STUDIES ON THE MECHANISM OF ACTION OF α-PHENYLBUTYRATE

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Following the appearance of reports by COTTET and co-workers that sodium α-phenylbutyrate* could lower serum cholesterol levels in normal rats and in hypercholesterolemic patients[1], studies were initiated in this laboratory on the mode of action of this and related compounds[2]. It has been shown in previous reports that α-phenylbutyrate inhibits the rate of incorporation of ^{14}C-labeled acetate into cholesterol and fatty acids by rat liver, both *in vitro* and *in vivo*[3]. Incorporation of acetate-^{14}C into acetoacetate by liver slices *in vitro* was inhibited to about the same extent as was incorporation into fatty acids and cholesterol. Oxidation of acetate-^{14}C to $^{14}CO_2$ was inhibited to a variable extent, but this effect was always less marked than the effects on lipide synthesis. *In vivo* animal studies and patient studies[4], like those since reported from several other laboratories[5], failed to demonstrate any effects on serum cholesterol levels, in conflict with the results of COTTET's group and of several other European laboratories[6,7,8]. These findings indicated that the site of action of α-phenylbutyrate might be limited to the earliest reactions in the biosynthetic pathway from acetate to cholesterol. The present studies, undertaken in an attempt to define more closely the mechanism of action, demonstrate that the compound inhibits acetate activation by aceto-CoA-kinase and suggest that most, if not all, of the observed metabolic effects may be attributed to this inhibition. Additional sites of action are not ruled out.

METHODS

Butyrate oxidation by Cl. kluyveri – (see Table I)

Dried cells of *Cl. kluyveri*, prepared by the method of STADTMAN AND BARKER[9], were kindly furnished by Dr. EARL STADTMAN. Acetyl phosphate was determined as the acethydroxamate[10,11]. Acetoacetate was determined manometrically after decarboxylation catalyzed by aniline citrate[12].

Oxidation of octanoate by rat-liver mitochondria

Rat-liver mitochondria were prepared by the abridged procedure of KENNEDY AND LEHNINGER[13]. Oxygen consumption was measured during incubation in Warburg vessels (see Table II).

Acetylation of sulfanilamide by pigeon-liver acetone powder

Pigeon-liver acetone powder was prepared according to the method of KAPLAN AND LIPMANN[14]. Following the incubation (see Table III) the protein was precipitated by addition of 4 ml of 15% trichloroacetic acid. One ml of supernatant fluid was used for the determination of free sulfanilamide by the method of BRATTON AND MARSHALL[15]. Optical density was determined with a Coleman spectrophotometer at 545 mμ.

Activation of acetate by aceto-CoA-kinase

Partially purified aceto-CoA-kinase from frozen baker's yeast (kindly furnished by Dr. SIMON

* In the European literature this compound is named phenylethylacetic acid, sodium salt.

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BLACK) was prepared according to the method of JONES AND LIPMANN[16]. Details of the reaction mixture are listed above Table IV. S-acetyl glutathione was used as a standard in estimating hydroxamate formation.

Effect of α-phenylbutyrate on metabolism of glucose-^{14}C and pyruvate-^{14}C by surviving rat-liver slices

Whole liver slices, 300–400 mg per flask, were prepared and incubated according to the procedure described in Table V. The nonsaponifiable fraction of the liver lipides was isolated by conventional procedures and radioactivity was determined using a liquid scintillation counter (Packard Tricarb). Aliquots of the nonsaponifiable fraction in heptane were taken to dryness in the counting vial and then taken up in 15 ml of toluene containing 400 mg % of diphenyloxazole.

RESULTS

STADTMAN AND BARKER have shown that the oxidation of butyrate by dried cells of *Cl. kluyveri* leads to the production of two moles of acetyl-S-CoA[9]. One mole of acetyl-S-CoA* is utilized for the activation of more free butyrate, thus making the oxidation autocatalytic with respect to CoA. The other is converted to acetylphosphate by the action of phosphotransacetylase. Equimolar amounts of free acetate and acetyl phosphate thus accumulate as end products of butyrate oxidation in this system[17].

From Table I, it can be seen that $1 \cdot 10^{-2} M$ α-phenylbutyrate had no effect on the metabolism of butyrate by dried cells of *Cl. kluyveri* with respect to O_2 consumption, acetyl phosphate formation or acetoacetate production.

