Biochem. J. (1976) **156**, 91–102
Printed in Great Britain

Phosphorylation State of Cytosolic and Mitochondrial Adenine Nucleotides and of Pyruvate Dehydrogenase in Isolated Rat Liver Cells

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(*Received* 3 *November* 1975)

1. Cytosolic and mitochondrial ATP and ADP concentrations of liver cells isolated from normal fed, starved and diabetic rats were determined. 2. The cytosolic ATP/ADP ratio was 6, 9 and 10 in normal fed, starved and diabetic rats respectively. 3. The mitochondrial ATP/ADP ratio was 2 in normal and diabetic rats and 1.6 in starved rats. 4. Adenosine increased the cytosolic and lowered the mitochondrial ATP/ADP ratio, whereas atractyloside had the opposite effect. 5. Incubation of the hepatocytes with fructose, glycerol or sorbitol led to a fall in the ATP/ADP ratio in both the cytosolic and the mitochondrial compartment. 6. The interrelationship between the mitochondrial ATP/ADP ratio and the phosphorylation state of pyruvate dehydrogenase in intact cells was studied. 7. In hepatocytes isolated from fed rats an inverse correlation between the mitochondrial ATP/ADP ratio and the active form of pyruvate dehydrogenase (pyruvate dehydrogenase a) was demonstrable on loading with fructose, glycerol or sorbitol. 8. No such correlation was obtained with pyruvate or dihydroxyacetone. For pyruvate, this can be explained by inhibition of pyruvate dehydrogenase kinase. 9. Liver cells isolated from fed animals displayed pyruvate dehydrogenase a activity twice that found *in vivo*. Physiological values were obtained when the hepatocytes were incubated with albumin–oleate, which also yielded the highest mitochondrial ATP/ADP ratio.

Since the occurrence of active and inactive forms of pyruvate dehydrogenase (EC 1.2.4.1) was demonstrated in mammalian tissues (Wieland *et al.*, 1971*a*), several studies have been performed to establish the factors acting on pyruvate dehydrogenase interconversion *in vivo*. According to the results so far accumulated, mainly from experiments with intact animals (Wieland *et al.*, 1971*a*; Söling *et al.*, 1971; Wieland *et al.*, 1972*a*), isolated perfused organs (Patzelt *et al.*, 1973; Wieland *et al.*, 1971*b*), isolated kidney tubules (Guder & Wieland, 1974) and mitochondria isolated from various sources (Portenhauser & Wieland, 1972; Martin *et al.*, 1972; Wieland & Portenhauser, 1974; Walajtys *et al.*, 1974; Whitehouse *et al.*, 1974; Cooper *et al.*, 1974; Chiang & Sacktor, 1975; Taylor *et al.*, 1975; Batenburg & Olson, 1975), the steady state between the dephospho- and phospho-forms of the pyruvate dehydrogenase complex is thought to be influenced by the concentrations of pyruvate (Portenhauser & Wieland, 1972; Martin *et al.*, 1972; Patzelt *et al.*, 1973; Guder & Wieland, 1974; Taylor *et al.*, 1975; Chiang & Sacktor, 1975), 2-oxoglutarate (Wieland & Portenhauser, 1974; Guder & Wieland, 1974) or long-chain acyl-CoA (Löffler *et al.*, 1975), by the mitochondrial redox state (Taylor *et al.*, 1975; Pettit *et al.*, 1975; Batenburg & Olson, 1975), the phosphorylation state of the mitochondrial adenine nucleotides (Martin *et al.*, 1972; Portenhauser & Wieland, 1972; Walajtys *et al.*, 1974; Whitehouse *et al.*, 1974; Cooper *et al.*, 1974; Taylor *et al.*, 1975; Chiang & Sacktor, 1975), the mitochondrial ratio of acetyl-CoA to CoA (Pettit *et al.*, 1975; Batenburg & Olson, 1975) and fluctuations of K^+ (Roche & Reed, 1974), Ca^{2+} and Mg^{2+} (Hucho, 1974; Randle & Denton, 1973). Isolated hepatocytes have been shown to provide a useful model for studies on pyruvate dehydrogenase regulation, especially since a method developed for the separation of the cytosolic and mitochondrial compartments (Zuurendonk & Tager, 1974) opened the possibility of investigating the interrelationship between the phosphorylation state of mitochondrial adenine nucleotides and that of the pyruvate dehydrogenase complex in its natural environment in the intact cell. In extension of a previous report (Siess & Wieland, 1975), in the present study pyruvate dehydrogenase activities and mitochondrial adenine nucleotides were measured in parallel in isolated hepatocytes kept under a variety of metabolic conditions. By this approach a more refined characteriza-

tion of the relative importance of the factors mentioned above for the regulation of pyruvate dehydrogenase phosphorylation in the intact cell seemed feasible.

