Generation of Extramitochondrial Reducing Power in Gluconeogenesis

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1. Kidney-cortex slices incubated with pyruvate formed glucose and lactate in relatively large and approximately equimolar quantities. The formation of these products involves two exclusively cytoplasmic NADH₂-requiring reductions, catalysed by lactate dehydrogenase and triose phosphate dehydrogenase. From the rates of glucose and lactate formation it can be calculated that over 1000 μ -moles of NADH₂ must have been produced in the cytoplasm/g. dry wt. of tissue/hr. 2. When lactate is a gluconeogenic precursor the required NADH₂ is generated in the cytoplasm, but, when a substrate more highly oxidized than glucose, such as pyruvate, is the precursor, there is no direct cytoplasmic source of NADH₂. Quantitative data on the fate of pyruvate are in accord with the conclusion that the NADH₂ was primarily formed intramitochondrially by the dehydrogenases of cell respiration, with pyruvate as the major substrate. 3. Similar observations and conclusions apply to experiments with mouse-liver slices incubated with pyruvate, serine or aspartate. 4. Addition of ethanol, which increases the formation of NADH₂ in the cytoplasm, increased the formation from pyruvate of lactate but not of glucose. 5. In view of the low permeability of mitochondria for NAD and NADH₂ it must be postulated that special carrier mechanisms transfer the reducing equivalents of intramitochondrially generated NADH₂ to the cytoplasm. Reasons are given in support of the assumption that the malate-oxaloacetate system acts as the carrier. 6. Various aspects of the generation of reducing power and its transfer from mitochondria to cytoplasm are discussed.

Gluconeogenesis from all amino acids, lactate, pyruvate and several other precursors involves a reductive step, namely the formation of glyceraldehyde phosphate from diphosphoglycerate, a reaction that occurs exclusively in the cytoplasm. This raises the question of the origin of the required reducing agent, NADH₂. When lactate is the gluconeogenic precursor, an obvious source of NADH₂ is the reaction lactate $\rightarrow$ pyruvate, which occurs in the cytoplasm in exact stoichiometry with the reaction diphosphoglycerate $\rightarrow$ glyceraldehyde phosphate.

However, the origin of the NADH₂ is not obvious when added pyruvate acts as gluconeogenic precursor, because its conversion into glucose does not involve a cytoplasmic NAD-linked dehydrogenation. Evidence presented in this paper indicates that in this situation NADH₂ is primarily formed by the intramitochondrial dehydrogenases of the tricarboxylic acid cycle and connected reactions. Since the internal mitochondrial membrane is virtually impermeable to NADH₂, the transfer of

NADH₂ to the cytoplasm requires a special carrier mechanism: NADH₂ reduces intramitochondrial oxaloacetate to malate, which diffuses into the cytoplasm and through the cytoplasmic malate dehydrogenase generates extramitochondrial NADH₂. This mechanism of generating NADH₂ in the cytoplasm must also operate for other precursors that are more highly oxidized than glucose, such as serine and glycerate.

EXPERIMENTAL

Methods. Slices of rat kidney cortex and mouse liver were incubated as described by Krebs, Bennett, de Gasquet, Gascoyne & Yoshida (1963a) and Krebs, Notton & Hems (1966). The phosphate-buffered saline of Krebs, Hems & Gascoyne (1963b) was used, with NaOH in the centre well of the manometer cups and O₂ in the gas space so that the O₂ consumption could be measured accurately. The methods used for the determination of metabolites were the same as in previous investigations in this Laboratory (Krebs, Dierks & Gascoyne, 1964; Gevers & Krebs, 1966; Hems, Ross, Berry & Krebs, 1966).

RESULTS

Products of pyruvate metabolism in rat kidney cortex. When pyruvate was added aerobically to slices of rat kidney cortex almost 1 mol. of pyruvate was removed/mol. of oxygen taken up (Table 1). The main products, apart from carbon dioxide,

were glucose and lactate, which appeared in approximately equal molecular quantities. Small amounts of malate, fumarate, glutamate, glutamine, α -glycerophosphate and ketone bodies were also formed.

The reducing equivalents required for the formation of glucose and lactate from pyruvate (2 mol.

