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Carbohydrate Synthesis from Lactate in Pigeon-Liver Homogenate

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Liver has long been known to be a major site of carbohydrate formation from lactate (Cori & Cori, 1929), but in the past it has not proved possible to reproduce the synthesis satisfactorily in isolated tissue *in vitro*. It is true that liver slices suspended in a saline medium synthesize some carbohydrate (Takane, 1926; Buchanan, Hastings & Nesbitt, 1942, 1949), but for reasons that are still obscure the rate of carbohydrate synthesis in slices is very much lower than the rate *in vivo*. There are no records of experiments in which the synthesis occurred in disrupted liver cells. Since cell-free material is a prerequisite for the detailed investigation of the process, efforts were made to obtain a cell-free liver preparation capable of synthesizing carbohydrate at high rates. A pigeon-liver homogenate is described below which readily forms carbohydrate from L-lactate.

EXPERIMENTAL

Analytical methods. Glucose and the sum of glycogen and glucose were determined enzymically by the glucose-oxidase method (see Krebs, Bennett, de Gasquet, Gascoyne & Yoshida, 1963). 'Bound' glycogen, i.e. glycogen insoluble in aqueous HClO_4 , was determined by washing the centrifuged and well-drained sediment from the HClO_4 treatment twice with 2% (w/v) HClO_4 solution. The washed precipitate was dissolved in 1 ml. of 30% (w/v) KOH and heated in a boiling-water bath for 20 min. The glycogen was isolated according to the method of Good, Kramer & Somogyi (1933) and determined as described by Krebs *et al.* (1963).

When glucose is determined by the glucose-oxidase

method in the presence of glycogen or maltose the values obtained by the method of Krebs *et al.* (1963) are too high, because glucose-oxidase preparations, even of high purity, are contaminated by amylase, maltase and other glucosidases (Dahlqvist, 1961). In previous work on kidney this interference by glycogen was negligible because the amounts of glycogen occurring in kidney are very low, but it is not negligible in the liver. The difficulty can be overcome by including in the reagent tris (Dahlqvist, 1961), which inhibits amylase and glucosidases (Larner & Gillespie, 1956; Dahlqvist, 1958). The previous procedure was therefore modified as follows: a 1 ml. sample, deproteinized with HClO_4 (if necessary diluted with 2% HClO_4), is incubated with 2.5 ml. of a solution containing 4 mg. of peroxidase, 12.5 mg. of glucose oxidase (Sigma), 0.5 ml. of 1% (w/v) o-dianisidine in 95% (v/v) ethanol, and phosphate-tris buffer, pH 7.3, to 100 ml. This buffer is prepared by dissolving 56.79 g. of Na_2HPO_4 (anhydrous), 15.6 g. of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 12.1 g. of tris and 43 ml. of 2N-HCl in a final volume of 1 l.

During the present work it became clear that an avian (and mammalian) liver contains not only glucose, glycogen and the phosphorylated hexoses of glycolysis but also significant quantities of oligosaccharides (see Sie & Fishman, 1958; Olavarria, 1960; Olavarria & Torres, 1962). The oligosaccharides would appear as glycogen in the method used. As the work was concerned with the formation of carbohydrate from non-carbohydrate precursors it was the main object to determine the sum of glucose, glycogen and the intermediate oligo- or poly-saccharides. A resolution of the carbohydrates formed into the various fractions is under investigation.

In several experiments the glucose-oxidase method was checked by the hexokinase-glucose 6-phosphate-dehydrogenase method (see Slein, 1963) and good agreement was found.

L(+)-Lactate was determined with crystalline lactate dehydrogenase from skeletal muscle (see Hohorst, 1963a).

Pyruvate was determined according to the method of Bücher, Czok, Lamprecht & Latzko (1963), malate according to the method of Hohorst (1963b), citrate according to the method of Taylor (1953), α -oxoglutarate according to the method of Bergmeyer & Bernt (1963), β -hydroxybutyrate according to the method of Williamson, Mellanby & Krebs (1962), glutamate according to the method of Bernt & Bergmeyer (1963), and the sum of glutamate and glutamine by the same procedure after acid hydrolysis for 5 min. at 100° in $N-H_2SO_4$.

Preparation of liver homogenates (standard method). The saline medium found to give optimum results after many variations of the composition contained 100 ml. of 0.154M-KCl, 2 ml. of 0.1M-MgCl₂, 4 ml. of 0.2M-potassium phosphate buffer, pH 7.4, 21 ml. of 0.154M-NaHCO₃ and 0.4 ml. of 0.1M-EDTA (neutral sodium salt). This solution was saturated with a gas mixture containing 5% of CO₂ and other gas(es) as stated in the text. Pigeon liver from a freshly killed animal was placed for 3 min. in ice-cold 0.154M-KCl solution and homogenized in a stainless-steel Potter-Elvehjem apparatus with 6.5 parts of the ice-cold basal saline. Ten up-and-down movements of the barrel, with the piston rotating at 2000 rev./min., were sufficient to obtain a homogenate that could be conveniently pipetted. The internal diameter of the barrel was 1 in. and the clearance between barrel and piston was between 26 and 37 μ . As the diameter of rat-liver cells is about 40 μ the forcing of the tissue through the gap of the homogenizer, together with the shearing forces, broke virtually every cell, as shown by phase-contrast microscopy. Prolonged homogenization in the same type of homogenizer, or additional homogenization by hand in a glass homogenizer with a clearance of 20–25 μ , sometimes diminished the capacity of the homogenate to form carbohydrate from lactate, mainly because in the more finely homogenized materials the rates fell more rapidly during incubation.

