benzamide, toluene, benzyl alcohol and benzaldehyde to benzoic acid also follow first-order reaction kinetics with velocity constants 0.32, 0.11, 1.00 and 0.33 hr.⁻¹ respectively.

4. Mathematical treatment of the observed kinetics gives expressions from which theoretical values can be derived which are in agreement with experimental observations.

5. The observed kinetics provide an explanation for the different proportions of benzoylglucuronide and hippuric acid excreted after administration of

benzoic acid at different dose levels either as sodium benzoate or as precursors.

6. The well-known species differences in the degree of conjugation of benzoic acid with glucuronic acid is also explained.

The authors wish to express their indebtedness to Dr G. J. Kynch of the department of Mathematical Physics in this University for helpful discussion of the mathematical treatment, to Dr M. E. Nutt and Miss D. M. Jackson for determining the blood volumes and to Mr J. Walker and Mr P. N. Wormald for co-operation in human experiments.

REFERENCES

- Bray, H. G., Humphris, B. G. & Thorpe, W. V. (1949). *Biochem. J.* **45**, 241.
 Bray, H. G., Neale, F. C. & Thorpe, W. V. (1946). *Biochem. J.* **40**, 134.
 Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1947). *Biochem. J.* **41**, 212.
 Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1948). *Biochem. J.* **43**, 561.
 Bray, H. G., Thorpe, W. V. & Wood, P. B. (1949). *Biochem. J.* **45**, 45.
 Courtice, F. C. & Gunton, R. W. (1949). *J. Physiol.* **108**, 405.
 Csonka, F. A. (1924). *J. biol. Chem.* **60**, 545.
 Griffith, W. H. (1938). *J. biol. Chem.* **123**, xlv.
 Griffith, W. H. & Lewis, H. B. (1923). *J. biol. Chem.* **57**, 1.
 Jaffé, M. (1878-79). *Hoppe-Seyl. Z.* **2**, 47.
 Lehmann, G. & Knoefel, P. K. (1938). *J. Pharmacol.* **63**, 453.
 Quick, A. J. (1931). *J. biol. Chem.* **92**, 65.
 Quick, A. J. (1932). *J. biol. Chem.* **97**, 403.
 Sammons, H. G. & Williams, R. T. (1946). *Biochem. J.* **40**, 223.
 Thorpe, W. V. (1950). *Brit. Sci. News*, **3**, 78.
 Williams, R. T. (1947). *Detoxication Mechanisms*, p. 95. London: Chapman and Hall.
 Wilson, P. W. (1949). *Respiratory Enzymes*, p. 16, ed. by Lardy, H. A. Minneapolis: Burgess Publ. Co.

The Influence of 2:4-Dinitrophenol on the Oxidative Breakdown of Fatty Acids

By P. FANTL, G. J. LINCOLN AND J. F. NELSON
Baker Medical Research Institute, Alfred Hospital, Melbourne, Australia

(Received 13 March 1950)

Certain substituted phenols have the property of increasing the oxygen uptake of tissue slices. Dodds & Greville (1933) and Elliott & Baker (1935) found that 4:6-dinitro-*o*-cresol in 10⁻⁵M concentration accelerated the respiration of slices of several mammalian organs *in vitro*, but other workers found that similar compounds did not have this influence on tissue extracts (for references see Peiss & Field, 1948). Pickett & Clifton (1941) and Clifton (1946) indicated that 2:4-dinitrophenol (DNP) in suitable concentrations inhibited synthetic processes without influencing oxidation, and Hotchkiss (1944) reported that DNP prevented the uptake of inorganic phosphate by respiring yeast. Experiments carried out by Loomis & Lipmann (1948) led them to the conclusion that DNP uncoupled phosphorylation from oxidation in rabbit kidney homogenates. An increased breakdown of glycogen in muscle under the stimulus of DNP has been observed by several

workers (for references see Pierce & Field, 1949). Since it is undecided whether phosphorylated compounds take part in the oxidative breakdown of fatty acids, the effect of DNP on this process has been investigated.

EXPERIMENTAL AND RESULTS

All experiments were carried out with liver slices taken from rats maintained on an adequate diet until required. The slices were incubated at 37° in Krebs-Ringer phosphate solution of pH 7.4 for 60 min., unless otherwise stated. The conventional Warburg technique was followed. In all experiments the gas phase was O₂.

Table 1 gives the average results of four experiments in which sodium octanoate was used as substrate. It will be observed that DNP increased the oxygen uptake in the absence and presence of octanoate. In order to establish whether the DNP

concentration used was sufficient to inhibit oxidative phosphorylation it was necessary to test simultaneously a system requiring phosphorylation. A

Table 1. *Influence of DNP on octanoate oxidation by rat liver slices*

(Krebs-Ringer phosphate, pH 7.4; total vol. 3.4 ml.; time 60 min. Q_{O_2} measured at 37°; based on dry wt. of tissue, which was 14.5–25.7 mg. $Q_{Acac.}$ refers to CO_2 liberated from acetoacetic acid by the aniline citrate technique of Edson, 1935.)

