5 %) of activity against either PPP or PPA due to other enzymes be detected. Indeed, from preliminary experiments with fractionation on DEAE-cellulose we have indications that small amounts of such esterases may be present.

SUMMARY

1. A method is described whereby from the relationship between concentration of di-isopropyl phosphorofluoridate (DFP) and esteratic activity of chicken brain against phenyl phenylacetate and phenyl 3-phenylpropionate, the ratio of the rates of hydrolysis of the two substrates by the two major esterase components may be calculated.

2. The results have been confirmed by a qualitative fractionation on DEAE-cellulose.

3. Chicken brain contains two esterases: one much more sensitive to DFP and *NN'*-di-isopropyl phosphorodiamidic fluoride (mipafox) and hydrolysing the phenylacetate faster than the phenylpropionate (phenylpropionate/phenylacetate ratio, 0.05) and the other hydrolysing the phenylpropionate faster than the phenylacetate (phenylpropionate/phenylacetate ratio, 9.75).

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REFERENCES

- Aldridge, W. N. (1950). *Biochem. J.* **46**, 451.
 Aldridge, W. N. (1953). *Biochem. J.* **53**, 110.
 Aldridge, W. N. (1954a). *Biochem. J.* **56**, 185.
 Aldridge, W. N. (1954b). *Biochem. J.* **57**, 692.
 Aldridge, W. N. (1956). *Rep. Progr. Chem.* **53**, 294.
 Aldridge, W. N. (1961). In *Biochemists' Handbook*, p. 275. Ed. by Long, C. London: E. and F. N. Spon Ltd.
 Aldridge, W. N. & Davison, A. N. (1952). *Biochem. J.* **51**, 62.
 Bergmann, F., Segal, R. & Rimon, S. (1957). *Biochem. J.* **67**, 481.
 Gottlieb, S. & March, P. B. (1946). *Analyt. Chem.* **18**, 16.
 Jansen, E. F., Fellows-Nutting, M. D. & Balls, A. K. (1948). *J. biol. Chem.* **175**, 975.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, B. J. (1951). *J. biol. Chem.* **193**, 265.
 Peterson, E. A. & Sober, H. A. (1962). In *Methods in Enzymology*, vol. 5, p. 6. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press Inc.
 Poulsen, E. & Aldridge, W. N. (1964). *Biochem. J.* **90**, 182.

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Reduced Nicotinamide-Adenine Dinucleotide as a Rate-Limiting Factor in Gluconeogenesis

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In previous experiments it was noted that in slices of kidney cortex gluconeogenesis was slower from oxaloacetate than from fumarate or malate (Krebs, Bennett, de Gasquet, Gascoyne & Yoshida, 1963), and that in pigeon-liver homogenates gluconeogenesis from pyruvate was slower than from lactate (Krebs, Dierks & Gascoyne, 1964). These findings are somewhat unexpected because oxaloacetate and pyruvate are intermediates in carbohydrate formation from fumarate (or malate) and lactate respectively, and as intermediates they should react at least as rapidly as the starting materials. The present paper is concerned with the clarification of this apparent discrepancy. It is shown that oxaloacetate and pyruvate, when added at substrate-level concentrations, cause a major fall in the concentration of NADH_2 , which is required in gluconeogenesis for the reduction of diphospho-

glycerate to triose phosphate. The fall in the concentration of NADH_2 is due to its rapid interaction with oxaloacetate and pyruvate to form malate and lactate respectively, and this can account for the decreased rate of gluconeogenesis. The inference, then, is that the added oxo acids successfully compete with diphosphoglycerate for the available NADH_2 .