Conditions of incubation. Incubations were carried out in conical Warburg manometer vessels of about 20 ml. volume, provided with a centre well and side arm. The gas space contained CO₂ + O₂ (5:95). The temperature was 40°. Each manometer vessel contained 2 ml. of the homogenate and 2 ml. of additional solution so that the final dilution of the homogenate was 1:15. The additions included either 0.8 ml. of 0.154M-NaCl or of 0.2M-sodium L-lactate and 0.2 ml. of 2.5 mM-NAD. Further additions of special reagents, the effect of which was to be tested, usually amounted to between 0.1 and 0.4 ml. The volume was made up to a total of 4 ml. with basal saline. Sodium ions are essential for maximal activity, and NaCl or sodium lactate must therefore not be replaced by the potassium salts. As the rate of oxygen consumption in the presence of lactate was very high, rapid shaking (100–120 cyc./min.) was essential. At the end of the incubation period the activities of the homogenate were stopped by the addition of 0.1 vol. of 20% (w/v) HClO₄.

RESULTS

Carbohydrate synthesis from lactate in pigeon-liver homogenate. An experiment demonstrating the formation of carbohydrate from lactate is shown in

Table 1. Lactate was rapidly removed (92 μ moles in 45 min.), and much glucose but very little glycogen was formed. The initial glycogen content fell in this experiment, especially in the blank, and this may have contributed to the formation of glucose. The real measure of net carbohydrate synthesis is therefore the change in the sum of glucose plus glycogen. The changes in the first 45 min. (10.1 μ moles of carbohydrate formed in the presence of lactate) were greater than those in the second 45 min. (7.3 μ moles). The rate of lactate removal fell from 92 μ moles to 38 μ moles in the second 45 min. This fall may have been partly due to the fall of the concentration of lactate; more than 80% had been used at the end of the experiment. Another factor contributing to the fall was the inactivation of some enzymes of the homogenate.

The initial amount of 'bound' glycogen was low, being less than 4% of the total carbohydrate. It fell to negligible values on incubation. Similar low percentages were found in livers of well-fed animals, and therefore bound glycogen was as a rule not measured (see also Koritz & Munck, 1960; Roe, Bailey, Gray & Robinson, 1961; Singh & Venkitasubramanian, 1963).

The negative pressure changes on the addition of lactate were very high and, like the formation of glucose and disappearance of lactate, also showed a gradual decline. These changes are the resultants of the oxygen uptake and carbon dioxide production. Since the conversion of lactate into carbohydrate causes the removal of H⁺ ions from the solution and an equivalent generation of OH⁻ ions, 2 molecules of carbon dioxide are converted into HCO₃⁻ ions for each molecule of glucose formed, and since the combustion of lactate yields 2 molecules of carbon dioxide and 1 HCO₃⁻ ion per molecule of lactate, the free carbon dioxide: oxygen ratio in the homogenates containing lactate is low (about 0.4–0.5). This, and the high rate of respiration, account for the great negative pressure changes.

The initial rate of carbohydrate synthesis of 10.1 μ moles in 45 min. for 267 mg. of tissue corresponds to a rate of 50 μ moles/g. wet wt./hr. Cori & Cori (1929) found a rate *in vivo* of 30 μ moles/g. wet wt./hr. on feeding starved rats with L-lactate by stomach tube. J. Ashmore (personal communication; see also Hastings, Teng, Nesbitt & Sinex, 1952) computed the rate of glucose production *in vivo* in a starved rat receiving lactate at 372 μ moles/100 g. body wt./hr. or about 90 μ moles/g. of liver/hr. Rat-kidney slices of animals on a normal diet form about 26 μ moles of glucose/g. wet wt./hr. from lactate (Krebs *et al.* 1963). A comparison of these values with those obtained for pigeon-liver homogenate indicate that the rates observed in the homogenate were of the order of magnitude expected for the living animal.

Of the 92 μ moles of lactate removed after 45 min., 20.2 μ moles (i.e. 22 %) were accounted for by the formation of carbohydrate; of the 130 μ moles removed after 90 min., 34.8 μ moles (i.e. 27 %) appeared as carbohydrate. Much of the remainder of the lactate underwent oxidation. Other products formed from lactate include small amounts of pyruvate, malate, citrate, glutamate, glutamine, α -oxoglutarate and β -hydroxybutyrate.

