Interactions of Acetate, Propionate and Butyrate in Sheep Liver Mitochondria

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1. Interactions in the rates of consumption of acetate, propionate and butyrate in sheep liver mitochondria were examined in the presence and absence of L-malate and \(\alpha\)-oxoglutarate. 2. Acetate was not consumed in absence of ancillary substrate but utilization of acetate (7.2 nmol/min per mg of protein) occurred in the presence of \(\alpha\)-oxoglutarate. This consumption was abolished by propionate or butyrate but the presence of acetate did not affect consumption of propionate or butyrate. 3. Propionate consumption (10.1 nmol/min per mg of protein) was unaffected by malate but was stimulated by 63% by butyrate or by 180% by \(\alpha\)-oxoglutarate. 4. Butyrate consumption (3.3 nmol/min per mg of protein) was stimulated by 117% by malate, by 151% by propionate and by 310% by \(\alpha\)-oxoglutarate. 5. In the absence of ancillary substrates the maximum rate of total volatile fatty acid utilization (24.7 nmol/min per mg of protein) occurred with a mixture of propionate and butyrate. When both propionate and butyrate were present total consumption was not affected by malate but was stimulated by 24% by \(\alpha\)-oxoglutarate. With \(\alpha\)-oxoglutarate present, propionate and butyrate each decreased the other's consumption by about 28%, but the total utilization was the greatest observed. 6. The inhibition of acetate consumption by propionate or butyrate is unexplained, but the remaining effects are consistent with an interaction of propionate and butyrate through oxaloacetate together with a general limitation imposed by a need for GTP to rephosphorylate AMP formed during activation of the volatile fatty acids.

Much of the energy requirement of ruminants is met by metabolism of volatile fatty acids produced by fermentation of the fodder in the rumen (for review see Warner, 1964). Substantial amounts of acetic acid, propionic acid and butyric acid with smaller amounts of higher fatty acids are formed in the rumen of sheep and are absorbed through the rumen wall (Kiddle, Marshall & Phillipson, 1951). The epithelium of the rumen wall is capable of metabolizing butyrate and propionate, and to some extent acetate (Pennington, 1952; Pennington & Sutherland, 1956) and the mixture of fatty acids that is transported to the liver in portal blood is depleted in butyrate as compared with the mixture in rumen fluid (Kiddle et al., 1951; Annison, Hill & Lewis, 1957; Cook & Miller, 1965). Studies with anaesthetized sheep (Annison et al., 1957; Cook & Miller, 1965) indicate that the propionate in portal blood is almost quantitatively removed during its passage through the liver, but the role of the liver in butyrate metabolism is less clear. Liver slices from sheep are capable of metabolizing all three fatty acids, however (Pennington, 1952; Leng & Annison, 1963), and in perfused goat liver when all three are present both propionate and butyrate (but not acetate) are extensively consumed (Holter, McCarthy & Kesler, 1963).

In general, acetate is the only short-chain volatile fatty acid present in significant concentration in the peripheral blood in sheep (Reid, 1950) and the conclusion seems justified that both propionate and butyrate in portal blood are largely removed during its passage through the liver, the former giving rise to lactate, glucose and carbon dioxide (Leng & Annison, 1963; Annison, Leng, Lindsay & White, 1963) and the latter producing chiefly \(\beta\)-hydroxybutyrate (Pennington, 1952; Leng & Annison, 1963; Holter et al., 1963). The question of how much acetate is metabolized by the liver in vivo is not resolved. Holter et al. (1963) found no metabolism of acetate in perfused goat liver when propionate and butyrate were present, whereas Cook & Miller (1965) with anaesthetized sheep found that more acetate than propionate was removed by the liver in some cases.

Some of the interactions in the metabolism of
acetate, propionate and butyrate by sheep liver slices have been studied by Pennington (1957), Pennington & Appleton (1958), Pritchard & Tove (1960) and Long & Annison (1963). These last workers studied the production of $^{14}$CO$_2$ from 1-$^{14}$C-labelled substrates and some of the interactions found differed in liver slices from fed and starved sheep. In all cases there was marked inhibition of acetate oxidation by either propionate or butyrate, with little or no effect of acetate on the oxidation of butyrate, but the effects of propionate on butyrate oxidation and of acetate or butyrate on propionate oxidation generally differed in liver slices from fed and starved sheep.