EXPERIMENTAL

Analytical procedures. The four forms of nicotinamide nucleotides (NAD , NADH_2 , NADP and NADPH_2) were determined in duplicate by the method of Bassham, Birt, Hems & Loening (1959), with the following modifications suggested by Dr K. Burton (see also Lindall & Lazarow, 1964). The alkaline extract of the tissue after adjustment to pH 8.2 (see Fig. 1 of Bassham *et al.* 1959) contained, apart from NADH_2 and NADPH_2 , variable amounts of NAD and NADP owing to autoxidation of the reduced forms during

neutralization. In order to have NAD solely in the oxidized form and NADP solely in the reduced form, the extract was treated as follows. To a sample (0.5 ml.) of the alkaline extract neutralized to pH 8.2 were added 10 μ l. of 0.1M-glucose 6-phosphate, 10 μ l. of glucose 6-phosphate dehydrogenase (0.15 i.u.), 10 μ l. of 0.01M-acetaldehyde, 10 μ l. of 0.1M-EDTA and 10 μ l. of alcohol dehydrogenase (5 i.u.). After standing at room temperature for 20 min., 25 μ l. samples were taken and the procedure was continued at the point referred to as 'Final separation of the individual enzymes' in Fig. 1 of Bassham *et al.* (1959). This modification allowed the omission of tubes 9-12 in the analytical scheme of Fig. 1 of Bassham *et al.* (1959).

L-Malate and L-lactate were determined according to the method of Hohorst (1963), and the sum of L-malate and fumarate was determined by the same procedure as for the malate determination except that fumarase (C. F. Boehringer und Soehne G.m.b.H.) (35 i.u.) was added.

Tissue preparations. Pigeon-liver homogenates from animals starved for 48 hr. were prepared as described by Krebs *et al.* (1964). They were supplemented with 0.500 μ -mole of NAD/4 ml. of dilute homogenate added immediately before the incubation started. Rat kidney-cortex slices were cut with a dry razor blade and incubated in conical Warburg vessels either in the presence of phosphate-buffered calcium-containing saline (Krebs & de Gasquet, 1964) with O₂ in the gas space or in bicarbonate-buffered saline (Krebs & Henseleit, 1932) with CO₂+O₂ (5:95) in the gas space. Reduced and oxidized nicotinamide nucleotides were determined in two sets of slices incubated separately. At the end of the incubation the slices were blotted on hard filter paper and placed in either the acid or alkaline medium.

RESULTS

Effects of L-lactate and pyruvate on the concentrations of nicotinamide nucleotides in pigeon-liver homogenates. The experiments shown in Table 1

confirm (see Krebs *et al.* 1964) that pyruvate decreases the loss of carbohydrate in the homogenate but does not give rise to an increase of glucose and glycogen, whereas lactate causes a synthesis of carbohydrate. Much of the added NAD disappeared within a few minutes, as observed in minced tissue by Birt & Bartley (1960). Of the 0.500 μ mole of NAD added, less than half was recovered after 10 or 20 min. under most conditions. The loss was greatest in the presence of pyruvate, smaller in the blank without substrate and least in the presence of lactate.

The most striking effect was that of lactate on the concentration of NADH₂. Without substrate or with pyruvate the NADH₂ concentration fell to very low values, but with lactate relatively high values were maintained. The effects of substrates on the concentrations of NAD, NADP and NADPH₂ were not very pronounced. Pyruvate caused a loss of NAD. It also gave rise to the formation of lactate: 5.2 μ moles of lactate were formed after 20 min. and 2.85 μ moles after 10 min.

Effect of lactate and pyruvate on the concentration of nicotinamide nucleotides in pigeon-liver slices. Table 2 records an experiment in which the sums of oxidized nicotinamide nucleotides and reduced nicotinamide nucleotides were measured in pigeon-liver slices after incubation with lactate and pyruvate. The amounts of material available for analysis were not sufficient for the separate determination of NAD (or NADH₂) and NADP (or NADPH₂). Pigeon-liver slices, as indicated by the results in Table 2, synthesize carbohydrate from lactate much faster than rat-liver slices [see the results for rat liver reported by Meyerhof, Lohmann & Meier (1925) and Takane (1926)], and as in the

Table 1. *Effect of L-lactate and pyruvate on the concentrations of nicotinamide nucleotides in pigeon-liver homogenates*

The incubation was for 10 min. in Expt. 1 and 20 min. in Expt. 2. The initial concentration of the added substrates was 40 mM. The results refer to 4 ml. of homogenate. Further details are given in the text.