Similar results were obtained in many other

experiments provided that the animals were deprived of their liver glycogen by starvation. Since pigeons fed on grain may store substantial amounts of food in their crops, a starvation period of less than 48 hr. is often inadequate for the glycogen depletion of the liver.

When a well-fed pigeon was used for this type of experiment and the initial carbohydrate content was high (in the example recorded in Table 2, 17 times higher than in the starved animal), there

Table 1. *Carbohydrate synthesis from L-lactate in pigeon-liver homogenate from a starved animal*

The conditions of incubation were as described in the text. The temperature was 40°, and the gas phase was CO₂ + O₂ (5:95). The results refer to 4 ml. of homogenate. One pair of vessels was incubated for 45 min., another for 90 min. The 4 ml. of homogenate contained 76 mg. dry wt. of tissue (267 mg. wet wt.). The pigeon was starved for 48 hr.

Substrate added ...	...	...	...	None		L-Lactate (40 mm)	
Gas pressure changes in vessels (mm.)							
Second 15 min.				-48		-280	
Third 15 min.				-41		-250	
Fourth 15 min.				-36		-234	
Fifth 15 min.				-33		-210	
Sixth 15 min.				-29		-191	
				Found	Change	Found	Change
				(μ moles)	(μ moles)	(μ moles)	(μ moles)
Glucose							
Initial				4.18	—	4.18	—
At 45 min.				1.34	-2.84	12.1	+7.9
At 90 min.				1.14	-3.04	20.1	+15.9
Glucose + soluble glycogen							
Initial				6.58	—	6.58	—
At 45 min.				3.39	-3.2	16.7	+10.1
At 90 min.				3.68	-2.9	24.0	+17.4
Soluble glycogen (by difference)							
Initial				2.40	—	2.4	—
At 45 min.				2.05	-0.3	4.6	+2.2
At 90 min.				2.54	+0.1	3.9	+1.5
Bound glycogen							
Initial				0.25	—	0.25	—
At 45 min.				0.05	—	0.07	—
At 90 min.				0.05	—	0.07	—
Lactate							
Initial				1.44	—	166	—
At 45 min.				0.08	-1.4	74	-92
At 90 min.				0.04	-1.4	36	-130

Table 2. *Carbohydrate and lactate metabolism in pigeon-liver homogenate from a well-fed animal*

The conditions were as described in Table 1, except that the animal was well fed.

Substrate added ...	...	...	...	None		L-Lactate (40 mm)	
Gas pressure changes in manometer vessels (mm.)							
Second 15 min.				-35		-143	
Third 15 min.				-55		-132	
				Found	Change	Found	Change
				(μ moles)	(μ moles)	(μ moles)	(μ moles)
Glucose							
Initial				17.6	—	17.8	—
At 45 min.				12.7	-4.9	18.1	+0.5
Glucose + glycogen							
Initial				112	—	112	—
At 45 min.				93	-19	96	-16
Lactate							
Initial				2.8	—	166	—
At 45 min.				0.8	-2.0	114	-52

Table 3. *Effect of nutritional state on the changes in the content of glucose plus glycogen in pigeon-liver homogenates on incubation with lactate*

The conditions of incubation were as described in Table 1. The period of incubation was 45 min.

Nutritional state	Glucose + glycogen (μ moles/g. dry wt.)					Lactate (μ moles/g. dry wt.) Change after incubation with 40 mm-L-lactate
	Initial	After incubation without added substrate		After incubation with 40 mm-L-lactate		
		Found	Change	Found	Change	
Starved for 48 hr.	95	33	-62	178	+83	-676
	178	77	-101	261	+83	—
	72	50	-22	209	+137	-651
	151	52	-99	214	+162	-983
	87	23	-64	202	+115	-911
	200	165	-35	355	+155	-987
	64	19	-45	197	+133	-932
	98	24	-74	253	+155	—
	174	103	-71	256	+82	-567
	68	12	-56	172	+104	—
Well fed	720	638	-82	736	+16	-615
	376	321	-47	413	+37	-613
	999	725	-174	819	-80	-459
	718	581	-137	720	+2	-384
	475	354	-121	433	-42	-376
	644	530	-114	649	+5	-718
	518	406	-112	524	+6	-494
	1460	1210	-250	1210	-250	-672

Table 4. *Effect of the omission of ethylenediaminetetra-acetate from the medium on the metabolism of lactate by pigeon-liver homogenate*

The conditions were as described in Table 1, except that in vessels 3 and 4 EDTA was omitted. The pigeon was starved for 48 hr. The results refer to 4 ml. of homogenate containing 75 mg. dry wt. of tissue. Incubation was for 45 min.