Table 1. *Fate of pyruvate and lactate in rat kidney-cortex slices*

Washed slices were incubated for 60 min. at 40° in 4 ml. of the phosphate-buffered saline of Krebs *et al.* (1963b). The gas space contained O₂. The animals had been starved for 24 hr. Disappearance of the metabolite is indicated by the — sign, formation by the + sign. All results in this and the other Tables represent averages of duplicates of single experiments.

Substrate added ...	Metabolic changes (μ moles/g. dry wt./hr.)		
	None	Pyruvate (10mm)	L-Lactate (10mm)
Metabolite			
O ₂	— 1060	— 2130	— 1865
Glucose	+ 19	+ 369	+ 197
Pyruvate	+ 2	— 2020	+ 120
Lactate	+ 40	+ 334	+ 736
Malate + fumarate	+ 1	+ 5	+ 2
Glutamate + glutamine	+ 8	+ 16	+ 23
α -Glycerophosphate	+ 1	+ 5	+ 2
Acetoacetate	+ 6	+ 16	
β -Hydroxybutyrate	+ 1	+ 4	

Table 2. *Analysis of data of Table 1*

It is assumed that the formation of malate and fumarate requires 1 mol. of pyruvate or lactate/mol., and the formation of glutamate, glutamine and ketone bodies 2 mol./mol.; it is further assumed that pyruvate yields 4 mol. of NADH₂/mol. and 1 mol. of reduced flavoprotein/mol. when undergoing degradation without electron transfer by the respiratory chain. No corrections were made for the small blanks observed in the control to which no substrate had been added, because the justification of any corrections is doubtful. The oxidations and reductions involved in the formation of glutamate, ketone bodies, lactate and pyruvate have been neglected.

Substrate added ...	Pyruvate	Lactate
Substrate removed accounted for as:		
Glucose (μ moles/g. dry wt.)	739	394
Lactate (μ moles/g. dry wt.)	334	—
Pyruvate (μ moles/g. dry wt.)	—	120
Malate + fumarate (μ moles/g. dry wt.)	5	2
Glutamate + glutamine (μ moles/g. dry wt.)	32	46
Ketone bodies (μ moles/g. dry wt.)	40	—
Total (μ moles/g. dry wt.)	1150	562
Pyruvate required for supply of NADH ₂ (μ moles/g. dry wt.)	268	—
Substrate removed not accounted for (μ moles/g. dry wt.)	602	174
O ₂ required for:		
(a) oxidation of substrate not accounted for (μ moles/g. dry wt.)	1505	522
(b) oxidation of reduced flavoprotein formed during the generation of NADH ₂ (μ moles/g. dry wt.)	134	—
Percentage of total O ₂ uptake accounted for by added substrate	77	28

of NADH₂/mol. of glucose, 1 mol. of NADH₂/mol. of lactate) equalled 1072 μmoles/g. dry wt. (uncorrected for the small changes in the control cup). No such amounts of hydrogen donors were available from preformed cell constituents because the concentrations of the substrates of NAD-linked dehydrogenases in the tissue were low; moreover, they decreased rather than increased on incubation with pyruvate. These quantitative considerations lead necessarily to the conclusion that the required NADH₂ was formed as the result of cell respiration, which in the experiment under discussion was mainly an oxidation of pyruvate, as the analysis of the data of Table 1 indicates (see Table 2). Much more pyruvate was removed than can be accounted for by the formation of glucose, lactate and the other products. These required 1150 μmoles of pyruvate/g. dry wt., whereas 2020 μmoles/g. dry wt. were removed. The excess, 870 μmoles/g. dry wt., is more than sufficient to allow the assumption that pyruvate was used to supply NADH₂ according to the reaction:

To obtain 1072 μmoles of reducing equivalents/g. dry wt. by this reaction 268 μmoles of pyruvate/g. dry wt. are required, leaving 602 μmoles of pyruvate/g. dry wt. not accounted for. This fraction must have served as a substrate of respiration and was sufficient to contribute 77% of the respiratory fuel. More pyruvate was oxidized than can be accounted for by the extra oxygen uptake due to added substrate; thus pyruvate replaced a major part of the endogenous substrates of respiration.