TABLE I

BUTYRATE OXIDATION IN DRIED CELLS OF *Cl. kluyveri*

α-Phenylbutyrate concentration	*Oxygen consumed (micromoles)*	*Acetyl phosphate formed (micromoles)*	*Acetoacetate formed (micromoles)*
None	16.3	8.2	1.2
$1 \cdot 10^{-2} M$	17.0	8.3	1.1

The incubation medium contained 0.1 ml of 1.0 *M* Tris buffer, pH 7.8, 0.05 ml of 0.5 *M* reduced glutathione, pH 7.5, 0.1 ml of 1 *M* phosphate buffer, pH 8, 0.1 ml of 1.0 *M* sodium butyrate, 0.5 ml of dried cells of *Cl. kluyveri*, 100 mg/ml and inhibitor as shown below. Final vol. 2 ml. Incubation time, 19 min.

Inhibition of the β-ketothiolase reaction in this system would be expected to lead to a decreased yield of acetyl phosphate and, possibly, an accumulation of acetoacetate. The absence of any such effects appears to rule out inhibition of β-ketothiolase. It should be noted also that the failure to inhibit overall butyrate oxidation rules out an inhibition of butyrate activation, catalyzed here by CoA transphorase, and suggests that none of the reactions for degradation of fatty acid CoA complexes are inhibited.

Studies on the effect of α-phenylbutyrate on the metabolism of octanoate by rat liver mitochondria are shown in Table II. There was marked inhibition of oxidation even at $1 \cdot 10^{-3} M$ α-phenylbutyrate. This result, combined with the above results in *Cl. kluyveri*, suggests inhibition at the level of octanoate activation, assuming there is no important difference in the systems for fatty acid degradation.

* The following abbreviations are used: CoA, coenzyme A; acetyl-CoA, S-acetylcoenzyme A; ATP, adenosine triphosphate; DPNH, reduced diphosphopyridine nucleotide; TCA, trichloroacetic acid.

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TABLE II

EFFECT OF α-PHENYLBUTYRATE ON OXYGEN CONSUMPTION BY RAT-LIVER MITOCHONDRIA WITH OCTANOATE AS SUBSTRATE

α-phenylbutyrate	*Octanoate*	*Oxygen consumed (microliters)*
0	$1.6 \cdot 10^{-3}$ *M*	201.2
0	0	67.8
$1 \cdot 10^{-3}$ *M*	$1.6 \cdot 10^{-3}$ *M*	79.2
$1 \cdot 10^{-2}$ *M*	$1.6 \cdot 10^{-3}$ *M*	67.4

The incubation medium contained 1 ml of a mitochondrial suspension in 0.15 *M* KCl, 0.3 ml of 0.016 *M* sodium octanoate, pH 7.4, 0.3 ml of 0.03 *M* DPNH, 0.3 ml of 0.01 *M* ATP, 0.3 ml of 0.05 *M* $MgCl_2$, 0.3 ml of 10^{-4} *M* cytochrome *c*, 0.2 ml of 0.15 *M* phosphate buffer, pH 7.4 and H_2O to a final vol. of 3 ml. The center well contained 0.3 ml 20% KOH. Flasks were incubated for 1 h at 37°C.

TABLE III

EFFECT OF α-PHENYLBUTYRATE ON ACETYLATION OF SULFANILAMIDE BY PIGEON-LIVER ACETONE POWDER

CoA (micromoles/ml)	*α-phenylbutyrate*	*Sulfanilamide acetylated (mg) × 10^{-3}*	*Percentage inhibition*
$0.72 \cdot 10^{-2}$	0	5.8	
$0.72 \cdot 10^{-2}$	$1 \cdot 10^{-2}$ *M*	3.5	39
$1.20 \cdot 10^{-2}$	0	6.8	
$1.20 \cdot 10^{-2}$	$1 \cdot 10^{-2}$ *M*	5.3	22
$2.4 \cdot 10^{-2}$	0	7.6	
$2.4 \cdot 10^{-2}$	$1 \cdot 10^{-2}$ *M*	6.7	12

The *reaction mixture* contained 10 ml of 0.004 *M* sulfanilamide, 2.5 ml of 1 *M* sodium acetate, 8.0 ml of 0.05 *M* ATP and 10 ml of 0.2 *M* sodium citrate.

The *incubation mixture* contained 0.3 ml of the above *reaction mixture*, 0.1 ml of 1 *M* $NaHCO_3$, 0.6 ml of aged enzyme[14], 0.1 ml of 1.0 *M* cysteine hydrochloride, 0.1 ml of 0.01 *M* $MgCl_2$, CoA and α-phenylbutyrate as indicated. Water was added to a final volume of 2 ml. Incubation time was 2 h at 37° C.