Vessel no.	...	...	...	...	...	0	1	2	3	4
						Initial values	Complete medium	Complete medium	Medium without EDTA	Medium without EDTA
Added L-lactate (μ moles)	...	...	...	...	...	—	0	167	0	167
Pressure changes in 45 min. (mm.)						—	-168	-782	-227	-379
Glucose										
Found (μ moles)						2.6	0.2	12.1	1.7	3.5
Change (μ moles)						—	-2.4	+9.5	-0.9	+0.9
Glucose + glycogen										
Found (μ moles)						6.8	1.8	15.8	4.2	7.4
Change (μ moles)						—	-5.0	+9.0	-2.6	+0.6
Lactate										
Found (μ moles)						1.5	0.2	9.6	2.9	115
Change (μ moles)						—	-1.3	-71	+1.4	-52
Accounted for as carbohydrate (μ moles)						—	—	28	—	6.4
Not accounted for as carbohydrate (μ moles)						—	—	43	—	46

was no net synthesis of carbohydrate on incubation but a loss that was slightly decreased by the addition of lactate. Lactate removal was decreased by 43 % and the extra pressure changes due to lactate were about halved (Table 2). The rate of carbohydrate synthesis in pigeon-liver homogenate thus depends on the nutritional state of the animal. This is further illustrated by Table 3.

Effect of ethylenediaminetetra-acetate. The effects of the omission of EDTA from the medium are shown in Table 4. All the measured metabolic

activities were smaller in the absence of EDTA except for lactate in the homogenate without added lactate. The endogenous lactate was partly removed in the complete medium but formation of lactate occurred in the absence of EDTA. The loss of endogenous glucose and glycogen was much smaller in the absence of EDTA and so was the removal of added lactate. The value for the sum of glucose and glycogen after incubation in the absence of EDTA was hardly greater than the initial value. The gas pressure changes in the

absence of EDTA were about half of those of the control in the presence of lactate. On the other hand, in the absence of lactate pressure changes were somewhat higher in the absence of EDTA. The amounts of lactate not accounted for by the formation of carbohydrate were about equal in the presence and absence of EDTA. This indicates that EDTA did not affect the reactions of lactate other than the conversion into carbohydrate. Evidently the addition of EDTA is essential for obtaining maximum rates of carbohydrate synthesis from lactate.

Several other chelating agents (see Raaflaub, 1955) related to EDTA (kindly supplied by J. R. Geigy S.A., Basel, Switzerland) were tested but none of them proved advantageous in relation to the formation of carbohydrate from lactate. Ethylene glycol bisaminoethyl ether-*NNN'*-tetra-acetic acid ('glycol EDTA') and in particular diaminocyclohexane-*NNN'*-tetra-acetic acid ('sequestrene CD') inhibited carbohydrate formation. $\beta\beta$ -Diaminodiethyl ether-*NNN'*-tetra-acetic acid ('sequestrene ME') and *N*-(hydroxycyclohexyl)ethylenediamine-*NNN'*-tri-acetic acid ('sequestrene OC') were almost as effective as EDTA.

Effect of centrifugation of homogenate. On centrifugation, even at very low force, supernatants were obtained that no longer converted lactate into carbohydrate. The sediment, when resuspended in the saline medium, was also inactive (Table 5). After centrifugation for 5 min. at 250g the supernatant still converted some lactate into pyruvate. The amounts of pyruvate that accumulated in the complete homogenate were relatively low (0.4 μ -mole/4 ml.). In the supernatant 18 times more

(7.2 μ moles) appeared. The pressure changes produced by the supernatant were small but lactate still caused a small increase. The lactate removed was almost completely accounted for by the pyruvate found.

Effect of the omission of nicotinamide-adenine dinucleotide. The omission of NAD slightly decreased the conversion of lactate into carbohydrate as well as the removal of lactate and the negative pressure changes in the presence of lactate (Table 6). It had no appreciable effects in the absence of lactate. The increase in the carbohydrate synthesis caused by 0.125 mM-NAD was 13.5 % and this was all due to extra glucose formation. The increase in the rate of lactate removal was somewhat greater. The optimum concentration of NAD for carbohydrate synthesis was 0.125 mM; this amount was added to the homogenate in the standard procedure. The NAD content of pigeon liver is considerably higher.

Effect of the omission of inorganic phosphate. When the medium contained no inorganic phosphate the rates of carbohydrate synthesis and lactate removal were low. Phosphate at a concentration of 2.5 mM more than trebled the rate of carbohydrate synthesis and the maximum effect was reached at about 4 mM-phosphate (Table 7). The standard medium, after dilution with substrates and supplements, contained 4.7 mM-phosphate.

Effect of the omission of magnesium ions. The omission of Mg^{2+} ions caused a slight decrease (10–20 %) in the rates of carbohydrate formation and lactate removal.

Effect of decreasing the bicarbonate concentration. When the bicarbonate-carbon dioxide buffer was

Table 5. *Effect of centrifugation on the metabolism of lactate in pigeon-liver homogenate*

The general conditions for the complete homogenate were as described in Table 1, except that NAD was omitted. Incubation was for 45 min. The homogenate was centrifuged for 5 min. at 250g. It contained originally 73 mg. dry wt. of tissue in 4 ml.; the supernatant contained 48 mg. The results refer to 4 ml. of homogenate or supernatant.

