pattern of incorporation of radioactivity into all the isoprene compounds was essentially similar in all the tissues.

When [2-^{14}C]mevalonate was used as the tracer, there was no inhibition of its incorporation into sterols in CPIB-fed animals (Table 5). There was an increase in radioactivity in ubiquinone in these animals compared with normal controls, and no

Table 2. *Incorporation of* [1-^{14}C]*acetate into liver isoprenoid compounds and fatty acids by CPIB-fed rats*

The rats were given CPIB (0.5% in the diet) for the specified periods. After this the rats were given [1-^{14}C]acetate (50 μCi/rat) and killed after 4 h. All the radioactivity and weight values given below are per liver and are the averages of independent analyses of three rats in each group. The values in parentheses are the percentage of the radioactivity found in each fraction, the total in non-saponifiable matter being taken as 100%.

	Mean liver weight	Non-saponifiable lipids	Hydrocarbons		Ubiquinone		Sterols		Fatty acids
Status of rats	(g)	(c.p.m.)	(c.p.m.)	(mg)	(c.p.m.)	(nmol)	(c.p.m.)	(mg)	(c.p.m.)
Normal	7.3	6340	750 (13)	2.2	290 (5)	510	4250 (67)	16.5	20550
CPIB-fed (10 days)	9.8	3910	620 (16)	2.3	430 (11)	1390	2210 (57)	15.5	42560
Normal	7.5	7560	670 (9)	2.0	310 (4)	640	5160 (68)	16.0	26500
CPIB-fed (25 days)	11.0	5120	560 (11)	2.1	360 (7)	3080	3170 (60)	17.5	33700

Table 3. *Incorporation of* [1-^{14}C]*acetate into non-saponifiable lipids and fatty acids by liver slices from CPIB-fed rats*

The rats were given CPIB (0.5% in the diet). After the experimental periods the rats were killed by stunning and decapitation, and each liver was sliced and transferred to 6ml of Krebs–Ringer 0.1M-phosphate buffer, pH7.4 (Krebs & Henseleit, 1932), and incubated for 3h at 37°C in an oxygen atmosphere in the presence of [1-^{14}C]acetate (10μCi/flask). Radioactivity and weight values are given per g of liver and represent the averages of independent determinations from slices taken from three rats in each group. The values in parentheses are the percentage of the radioactivity found in each fraction, the total in non-saponifiable matter being taken as 100%.

Status of rats	Non-saponifiable lipids (c.p.m.)	Hydrocarbons (c.p.m.)	Ubiquinone (c.p.m.)	Ubiquinone (nmol)	Sterols (c.p.m.)	Sterols (mg)	Fatty acids (c.p.m.)
Normal	9830	1020 (10)	312 (3)	73	6430 (65)	2.4	25400
CPIB-fed (10 days)	4200	630 (15)	240 (6)	132	2640 (63)	2.2	24750
Normal	15650	1270 (8)	330 (2)	75	10040 (66)	2.3	21770
CPIB-fed (25 days)	9780	840 (9)	290 (3)	210	6170 (63)	2.1	19680

Table 4. *Incorporation of* [1-^{14}C]*acetate into non-saponifiable lipids of liver, kidney and intestine*

The rats were given CPIB (0.5% in the diet) for 10 days. After this period [1-^{14}C]acetate (50μCi/rat) was given orally and the animals were killed after 4h. Radioactivity and weight values are given per liver and represent the averages of independent analyses from three rats in each group; the values in parentheses are the percentage of the radioactivity found in each fraction, the total in non-saponifiable matter being taken as 100%.

Tissue	Status of rats	Non-saponifiable lipids (c.p.m.)	Hydrocarbons (c.p.m.)	Hydrocarbons (mg)	Ubiquinone (c.p.m.)	Ubiquinone (nmol)	Sterols (c.p.m.)	Sterols (mg)
Liver	Normal	8540	570 (7)	2.2	280 (3)	875	6065 (71)	13.8
	CPIB-fed	4970	400 (8)	2.4	160 (3)	1715	3450 (69)	15.7
Kidney	Normal	2580	150 (6)	1.0	100 (4)	72	1760 (68)	3.0
	CPIB-fed	2700	165 (6)	1.2	105 (4)	75	1840 (68)	2.8
Intestine	Normal	47460	4150 (9)	6.2	850 (2)	30	37500 (79)	13.9
	CPIB-fed	51380	4500 (9)	8.0	810 (2)	38	39460 (78)	14.8

change in the hydrocarbon fraction. As explained below, the increase in radioactivity in ubiquinone in CPIB-fed animals appears to be due to increased rate of synthesis.