In the present work the rates of consumption of acetate, propionate and butyrate separately and in mixtures were studied with sheep liver mitochondria in the presence and absence of L-malate or $\alpha$-oxoglutarate as stimulants.

MATERIALS AND METHODS

Mitochondria were prepared and washed twice in 0.25 M-sucrose as described by Smith, Osborne-White & Russell (1965a). Mitochondria (about 9 mg of protein) were incubated for 36 min at 37°C in 6 ml of a medium containing (final concentrations) K$_2$HPO$_4$ (16.4 mM), KH$_2$PO$_4$ (3.6 mM), NaHCO$_3$ (25.0 mM), KCl (85 mM), MgCl$_2$ (5.0 mM), sucrose (46 mM), cytochrome c (11 $\mu$M), ATP (1.3 mM), potassium acetate, propionate or butyrate (5.0 mM) and where appropriate potassium L-malate or $\alpha$-oxoglutarate (1.67 mM) in equilibrium with a gas phase of O$_2$+CO$_2$ (95:5). The final pH was 7.4. Incubations were done in Pyrex tubes with side-arms, and substrate and ancillary substrates were added from the side-arms after 2 min in the water-bath and the incubations were performed and terminated as previously described (Smith et al. 1965a).

Two identical experiments were carried out, each involving duplicate incubations of all combinations of substrates with and without the ancillary substrates. The total residual volatile fatty acid in each reaction tube was determined after steam distillation, by titration under N$_2$ (Smith & Osborne-White, 1965). Samples of titrated distillate (1–7 $\mu$mol of total volatile acid in 50 ml) were made alkaline with one drop of 0.1 M-NaOH, evaporated to dryness under N$_2$ and dissolved in 125 $\mu$l of water. Great care was necessary to avoid contamination of samples with acetic acid from any of several sources and for this reason all the glass-distilled water that was used was first passed through a column of Dowex 1 (OH$^-$ form) into a Pyrex container and protected from the atmosphere with a trap containing the alkaline CO$_2$ absorbent, Sofnolite (Sofnol Ltd., Greenwich, London, U.K.); the N$_2$ used for evaporation was passed through Sofnolite and NaOH pellets and led through an all-glass gas line; evaporated samples were kept in desiccators over NaOH pellets until analysed.

The relative quantities of the three acids present were determined by g.l.c. of duplicate 25 $\mu$l samples by using a Pye Panchromatograph fitted with a flame-ionization detector (W. G. Pye and Co., Cambridge, U.K.). Chromatograms were recorded on a Honeywell Electronic recorder fitted with a Disc integrator as supplied by W. G. Pye and Co. The column (5ft $\times$ 0.25 in internal diameter) was packed with Poropak (80–100 mesh, type Q) (Waters Associates, Framingham, Mass., U.S.A.) preceded by a mixture of equal weights of Celite and finely crystalline NaHSO$_4$. Chromatograms were run at 195°C with a mobile phase of N$_2$ (40 ml/min). The instrument was standardized with known solutions and mixtures of acetic acid, propionic acid and butyric acid. Integrated peak responses were proportional to the amount of acid present over the range used. The residual amounts of each acid in the flask contents were calculated from the amount of total residual volatile fatty acid together with the molecular proportions of the three acids determined from the chromatograms. Initial values for each of the seven substrate combinations were determined similarly from duplicate flasks to which protein precipitant was added at the time of addition of substrate, and the utilization of each acid was calculated by difference.

Acetic acid, propionic acid and butyric acid were Laboratory Reagent-grade chemicals from BDH Chemicals Ltd., Poole, Dorset, U.K., and were fractionally distilled and shown chromatographically to be more than 99.5% pure. Other materials were as previously described (Smith, Osborne-White & Russell, 1967).

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

The results of the two experiments agreed closely and have been combined in Table 1, which shows mean consumption for each substrate–ancillary substrate combination. As Table 1 shows the experiments were designed on the basis of a factorial structure and the statistical analysis examined the factorial effects by using the $t$ test (Cochran & Cox, 1957).