	Complete homogenate			Supernatant of centrifuged homogenate		
	Initial value	After incubation without substrate	After incubation with 160 μ moles of lactate	Initial value	After incubation without substrate	After incubation with 160 μ moles of lactate
Gas pressure changes in 45 min. (mm.)	—	– 175	– 870	—	– 25	– 45
Glucose						
Found (μ moles)	6.5	3.8	21.1	6.7	7.0	6.8
Change (μ moles)	—	– 2.7	+ 14.6	—	+ 0.3	+ 0.1
Glucose + glycogen						
Found (μ moles)	9.2	6.3	24.6	9.9	10.0	8.8
Change (μ moles)	—	– 2.9	+ 15.4	—	+ 0.1	– 1.1
Lactate						
Found (μ moles)	1.7	0.1	83	2.5	3.1	154
Change (μ moles)	—	– 1.6	– 79	—	+ 0.6	– 8.5
Pyruvate						
Found (μ moles)	0.1	0.1	0.4	0.1	0.4	7.2

Table 6. *Effect of nicotinamide-adenine dinucleotide on the conversion of lactate into carbohydrate in pigeon-liver homogenate*

The conditions were as described in Table 1, except that the concentration of added NAD was varied. The pigeon was starved for 48 hr. Incubation was for 45 min. The final concentrations of the added substances are recorded. The amounts of metabolites refer to 4 ml. of homogenate containing 68.6 mg. dry wt. of tissue. The initial values for the metabolites were 7.0 μ moles of glucose, 6.7 μ moles of glycogen and 2.6 or 16.3 μ moles of lactate.

Final concn. of added L-lactate (mm)	...	0	40	0	40	0	40	0	40	0	40	0	40	0	40
Final concn. of added NAD (mm)	...	0	0	0.031	0.063	0.063	0.063	0.063	0.063	0.125	0.125	0.125	0.125	0.25	0.25
Pressure changes in 45 min. (mm.)		-128	-560	-127	-580	-126	-630	-630	-690	-126	-690	-126	-730	-730	-730
Glucose															
Found (μ moles)		7.0	17.8	7.1	17.9	6.9	18.3	6.6	19.4	6.7	19.4	6.7	18.6	6.7	18.6
Change (μ moles)		0	+10.8	+0.1	+10.9	-0.1	+11.3	-0.4	+12.4	-0.3	+12.4	-0.3	+11.6	-0.3	+11.6
Glycogen + glycogen															
Found (μ moles)		12.6	21.5	11.5	22.5	10.7	23.5	11.4	24.4	11.4	24.4	11.4	22.5	11.4	22.5
Change (μ moles)		-1.1	+7.8	-2.2	+8.8	-3.0	+9.8	-2.3	+10.7	-2.3	+10.7	-2.3	+8.8	-2.3	+8.8
Glycogen															
Found (by difference) (μ moles)		5.6	3.7	4.4	4.6	3.8	5.2	4.8	5.0	4.7	5.0	4.7	3.9	4.7	3.9
Change (by difference) (μ moles)		-1.1	-3.0	-3.3	-3.1	-2.9	-1.5	-1.9	-1.7	-2.0	-1.7	-2.0	-2.1	-2.0	-2.1
Lactate															
Found (μ moles)		0.74	115	0.32	107	0.27	96	0.29	96	0.24	96	0.24	81	0.24	81
Change (μ moles)		-1.9	-48	-2.3	-56	-2.3	-67	-2.3	-67	-2.4	-67	-2.4	-82	-2.4	-82

replaced by a phosphate buffer the rate of gluconeogenesis dropped to less than one-third (Table 8). The phosphate buffer medium contained 100 ml. of 0.154M-potassium chloride, 2 ml. of 0.1M-magnesium chloride, 4 ml. of 0.154M-sodium bicarbonate, 20 ml. of 0.1M-sodium phosphate buffer, pH 7.3, and 0.4 ml. of 0.1M-EDTA. The gas phase over the phosphate-buffered homogenate was oxygen without carbon dioxide and the centre well contained 0.3 ml. of 2N-sodium hydroxide. There was thus some bicarbonate in the medium. This increased during the incubation as a result of the oxidation of lactate. In another experiment the above medium was modified by decreasing the amount of phosphate buffer from 20 ml. to 4 ml. The results were very similar. Bicarbonate and carbon dioxide are known to be reactants in gluconeogenesis at the stage of the carboxylation of pyruvate. The K_m value for HCO_3^- ions in this reaction is 1 mM (Keech & Utter, 1963), and the bicarbonate concentration is therefore not expected to affect the rate of the carboxylation of pyruvate within the variations occurring in the above experiments. The observed effects may be due to adverse effects of high phosphate concentrations and, when bicarbonate is not replaced by another buffer, to inadequate buffering. The rapid production of bicarbonate from lactate makes effective buffering essential.