Materials and Methods

Animals

Male Sprague–Dawley rats (W. Gassner, Sulzfeld, Germany) weighing 190–260g, kept under standard conditions, were used. Rats were made diabetic with streptozotocin by intraperitoneal injection of 100mg/kg body wt. in 0.1M-sodium citrate buffer, pH4.0, or with alloxan as described by Wieland *et al.* (1971*a*). The diabetic rats were treated with insulin for 5–6 days essentially as described by Wieland *et al.* (1971*a*), and used 24h after withdrawal of the hormone. Although blood glucose determined on the day of the experiment was around 400mg/100ml in both groups, ketonuria (measured with Acetest tablets; Ames, Frankfurt/M, Germany) was displayed only by the alloxan-diabetic rats.

Chemicals

Bovine serum albumin was purchased from Behringwerke (Marburg/Lahn, Germany) and defatted as described by Chen (1967). Pyruvate and adenosine came from Boehringer (Mannheim, Germany). No ATP or ADP was detectable in the adenosine preparation. Fructose, glycerol, ethanol, NH_4Cl and oleic acid were products of Merck (Darmstadt, Germany). Sorbitol was supplied by Schuchardt (München, Germany). Dihydroxyacetone and Scintigel were from Roth (Karlsruhe, Germany). 2-Oxoglutarate dehydrogenase (EC 1.2.4.2) from pig heart muscle was kindly supplied by Dr. H. J. Kolb of our laboratory. [^{3}H]ATP (sp. radioactivity 5.56Ci/mmol) and [^{14}C]ADP (sp. radioactivity 48mCi/mmol) were products of The Radiochemical Centre (Amersham, Bucks., U.K.). The NCS tissue solubilizer was bought from Nuclear Chicago (Heusenstamm, Germany). Other chemicals were those described by Siess & Wieland (1975).

Methods

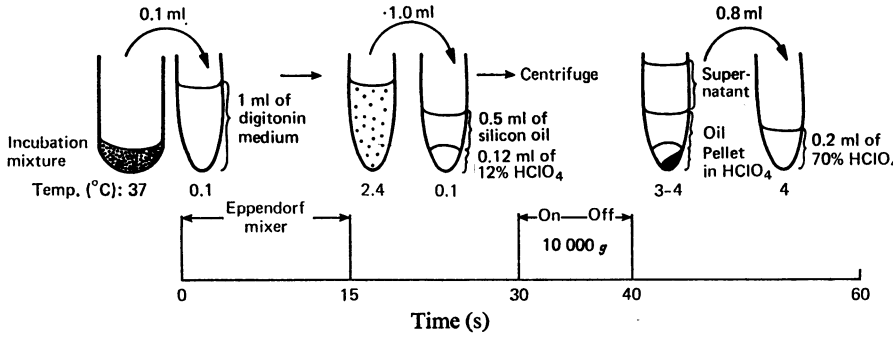
The liver cell preparation, the incubation medium and the digitonin treatment of cells were performed as described previously (Siess & Wieland, 1975). At least 90% of the cells from normal fed or diabetic rats, and 97% of the cells from starved rats, excluded 0.2% Trypan Blue. The cells were incubated for 10min at 37°C unless otherwise stated. For determination of long-chain acyl-CoA in unfractionated liver cells, 0.4ml of the incubation mixture was mixed with 0.08ml of ice-cold 70% (w/v) $HClO_4$. After centrifugation for 2min in an Eppendorf centrifuge (model 3200) the supernatant was discarded and the precipitate washed with 2×1ml of 6% $HClO_4$. The washed precipitate was suspended in 0.2ml of 10M-KOH containing 30mM-dithiothreitol, and incubated for 5min at 37°C. After addition of 0.06ml of 20% $HClO_4$ and centrifugation as above, the precipitate was washed twice with water, 0.25ml being used each time. The three supernatants were pooled and analysed for CoA (Garland, 1974). For determination of long-chain acyl-CoA in the supernatant and pellet fractions of digitonin-treated hepatocytes the precipitates of triplicates were combined and treated as described above. Acetoacetate and 3-hydroxybutyrate were determined by the methods of Mellanby & Williamson (1974) and Williamson & Mellanby (1974) respectively. The other analytical procedures were performed as described earlier (Siess & Wieland, 1975). For electron microscopy, pellets from liver cells treated with or without digitonin were fixed in 1% OsO_4 in 0.15M-potassium phosphate buffer, pH7.4, for 2h. Dehydration and embedding were performed by the method of Luft (1961). Thin sections obtained with a LKB Ultrotome III with glass knives were stained with uranyl acetate and Reynolds' (1963) lead citrate and examined in a Siemens Elmiskop 101 instrument at 80kV.