When lactate was the added substrate there was also more lactate removed than could be accounted

for by the products, but in this case the extra substrate used supplied only 28% of the total fuel for respiration and this was not sufficient for the extra oxygen consumption caused by the addition of lactate. Thus some of the extra respiration was due to an increased utilization of endogenous substrates.

The extra oxygen uptake caused by the addition of pyruvate or lactate was rather greater than might be expected on the basis of extra ATP requirements of gluconeogenesis, namely 6 mol. of ATP/mol. of glucose. With pyruvate as the substrate the extra ATP requirements were 369 × 6 = 2214 μmoles/g. dry wt., corresponding to an extra oxygen uptake of about 400 μmoles/g. dry wt., depending on the efficiency of oxidative phosphorylation. The observed extra oxygen uptake was 1070 μmoles/g. dry wt. With lactate as the substrate the expected extra oxygen uptake was about 200 μmoles/g. dry wt. and the observed value 805 μmoles/g. dry wt.

Products of pyruvate metabolism in mouse liver. When mouse liver was incubated with pyruvate analogous results were obtained (Table 3). Again much lactate was formed in addition to carbohydrate, and further NADH₂ was required for the formation of malate, fumarate and β-hydroxybutyrate. The analysis of the results is not as simple as for the kidney cortex because the changes in the control without pyruvate were much greater than in the kidney. In the latter tissue, in contrast with liver, the calculations are not much affected by deducting the control values. The sum of the metabolites formed in the presence of pyruvate in the liver was greater than the amount of pyruvate removed, which indicates that addition of pyruvate did not suppress the basic metabolic activities, as

Table 3. Fate of pyruvate in mouse liver

The general experimental conditions were as described in Table 1. To deplete the liver of glycogen, the mouse was treated with phlorrhizin (see Krebs *et al.* 1966) and left without food for 3 hr. Slices were cut dry and not washed. Analyses were carried out on slices plus medium.

Metabolite	Initial values (μmoles/g. dry wt.)	Values after 60 min. incubation (μmoles/g. dry wt.)	
		Without added substrate	With pyruvate (10 mm)
O ₂ used	—	355	532
Glucose + glycogen found	0.7	21	72
Pyruvate removed	—	—	456
Lactate found	7	27	131
Malate + fumarate found	0.3	1.6	42
Glutamate + glutamine found	44	7.7	5.6
Acetoacetate found	12	55	49
β-Hydroxybutyrate found	9	17	66
Total ketone bodies found	21	72	115
Acetoacetate/β-hydroxybutyrate ratio	1.33	3.2	0.74

it did to a large extent in kidney cortex. The increments over the control, in terms of NADH₂ requirements and expressed per g. dry wt., were 102 μ moles for glucose, 104 μ moles for lactate, 40 μ moles for malate plus fumarate and 49 μ moles for β -hydroxybutyrate, a total of 295 μ moles/g. dry wt. The findings are in agreement with the postulate that the NADH₂ was formed as a result of mitochondrial dehydrogenase activity. The theoretical extra oxygen uptake due to ATP requirements was, as in the kidney experiments, much exceeded. A noteworthy observation is the fall of the acetoacetate/ β -hydroxybutyrate ratio caused by pyruvate. It should be mentioned that the phosphate-buffered saline used to facilitate the measurement of the oxygen uptake was not the optimum medium for gluconeogenesis because of its low bicarbonate and carbon dioxide concentrations. For this reason the rate of glucose formation from pyruvate was lower than that reported by Krebs *et al.* (1966).

Metabolism of aspartate and serine. In principle the fate of serine in mouse liver (Table 4) and of L-aspartate in rat kidney cortex (Table 5) shows the same characteristics as that of pyruvate,

though on a smaller scale because the rates at which these two substrates are metabolized are lower than those of pyruvate. Both carbohydrate and lactate were formed and the oxygen uptake was increased, particularly with aspartate. Since the degradation of serine and aspartate is not accompanied by cytoplasmic dehydrogenations, the reducing equivalents required for the formation of glucose and lactate must have arisen from intramitochondrial dehydrogenases.