Oxygen consumption. In the experiment recorded in Table 8, the oxygen consumption in the flasks containing the phosphate-buffered homogenate and alkali in the centre well was obtained directly from the manometric readings. The oxygen consumption of the bicarbonate-carbon dioxide-buffered homogenate was measured by the method of Warburg & Krippahl (1960) and Warburg, Geissler & Lorenz (1961), in a 1 ml. sample placed in the outer compartment of a vessel of the type described by Dickens & Šimer (1931) of 30 ml. capacity. The centre well contained 5 ml. of 3M-potassium carbonate (24 parts)-bicarbonate buffer (76 parts), titrated according to the method of Kolthoff & Sandell (1952), together with carbonic anhydrase and five small glass beads. The gas space was filled with carbon dioxide+oxygen (5:95). This buffer kept the carbon dioxide pressure at about 470 mm. manometric fluid. The values for the oxygen uptake obtained were used to calculate the formation of carbon dioxide from the pressure changes observed in the experiment recorded in Table 8.

In the bicarbonate-buffered homogenate the oxygen consumption was 49.5 μ moles in 45 min. without lactate and 92.0 μ moles with lactate. The corresponding values for the phosphate-buffered homogenate, corrected for the same amount of tissue, were 45.0 and 83.5 μ moles. The Q_{O_2} values

(μ l. of oxygen used/mg. dry wt./hr.) were thus 18.7 and 36.6 in the bicarbonate-buffered homogenate.

The amounts of carbon dioxide formed were 43.5 μ moles in the absence of lactate and 50.6 μ moles in the presence of lactate. The carbon dioxide: oxygen ratio was thus 0.88 in the control and 0.55 with lactate. The low value for this ratio in the presence of lactate is due to the fact that one-third of the respiratory carbon dioxide formed from lactate is expected to appear as bicarbonate, and that the conversion of lactate into carbohydrate forms alkali which converts free carbon dioxide into bicarbonate.

Effect of oxygen pressure. Since oxygen of 1 atm. pressure is known to be toxic to some respiratory enzymes (Dickens, 1946; Mann & Quastel, 1946; Stadie, Riggs & Haugaard, 1944), it might be thought that the gradual decline of the homogenate activity is due to the use of oxygen in the manometer vessels. However, the fall of activity was the same with carbon dioxide + oxygen + nitrogen (5:47.5:47.5) and with carbon dioxide + oxygen (5:95), and the rates of the gas pressure changes were the same, indicating that the

oxygenation was still adequate in 47.5 % oxygen. The amounts of lactate removed and carbohydrate formed were also the same. When carbon dioxide + air (5:95) was used the metabolic rates were about 30 % lower.

Effect of the sodium ion and potassium ion concentrations in the medium. Wide variations in the concentrations of Na^+ and K^+ ions in the suspension medium had no major effect on the initial rate of carbohydrate synthesis. The complete omission of Na^+ or K^+ ions did not affect the initial rate of synthesis of carbohydrate (sum of glucose plus glycogen), but the share of glycogen in the total was somewhat greater in the all- K^+ ion medium. The standard medium used in the present work gave the highest rates on prolonged incubation, especially beyond 45 min. because the gradual fall of activity was less in this medium than in others (see also Buchanan *et al.* 1949). The complete omission of K^+ ions with replacement by Na^+ ions greatly decreased the fall of the carbohydrate content on incubation in the absence of lactate, but carbohydrate formation from lactate was lower than in media containing 40 mM- Na^+ ion.

Table 7. *Effect of inorganic phosphate on gluconeogenesis from lactate in pigeon-liver homogenate*

The general conditions were as described in Table 1, except that the phosphate concentration was varied as shown below. Incubation was for 45 min. The initial lactate content was 163 μ moles in all samples. The results refer to 4 ml. of homogenate containing 72.7 mg. dry wt. of tissue. The initial content of glucose was 5.26 μ moles, and that of glucose plus glycogen 11.1 μ moles.

Final concn. of added phosphate (mM) ...	0	2.5	5	10
Glucose				
Found (μ moles)	6.91	12.2	14.3	15.3
Change (μ moles)	+1.65	+6.9	+9.0	+10.0
Glucose + glycogen				
Found (μ moles)	12.6	19.3	20.6	20.6
Change (μ moles)	+1.5	+8.2	+9.5	+9.5
Lactate				
Found (μ moles)	131	106	95	97
Change (μ moles)	-31	-57	-68	-66

Table 8. *Effect of replacing bicarbonate-carbon dioxide buffer by phosphate buffer on gluconeogenesis from lactate in pigeon-liver homogenate*

The compositions of the two homogenates are given in the text. The samples were incubated for 45 min. with and without 40 μ moles of lactate. The results refer to 4 ml. of homogenate. The bicarbonate- CO_2 -buffered homogenate contained 75.0 mg. dry wt. in 4 ml., and the phosphate-buffered homogenate 81.6 mg. dry wt. of tissue in 4 ml. The pigeon was starved for 48 hr.