Results

Compartmentation of adenine nucleotides by the digitonin method

The experimental approach for adenine nucleotide measurements in the supernatant and the pellet fraction, which are regarded as the cytosolic and mitochondrial compartments respectively, is summarized in Scheme 1. Optimal conditions with respect to digitonin concentration and time of incubation as already documented (Siess & Wieland, 1975) were found to be the same for cells incubated under the various conditions described below. Table 1 illustrates how the adenine nucleotide distribution is influenced when the liver cells are treated with digitonin at various temperatures. A rise in temperature above 6°C caused a decrease in ATP, especially in the pellet fraction, and an increase in ADP. Results similar to those shown in Table 1 were obtained with hepatocytes from a fed rat.

The carry-over of adenine nucleotides from the supernatant to the pellet was tested by the addition of [^{3}H]ATP and [^{14}C]ADP to the standard digitonin medium. As shown in Table 2, not more than 6 and 13% of the added ATP and ADP respectively were recovered from the pellet fraction. Since the adenine nucleotide translocase may still display some activity under the conditions used, these values are presumably somewhat too high. Only 1% of the radioactivity was found in the pellet if the experiment was carried out with labelled acetyl-CoA or citrate instead of adenine nucleotides.



Scheme 1. *Schematic view of the separation by digitonin treatment of soluble and pellet fractions of isolated hepatocytes*

Table 1. *Effect of temperature during digitonin treatment on ATP and ADP concentrations in the supernatant and pellet fractions of liver cells isolated from a 24 h-starved rat*

For experimental details see the Materials and Methods section. Temperature was measured 10–15 s after starting digitonin incubations by a thermoelement as described by Siess & Wieland (1975).

Temperature (°C)	Supernatant (μmol/10⁸ cells)				Pellet (μmol/10⁸ cells)			
	ATP	ADP	ATP+ADP	ATP/ADP	ATP	ADP	ATP+ADP	ATP/ADP
2.6	1.79	0.14	1.93	12.8	0.58	0.41	0.99	1.41
6.0	1.71	0.14	1.85	12.2	0.54	0.42	0.96	1.28
11.8	1.73	0.18	1.91	9.6	0.45	0.42	0.87	1.07
21.9	1.56	0.40	1.96	3.9	0.26	0.65	0.91	0.40

Table 2. *Recovery of [³H]ATP and [¹⁴C]ADP in the supernatant and pellet fractions of hepatocytes isolated from a normal fed rat*

The ATP and ADP contents of a sample run in parallel were in the supernatant 13.7 and 2.1 nmol respectively, and in the pellet fraction (=$HClO_4$ phase plus pellet extract) 4.3 and 2.4 nmol respectively. More than 99.5% of [³H]ATP and [¹⁴C]ADP were recovered in the supernatant of a sample treated with digitonin-free medium.

Nucleotides added ...	[³H]ATP		[¹⁴C]ADP	
Fraction	(pmol)	(%)	(nmol)	(%)
Before centrifugation	44.7	100	1.97	100
1. Supernatant	43.9	98.2	1.78	90.4
2. Oil phase	0	0	0	0
3. (a) $HClO_4$ phase	0.9	2.0	0.12	6.1
(b) Pellet (solubilized)	1.9	4.3	0.14	7.1
Recovery (1+2+3)	46.7	104.5	2.04	103.6

Adenine nucleotides in the supernatant and pellet fractions of hepatocytes from fed rats

