- Ellis, S. & Walker, B. S. (1942). *J. biol. Chem.* **142**, 291.
 El Masry, A. M., Smith, J. N. & Williams, R. T. (1956). *Biochem. J.* **64**, 50.
 Fishman, W. H. & Green, S. (1955). *J. biol. Chem.* **215**, 527.
 Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.
 Gaffney, G. W., Schreier, K., DiFerrante, N. & Altman, K. I. (1954). *J. biol. Chem.* **206**, 695.
 Kamil, I. A., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 235.
 Levvy, G. A. (1946). *Biochem. J.* **40**, 396.
 Mead, J. A. R., Smith, J. N. & Williams, R. T. (1958). *Biochem. J.* **68**, 61.
 Mitchell, L. C. (1957). *J. Ass. off. agric. Chem., Wash.* **40**, 909.
 Parke, D. V. & Williams, R. T. (1951). *Biochem. J.* **48**, 624.
 Phillips, M. A. & Hinkel, R. D. (1957). *J. agric. Food Chem.* **5**, 397.
 Robinson, D. & Williams, R. T. (1955). *Biochem. J.* **59**, 159.
 Shaffer, P. A. & Hartmann, A. F. (1921). *J. biol. Chem.* **45**, 349.
 Sperber, I. (1948). *J. biol. Chem.* **172**, 441.
 Stekol, J. A. (1936). *J. biol. Chem.* **113**, 279.
 Tollenaar, F. D. & Vos, H. J. (1956). *Fette Seifen Anstrichmittel*, **58**, 112.
 Williams, R. T. (1959). *Detoxication Mechanisms*, 2nd ed. London: Chapman and Hall Ltd.
 Yohe, G. R., Dunbar, J. E., Pedrotti, R. L., Scheidt, F. M., Lee, F. G. H. & Smith, E. C. (1956). *J. org. Chem.* **21**, 1289.

Biochem. J. (1961) **78**, 766

Metabolic Effects of α -Oxobutyric Acid: Effect on Respiration of Rat-Liver Homogenates

By G. Y. N. IYER, M. S. P. NAIR AND M. SUKUMARAN
Department of Biochemistry, Medical College, Trivandrum, India

(Received 3 May 1960)

The observation that the plasma level of α -amino-*n*-butyric acid is elevated in several pathological states (Iyer, 1956) stimulated interest in the metabolism of the amino acid. This amino acid has been shown to participate in transamination reactions (Iyer & Sukumaran, 1959). It was felt that a study of the metabolism of the corresponding keto acid, α -oxobutyric acid, would help to explain some of the observations.

α -Oxobutyric acid arises chiefly from methionine (Greenberg, 1954) and, probably, also from threonine (Lien & Greenberg, 1952). Long (1942, 1943) has demonstrated that pyruvic-oxidase preparations from brain oxidize α -oxobutyric acid at the same rate as pyruvate. Kinnory, Takeda & Greenberg (1955) have shown that the first step in the catabolism of α -oxobutyric acid is its oxidative decarboxylation to propionic acid. One would therefore expect α -oxobutyric acid to cause an increase in oxygen uptake of liver tissue, which would be a measure of the keto acid catabolized. On this premise we made a manometric study of the metabolism of α -oxobutyric acid. Contrary to expectation, inhibition rather than stimulation of oxygen consumption occurred on the addition of α -oxobutyric acid to rat-liver homogenate.

Further, α -oxobutyric acid causes an appreciable depression in oxygen uptake of rat liver when α -oxoglutarate, succinate, fumarate, malate, oxalo-

acetate, pyruvate, glutamate or aspartate is present as a substrate. α -Oxobutyric acid has been shown to inhibit the formation of citrate from added oxaloacetate and also the utilization of α -oxoglutarate.

MATERIALS AND METHODS

Adult rats were killed by a blow on the head and the liver was dissected out. The liver was rinsed free of blood in ice-cold Krebs-Ringer phosphate solution (Krebs & Eggleston, 1940) and then homogenized in ice-cold Krebs-Ringer phosphate solution containing cytochrome *c* and diphosphopyridine nucleotide (DPN), or in 1.15% (w/v) potassium chloride solution, in a Potter-Elvehjem grinder. Preliminary observations on the depression of endogenous respiration were made with a 30% homogenate in potassium chloride medium. A 10% homogenate in the other medium was subsequently used for studying the effect of α -oxobutyric acid on substrate-stimulated respiration, since such a preparation had the advantage of a relatively low endogenous respiration and a fairly high substrate-stimulated respiration.