Substrate added	...	...	...	...	...	...	None	Pyruvate	L-Lactate
Expt. 1	Glucose change (μ moles)						-1.34	-0.56	+0.36
	Glucose + glycogen change (μ moles)						-1.53	-0.77	+0.58
	Pyruvate change (μ moles)						+0.10	-46.1	+2.10
	Lactate change (μ moles)						-1.27	+2.85	-29.1
	NAD (μ mole)						0.141	0.094	0.117
	NADH ₂ (μ mole)						< 0.002	0.006	0.040
	NADP (μ mole)						0.027	0.022	0.023
	NADPH ₂ (μ mole)						0.062	0.059	0.068
	Total nicotinamide nucleotides (μ mole)						0.230	0.181	0.248
Expt. 2	Glucose change (μ moles)						-3.64	0.14	+3.48
	Glucose + glycogen change (μ moles)						-2.83	0.28	+4.80
	Pyruvate change (μ moles)						0	-91	+1.0
	Lactate change (μ moles)						-0.92	+5.2	-44.2
	NAD (μ mole)						0.106	0.094	0.176
	NADH ₂ (μ mole)						0.005	0.005	0.024
	NADP (μ mole)						0.062	0.046	0.083
	NADPH ₂ (μ mole)						0.064	0.038	0.073
	Total nicotinamide nucleotides (μ mole)						0.237	0.183	0.356

Table 2. *Oxidized and reduced nicotinamide nucleotides in pigeon-liver slices incubated with lactate or pyruvate*

The pigeon was starved for 48 hr. Incubation was for 90 min. at 40°. Details are given in the text. The results are expressed as μ moles/g. wet wt.

Substrate added	None	L-Lactate	Pyruvate	None; not incubated (initial values)
O ₂ used in 90 min.	104	160	100	—
Glucose + glycogen found	37.0	89	29.2	29.6
NAD + NADP found	0.299	0.215	0.277	0.301
NADH ₂ + NADPH ₂ found	0.085	0.107	0.021	0.231
(NAD + NADP)/ (NADH ₂ + NADPH ₂) ratio	3.52	2.01	13.2	1.30
Total nicotinamide nucleotides	0.384	0.322	0.298	0.532

homogenates the synthesis from pyruvate was negligible. The fall of the total nicotinamide nucleotide concentration was rather less in slices than in the homogenates. Pyruvate caused a major fall of the concentration of the reduced nicotinamide nucleotides. This was less marked than the fall in the concentration of NADH₂ in homogenates, presumably because the concentration of NADPH₂ was less affected than that of NADH₂. The results thus conform to those obtained with the homogenates.

Effects of oxaloacetate and fumarate on nicotinamide nucleotide concentrations in rat kidney-cortex slices. In order to have sufficient material for the separate determination of the four nicotinamide nucleotides a rat was treated intraperitoneally with nicotinamide (500 mg./kg.) 11 hr. before being killed. As shown by Kaplan, Goldin, Humphreys & Stolzenbach (1957), this greatly increases the nicotinamide nucleotide concentration in various tissues of mice and rats (see also Kaplan, 1961). Normal rat kidney cortex incubated as described in the present paper was found to contain 0.5–0.8 μ mole of total nicotinamide nucleotide/g. wet wt.; about 2.0 μ moles/g. were found in the rat treated with nicotinamide. After incubation with oxaloacetate the concentration of NADH₂ was less than one-fifth of that found on incubation with fumarate. The rate of glucose formation from oxaloacetate was about 40 % of that from fumarate (Table 3).