Table 3 summarizes the adenine nucleotide contents and the ATP/ADP ratios in the supernatant and pellet of hepatocytes from normal fed rats incubated in the presence of various substrates. With each substrate, ATP in the supernatant exceeds that in the pellet by a factor of about 2.5, whereas ADP is almost equally distributed between the two spaces. The adenine nucleotide contents were markedly influenced by the substrate used. Fructose has been shown to deplete hepatic ATP when applied *in vivo* (Mäenpää *et al.*, 1968; Burch *et al.*, 1969; Söling *et al.*, 1971) or in liver perfusion (Woods *et al.*, 1970), and a similar but smaller effect was caused by glycerol (Woods & Krebs, 1973). In agreement with these observations,

Table 3. *Distribution of adenine nucleotides in isolated hepatocytes from fed rats in the presence of various substrates*

Mean values ± S.E.M. are given for the numbers of different cell preparations in parentheses. Statistical significance against relevant control was measured by Student's *t* test: *$P<0.025$; **$P<0.01$; ***$P<0.0025$.

		Supernatant			Pellet		
		(μmol/10^8 cells)			(μmol/10^8 cells)		
Substrate added	Concn. (mM)	ATP	ADP	ATP/ADP	ATP	ADP	ATP/ADP
1. None		2.21±0.10 (22)	0.40±0.04 (17)	5.97±0.58	0.73±0.04 (22)	0.39±0.02 (20)	2.00±0.10
2. Glycerol	12	1.56±0.17*** (7)	0.42±0.01 (5)	3.59±0.46	0.54±0.05** (7)	0.47±0.04 (6)	1.24±0.12
	6	1.93±0.13 (3)	0.40 (2)	4.81	0.58±0.08 (3)	0.43±0.07 (3)	1.38±0.05
	3	2.09±0.19 (3)	—	—	0.68±0.07 (4)	0.40±0.06 (3)	1.9 ±0.24
3. Sorbitol	24	1.38±0.24** (3)	0.67±0.11** (3)	2.09±0.26	0.42±0.04** (3)	0.63±0.03*** (3)	0.67±0.09
	12	1.36±0.23*** (8)	0.41±0.05 (7)	3.43±0.5	0.47±0.04*** (8)	0.54±0.05*** (7)	0.94±0.06
4. Fructose	12	0.65±0.07*** (7)	0.41±0.04 (6)	1.55±0.21	0.27±0.03*** (7)	0.52±0.04*** (7)	0.54±0.08
5. Dihydroxy-acetone	12	1.98±0.19 (8)	0.39±0.02 (8)	5.19±0.64	0.64±0.03 (9)	0.43±0.03 (8)	1.60±0.10
6. Pyruvate	12	2.77±0.17** (6)	0.34±0.04 (4)	7.68±0.77	0.44±0.07*** (6)	0.33±0.03 (6)	1.35±0.18
7. Ethanol	12	2.21±0.29 (6)	0.36±0.06 (6)	5.72±0.87	0.55±0.07* (7)	0.22±0.03*** (7)	2.32±0.32
8. Albumin–NaCl		2.72±0.22 (8)	0.31±0.05 (8)	9.95±1.28	0.81±0.06 (8)	0.39±0.02 (8)	2.13±0.2
9. Albumin–oleate	1.26	2.66±0.16 (8)	0.33±0.05 (8)	9.63±1.76	0.86±0.05 (8)	0.36±0.03 (8)	2.48±0.27
10. Alanine†	10	2.27±0.12 (11)	0.41±0.09 (11)	6.34±0.89	0.64±0.05 (11)	0.38±0.03 (11)	1.70±0.11
11. Adenosine	6.2	3.56±0.19* (4)	0.35±0.07 (3)	10.3	0.53±0.07* (4)	0.48±0.04 (4)	1.14

† Incubation time was 5 min.

fructose, sorbitol and glycerol diminished total cellular ATP by 69, 38 and 29% respectively. Providing additional information, our experiments show that the fall in ATP occurs in both the extramitochondrial and the mitochondrial compartments of the liver cell (Table 3). Dihydroxyacetone had no effect on the adenine nucleotide content of isolated liver cells, in agreement with reports on dihydroxyacetone loading *in vivo* (Burch *et al.*, 1969; Williamson *et al.*, 1969) and in liver perfusion (Woods & Krebs, 1973).