Exactly similar results were obtained with liver slices obtained from CPIB-fed animals when [2-^{14}C]mevalonate was used as the tracer: lack of inhibition of isoprene synthesis, increase in ubiquinone synthesis and no change in the hydrocarbon fraction (Table 6).

Effect of addition of CPIB in vitro *on the incorporation of tracers by the tissue slices.* The results in Table 7 show that when CPIB was added at concentrations of up to 100μg in the incubation medium (6ml) there was no inhibition of synthesis of sterols and fatty acids from [1-^{14}C]acetate. However, a higher concentration (200μg/6ml) showed small inhibitory effects on isoprene synthesis and 'fatty acid' synthesis. It is therefore possible that CPIB, when added *in vitro* at higher concentrations, has an inhibitory effect on the activation of acetate.

It was decided to test whether CPIB could activate ubiquinone synthesis from [2-^{14}C]mevalonate when added *in vitro* to the normal liver slices. The results in Table 7 show that there was no

Table 5. *Incorporation of* [2-^{14}C]*mevalonate into non-saponifiable lipids of CPIB-fed rats* in vivo

The rats were given CPIB (0.5% in the diet). After the experimental period the rats were dosed orally with radioactive mevalonate (2 μCi/rat) and killed after 4 h. All the radioactivity and weight values are given per liver and represent the averages of independent analyses from three rats in each group. The values in parentheses are the percentage of the radioactivity found in each fraction, the total in non-saponifiable matter being taken as 100%.

Status of rat	Liver weight (g)	Non-saponifiable lipids (c.p.m.)	Hydrocarbons (c.p.m.)	Ubiquinone (c.p.m.)	Ubiquinone (nmol)	Sterols (c.p.m.)	Sterols (mg)
Normal	7.4	40300	3170 (8)	2400 (6)	600	22800 (57)	21.0
CPIB-fed (10 days)	9.7	39380	2950 (7)	3000 (7)	1400	20500 (52)	19.0
Normal	10.0	40670	3590 (9)	1690 (4)	860	22740 (57)	23.6
CPIB-fed (25 days)	13.5	42600	3190 (7)	2740 (7)	2600	26360 (62)	18.5

Table 6. *Incorporation of* [2-^{14}C]*mevalonate into non-saponifiable lipids by liver slices of CPIB-fed rats*

The rats were given CPIB (0.5% in the diet) for 10 days. The experimental conditions are the same as in Table 3. [2-^{14}C]Mevalonate (2 μCi/flask) was added to the slices. The radioactivity and weight values are per g of liver and represent the averages of independent analyses of slices from three rats in each group. The values in parentheses are the percentage of the radioactivity found in each fraction, the total in non-saponifiable matter being taken as 100%.

Status of rats	Non-saponifiable lipids (c.p.m.)	Hydrocarbons (c.p.m.)	Ubiquinone (c.p.m.)	Ubiquinone (nmol)	Sterols (c.p.m.)	Sterols (mg)
Normal	71250	12130 (17)	520	72	38540 (54)	2.54
CPIB-fed	100300	17150 (17)	1000	128	45450 (45)	2.20

increase in radioactivity in ubiquinone when CPIB was added, except a small activation at the concentration of 10 μg/6 ml, which may be due to higher incorporation of the tracer into total non-saponifiable lipids. In fact an inhibition of synthesis of ubiquinone as well as of non-saponifiable lipids was obtained at high concentration of CPIB. The results suggest that CPIB acts indirectly in increasing the synthesis and concentration of ubiquinone *in vivo*.

Incorporation of [2-^{14}C]*mevalonate into ubiquinone in CPIB-fed rats: time study.* To find whether ubiquinone synthesis could be activated in experiments *in vivo* progressively with the time of feeding with CPIB, the incorporation of [2-^{14}C]mevalonate was studied in rats fed with CPIB for various times. Fig. 1 shows that the increase in synthesis was maximal at 20 days, after which it decreased and returned to the normal value at 50 days. The increase in ubiquinone content was progressive with the time of feeding CPIB to the animals. These results showed that in the initial periods of feeding the synthesis increased till sufficient quantities of ubiquinone were built up. This high ubiquinone content would be expected to inhibit its own synthesis (Krishnaiah *et al.* 1967*a*), which may be the reason for the lower ubiquinone synthesis at later times.