Of the volatile fatty acids propionate was metabolized at the greatest rate under all conditions, followed in magnitude by butyrate and acetate. Maximum rates for all three acids were obtained in the presence of $\alpha$-oxoglutarate, which significantly stimulated consumption of acetate ($P<0.01$), propionate ($P<0.001$) and butyrate ($P<0.001$).

No metabolism of acetate was detected in the absence of ancillary substrate and the small utilization in the presence of malate was not statistically significant. The significant ($P<0.01$) consumption of acetate in the presence of $\alpha$-oxoglutarate was abolished by either propionate or butyrate. However, acetate had no significant effect on utilization of propionate or butyrate under any of the conditions studied. (The apparent stimulation of butyrate consumption by acetate in the presence of malate and absence of propionate was non-significant and is not regarded as meaningful.)

Propionate consumption was not affected by
malate but was greatly stimulated by \(\alpha\)-oxoglutarate \((P<0.001)\). Propionate consumption was also stimulated by butyrate both in the absence of ancillary substrate \((P<0.001)\) and in the presence of malate \((P<0.05)\). In the presence of \(\alpha\)-oxoglutarate propionate utilization was decreased by 27% by butyrate \((P<0.001)\).

Butyrate consumption was stimulated by propionate \((P<0.001)\) or by malate \((P<0.05)\). The magnitude of the effects of propionate and malate on butyrate consumption were not significantly different and the effects were not additive. The effect of \(\alpha\)-oxoglutarate on butyrate consumption, however, was significantly greater than that of either malate or propionate \((P<0.01)\). In the presence of \(\alpha\)-oxoglutarate butyrate utilization was decreased by 26% by propionate \((P<0.01)\).

For each of the ancillary substrates a maximum total utilization of volatile fatty acids occurred with the combination propionate plus butyrate. In the absence of ancillary substrate this utilization was characterized by a mutual stimulation by the two acids of one another’s consumption. There was then no further effect of malate on the consumption of either acid and only a moderate stimulation \((21\% - 26\%)\) by \(\alpha\)-oxoglutarate on the consumption of both. Under the latter conditions the individual rates of utilization of both propionate and butyrate were 73%–74% of those found when they were incubated separately with \(\alpha\)-oxoglutarate. This effect may indicate saturation of the system with respect to the rate of total volatile fatty acid utilization. In the presence of \(\alpha\)-oxoglutarate the combined utilization of propionate and butyrate incubated together was only slightly \((9\%)\) greater than that of propionate incubated alone with \(\alpha\)-oxoglutarate.

**DISCUSSION**

The results show two features for which an explanation is not immediately apparent. These are the complete inhibition of acetate metabolism by propionate or butyrate and the markedly greater stimulation of acetate or butyrate utilization by \(\alpha\)-oxoglutarate as compared with malate.

The unique effectiveness of \(\alpha\)-oxoglutarate in promoting mitochondrial oxidation of fatty acids of longer chain length (Lehninger, 1945) has been attributed to its capacity to supply GTP for a membrane-bound acid–CoA ligase (GPD) (Rossi & Gibson, 1964). This enzyme is severely inhibited by phosphate both in intact mitochondria (van den Bergh, 1966) and after purification (Galzigna, Rossi, Sartorelli & Gibson, 1967). The presence of 20 mM-phosphate in the experiments reported makes it highly unlikely that the effects of \(\alpha\)-oxoglutarate were due to activation of the volatile fatty acids by the GTP-linked enzyme. The relative rates of consumption of the three acids in the presence of \(\alpha\)-oxoglutarate (acetate:propionate:butyrate, 1:0:3.9:1.9) correspond quite closely with their relative rates of activation by ATP in sheep liver mitochondria (acetate:propionate:butyrate, 1:0:2.8:2.1), but show no correlation with the low rates of activation by GTP (Smith & Russell, 1967a). Under the conditions used there is little doubt that ATP and not GTP was primarily responsible for activation of the volatile fatty acids.