Effect of adenosine on adenine nucleotide content and distribution in isolated hepatocytes

Adenosine administered to the intact rat (Chagoya de Sánchez *et al.*, 1972) or the isolated perfused liver (Wilkening *et al.*, 1975) raises the hepatic ATP content. Therefore the effect of adenosine on the adenine nucleotide contents in both compartments of isolated hepatocytes was studied. As shown in Table 3 the increase in the ATP content of isolated hepatocytes incubated with adenosine resulted from a marked increase of the nucleotide in the supernatant and a decrease in the pellet fraction.

Effect of carbonyl cyanide m-chlorophenylhydrazone and of atractyloside on adenine nucleotides in hepatocytes from fed rats incubated with alanine

In previous experiments the effect of inhibitors of oxidative phosphorylation (10 μM-carbonyl cyanide *m*-chlorophenylhydrazone) or of mitochondrial adenine nucleotide translocation (0.29 mM-carboxyatractyloside) on the subcellular distribution of ATP and ADP in liver cells incubated without exogenous substrate was determined (Siess & Wieland, 1975). Identical results were obtained in this study when the same experiments were performed in the presence of 10 mM-alanine (results not shown).

Table 4. *Compartmentation of adenine nucleotides in liver cells isolated from 24–36 h-starved rats*

Mean values ± S.E.M. are given for the numbers of different cell preparations in parentheses.

Incubation time (min)	Substrate added	Supernatant (μmol/10^8 cells) ATP	Supernatant (μmol/10^8 cells) ADP	Supernatant ATP/ADP	Pellet (μmol/10^8 cells) ATP	Pellet (μmol/10^8 cells) ADP	Pellet ATP/ADP
10	None	1.99±0.15 (4)	0.28±0.07 (4)	9.10±1.85	0.70±0.07 (4)	0.46±0.03 (4)	1.52±0.06
	Lactate (10mM)	2.70±0.23 (4)	0.18±0.04 (4)	16.78±3.17	0.73±0.11 (4)	0.35±0.07 (4)	2.18±0.15
20	None	2.21 (2)	0.21 (2)	10.60	0.74 (2)	0.48 (2)	1.57
	Lactate* (10mM)	2.33±0.14 (7)	0.29±0.04 (7)	8.78±1.14	0.65±0.07 (7)	0.38±0.02 (7)	1.72±0.12

* Glucose formation was 10.4 μmol/10^8 cells.

Table 5. *Compartmentation of adenine nucleotides in hepatocytes from alloxan-diabetic rats*

Mean values ± S.E.M. are given for the numbers of different cell preparations in parentheses.

Substrate added	Concn. (mM)	Supernatant (μmol/10^8 cells) ATP	Supernatant (μmol/10^8 cells) ADP	Supernatant ATP/ADP	Pellet (μmol/10^8 cells) ATP	Pellet (μmol/10^8 cells) ADP	Pellet ATP/ADP
None		1.29±0.11 (5)	0.13±0.01 (5)	10.23±1.26	0.54±0.07 (5)	0.30±0.05 (5)	1.98±0.38
Glycerol	12	1.42±0.10 (4)	0.18±0.02 (5)	7.37±0.61	0.52±0.07 (4)	0.40±0.05 (5)	1.36±0.26
Sorbitol	12	1.23±0.18 (5)	0.21±0.03 (5)	6.38±1.29	0.43±0.06 (5)	0.35±0.02 (5)	1.24±0.16
Fructose	12	0.82±0.11 (5)	0.18±0.04 (5)	5.33±0.91	0.16±0.03 (5)	0.46±0.04 (5)	0.37±0.09
Albumin–NaCl		1.41±0.12 (5)	0.14±0.02 (5)	11.11±1.99	0.53±0.06 (5)	0.29±0.05 (5)	2.10±0.44
Albumin–oleate	1.26	1.43±0.10 (5)	0.13±0.01 (5)	11.03±0.59	0.46±0.03 (5)	0.24±0.02 (5)	1.99±0.21

Compartmentation of adenine nucleotides in hepatocytes from starved and diabetic rats

Except for ADP in the supernatant, the adenine nucleotide distribution in hepatocytes from 24–36h-starved rats incubated without exogenous substrate did not differ significantly from that in fed rats (Table 4). Owing to the marked decrease in ADP, to 70% of that found in fed animals, the cytosolic ATP/ADP ratio rose from 5.9 to 9.1.