Aerobic formation of malate from oxaloacetate. If the inhibitory effect on gluconeogenesis of oxaloacetate were due to a competition of oxaloacetate for NADH₂, malate should accumulate in the presence of oxaloacetate, and if there were no complicating circumstances the formation of two mol. of malate would account for the non-formation of one mol. of glucose. In an experiment in which rat kidney-cortex slices were incubated with fumarate (10 mM) or oxaloacetate (20 mM) for 30 min. in bicarbonate-buffered saline, fumarate yielded 118 μ moles of glucose/g. dry wt. and oxaloacetate 31 μ moles/g. dry wt. The latter also formed 145 μ moles of malate plus fumarate/g. dry wt. Thus

Table 3. *Effect of fumarate and oxaloacetate on the tissue concentrations of reduced and oxidized nicotinamide nucleotides in rat kidney-cortex slices*

The rat received nicotinamide (500 mg./kg.) intraperitoneally 11 hr. before being killed. Incubation was for 60 min. The substrate concentrations were 10 mM. The initial values for NAD + NADP were 2.21 μ moles and for NADH₂ + NADPH₂ 0.56 μ mole/g. wet wt.

Substrate added	None	Fumarate	Oxaloacetate
O ₂ used (μ moles/g. wet wt.)	112	217	244
Glucose found (μ moles/g. wet wt.)	2.3	51.4	21.5
NAD + NADP found (μ moles/g. wet wt.)	2.03	1.59	1.43
NADH ₂ + NADPH ₂ found (μ mole/g. wet wt.)	0.12	0.54	0.096

oxaloacetate yielded 87 μ moles less of glucose/g. dry wt. than fumarate and formed 142 μ moles of malate plus fumarate/g. dry wt. It is not surprising that the formation of malate was somewhat lower than expected in an ideal system because additional reactions, e.g. the oxidation of malate to oxaloacetate, were likely to take place.

Effect of γ -methylthio- α -oxobutyrate on the concentration of nicotinamide nucleotides in rat kidney-cortex slices. The observations on the 'inhibition' of gluconeogenesis by pyruvate and oxaloacetate raise the question whether inhibitory effects on gluconeogenesis by γ -methylthio- α -oxobutyrate and certain other α -oxo acids described by Krebs & de Gasquet (1964) are also due to a removal of NADH₂. This was tested and the results (Table 4) show that the inhibition of gluconeogenesis is not accompanied by a fall of the concentration of NADH₂.

DISCUSSION

Under all conditions tested—in pigeon-liver homogenates, pigeon-liver slices and in rat kidney-cortex slices—the relatively low rate or 'inhibition' of gluconeogenesis in the presence of oxaloacetate

Table 4. *Effect of γ -methylthio- α -oxobutyrate on glucose formation and the concentration of nicotinamide nucleotides in rat kidney-cortex slices*

Slices were incubated for 30 min. at 40° in phosphate-buffered saline. The rat received nicotinamide (1 g./kg.) intraperitoneally 12 hr. before being killed. The initial values for NAD, NADH₂, NADP, NADPH₂ and total nicotinamide nucleotides were 0.44, 0.28, 0.096, 0.15 and 0.97 μ mole/g. wet wt. respectively.

Substances added	None	L-Lactate (10 mm)	L-Lactate (10 mm) + γ -methylthio- α -oxobutyrate (2 mm)
O ₂ used (μ moles/g. wet wt.)	112	172	164
Glucose formed (μ moles/g. wet wt.)	6.2	18.6	5.4
NAD (μ mole/g. wet wt.)	0.43	0.50	0.55
NADH ₂ (μ mole/g. wet wt.)	0.20	0.21	0.20
NADP (μ mole/g. wet wt.)	0.10	0.064	0.13
NADPH ₂ (μ mole/g. wet wt.)	0.13	0.13	0.14
Total nicotinamide nucleotides (μ moles/g. wet wt.)	0.86	0.90	1.02