Acetate activation and acetyl transfer were studied directly using the sulfanilamide acetylating system of pigeon liver[14]. As shown in Table III, α-phenylbutyrate at a concentration of $1 \cdot 10^{-2}$ mole effected a 39% inhibition of sulfanilamide acetylation. At higher levels of CoA, the extent of this inhibition was less marked, falling to 12% at a CoA concentration of $2.4 \cdot 10^{-2}$ μmole/ml. At still higher levels of CoA there was essentially no inhibition. At concentrations above $7 \cdot 10^{-2}$ μmole/ml, CoA itself was observed to inhibit slightly. On the other hand, the percentage inhibition of sulfanilamide acetylation by α-phenylbutyrate was independent of acetate concentrations from 0.12 to 12.5 μmoles/ml.

Because sulfanilamide acetylation involves at least two enzymes[18], it was desirable to study the effect of α-phenylbutyrate on the isolated first step, activation of acetate to acetyl-S-CoA[14]. As shown in Table IV, acetate activation by the aceto-CoA-kinase of yeast[16] was inhibited 70–80% by $1 \cdot 10^{-2}$ *M* α-phenylbutyrate.

Further experiments were carried out in an attempt to determine whether the inhibitory effect of α-phenylbutyrate could be entirely attributable to inhibition of

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TABLE IV

EFFECT OF α-PHENYLBUTYRATE ON ACTIVATION OF ACETATE BY ACETO-CoA-KINASE

α-Phenylbutyrate	*Acethydroxamate formed (micromoles)*	*Percentage inhibition*
0	0.34	70.5%
$1 \cdot 10^{-2}$ *M*	0.1	
0	0.25	80%
$1 \cdot 10^{-2}$ *M*	0.05	

The incubation mixture contained 0.1 ml of 0.2 *M* potassium acetate, 0.1 ml of 1 *M* phosphate buffer, pH 7.5, 0.05 ml of 1 *M* KF, 0.05 ml of 0.2 *M* $MgCl_2$, 0.05 ml of 0.2 *M* reduced glutathione adjusted to pH 4.5 with KOH, 0.1 ml of 0.1 *M* ATP at pH 7.5, 0.1 ml of $8.1 \cdot 10^{-4}$ *M* CoA, 0.2 ml of an ammonium sulfate fraction of aceto-CoA-kinase, 0.1 ml of freshly prepared solution of 2 *M* hydroxylamine, pH 7.4. Water was added to make the final volume 1.0 ml. Incubation time was 20 or 30 min at 37°C. Acethydroxamate was determined according to JONES AND LIPMANN[16].

TABLE V

EFFECT OF α-PHENYLBUTYRATE ON THE INCORPORATION OF GLUCOSE-U-^{14}C AND PYRUVATE-2-^{14}C INTO CHOLESTEROL

Experiment	*α-Phenylbutyrate*	*Labeled substrate*	*Radioactivity in nonsaponifiable lipide (c.p.m./100 mg liver)*
I	0	Acetate	508
	$1 \cdot 10^{-3}$ *M*	Acetate	222
	0	Glucose	61.3
	$1 \cdot 10^{-3}$ *M*	Glucose	96.3
II	0	Acetate	352
	$1 \cdot 10^{-3}$ *M*	Acetate	250
	0	Glucose	48.6
	$1 \cdot 10^{-3}$ *M*	Glucose	52.0
III	0	Acetate	1899
	$1 \cdot 10^{-3}$ *M*	Acetate	690
	0	Glucose	110.1
	$1 \cdot 10^{-3}$ *M*	Glucose	106.2
IV	0	Glucose	91.6
	$1 \cdot 10^{-3}$ *M*	Glucose	42.0
V	0	Glucose	48.2
	$1 \cdot 10^{-3}$ *M*	Glucose	12.6
VI	0	Pyruvate	257.6
	$1 \cdot 10^{-3}$ *M*	Pyruvate	224.2
VII	0	Pyruvate	154.8*
	$1 \cdot 10^{-3}$ *M*	Pyruvate	83.6*

* Digitonin precipitable lipides counted.

300–400 mg of rat liver slices were incubated under oxygen in 3 ml of a modified Krebs-Ringer phosphate buffer, pH 7.4, for 4 h at 37°C. Five μc of each of the labeled substrates was added as indicated. Final substrate concentrations used were: acetate, 38.8 μmoles/ml; glucose, 7 μmoles/ml; pyruvate, 1.25 μmoles/ml.

acetate activation, or if there might be, in addition, inhibition at later stages in cholesterol biosynthesis. KORKES *et al.*[19,20,21] have shown that acetyl-S-CoA can be generated directly from pyruvate, without the participation of aceto-CoA-kinase. By using uniformly labeled glucose-^{14}C as substrate, it should be possible to generate labeled acetyl-S-CoA without obligatory participation of aceto-CoA-kinase. Inhibition by α-phenylbutyrate of incorporation of glucose-U-^{14}C into cholesterol would then point to an additional site of action in the biosynthetic pathway.