As decreases in hepatic ATP in diabetes have been reported (Tarnowski & Seemann, 1967; Wieland, 1968), the adenine nucleotide distribution between the cytosolic and mitochondrial fractions of hepatocytes from alloxan-diabetic rats was studied (Table 5). Without substrate added, cytosolic ATP and ADP were decreased by 41 and 67% respectively. No effect of glycerol on the ATP content was seen, perhaps because of the lowered glycerokinase activity in alloxan diabetes (Kampf *et al.*, 1968). In the pellet, adenine nucleotides appeared to be decreased by about 25% when calculated on the basis of cell number. However, this fall becomes insignificant if one relates the adenine nucleotide content of the pellet to glutamate dehydrogenase activity, which was decreased from 293±13 units/10^8 cells in normal rats ($n = 21$) to 225±11 units/10^8 cells in diabetic rats ($n = 5$). Also the mitochondrial nucleotide content in hepatocytes from streptozotocin-diabetic rats (Table 6) are not different from those of non-diabetic animals. Thus it appears that in the diabetic liver only the cytosolic but not the mitochondrial content of the adenine nucleotides is lowered. As to the conflict-

Table 6. *Effect of incubation with various substrates on ATP and ADP concentrations in the pellet of hepatocytes from streptozotocin-diabetic rats*

Mean values±S.E.M. in μmol/10^8 cells are given for the numbers of different cell preparations in parentheses.

Substrate added	Concn. (mM)	ATP	ADP	ATP/ADP
None		0.84±0.03 (4)	0.45±0.02 (4)	1.90±0.15
Fructose	12	0.38±0.02 (3)	0.66±0.05 (3)	0.59±0.09
Glycerol	12	0.57±0.06 (3)	0.61±0.03 (3)	0.95±0.08
Albumin–NaCl		0.79±0.05 (4)	0.45±0.01 (4)	1.79±0.14
Albumin–oleate	1.26	0.75±0.07 (4)	0.37±0.02 (4)	2.00±0.15

Table 7. *Active form and total pyruvate dehydrogenase in liver cells isolated from normal fed rats*

Mean values±S.E.M. are given, for the numbers of different cell preparations in parentheses.

Substrate added	Concn. (mM)	Pyruvate dehydrogenase activity (munits/10^8 cells) a	Pyruvate dehydrogenase activity (munits/10^8 cells) a+b	Pyruvate dehydrogenase a (% of total)
1. None		424± 39	1211± 61 (19)	34.8±2.7
2. Pyruvate	12	989±117	1195±124 (5)	82.8±5.4*
3. Dihydroxyacetone	12	778± 73	1280± 83 (11)	60.1±3.1*
4. Fructose	12	763± 59	1248± 70 (10)	60.6±2.2*
5. Sorbitol	24	528	1123 (3)	48.3±3.4
	12	476± 43	1122±137 (11)	38.4±2.5
6. Glycerol	12	450± 62	1220± 75 (9)	36.1±3.8
7. Ethanol	12	393± 74	1194±113 (8)	35.4±5.7
8. Albumin–NaCl		327± 35	1048± 72 (7)	31.0±1.9
9. Albumin–oleate	1.26	215± 23	975± 72 (7)	22.2±2.1*

* $P<0.005$ versus relevant control.

ing reports of decreased (Vester & Stadie, 1957; Hall *et al.*, 1960; Matsubara & Tochino, 1969, 1970) or normal (Parks *et al.*, 1955; Beyer & Shamoian, 1961; Boveris *et al.*, 1969; Mackerer *et al.*, 1971) ATP synthesis in mitochondria from acutely diabetic rats, our results on a normal mitochondrial ATP concentration in liver cells from diabetic rats are hardly compatible with the view of an impaired oxidative phosphorylation in diabetes.