Heldt & Schwalbach (1967) have shown that the
terminal phosphate bond of GTP arising from the substrate-level phosphorylation is transferred rapidly and preferentially to endogenous AMP to form ADP in the mitochondrial matrix. Because of the intramitochondrial distribution of phosphotransferases they suggested that rephosphorylation of endogenous AMP in the matrix may depend on the activity of GTP–AMP phosphotransferase. In sheep liver mitochondria α-oxoglutarate has been found to reverse the strong inhibition of propionate metabolism caused either by exogenous AMP (Smith & Russell, 1967b) or by aging the mitochondria at 37°C without substrate (Smith, Osborne-White & Russell, 1965a,b). The superiority of α-oxoglutarate over malate as a stimulus of acetate or butyrate utilization may also be because of the activity of GTP–AMP phosphotransferase, since activation of these acids by ATP leads to release of AMP. A GTP–AMP phosphotransferase has been isolated from calf liver by Hoppe, Strominger & Maxwell (1959).

Because succinyl-CoA is an intermediate of propionate metabolism and may itself give rise to GTP the stimulation of propionate consumption by α-oxoglutarate requires a different explanation. De-inhibition of succinate oxidase activity by the GTP-dependent conversion of intramitochondrial oxaloacetate into phosphoenolpyruvate adequately explains the effects of α-oxoglutarate on propionate utilization in the present experiments with fresh mitochondria (Smith & Russell, 1967c; Smith & Osborne-White, 1971). Stimulation of propionate utilization by butyrate may also be attributed to removal of oxaloacetate. Like pyruvate (Smith & Osborne-White, 1971), butyrate may serve as a source of acetyl-CoA to convert intramitochondrial oxaloacetate into citrate. Both pyruvate and α-oxoglutarate stimulate propionate utilization to an equal extent in fresh mitochondria (Smith & Osborne-White, 1971) but, as shown in Table 1, the effect of butyrate was smaller than that of α-oxoglutarate. This is to be expected, because activation of butyrate leads to release of AMP whereas formation of acetyl-CoA from pyruvate does not. When butyrate is present in addition to propionate, production of succinyl-CoA is no longer equivalent to the total amount of volatile fatty acids consumed, and although butyrate will have released propionate utilization from the constriction imposed by succinate oxidase activity, the rate of production of GTP for rephosphorylation of AMP emerges as a second limitation. Examination of Table 1 suggests that this limitation was overcome by adding α-oxoglutarate when the system reached the maximum rate observed for total volatile fatty acid utilization.

Stimulation of butyrate utilization by the presence of malate was most probably due to provision of oxaloacetate, and the equivalent effect of propionate may be explained similarly. Malate is the major product of propionate metabolism and the two effects were not additive. The synergism of the effect of propionate and butyrate would appear to be mediated via the production and removal of oxaloacetate.

Although the strong inhibition of acetate metabolism by propionate or butyrate has been frequently observed its mechanism remains obscure. Both acetate and propionate penetrate sheep liver mitochondria very readily (Smith & Osborne-White, 1971) and a complete blockage of acetate entry by propionate seems improbable. Sequestration of available CoA by propionate or butyrate is also unlikely in view of the mutual stimulation by these two acids. Pearson & Tubbs (1967) showed that propionate greatly decreased acetyl-CoA concentrations in rat hearts perfused with acetate, pyruvate, β-hydroxybutyrate or palmitate. They pointed out that inhibition of acetyl-CoA synthetase could not have been the common factor responsible and they demonstrated that the effect of propionate, was not due either to sequestration of CoA or to removal of acetyl-CoA to form citrate. In sheep liver mitochondria propionate strongly inhibits utilization of pyruvate (Smith & Osborne-White, 1971) as well as of acetate, but it stimulates consumption of butyrate. If the synergism of propionate and butyrate is mediated via oxaloacetate, then propionate did not inhibit acetyl-CoA formation from butyrate but it entirely prevented acetyl-CoA formation from acetate.

Whatever the mechanism of this effect, the results show clearly that of the volatile fatty acids produced most abundantly in the rumen, liver mitochondria most effectively metabolized a mixture of propionate and butyrate. Acetate was metabolized only in the absence of the other two, but the presence of acetate did not modify the metabolism of propionate or butyrate. The results are consistent with the appearance only of acetate in significant quantities in peripheral blood and may provide, in part, an explanation of the apparently conflicting reports as to whether or not acetate is metabolized by sheep liver.

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REFERENCES