Effect of ethanol. It was thought that the supply of extramitochondrial reducing equivalents is a factor limiting the rate of gluconeogenesis from pyruvate. Ethanol was added to increase the rate of formation of extramitochondrial NADH₂, because liver alcohol dehydrogenase is a cytoplasmic enzyme. In fact, there was a striking increase (more than doubling) of the lactate formation from pyruvate (Table 6), but the formation of glucose was not affected. Thus the additional NADH₂ reacted preferentially to reduce pyruvate rather than diphosphoglycerate.

Analogous observations were made in experiments on pigeon-liver homogenates (Table 7) in which ethanol together with crystalline liver

Table 4. *Fate of L-serine in mouse liver*

The general experimental conditions were as described in Table 1 except that pyruvate was replaced by L-serine.

Metabolite	Initial values (μ moles/g. dry wt.)	Values after 60 min. incubation (μ moles/g. dry wt.)	
		Without added substrate	With L-serine (10 mm)
O ₂ used	—	216	344
Glucose + glycogen found	1	22	44
Pyruvate found	0.4	~0	15
Lactate found	7	20	39
Malate + fumarate found	1.3	4.8	5.1
Acetoacetate found	10	38	31
β -Hydroxybutyrate found	5.8	21	21
NH ₃ + urea (as NH ₃)	68	157	249

Table 5. *Fate of L-aspartate in slices of rat kidney cortex*

Washed slices were incubated in phosphate-buffered saline as described in Table 1. The rat had been fed on the casein-margarine diet specified by Krebs *et al.* (1963a).

Metabolite	Initial values (μ moles/g. dry wt.)	Values after 60 min. incubation (μ moles/g. dry wt.)	
		Without added substrate	With L-aspartate (10 mm)
O ₂ used	—	936	2030
Glucose found	21	18	48
Pyruvate found	2	3	76
Lactate found	18	23	57
Malate + glutarate found	0.3	~0	12

Table 6. *Effect of ethanol on gluconeogenesis from pyruvate in mouse-liver slices*

The conditions of incubation and the treatment of the mouse were as described in Table 3.

Metabolite	Initial values (μ moles/g. dry wt.)	Values after 60 min. incubation (μ moles/g. dry wt.)			
		Without added substrate	With ethanol (10 mm)	With pyruvate (10 mm)	With pyruvate (10 mm) + ethanol (10 mm)
O ₂ used	—	296	236	425	348
Glucose + glycogen found	0.4	25	17	57	60
Lactate found	7.3	15	34	92	198
Pyruvate used	—	—	—	343	398
Malate + fumarate found	—	—	—	4	4
Acetoacetate found	0.4	49	33	43	35
β -Hydroxybutyrate found	11.5	20	25	34	44

Table 7. *Effect of ethanol and alcohol dehydrogenase on the fate of pyruvate in pigeon-liver homogenate*

The homogenate was prepared as described by Krebs *et al.* (1964) from the liver of a starved (48 hr.) pigeon. The final concentrations of added L-lactate and pyruvate were 40 mM, of added NAD 0.5 mM, of ethanol 20 mM and of crystalline liver alcohol dehydrogenase (Dalziel, 1961) 0.25 mg./ml. (0.003 mM). Incubation was for 1 hr. at 40°. The initial amounts of glucose + glycogen were 36 μ moles/g. dry wt. and of lactate 20 μ moles/g. dry wt. For general experimental details see Krebs *et al.* (1964).

Metabolite	Substrate added ...	Values after 60 min. incubation (μ moles/g. dry wt.)				
		None	Ethanol	Lactate	Pyruvate	Pyruvate + ethanol
Glucose + glycogen found		1.4	1.3	239	49	41
Lactate found		0.3	16	—	232	1260

alcohol dehydrogenase was added (because the activity of alcohol dehydrogenase of pigeon liver is low). These additions increased the yield of lactate over fivefold without affecting the yield of glucose. The relatively low rate of glucose formation from pyruvate in pigeon-liver homogenate was thus not due to lack of NADH₂.