and pyruvate was paralleled by a fall in the concentrations of NADH₂ or the sum of NADH₂ plus NADPH₂. They were also accompanied by a rise in the concentration of malate and of lactate respectively. The increase in the concentration of lactate in pigeon-liver homogenates (Table 1) was comparable with the difference in glucose formation: in Expt. 1 the deficit of glucose in the presence of pyruvate (glucose change with lactate minus glucose change with pyruvate) was 1.35 μ moles. The lactate formed from pyruvate was, as expected, about twice this amount (2.85 μ moles). In Expt. 2 (after a longer period of incubation) the extra lactate found was less than expected, presumably as a result of the reoxidation of some lactate. As stated in the Results section, similar quantitative relations applied to the malate and glucose formation in kidney cortex in the presence of oxaloacetate. These findings directly demonstrate the competition of oxaloacetate and pyruvate for NADH₂. Owing to the high activities of lactate dehydrogenase and malate dehydrogenase in liver and in kidney, and an equilibrium position favourable to the reaction with NADH₂ of oxaloacetate and pyruvate, the results obtained are not unexpected. They show how an apparently irregular behaviour of an intermediate can be due to the special situation created by adding an intermediate in large excess. This unphysiological excess causes the occurrence of secondary reactions of the intermediate.

In kidney cortex, as shown by Krebs *et al.* (1963), glucose formation from pyruvate is much more rapid than from lactate. That excess of pyruvate is not inhibitory in kidney is not surprising, because the rate of pyruvate oxidation in this tissue is exceptionally rapid and this implies that pyruvate can cause a rapid reduction of NAD. This is expected to shift the steady-state concentration in favour of NADH₂. The effect of pyruvate on nicotinamide

nucleotides depends on the balance of the various reductions and oxidations initiated by pyruvate.

The observations can thus be explained by the assumption that the concentration of NADH₂ can limit the rate of gluconeogenesis under clearly defined experimental conditions, but there is no evidence that it is rate-limiting under physiological conditions *in situ*.

SUMMARY

1. The reason why oxaloacetate or pyruvate when added as substrates to liver and kidney preparations are less effective as gluconeogenic precursors than malate or lactate (although they are intermediates in the glucose formation from the latter two substances respectively) has been investigated.

2. Determinations of the concentrations of NAD, NADH₂, NADP and NADPH₂ in liver and kidney preparations show that oxaloacetate or pyruvate can cause a major fall in the concentration of NADH₂ and that this fall is accompanied by a formation of malate and lactate respectively. As NADH₂ is required in gluconeogenesis to reduce diphosphoglycerate to glyceraldehyde phosphate, the inhibition of gluconeogenesis by the α -oxo acids can be explained by their competition for NADH₂.

3. Though NADH₂ can limit the rate of gluconeogenesis under well-defined experimental conditions, there is no evidence that it can do so *in vivo*.

4. The inhibition of gluconeogenesis by other α -oxo acids (e.g. γ -methylthio- α -oxobutyrate) is not accompanied by changes in the concentration of NADH₂.

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REFERENCES

- Bassham, J. A., Birt, L. M., Hems, R. & Loening, U. F. (1959). *Biochem. J.* **73**, 491.
- Birt, L. M. & Bartley, W. (1960). *Biochem. J.* **76**, 328.
- Hohorst, H. J. (1963). In *Methods of Enzymatic Analysis*, pp. 266, 328. Ed. by Bergmeyer, H. U. New York and London: Academic Press Inc.
- Kaplan, N. O. (1961). *Metabolic Pathways*, vol. 2, p. 627.
- Kaplan, N. O., Goldin, S. R., Humphreys, M. M. & Stolzenbach, F. E. (1957). *J. biol. Chem.* **226**, 365.
- Krebs, H. A., Bennett, D. A. H., de Gasquet, P., Gascoyne, T. & Yoshida, T. (1963). *Biochem. J.* **86**, 22.
- Krebs, H. A. & de Gasquet, P. (1964). *Biochem. J.* **90**, 149.
- Krebs, H. A., Dierks, C. & Gascoyne, T. (1964). *Biochem. J.* **93**, 112.
- Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
- Lindall, A. W. & Lazarow, A. (1964). *Metabolism*, **13**, 259.
- Meyerhof, O., Lohmann, K. & Meier, R. (1925). *Biochem. Z.* **157**, 459.
- Takane, R. (1926). *Biochem. Z.* **171**, 403.