Metabolite in 4 ml. of homogenate	In bicarbonate- CO_2 - buffered homogenate		In phosphate-buffered homogenate	
Lactate				
Initial (μ moles)	1.25	158	1.31	158
Change (μ moles)	-0.60	-46	+0.2	-60
Glucose				
Initial (μ moles)	2.90	2.90	3.30	3.30
Change (μ moles)	-2.81	+7.3	-3.24	+2.99
Glucose + glycogen				
Initial (μ moles)	4.93	4.93	5.34	5.34
Change (μ moles)	-3.57	+7.39	-4.26	+2.36

Effects of adenosine triphosphate and adenosine monophosphate. The addition of ATP, which accelerates many metabolic activities of tissue homogenates, had no beneficial effect on gluconeogenesis. On the contrary, at physiological concentrations (2.5 mM) it drastically decreased the formation of carbohydrate (Table 9), without affecting the rate of lactate disappearance. AMP at very low concentrations had similar effects (Table 10).

Effects of other additions. Many substances were tested to determine whether their addition causes higher rates of carbohydrate synthesis. No beneficial effects were observed with glutathione, mercaptoethanol, succinate, acetoacetate, β -hydroxybutyrate, nicotinamide or ethanol.

Gluconeogenesis from different substrates. Of the substrates tested lactate gave the highest rates of carbohydrate synthesis (Table 11). All other sub-

strates prevented the loss of preformed carbohydrate. With pyruvate there was no increase over the initial carbohydrate value but all other substrates raised the initial value. As pyruvate is an intermediate in the conversion of lactate into carbohydrate, it may appear surprising that it is a less efficient precursor than lactate. Unpublished work indicates that excess of pyruvate competes with diphosphoglycerate for NADH₂ and thereby inhibits the triose phosphate-dehydrogenase step of gluconeogenesis.

Gluconeogenesis in liver homogenates of other species. Homogenates from the livers of rat, mouse, sheep, cattle, domestic fowl, Japanese quail and duckling, prepared in the same way as the pigeon-liver homogenate, formed little or no carbohydrate from lactate. Increasing the tissue concentration did not improve the gluconeogenic capacity.

Table 9. *Effect of adenosine triphosphate on gluconeogenesis from lactate in pigeon-liver homogenate*

The general conditions were as described in Table 1. The results refer to 4 ml. of homogenate which contained 75.5 mg. dry wt. of tissue. The initial content of glucose was 4.1 μ moles and that of glucose plus glycogen 5.87 μ moles. The amount of the added lactate was 160 μ moles and that of the added ATP 10 μ moles.

Vessel no.	...	...	1	2	3	4
Additions to homogenate	...		None	ATP	L-Lactate	L-Lactate + ATP
Metabolic changes						
Glucose (μ moles)			-3.12	-3.78	+10.8	-0.81
Glucose + glycogen (μ moles)			-3.60	-3.91	+11.7	-1.47
Lactate (μ moles)			-1.53	-1.43	-82.0	-85.4

Table 10. *Effect of adenosine monophosphate on gluconeogenesis from lactate in pigeon-liver homogenate*

The general conditions were as described in Table 1. The results refer to 4 ml. of homogenate containing 86 mg. dry wt. of tissue. The initial content of glucose was 6.44 μ moles and that of glucose plus glycogen 9.42 μ moles.

Vessel no.	...	...	1	2	3	4	5	6	7	8
Added lactate (μ moles)	...	...	0	160	0	160	0	160	0	160
Final concn. of added AMP (mM)			0	0	0.313	0.313	0.625	0.625	1.25	1.25
Metabolic changes										
Glucose (μ moles)			-6.1	+2.41	-6.2	-3.62	-6.2	-3.87	-6.3	-3.51
Glucose + glycogen (μ moles)			-7.0	+6.33	-8.9	-2.98	-8.5	-4.98	-7.8	-3.80
Lactate (μ moles)			-1.3	-59	-0.4	-67	-0.3	-68	-0.5	-62

Table 11. *Gluconeogenesis from various precursors in pigeon-liver homogenate*

The general conditions were as described for Table 1. The pigeon was starved for 48 hr. Incubation was for 45 min. The results refer to 4 ml. of homogenate containing 69.7 mg. dry wt. of tissue. All substrate concentrations were 40 mM. The acids were added as sodium salts. Initially the homogenate contained 5.31 μ moles of glucose and 7.13 μ moles of glucose plus glycogen.

Substrate added	Glucose (μ moles)		Glucose + glycogen (μ moles)	
	Found	Change	Found	Change
None	0.26	-5.05	1.58	-5.55
L-Lactate	9.73	+4.42	13.6	+6.5
Pyruvate	4.97	-0.29	7.21	-0.1
Fumarate	7.79	+2.48	12.7	+5.6
α -Oxoglutarate	6.21	+0.90	8.48	+1.35
L-Glutamate	6.80	+1.49	8.59	+1.46
α -Glycerophosphate	7.77	+2.46	9.96	+2.83
Glycerol	4.93	-0.38	7.94	+0.81

DISCUSSION

The cell-free homogenate described above performs the complete series of glucogenic reactions leading from lactate and other precursors to carbohydrate, as well as the required energy-supplying processes with which gluconeogenesis is linked. Such a system should prove valuable in further work, especially in studies on the control mechanisms that determine the overall rate of gluconeogenesis (see Krebs, 1964).