Solutions of the potassium salts of citric acid, α -oxoglutaric acid, succinic acid, fumaric acid, L-malic acid, L-glutamic acid, L-aspartic acid, pyruvic acid and α -oxobutyric acid (L. Light and Co. Ltd.) were prepared by neutralizing their aqueous solutions and adjusting the pH to 7.3 with *N*-potassium hydroxide in the cold. The final concentrations used are given in the tables.

The oxygen consumption was measured by conventional Warburg manometry at 38° after allowing 3 min. for

equilibration. The gas phase was oxygen. Respiration was linear for the first 45 min. of incubation.

α -Oxoglutarate was determined by the method of McArdle (1957). Immediately after the measurement of oxygen consumption, 1 ml. of the reaction mixture was added to 20 ml. of 2.5% metaphosphoric acid, the volume was made to 25 ml., and α -oxoglutarate in 20 ml. of the protein-free filtrate was determined as its dinitrophenylhydrazone. The dinitrophenylhydrazone of α -oxobutyric acid resolves itself into two bands (R_F 0.86 and 0.64), which are well separated from that of α -oxoglutaric acid (R_F 0.36). The solvent run was 15 cm.

Citric acid was estimated by the method of Ettinger, Goldbaum & Smith (1952) with light petroleum (b.p. 40–60°) instead of heptane for the extraction of pentabromacetone.

RESULTS

Respiration of liver homogenates. α -Oxobutyric acid depressed the endogenous oxygen consumption of liver homogenates (Table 1). Oxygen consumption stimulated by the addition of citrate, α -oxoglutarate, succinate, fumarate, malate, oxaloacetate, pyruvate, glutamate or aspartate was also depressed (Table 2). With the Krebs-cycle intermediates, the percentage inhibition varied in a somewhat regular fashion with the substrate used. The maximum inhibition (88%) was observed with oxaloacetate as the substrate and the minimum (7%) with citrate. The inhibitory effect of α -oxo-

Table 1. *Effect of α -oxobutyrate on oxygen uptake by liver homogenates*

Volume of 30% homogenate in each flask, 1 ml.; final volume of the reaction mixture was made to 2 ml. with water; final concentration of additions are given in parentheses. Duration 30 min. Temperature of incubation 38°. Gas phase O_2 .

Additions	Consumption of oxygen (μ l./mg. of dry tissue/hr.)		Inhibition (%)
	Range	Mean (6 expts.)	
None	4.1–6.5	5.2	—
Oxobutyrate (25 mM)	1.9–4.0	2.9	44
Oxaloacetate (12.5 mM)	4.9–12.2	7.7	—
Oxaloacetate (12.5 mM) + oxobutyrate (25 mM)	1.0–2.6	1.6	79

Table 2. *Effect of α -oxobutyrate on oxygen uptake by liver homogenates in the presence of added substrates*

Each flask contained 1 ml. of 10% homogenate containing cytochrome c and DPN at final concentrations 0.05 mM and 1 mM respectively; water was added to make the final volume 2 ml.; final concentrations of added substrate and of oxobutyrate, 12.5 mM; duration 60 min. Temperature of incubation 38°. Gas phase O_2 .

Additions	Uptake of oxygen (μ l./mg. of dry tissue/hr.)		Inhibition (%)
	Range	Mean (6 expts.)	
None	3.0–6.6	4.5	—
Oxobutyrate	1.6–3.2	2.4	47
Citrate	9.5–18.1	14.5	—
Citrate + oxobutyrate	9.0–15.0	13.5	7
α -Oxoglutarate	10.1–23.1	17.0	—
α -Oxoglutarate + oxobutyrate	9.5–17.7	13.4	21
Succinate	10.7–20.1	17.7	—
Succinate + oxobutyrate	5.3–10.2	8.3	53
Fumarate	9.9–18.6	15.0	—
Fumarate + oxobutyrate	2.8–4.1	3.5	77
Malate	10.0–19.2	15.3	—
Malate + oxobutyrate	2.9–4.0	3.5	77
Oxaloacetate	9.8–22.1	15.8	—
Oxaloacetate + oxobutyrate	1.5–2.4	1.9	88
Glutamate	13.6–17.7	15.2	—
Glutamate + oxobutyrate	10.4–14.6	12.8	16
Aspartate	11.9–14.4	13.2	—
Aspartate + oxobutyrate	1.6–2.8	2.2	83
Pyruvate	13.8–19.5	16.0	—
Pyruvate + oxobutyrate	3.6–4.6	4.1	74

Table 3. *Effect of α -oxobutyrate on (a) citrate synthesis and disappearance and (b) α -oxoglutarate formation and disappearance*

Experimental conditions were as given in Table 2. Final concentration of oxobutyrate, 12.5 mM; concentrations of substrate are given in parentheses.