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The Chemistry of Xanthine Oxidase

11. ULTRACENTRIFUGE AND GEL-FILTRATION STUDIES ON THE MILK ENZYME*

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The molecular weight of xanthine oxidase from cow's milk, as measured by sedimentation and diffusion, has been given as 290 000 (Avis, Bergel, Bray, James & Shooter, 1956*a*). The value from chemical analysis, on the basis of 2 moles of FAD/mole, namely 308 000, was in agreement with this (Avis, Bergel & Bray, 1956*b*). Recently Brumby (1963) and Brumby & Massey (1963) purified pig-liver xanthine oxidase and showed it to be generally similar to the milk enzyme. However, though Brumby (1963) found a molecular weight of 288 000 by the sedimentation and diffusion method, Brumby & Massey (1963) (see also Brumby, 1963) give values of 385 000 obtained by the Archibald method and 190 000 by gel filtration, suggesting that reversible dissociation of a dimer might be occurring. In the present paper we report further studies on the molecular weight of the milk enzyme which confirm our original value, and we show also that the molecule is broken down only under relatively drastic conditions.

MATERIALS AND METHODS

Xanthine oxidase solutions. The enzyme was prepared from milk as described previously (Palmer, Bray & Beinert, 1964; cf. Gilbert & Bergel, 1964). A single batch of twice-chromatographed xanthine oxidase (sample A) was used for all the experiments, apart from one gel-filtration run, for which cruder material (sample B) was used. This sample had not been chromatographed and had lost activity during

storage. At the time of use sample A had an activity/ E_{450} of about 80 (cf. Bray, Pettersson & Ehrenberg, 1961), whereas sample B had a value of only 35. Xanthine oxidase concentrations were determined either directly from E_{450} (Avis *et al.* 1956*b*) or indirectly, in some ultracentrifuge runs, by comparison of the areas under the peaks with those given by samples of known concentration. All solutions used in the ultracentrifuge work contained sodium salicylate (1 mM) and EDTA (1 mM) to stabilize the enzyme (Bergel & Bray, 1959).

Proteins for gel filtration. These are listed in Table 3. Apoferritin and lactoperoxidase were kindly provided by Dr Pauline M. Harrison and Mr A. Pickering respectively.

Sedimentation experiments. These experiments were performed in a Spinco model E analytical ultracentrifuge. The temperature was controlled near to 20° unless otherwise stated. For the determinations of molecular weight solutions of xanthine oxidase in 0.2M-NaCl buffered to pH 6.0 with 0.01M-phosphate were used. At the highest enzyme concentration (0.89%) about 2% of a contaminant with a sedimentation coefficient of about 16–17S was observed. This was presumably the component *fa* described by Avis *et al.* (1956*a*) and by Palmer *et al.* (1964). The presence of this small amount of impurity will not affect the calculations of molecular weight reported below. For the measurements of the diffusion coefficient a 0.89% solution was used. Solvent against which the enzyme had been dialysed was layered on top of the solution in a synthetic-boundary cell. Photographs were taken every 8 min. for 80 min. with the rotor spinning at 7852 rev./min. The schlieren diagrams were projected on to squared paper and tracings were made of the inside and outside regions of the curves by two different operators. The diffusion coefficient was calculated from the regression of $(A/H)^2$ on t , by using the formula:

$$(A/H)^2 = 4\pi Dt$$

* Part 10: Gilbert (1964).