Sodium octanoate	Without DNP		$4.5 \times 10^{-5} M$ -DNP	
	Q_{O_2}	$Q_{Acac.}$	Q_{O_2}	$Q_{Acac.}$
0.01 M	6.5	0.96	9.4	1.37
0	4.4	0.24	5.4	0.36
Differences	2.1	0.72	4.0	1.01

Table 2. *Comparison between fructose and octanoate metabolism of rat liver slices under the influence of DNP*

(Conditions similar to those given in Table 1. Dry weight of slices 20–37 mg.; fructose, 0.011 M; sodium octanoate, 0.01 M.)

Substrate	Without DNP			$4.5 \times 10^{-5} M$ -DNP		
	Q_{O_2}	$Q_{Acac.}$	Fructose after incubation (mg./100 ml.)	Q_{O_2}	$Q_{Acac.}$	Fructose after incubation (mg./100 ml.)
Fructose	8.4	—	106	10.3	—	178
None	6.9	—	—	10.0	—	—
Sodium octanoate	9.6	2.4	—	14.8	4.0	—
None	6.9	0.2	—	10.0	0.6	—

suitable reaction is the conversion of fructose to glucose (Cori & Shine, 1936; Goda, 1937), and Table 2 gives results of experiments on this point. Fructose was estimated by the Seliwanoff reaction as modified by Roe (1934).

Table 3. *Effect of DNP on the production of β -hydroxybutyrate and acetoacetate from 0.01 M-octanoate*

(Rat liver slices, 1.9 g., were incubated in 30 ml. of Krebs-Ringer phosphate, pH 7.4, in 250 ml. Erlenmeyer flasks. Gas phase O_2 . Temp. 37°. Time 90 min.)

$4.5 \times 10^{-5} M$ -DNP	β -Hydroxybutyric acid (mg.)	Acetoacetic acid (mg.)
Present	0.51	3.96
Absent	2.22	1.83

The results show that the liver slices utilized 45% of the fructose in the absence of DNP whilst less than 10% disappeared in its presence. In contrast to this inhibition DNP enhanced the metabolism of octanoate with regard to both oxygen uptake and acetoacetate formation. After incubation with octanoate the reaction products were examined by the steam-distillation technique of Leloir & Munoz (1939). It was found that the amount of substrate disappearing was 25% in the absence of DNP and 27% in its presence. The almost identical utilization of octa-

noate under either condition suggests that the augmented respiration was connected with some intermediate breakdown product.

It is known from the work of Jowett & Quastel (1935) and Edson (1935) that β -hydroxybutyrate and acetoacetate can be interconverted by slices of mammalian liver. The effect of DNP on the formation of these products was investigated. The estimations were carried out according to the technique of Edson (1935). From the figures given in Table 3 it is quite apparent that DNP altered the ratio between β -hydroxybutyrate and acetoacetate to yield a preponderance of the latter. Similar results were obtained from experiments in which synthetic DL- β -hydroxybutyrate and acetoacetate were incubated with rat liver slices (Table 4). The results show that whilst the enzymic dehydrogenation of β -hydroxy-

butyrate was enhanced by DNP the reduction of acetoacetate was inhibited.

Table 4. *Effect of DNP on the ratio of DL- β -hydroxybutyrate to acetoacetate*

(Conditions similar to those given in Table 1. Each result is the mean of four experiments. Δ indicates that the results have been corrected for control values.)

$4.5 \times 10^{-5} M$ -DNP	$\Delta Q_{Acac.}$	
	0.01 M-Sodium DL- β -hydroxybutyrate	0.01 M-Sodium acetoacetate
Present	1.75	-0.24
Absent	1.0	-1.35

Table 5. *Effect of DNP on β -keto-acid production from pyruvate by rat liver slices*

(Sodium pyruvate, 0.002 M. Conditions similar to those given in Table 1. The figures for β -keto-acid were calculated from estimations by the aniline citrate method.)

$4.5 \times 10^{-5} M$ -DNP	Pyruvic acid disappearance (mg./100 ml.)	Pyruvic acid accounted for as β -keto-acid (mg./100 ml.)	Recovery (%)
Present	11.0	12.5	114
Absent	10.5	6.2	59
Present	12.0	13.6	114
Absent	12.0	4.4	37

Since pyruvic acid leads to the same end products as fatty acids when offered to isolated liver (Embsen & Oppenheimer, 1913) it was included in this study. Pyruvate determinations were carried out by the method of Bueding, Stein & Wortis (1941). From the amounts of β -keto-acid formed (Table 5) it would appear that, whilst complete conversion of pyruvate into acetoacetate occurred in the presence of DNP, part of the keto-acid was reduced to β -hydroxybutyrate in its absence.