Experiments on perfused organs. Experiments on the isolated perfused rat liver and isolated perfused rat kidney in which pyruvate was added to the perfusion medium also indicated a substantial formation of lactate in addition to glucose. These experiments are not reported in detail in the present paper.

DISCUSSION

Appearance of cytoplasmic reducing power in the presence of pyruvate. The experiments show that large amounts of NADH₂, required for the conversion of diphosphoglycerate into glyceraldehyde phosphate and of pyruvate into lactate, are available in the cytoplasm when pyruvate is metabolized in kidney or liver slices. The location of these two reactions exclusively in the cytoplasm and the fact

that they require NADH₂ as an obligatory reducing agent leave no doubt of this. The quantities (1072 μ moles/g. dry wt./hr. in the experiments recorded in Table 1) are very high: they are of the same order as the basal oxygen uptake of kidney (1060 μ moles/g. dry wt./hr.) and about 50% of the oxygen uptake in the presence of pyruvate. This means that in the presence of pyruvate about one-quarter of the total NADH₂ formed serves as a reducing agent and two-thirds as substrate of oxidative phosphorylation.

Since the permeability of mitochondria to the nicotinamide-adenine dinucleotides is very low (Lehninger, 1951; Kaufman & Kaplan, 1960; Purvis & Lowenstein, 1961), special mechanisms must be postulated for the supply of extramitochondrial NADH₂ in gluconeogenesis from substrates other than lactate, this being the only gluconeogenic precursor that forms NADH₂ in the cytoplasm in the required stoichiometric proportions. Precursors more highly oxidized than lactate, such as pyruvate, glycerate, serine and oxaloacetate, generate no NADH₂ during their conversion into carbohydrate. Other precursors,

such as glutamate, aspartate, alanine, proline and ornithine, generate NADH_2 , but solely in the mitochondria, since all the dehydrogenases involved in the conversion of these precursors into oxaloacetate are located intramitochondrially. This holds also for glutamate dehydrogenase, which is indirectly concerned with the degradation of many amino acids from which it arises by transamination.

There is an abundant supply of intramitochondrial NADH_2 in all respiring cells, formed by the dehydrogenases of the tricarboxylic acid cycle and the related reactions. But special carrier mechanisms must operate, transferring NADH_2 to the cytoplasm. How rapidly these mechanisms must operate is illustrated by the following calculations. The total NADH_2 content of rat kidney is less than $1.5 \mu\text{moles/g. dry wt.}$ (Glock & McLean, 1955). Since $1072 \mu\text{moles of } \text{NADH}_2/\text{g. dry wt./hr.}$ were used (Table 1), an amount equal to the total NADH_2 content of the liver was supplied, and must have traversed the mitochondrial membrane, every 5 sec.

Hydrogen carriers from mitochondria to cytoplasm. The postulated carrier systems must meet three requirements. They must readily accept hydrogen atoms from mitochondrial NADH_2 . They must readily traverse the mitochondrial membrane. They must readily donate hydrogen atoms to extramitochondrial NAD. Thus they must be substances for which there are highly active NAD-linked dehydrogenases in both the intra- and extra-mitochondrial space. The only substrate couple that meets these three requirements in liver cells is the malate-oxaloacetate system. The isocitrate-oxalosuccinate system can be ruled out on account of the instability of oxalosuccinate and the virtual absence of an active NAD-linked isocitrate dehydrogenase in the cytoplasm. The conclusion is therefore inescapable that the malate-oxaloacetate system is the carrier converting intramitochondrial NADH_2 into extramitochondrial NADH_2 . This conclusion is supported by entirely independent evidence obtained by Hoberman & D'Adamo (1960), who followed the fate of deuterium of 2-2'-deuteriofumarate in starved rats. As expected, the deuterium appeared mainly in position 4 of glucose, with smaller quantities in position 6.