Effect of feeding with CPIB on ubiquinone in the liver: concentration and time study. Progressively with the time of feeding with CPIB (0.3% in the diet), wet weight and ubiquinone concentration of the liver increased (Table 8). At later times the concentration of liver sterols decreased. With increase in the dose of CPIB given for 30 days the wet weight and ubiquinone content of the liver increased (Table 8).

In the above set of experiments the retention of orally administered [^{14}C]ubiquinone was also tested. Orally administered ubiquinone is exclusively absorbed into the liver and catabolized and removed therefrom over a period of days (Jayaraman, Joshi & Ramasarma, 1963). If the rate of catabolism were lowered it may be expected

Table 7. *Effect of adding CPIB* in vitro *on the incorporation of* [1-^{14}C]*acetate and* [2-^{14}C]*mevalonate by rat liver slices*

Liver slices from normal rats were prepared and incubated in Krebs–Ringer 0.1 M-phosphate buffer, pH 7.4, at 37°C for 2h in the presence of added neutralized CPIB acid. [1-^{14}C]Acetate (10 μCi/flask) or [2-^{14}C]mevalonate (2 μCi/flask) was added to appropriate flasks as described below. The values in parentheses are the percentages of the radioactivity found in each fraction, the total in non-saponifiable matter being taken as 100%.

Additions		Incorporation (c.p.m./g of liver)				
Tracer	CPIB (μg)	Non-saponifiable lipids	Hydrocarbons	Ubiquinone	Sterols	Fatty acids
Acetate	0	8780	740 (9)	250	6570 (75)	28600
	10	7000	670 (10)	270	5940 (85)	27600
	100	8380	780 (9)	260	6370 (76)	31560
	200	5410	380 (7)	200	4860 (89)	21300
Mevalonate	0	55940	1550 (3)	390	37690 (67)	—
	10	64440	1420 (2)	460	45880 (71)	—
	50	53260	4780 (9)	320	47140 (88)	—
	200	39170	2700 (7)	130	31500 (80)	—

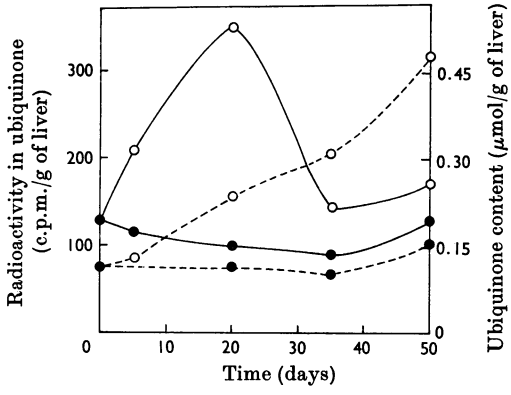

Fig. 1. Incorporation of [2-^{14}C]mevalonate into ubiquinone in the livers of rats fed with CPIB for various times. The rats were given CPIB (0.5% in the diet). After each experimental period the rats were orally dosed with [2-^{14}C]mevalonate and killed after 4h. The radioactivity (——) and concentration (----) of liver ubiquinone were determined. Each value is an average of independent analyses of two rats. ●, Normal rats; ○, CPIB-fed rats.

that a larger amount of the absorbed ubiquinone would be retained in the liver. Thus, it was considered that an increase in the amount of radioactivity (compared with the control animals) retained in the liver 72h after administration of a single dose of [^{14}C]ubiquinone would be indicative of a decreased rate of catabolism (Joshi & Ramasarma, 1966). The results in Table 8 show that, at 30 days and at dietary concentrations above 0.3% of CPIB, there was a considerable increase in the retention of [^{14}C]ubiquinone, suggesting decreased catabolism. A similar conclusion was reached by Lakshmanan *et al.* (1968), who studied the incorporation of [2-^{14}C]mevalonate into ubiquinone at 72h after administration of the tracer.

The relationship between the concentration of ubiquinone in the liver and sterols in the serum in the above two experiments with feeding with CPIB is shown in Fig. 2. An inverse relationship between liver ubiquinone and serum sterol concentration was observed.