In large-scale experiments rat liver slices were incubated with sodium pyruvate, and ketone bodies were estimated by the gravimetric technique described by Peters & Van Slyke (1932). It was ascertained in control experiments that the final pyruvate concentration was sufficiently low not to interfere with the estimation of ketone bodies. It was also shown that DNP had no influence on the estimation of β -hydroxybutyrate. The results of two experiments are recorded (Table 6).

pH of 4.5–4.8. The observation in the present work that octanoate and pyruvate oxidation by rat liver slices is not impaired by DNP at a pH near the physiological range, and at a concentration which inhibits fructose metabolism, speaks against a process of oxidative phosphorylation in the fatty acid breakdown. The differing results obtained by Cross *et al.* (1949) can perhaps be explained by the fact that, according to Potter (1946), the system required for octanoate oxidation prepared from rodent livers is very unstable, and addition of DNP often reduces the oxygen uptake of organ homogenates.

In contrast to the inhibition of oxidative phosphorylation processes by DNP sufficient experimental evidence is available to show that reactions requiring anaerobic phosphorylation are not affected by DNP, e.g. the glycogen breakdown in muscle occurs at an accelerated rate in the presence of DNP. Therefore no conclusion can be drawn regarding

Table 6. *Effect of DNP on the production of β -hydroxybutyrate and acetoacetate from pyruvate*

(Rat liver slices, 2.5 g., were incubated in 150 ml. Erlenmeyer flasks containing 52 ml. of 0.004M-sodium pyruvate in Krebs-Ringer phosphate, pH 7.4. Gas phase O_2 . Temp. 37°. Time 60 min. Ketone bodies estimated gravimetrically.)

4.5 $\times$ 10 ⁻⁵ M-DNP	Pyruvic acid disappearance (mg.)	β -Hydroxybutyric acid formation (mg.)	Acetoacetic acid formation (mg.)	Pyruvic acid accounted for as ketone bodies	
				(mg.)	(%)
Present	11.25	1.1	4.5	9.7	87
Absent	11.25	2.9	2.8	9.83	87
Present	6.7	0.6	3.0	6.15	92
Absent	6.7	1.38	2.26	6.24	93

The results given in Tables 3 and 6 establish that DNP increased the ratio of keto-acid to hydroxy-acid.

DISCUSSION

Confirmation of the hypothesis that phosphorylation participates in the intracellular breakdown of fatty acids has been attempted by several workers. No phosphorylated intermediate product has been isolated. Lehninger & Kennedy (1948) and Cross, Taggart, Covo & Green (1949) observed that fatty acid metabolism proceeds only with the simultaneous oxidation of some member of the citric acid cycle, and this may involve phosphorylation. DNP was found to block oxidation of acetate, butyrate or β -keto-octanoate in the cyclophorase system. On the other hand, Clifton (1946) has shown that acetate or butyrate is oxidized by *Pseudomonas calco-acetica* in the presence of DNP at a physiological pH. Stoppani (1949) found an inhibition of acetate oxidation by yeast in the presence of DNP at the unphysiological

anaerobic phosphorylations during the chain of reactions linked with the breakdown of fatty acids.

SUMMARY

1. Dinitrophenol (DNP) in a concentration of 4.5×10^{-5} M and at pH 7.4 enhanced the respiration of rat-liver slices metabolizing octanoate and pyruvate. The end products in both reactions contained a much greater proportion of acetoacetate than was found in control experiments.

2. Under similar experimental conditions DNP inhibited oxidative phosphorylation of fructose, as indicated by its effect on the aerobic utilization of this sugar.

3. It is suggested that oxidative phosphorylation is not involved in the intracellular breakdown of octanoate or pyruvate.

Thanks are due to The Australian National Health and Medical Research Council for a personal grant to one of us (G.J.L.).