As expected, the fractionation of the homogenate into supernatant and insoluble particles abolishes the glucogenic capacity. Gluconeogenesis from lactate requires at least 12 enzymes for converting the carbon skeleton of lactate into that of glucose. Nine of these (lactate dehydrogenase, enolase, phosphoglycerate phosphomutase, phosphoglycerate kinase, triose phosphate dehydrogenase, triose phosphate isomerase, aldolase, fructose 1,6-diphosphatase and glucose phosphate isomerase) are exclusively or predominantly in the soluble fraction (de Duve, Wattiaux & Baudhuin, 1962). Two enzymes (pyruvate carboxylase and phosphopyruvate carboxylase) are located in the mitochondria (Utter, 1959; see also Nordlie & Lardy, 1963). One (glucose 6-phosphatase) is in the microsomal fraction. The three additional enzymes needed for the formation of glycogen (phosphoglucose mutase, UDP-glucose pyrophosphorylase and UDP-glucose transglucosylase) are all located in the supernatant. In addition, ATP is required, 6 molecules for each glucose molecule formed and 7 for each glucose equivalent of glycogen. This is mainly formed in the mitochondria. Other necessary enzymes are the nucleoside diphosphokinases that supply GTP for the phosphopyruvate-carboxykinase reaction and UTP for the formation of UDP-glucose. These are located in the soluble fraction (Leloir & Goldemberg, 1960; de Duve *et al.* 1962). Evidently gluconeogenesis is dependent on an interplay of mitochondrial, soluble and microsomal enzymes (see also Krinsky, 1959; Mendicino & Utter, 1962; Mendicino, 1962).

Rather high initial lactate concentrations are required under the test conditions, mainly because the rate of lactate utilization is very rapid. In most experiments more than half of the added lactate was removed during the period of incubation. Additional reasons may be the fact that the conversion of lactate into pyruvate may be inhibited by very low concentrations of pyruvate (see Zewe & Fromm, 1962), and that the apparent K_m for lactate is high (see Nisselbaum & Bodansky, 1963).

Nature of the effect of ethylenediaminetetra-acetate. It is very probable that the action of EDTA is due to chelation, but it is not yet clear which metal ion

is involved. EDTA is known to activate several enzymes. Among these is fructose 1,6-diphosphatase (E. A. Newsholme & A. H. Underwood, personal communication). This effect may be connected with the observations reported in the present paper.

Oxygen uptake and carbohydrate synthesis. On the basis of the ATP requirements and a P:O ratio of 3 for oxidative phosphorylation, an uptake of 1 molecule of oxygen would be needed for the synthesis of 1 molecule of glucose. One glucose equivalent of glycogen would require 1.17 molecules of oxygen. The observed rate:

$$\frac{\text{extra O}_2 \text{ used on addition of lactate}}{\text{glucose equivalents formed}}$$

was much higher, e.g. $42.5/7.4 = 5.7$ (see Table 8 and values in the text). The high oxygen uptake is probably due to other energy-requiring processes taking place on the addition of lactate. The oxygen consumption was much lower than was needed for the complete oxidation of that fraction of lactate that cannot be accounted for by the formation of carbohydrate, or by the products of incomplete oxidation such as pyruvate. This indicates that lactate serves as a precursor in other biosyntheses. One of these is the formation of glutamine.

Miscellaneous points. Many observations made during the present work require further investigation before they can be discussed profitably. These include the fate of the glucose that disappears in the homogenates not supplemented with lactate, especially in liver from the well-fed animal; the question why glucose rather than glycogen is the main product of carbohydrate synthesis [this may be due to an activation of the lysosomal amylase in the homogenate (see Rutter & Brosemer, 1961; Brosemer & Rutter, 1961)]; the reasons for the failure of the liver of other species to yield an effective homogenate and the role of Na^+ ions in promoting gluconeogenesis.

SUMMARY

1. A cell-free pigeon-liver homogenate is described that readily synthesizes carbohydrate from lactate (about $50 \mu\text{moles/g. of liver/hr.}$). Essential components for maximum rates are Na^+ and K^+ ions, EDTA, phosphate, Mg^{2+} ions, bicarbonate and NAD. Glucose is the main carbohydrate formed; glycogen appears in minor quantities.

2. The rates of carbohydrate synthesis are higher in the livers of starved than in those of well-fed animals.

3. Fumarate, glutamate, α -oxoglutarate, glycerol and glycerophosphate also form carbohydrate in the pigeon-liver homogenates, but the rates are lower than with lactate.

4. ATP and AMP inhibit gluconeogenesis and accelerate the disappearance of carbohydrate in the absence of lactate.

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The Autodegradation of ³²P-Labelled Ribosomes from *Escherichia coli*

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Two features of a RNA in bacteria have led to its wide acceptance as a messenger of information between DNA and sites of protein synthesis. This 'messenger RNA', which has been the subject of

several recent reviews (Jacob & Monod, 1961; Burma, 1962; Canellakis, 1962; Grunberg-Manago, 1962; Volkin, 1963), has a base composition similar to concomitantly synthesized DNA and exhibits a