Additions	$10^3 \times \text{Changes } (\mu\text{moles/mg. of dry tissue/hr.})$			
	(a) Citrate		(b) α -Oxoglutarate	
	Range	Mean	Range	Mean
Oxaloacetate (12.5 mM)	4.15-13.0	+ 7.32	—	—
Oxaloacetate (12.5 mM) + oxobutyrate	0.85- 3.3	+ 2.0	—	—
Oxaloacetate (25 mM)	12.10-24.0	+18.10	—	—
Oxaloacetate (25 mM) + oxobutyrate	2.76- 4.50	+ 3.85	—	—
Oxaloacetate (50 mM)	30.93-44.10	+36.07	—	—
Oxaloacetate (50 mM) + oxobutyrate	7.98-14.14	+10.90	—	—
Citrate (12.5 mM)	41.75-48.75	-45.0	6.1- 8.36	+ 7.05
Citrate (12.5 mM) + oxobutyrate	50.0 -62.0	-56.75	13.5-31.4	+25.0
α -Oxoglutarate (12.5 mM)	—	—	62.0-76.50	-67.56
α -Oxoglutarate (12.5 mM) + oxobutyrate	—	—	40.0-67.50	-54.60

butyrate on the oxygen consumption appeared to be enhanced by added oxaloacetate, for the oxygen consumption in the presence of both oxobutyrate and oxaloacetate was smaller than with oxobutyrate alone. This effect was very marked with the 30% homogenate (Table 1). α -Oxobutyrate depressed the oxygen uptake of liver homogenates stimulated by glutamate and α -oxoglutarate to nearly the same extent. A similar observation was made with aspartate and oxaloacetate.

Citrate synthesis. The amount of citrate formed from oxaloacetate, under the conditions of these experiments, increased in proportion to the concentration of added oxaloacetate (Table 3). α -Oxobutyrate inhibited citrate formation and at a constant concentration of the inhibitor (12.5 mM) in the medium, the citrate synthesized was directly related to the concentration of oxaloacetate.

Utilization of citrate and α -oxoglutarate. Aerobic incubation of liver homogenates with citrate produced α -oxoglutarate. When the incubation was carried out in the presence of α -oxobutyrate (12.5 mM), larger amounts of α -oxoglutarate were found (Table 3). α -Oxobutyrate (12.5 mM) inhibited the removal of added α -oxoglutarate (Table 3), and also enhanced the disappearance of citrate.

DISCUSSION

The principal finding in this investigation is that α -oxobutyrate depresses the endogenous respiration of rat-liver homogenates. It also inhibits respiration stimulated by several added substrates. The question arises whether α -oxobutyrate interferes with oxidation at many sites or at one single site. That oxobutyrate has been shown here to inhibit respiration stimulated by various substrates strongly suggests that it acts at a number of different sites. However, the possibility of a single, or

major, site of action must also be considered. Bearing on this possibility are the two observations that: (1) it inhibits citrate formation from added oxaloacetate, and (2) among the Krebs-cycle intermediates there is a progressive increase in the inhibitory action of oxobutyrate on the oxidation, in the order citrate, α -oxoglutarate, succinate, fumarate, malate, oxaloacetate; the inhibition was greatest with oxaloacetate.

Oxaloacetate has been shown to have the capacity to inhibit a number of reactions within the citric acid cycle. Potter (1946) demonstrated its ability to inhibit malic dehydrogenase and its inhibitory action on succinic dehydrogenase is well established (Das, 1937; Pardee & Potter, 1948; Brummond & Burris, 1953; Tyler, 1955*a, b*; Wolff & Ball, 1957; Avron & Biale, 1957; Corwin & Schwarz, 1959). Tyler (1960) has shown that it also inhibits the removal of α -oxoglutarate. Such data suggest that α -oxobutyrate might exert its effect by inhibiting the synthesis of citrate and thereby the removal of oxaloacetate rather than by inhibiting each individual step in the citric acid cycle. The accumulation of oxaloacetate, in turn, would inhibit oxidative processes in the cycle. Such a rate-regulating function for oxaloacetate has been recently proposed by Tyler (1957, 1960). He suggested that substances affecting succinate oxidation and so oxidative activity through the cycle do so indirectly by affecting oxaloacetate metabolism. These observations here support this possibility, particularly since it is now well established that very small changes in oxaloacetate concentration have profound effects on succinate oxidation (Pardee & Potter, 1948; Tyler, 1955*b*). On this basis one would expect a more marked depression of oxygen uptake by a combination of oxaloacetate and α -oxobutyrate than by the latter alone; this is borne out by the data in Tables 1 and 2.