REFERENCES

- Bueding, E., Stein, M. H. & Wortis, H. (1941). *J. biol. Chem.* **140**, 697.
- Clifton, C. E. (1946). *Advanc. Enzymol.* **6**, 269.
- Cori, C. F. & Shine, W. M. (1936). *J. biol. Chem.* **114**, xxi.
- Cross, R. J., Taggart, J. V., Covo, G. A. & Green, D. E. (1949). *J. biol. Chem.* **177**, 655.
- Dodds, E. C. & Greville, G. D. (1933). *Nature, Lond.*, **132**, 966.
- Edson, N. L. (1935). *Biochem. J.* **29**, 2082.
- Elliott, K. A. C. & Baker, Z. (1935). *Biochem. J.* **29**, 2396.
- Embden, G. & Oppenheimer, M. (1913). *Biochem. Z.* **55**, 335.
- Goda, T. (1937). *Biochem. Z.* **294**, 259.
- Hotchkiss, R. D. (1944). *Advanc. Enzymol.* **4**, 153.
- Jowett, M. & Quastel, J. H. (1935). *Biochem. J.* **29**, 2181.
- Lehninger, A. L. & Kennedy, E. P. (1948). *J. biol. Chem.* **173**, 753.
- Leloir, L. F. & Munoz, J. M. (1939). *Biochem. J.* **33**, 734.
- Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.
- Peiss, C. N. & Field, J. (1948). *J. biol. Chem.* **175**, 49.
- Peters, J. P. & Van Slyke, D. D. (1932). *Quantitative Clinical Chemistry*, 1st ed., vol. ii (*Methods*), p. 625. Baltimore: Waverly Press.
- Pickett, M. J. & Clifton, C. E. (1941). *Proc. Soc. exp. Biol., N.Y.*, **46**, 443.
- Pierce, F. T. & Field, J. (1949). *J. biol. Chem.* **180**, 895.
- Potter, V. R. (1946). *J. biol. Chem.* **165**, 311.
- Roe, J. H. (1934). *J. biol. Chem.* **107**, 15.
- Stoppani, A. O. M. (1949). *Nature, Lond.*, **164**, 1096.

A New Volumetric Method for the Determination of Histaminase Activity in Biological Fluids

BY R. KAPPELLER-ADLER

Department of Pharmacology, University of Edinburgh

(Received 26 May 1950)

Different methods for the estimation of histaminase activity in biological fluids and many different units have been proposed by various workers. The methods described were either chemical (Zeller, 1940; Zeller, Stern & Wenk, 1941; Koloszynski, 1946), manometric (Zeller, Birkhäuser, Mislin & Wenk, 1939; Zeller & Birkhäuser, 1940; Laskowski, 1942; Stephenson, 1943; Swedin, 1943*a, b*, 1944) or biological ones (Marcou, Chiriceanu, Cosma, Gingold & Parhon, 1938; Ungar & Parrot, 1939; Albus, 1939; Werle & Effkemann, 1940; Ahlmark, 1944; Anrep, Barsoum & Ibrahim, 1947*a, b*; Wicksell, 1949). These methods were applied either to purified histaminase preparations, made from hog kidneys, or to the serum, plasma or placenta of pregnant women. Since the conditions under which these tests were carried out varied widely, the results obtained by various workers can hardly be compared.

In the present investigation a simple volumetric method for the determination of histaminase activity in biological media has been developed and a new histaminase unit proposed. Moreover, an attempt has been made to correlate the results obtained for histaminase activity with chemical, manometric and biological methods by applying these methods simultaneously to a purified preparation of histaminase made from hog kidneys. Finally, the discrepancy in the behaviour of histaminase towards histamine on the one hand and towards cadaverine on the other hand, described and discussed in previous work (Kapeller-Adler, 1944, 1949),

was confirmed by means of this new volumetric method. The substance of this paper was communicated at the First International Congress of Biochemistry at Cambridge in August 1949.

METHODS

New volumetric method for the estimation of histaminase in biological media

This method is a modification of Zeller's qualitative indigo test (Zeller *et al.* 1941); it is based on the theory that during the action of histaminase 1 mol. of H_2O_2 is formed for each mol. of histamine oxidized (Kiese, 1940; Zeller, 1938*a*, 1942; Stephenson, 1943; Swedin, 1943*a, b*, 1944; Kapeller-Adler, 1949).

A purified preparation of histaminase made from hog kidneys (Kapeller-Adler, 1949) was used. The activity of this preparation was expressed in Laskowski units (L.U.), one unit corresponding to the amount of enzyme which at 37°, pH 7.2, in air, with 1 mg. of histamine dihydrochloride as substrate, utilizes 1 μ l. O_2 /min. (Laskowski, 1942).

Reagents required: (1) M/15-phosphate buffer (Sørensen) pH 7.2. (2) Histamine solution in M/15-phosphate buffer, containing 10 mg. of histamine dihydrochloride/ml. (3) Indigo disulphonate solution. Indigo Carmine (200 mg.) is dissolved in 300 ml. water and the solution stored in a brown bottle. (4) 0.002N-KMnO₄ solution. (5) CHCl₃.

Procedure. To increasing amounts of enzyme solution, placed in Pyrex test tubes (0.1–1 ml. of a solution of the histaminase preparation in phosphate buffer, corresponding to 0.0025–0.025 L.U.), 1 ml. of the indigo solution and 0.1 ml. of the histamine solution are added and the volume made up with phosphate buffer to 4 ml. CHCl₃ (1 drop) is added as a