DISCUSSION

The lack of activation of ubiquinone synthesis by CPIB added *in vitro* suggests that CPIB acts in the intact animal in an indirect way, probably through a metabolic alteration. However, in the system *in vitro* when CPIB was added there was an increase in radioactivity in the 5% diethyl ether fraction (not shown in results) that was not associated with ubiquinone on further purification on

Table 8. *Effect of feeding with CPIB on concentration of ubiquinone in the liver*

The rats were given CPIB at the specified concentration in the diet for the periods indicated, and 72h before being killed each rat received orally [^{14}C]ubiquinone-9 (0.5μCi). Six rats were used in each group and the means ± S.D. are given.

Time of feeding (days)	Concn. of CPIB in the diet (%)	Liver weight (g)	Ubiquinone (nmol/g)	Sterols (mg/g)	Radioactivity in ubiquinone (c.p.m./liver)
Control	0	6.7±0.7	131±19	2.40±0.23	3370±540
5	0.3	7.1±0.9	158±20†	2.20±0.23	3490±180
10	0.3	7.7±0.8	180±13*	2.00±0.16	3440±340
20	0.3	8.1±0.4	206±17*	1.90±0.18	3810±600
30	0.3	8.5±0.8†	238±30*	1.90±0.13*	4990±530*
30	0.1	7.2±0.9	186±21†	2.00±0.21	3710±510
30	0.5	9.6±0.9	260±18*	1.70±0.14*	6600±140*

* $P<0.001$; † $P<0.01$ (compared with controls).

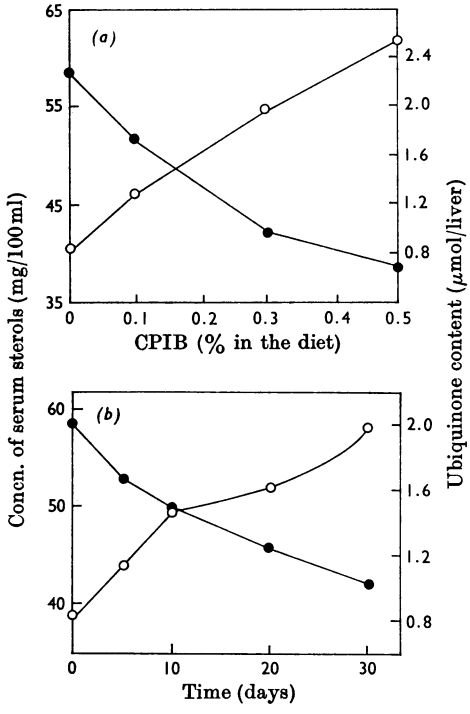

Fig. 2. Inverse relationship between liver ubiquinone content and serum sterol concentration in CPIB-fed rats. The rats were given CPIB at various concentrations in the diet for 30 days (*a*) and at 0.3% in the diet for various times (*b*). Samples of the liver and serum from six rats in each group were independently analysed. For clarity, only the mean values are plotted. ○, Liver ubiquinone content; ●, serum sterol concentration.

t.l.c. on alumina, the technique always employed in the analysis of radioactivity in ubiquinone. This needs further investigation. The possibility exists that CPIB *in vivo* may be activating the aromatic pathway synthesizing the quinone ring of the ubiquinone molecule, which may result in increased synthesis of ubiquinone in the animals given the drug.

The increase in ubiquinone concentration in CPIB-fed animals appears to be due to increased synthesis as well as decreased catabolism. Phillips *et al.* (1968) argued that the actual ubiquinone synthesis was inhibited in CPIB-fed animals, and that what was observed was only a dubious increase of radioactivity from mevalonate due to the block before mevalonate resulting in an increased specific radioactivity of the mevalonate pool. If this were true, only a twofold increase in radioactivity should be found in ubiquinone in view of the 50% maximum inhibition observed, but the several-fold increase at an intermediate stage (20 days) clearly demonstrates that ubiquinone synthesis was in fact increased. At late times of feeding with CPIB, as used by Phillips *et al.* (1968), catabolism of ubiquinone was lowered and synthesis returned to normal. Hence there is no essential disagreement between the results of Phillips *et al.* (1968) and the present studies.

Several lines of evidence (summarized by Ramasarma, 1968) suggest that CPIB probably acts by increasing the concentration of ubiquinone in the liver, which in turn produces the inhibition of sterol synthesis and decreases serum sterol concentrations:

CPIB → liver ubiquinone increased → serum sterols decreased

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