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As shown in Table V, the results with glucose-U-^{14}C were variable. In Experiments I, II and III α-phenylbutyrate inhibited 1-^{14}C-acetate incorporation into cholesterol by 29–64%, but there was no inhibition of glucose-U-^{14}C incorporation. On the other hand, in Experiments IV and V there was marked inhibition in the conversion of glucose-U-^{14}C to cholesterol. The possibility that inhibition of glycolysis rather than inhibition in the cholesterol biosynthetic pathway *per se* was responsible had to be considered. The effect of α-phenylbutyrate on the metabolism of 2-^{14}C pyruvate was tested in an attempt to clarify the result. Here again, the results were variable but there was inhibition of 13 and 43% in the incorporation of ^{14}C from pyruvate into cholesterol in two experiments shown in Table V.

DISCUSSION

The present study indicates that α-phenylbutyrate inhibits the activation of acetate by aceto-CoA-kinase. This is shown directly by the studies on acetyl-CoA synthesis by yeast aceto-CoA-kinase and indirectly by the studies on sulfanilamide acetylation. GARATTINI *et al.*[22] and MILHAUD AND AUBERT [3] have also demonstrated inhibition of sulfanilamide acetylation by α-phenylbutyrate *in vitro* and *in vivo*. The failure to affect butyrate oxidation in *Cl. kluyveri* suggests that β-ketothiolase is not inhibited. In this discussion we assume that similar enzymes isolated from different organisms respond similarly to α-phenylbutyrate. Inhibition of acetate activation alone would adequately explain the observed effects of this compound on lipide synthesis previously reported[2,3] and would be compatible with failure to influence net cholesterol metabolism or serum cholesterol levels[4] since acetyl-CoA could be generated at normal rates from other metabolic precursors. On the other hand, it cannot be stated from these studies alone whether or not this is the only site of action.

If aceto-CoA-kinase were the only enzyme affected by it, α-phenylbutyrate should not inhibit the incorporation of ^{14}C from labeled glucose into cholesterol. In several experiments this was indeed the case: incorporation of acetate-1-^{14}C was decreased but incorporation of glucose-U-^{14}C was not. In other experiments, however, there was significant suppression of glucose-1-^{14}C incorporation. The reasons for the equivocal results obtained are not clear but may relate to the dietary state of the animals at the time of sacrifice. The presence of inhibition at times with both glucose-U-^{14}C and pyruvate-2-^{14}C suggests that either the pathway from glucose and pyruvate to acetyl-CoA is inhibited by the action of α-phenylbutyrate or that the latter exerts its effect at more than one site in the cholesterol biosynthetic pathway.

The conclusion of ADAMSON AND GREENBERG[24] that α-phenylbutyrate exerts an inhibitory effect on cholesterol biosynthesis beyond the five- or six-carbon stage is not incompatible with these results. However, their conclusion that α-phenylbutyrate does not inhibit the formation of acetyl-CoA is not compatible with the present findings or with the results of other workers[22,23]. The inhibitory effects of α-phenylbutyrate on the incorporation of ^{14}C-acetate into fatty acids, generally of the same magnitude as the effect on incorporation into cholesterol and on the oxidation of ^{14}C-acetate[3], is not explained by their postulate of inhibition beyond the five- or six-carbon stage. TAVORMINA AND GIBBS[25] have shown that in liver homogenates α-phenylbutyrate inhibits the incorporation of 1-^{14}C acetate into cholesterol to a much greater extent (83%) than the incorporation of ^{14}C-labeled mevalonic acid (33%). Their studies show again that a

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major site of action is at an early stage in the biosynthetic pathway but the results are also compatible with a second site of action at a later stage.

SUMMARY

1. α-Phenylbutyrate inhibits the acetylation of sulfanilamide in a pigeon-liver system. This inhibition is reversed by higher concentrations of coenzyme A but is independent of acetate concentration over a wide range.

2. α-Phenylbutyrate is without effect on the metabolism of butyrate by dried cells of *Cl. kluyveri* indicating that the enzymes catalyzing fatty acid degradation, including β-ketothiolase, are not inhibited.

3. Studies utilizing the aceto-CoA-kinase of baker's yeast demonstrate directly that α-phenylbutyrate inhibits the activation of acetate to acetyl-coenzyme A.

4. The incorporation of glucose-U-^{14}C and pyruvate-2-^{14}C into cholesterol by rat-liver slices was unaffected in some experiments in which the incorporation of 1-^{14}C-acetate was depressed. In other experiments, however, there was significant inhibition.

5. A major site of action of α-phenylbutyrate is in the activation of acetate but additional sites of action are not ruled out.

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