Physiological significance of the dicarboxylic acid shuttle. Evidence that malate is formed during gluconeogenesis from lactate and pyruvate was provided by the ^{14}C -labelling data of Topper & Hastings (1949) and of Lorber, Lifson, Wood, Sakami & Shreeve (1950). Their experiments with $^{14}\text{CO}_2$ and lactate labelled in position 2 or 3 indicated that at least 85% of C-2 and C-3 of lactate is randomized during the conversion into glucose, which indicates that it passes through the stage of

fumarate. What had not been clear was the significance of this 'shuttle'. Earlier workers had assumed that it is an unavoidable side reaction of oxaloacetate due to the high activity of malate dehydrogenase. Later it was thought that 'malic' enzyme [malate dehydrogenase (decarboxylating) (NADP)] was the major catalyst in the formation of dicarboxylic acids from pyruvate (see Krebs, 1954), but this view had to be abandoned when evidence became available indicating that under physiological conditions 'malic' enzyme mainly reacts in the direction malate $\rightarrow$ pyruvate (Utter, Keech & Scrutton, 1964) and that its activity is relatively low under conditions of gluconeogenesis (Fitch & Chaikoff, 1960), and when it became clear that the formation of oxaloacetate is brought about by pyruvate carboxylase (Utter & Keech, 1960, 1963). The concept discussed in the present paper offers a satisfactory explanation for the fact that malate is formed during the gluconeogenesis from lactate: it is not formed by a useless 'shuttle' reaction but is an essential link in the transfer of hydrogen atoms to the glyceraldehyde phosphate-dehydrogenase system.

Sources of malate required as hydrogen carrier from mitochondria to cytoplasm. When substances more highly oxidized than glucose, such as pyruvate, are precursors the malate must be formed by the intramitochondrial reduction of oxaloacetate, the NADH_2 being supplied by the intramitochondrial dehydrogenases and their substrates, i.e. by the tricarboxylic acid cycle and the related reactions. When substances more reduced than glucose, such as glutamate, proline, propionate, citrate and succinate, are precursors, the malate is formed intramitochondrially during the oxidative degradation of the precursor and can diffuse into the cytoplasm. As mentioned above, no hydrogen transfer is required when lactate is the precursor. In this case oxaloacetate formed intramitochondrially from pyruvate may be transported to the cytoplasm either by direct diffusion or by the transamination mechanisms proposed by Lardy, Paetkau & Walter (1965).

Formation of lactate from pyruvate. In both kidney cortex and liver slices gluconeogenesis from an excess of added pyruvate was always accompanied by the formation of relatively large quantities of lactate. This is not surprising. Pyruvate is bound to compete with diphosphoglycerate for any NADH_2 available in the cytoplasm. Since the activity of lactate dehydrogenase is high, a formation of lactate, i.e. a diversion of the reducing power available in the cytoplasm from gluconeogenesis, is expected when an excess of pyruvate is present.

Source of reducing power for the formation of β -hydroxybutyrate from acetoacetate. β -Hydroxybutyrate dehydrogenase, being an intramito-

chondrial enzyme, requires intramitochondrial NADH_2 for the reduction of acetoacetate. The mechanism by which this NADH_2 is generated must be the same as in gluconeogenesis, i.e. by the intramitochondrial dehydrogenases of cell respiration, except that no transfer to the cytoplasm is required (Kulka, 1960; Kulka, Krebs & Eggleston, 1961; Krebs, Eggleston & D'Alessandro, 1961).

Role of energy-linked reduction of NAD in the generation of reducing power. It may be asked whether the energy-linked reduction of NAD, a well-established mitochondrial reaction, is of importance in the supply of NADH_2 for reductive syntheses. The following considerations show that the answer is essentially in the negative.

The energy-linked reduction of NAD involves a reversal of oxidative phosphorylation at the coupling stage between NADH_2 and flavoprotein, which may be formulated thus:

where FP represents an electron carrier, e.g. flavoprotein, and $\sim \text{P}$ an 'energy-rich' intermediate (or ATP). It may be left open whether FP and FPH_2 are in fact flavoproteins or electron carriers of a similar redox potential, such as ubiquinones. Reaction (1) implies that the energy-linked reduction of NAD, i.e. reaction (1) from right to left, depends on the stoichiometric supply of $\sim \text{P}$ and FPH_2 . Since $\sim \text{P}$ is plentiful in mitochondria the amount of FPH_2 is the major factor limiting the scope of the process. NADH_2 , normally the most rapid supplier of FPH_2 , cannot be a source, if reaction (1) proceeds from right to left. There remain as possible sources of FPH_2 the flavoprotein-linked dehydrogenases of succinate, fatty acyl-CoA esters, α -glycerophosphate, proline and choline. Of these substrates succinate is experimentally by far the most effective one because of the high activity of succinate dehydrogenase, and most of the experimental work demonstrating an energy-linked reduction has in fact been carried out in the presence of succinate (see Ernster & Lee, 1964). However, the amounts of succinate or acyl-CoA ester available *in vivo* are relatively small. Thus, when glucose is oxidized, one out of six dehydrogenations does not involve NAD but FP, and when fatty acids are oxidized the ratio is two out of six. Moreover, succinate and acyl-CoA esters must not only supply FPH_2 , but also $\sim \text{P}$. Hence some of the FPH_2 must be oxidized by oxygen. Maximally 2 mol. of $\sim \text{P}$ could be formed/mol. of succinate or acyl-CoA, and only two-thirds of the succinate or acyl-CoA could take part in reaction (1). It follows that during carbohydrate oxidation one-ninth at most and during fatty acid oxidation two-ninths at most of the potential NADH_2 can be generated by energy-

linked reduction. These are maximal values of what is theoretically feasible. The actual contribution of these systems to the production of NADH_2 is likely to be zero in animal tissues. The α -glycerophosphate oxidase cannot make a net contribution because the formation of α -glycerophosphate from triose phosphate requires the consumption of NADH_2 .

The situation is entirely different in certain autotrophic micro-organisms, which can obtain all their energy from substrates the redox potentials of which are much more positive than those of the NAD couple, e.g. *Ferrobacillus* (Blaylock & Nason, 1963) or *Nitrosomonas* (Aleem, 1966). These organisms have an unlimited supply of substrates for oxidative phosphorylation at coupling sites between flavoproteins and oxygen. They can therefore generate bulk quantities of FPH_2 and $\sim \text{P}$. The organisms readily reduce NAD and it appears that an energy-linked reduction is the only mechanism available to them.

Even though the reversal of oxidative phosphorylation in animal tissues may not be an effective mechanism for generating NADH_2 , it may nevertheless be useful to the economy of the cell by making possible a 100% utilization of energy.

Role of transamination in the transfer of oxaloacetate from mitochondria to cytoplasm. Lardy *et al.* (1965) and Haynes (1965) have discussed the concept of the diffusion of intramitochondrial malate into the cytoplasm with reference to the transfer of the carbon 'skeleton' of intramitochondrial oxaloacetate to the extramitochondrial space. They were not concerned with the transfer of reducing equivalent, a problem that arises with a limited number of glucogenic precursors of which pyruvate is the prototype. Lardy *et al.* (1965) also suggest that the intra- and extra-mitochondrial glutamate-oxaloacetate transaminase may play a role in transporting oxaloacetate from one compartment to another. Oxaloacetate is assumed to transaminate with glutamate in one compartment, and the products of transamination are postulated to diffuse into the other compartment where they react in the reverse direction. Such a mechanism can be visualized as transporting oxaloacetate from mitochondria to the cytoplasm without transporting reducing equivalents. It might therefore operate when carbon 'skeletons' but not reducing equivalents are to be transferred, as is the case when lactate is the precursor. A difficulty in accepting the hypothesis is the postulate that mitochondria are impermeable to oxaloacetate but readily permeable to α -oxoglutarate and aspartate. Evidence in support of the supposition of highly specific differential permeability is not entirely lacking but cannot be regarded as adequate (see Chappell & Haarhoff, 1966).

Cytoplasmic reducing power in fatty acid synthesis. When fatty acids are synthesized reducing equivalents have to be supplied in the cytoplasm in the form of NADPH₂. Most of this is generated by the pentose phosphate cycle and 'malic' enzyme (Young, Shrago & Lardy, 1964; Kornacker & Ball, 1965; Rognstad & Katz, 1966). These enzyme systems cannot play a major role in the generation of reducing power in gluconeogenesis because the activities of dehydrogenases of the pentose phosphate cycle and 'malic' enzyme are lower when gluconeogenesis is high and vice versa (Fitch & Chaikoff, 1960).

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