Interference with the removal of oxaloacetate through citrate formation would naturally impair the catalytic oxidation of pyruvate. The inhibition of oxygen uptake stimulated by aspartate may well be another consequence of the same effect. A similar explanation can be offered for the inhibitory effect on glutamate oxidation, since oxobutyrate inhibits the removal of α -oxoglutarate as well.

α -Oxobutyrate, which inhibits the utilization of α -oxoglutarate, presumably enhances its formation from added citrate. Montgomery & Webb (1956) have reported a similar effect with another inhibitor of oxoglutarate oxidation, namely parapyrivate, in the presence of which the unaffected sections of the Krebs cycle proceed at a more rapid pace.

SUMMARY

1. α -Oxobutyric acid depresses the endogenous oxygen consumption of rat-liver homogenates.

2. Respiration stimulated by citrate, α -oxoglutarate, succinate, fumarate, malate, oxaloacetate, glutamate, aspartate or pyruvate is also depressed, the extent of this varying with the substrate used. It is maximal with oxaloacetate and minimal with citrate.

3. Inhibition has been observed at two sites in the cycle: (i) between oxaloacetate and citrate and (ii) between α -oxoglutarate and succinate. α -Oxobutyrate decreases citrate formation from added oxaloacetate and also removal of added α -oxoglutarate.

4. The implications of these findings are discussed.

The authors appreciate the kindness of Professor D. B. Tyler in making available a typescript of his paper whilst it was in the press. They also acknowledge the encouragement of Dr M. Thangavelu, the Principal of this College.

REFERENCES

- Avron, M. & Biale, J. (1957). *J. biol. Chem.* **225**, 699.
 Brummond, D. O. & Burris, R. H. (1953). *Proc. nat. Acad. Sci., Wash.*, **39**, 754.
 Corwin, L. M. & Schwarz, K. (1959). *J. biol. Chem.* **234**, 191.
 Das, N. B. (1937). *Biochem. J.* **31**, 1124.
 Ettinger, R. H., Goldbaum, L. R. & Smith, L. H., jun. (1952). *J. biol. Chem.* **199**, 531.
 Greenberg, D. M. (1954). In *Chemical Pathways of Metabolism*, vol. 2, p. 73. Ed. by Greenberg, D. M. New York: Academic Press Inc.
 Iyer, G. Y. N. (1956). *Curr. Sci.* **25**, 224.
 Iyer, G. Y. N. & Sukumaran, M. (1959). *Canad. J. Biochem. Physiol.* **37**, 599.
 Kinnory, D. S., Takeda, Y. & Greenberg, D. M. (1955). *Biochim. biophys. Acta*, **17**, 561.
 Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **34**, 442.
 Lien, O. G., jun. & Greenberg, D. M. (1952). *J. biol. Chem.* **195**, 637.
 Long, C. (1942). *Biochem. J.* **36**, 807.
 Long, C. (1943). *Biochem. J.* **37**, 215.
 McArdle, B. (1957). *Biochem. J.* **66**, 144.
 Montgomery, C. M. & Webb, J. L. (1956). *J. biol. Chem.* **221**, 359.
 Pardee, A. B. & Potter, V. R. (1948). *J. biol. Chem.* **176**, 1085.
 Potter, V. R. (1946). *J. biol. Chem.* **165**, 311.
 Tyler, D. B. (1955a). *Proc. Soc. exp. Biol., N.Y.*, **88**, 333.
 Tyler, D. B. (1955b). *J. biol. Chem.* **216**, 395.
 Tyler, D. B. (1957). *Fed. Proc.* **16**, 130.
 Tyler, D. B. (1960). *Biochem. J.* **76**, 293.
 Wolff, E. C. & Ball, E. G. (1957). *J. biol. Chem.* **224**, 1083.

Biochem. J. (1961) **78**, 769

The Determination of Human-Serum-Cholinesterase Activity with *o*-Nitrophenyl Butyrate

By A. R. MAIN, K. E. MILES AND P. E. BRAID*

Occupational Health Division, Department of National Health and Welfare,
 45 Spencer Street, Ottawa, Ontario, Canada

(Received 5 May 1960)

The increasing use of organophosphorus compounds in agriculture, forestry and medicine has placed great emphasis on the need for a rapid and reasonably accurate method for determining human-serum-cholinesterase activity. The present paper describes such a method based on the use of *o*-nitrophenyl butyrate as substrate. Cholinesterase liberates *o*-nitrophenol under easily reproduced,

relatively non-critical conditions. The *o*-nitrophenol can be measured spectrophotometrically, and only a small volume (0.004 ml.) of serum is needed for each determination.

The effect of the orientation of the nitro group of nitrophenyl carboxylic esters on the cholinesterase activity was studied. Previously it had been shown that the A-type esterases in sheep serum hydro-