Active and inactive forms of pyruvate dehydrogenase in hepatocytes isolated from normal rats

On fractionation by the digitonin method at least 95% of the pyruvate dehydrogenase activity of the hepatocytes was recovered in the pellet. During incubation of the pellet extract with purified pyruvate dehydrogenase phosphatase (Siess & Wieland, 1972) and Mg^{2+}, pyruvate dehydrogenase activity rose and, under the conditions used, reached a plateau within 10–15min. This maximal pyruvate dehydrogenase activity is referred to as 'total' pyruvate dehydrogenase activity, in contrast with pyruvate dehydrogenase a, which represents dephosphopyruvate dehydrogenase before activation. The conclusion that the increase in pyruvate dehydrogenase activity was in fact due to dephosphorylation of phosphopyruvate dehydrogenase (pyruvate dehydrogenase b) has been confirmed (Wieland *et al.*, 1972*b*). According to Table 7, both molecular forms of the enzyme are present in isolated hepatocytes. Further, Table 7 documents large variations in the amount of pyruvate dehydrogenase a depending on the nature of the substrate added; total pyruvate dehydrogenase activities remained constant. Pyruvate dehydrogenase a was raised the most by pyruvate, followed by dihydroxyacetone and fructose. Small or no changes were observed with sorbitol, glycerol or ethanol, and a significant decrease in the amount of pyruvate dehydrogenase a was caused by oleate.

Active and inactive forms of pyruvate dehydrogenase in hepatocytes isolated from starved or diabetic rats

The amounts of active form and total enzyme in isolated liver cells from 24–36h-starved rats and from diabetic rats are summarized in Table 8. In contrast with about 35% pyruvate dehydrogenase a present in isolated liver cells from normal fed rats, only about half that amount was found in starved and diabetic animals. These values remained essentially unchanged during gluconeogenesis from lactate or in the presence of oleate (Table 8). Among the other substrates tested, only fructose was able to give a small increase in pyruvate dehydrogenase a in the diabetic groups.

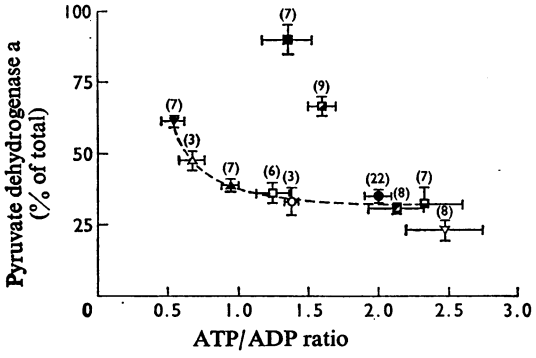


Fig. 1. *Correlation between the state of the pyruvate dehydrogenase system and the ATP/ADP ratio in the pellet fraction of digitonin-treated liver cells from fed rats*

▼, 12mM-fructose; △, 24mM-sorbitol; ▲, 12mM-sorbitol; □, 12mM-glycerol; ○, 6mM-glycerol; ◩, albumin–NaCl; ▽, albumin–1.26mM-oleate; ⬓, 12mM-ethanol; ■, 12mM-pyruvate; ◪, 12mM-dihydroxyacetone; ●, no addition. The numbers of experiments are given in parentheses.

Relationship between the mitochondrial ATP/ADP ratio and the amount of pyruvate dehydrogenase a

By plotting the amount of active form as a percentage of total pyruvate dehydrogenase (see Table 7) against the ATP/ADP ratio found in the pellet of hepatocytes from normal fed rats (see Table 3) the pattern shown in Fig. 1 results. The values for pyruvate dehydrogenase a observed with fructose, sorbitol, glycerol or ethanol, but not with pyruvate, dihydroxyacetone or oleate fit well in a curve, suggesting an inverse interrelationship between the amount of pyruvate dehydrogenase a and the mitochondrial ATP/ADP ratio. The curve in Fig. 1 shows that the response of pyruvate dehydrogenase a is most pronounced in the range of ATP/ADP values between 0.5 and 1.0, and reaches a plateau at values higher than 1.5. Hepatocytes from starved or diabetic rats displayed much lower amounts of pyruvate dehydrogenase a (see Table 8), yet their mitochondrial ATP/ADP ratios were not higher than in cells from normal rats in either the absence or the presence of various substrates (see Tables 3, 4, 5 and 6). In the cells from diabetic animals a significant increase in the amount of pyruvate dehydrogenase a was observed only with fructose, but this was much smaller than in normal hepatocytes.

Effect of NH_4Cl on the amount of pyruvate dehydrogenase a and the mitochondrial ATP/ADP ratio

In liver-perfusion studies NH_4Cl has been shown to

Table 8. *Active form and total pyruvate dehydrogenase activity in hepatocytes from starved or diabetic animals*

Mean values ± S.E.M. are given for the numbers of different cell preparations in parentheses.

Experimental group	Substrate added	Concn. (mM)	Pyruvate dehydrogenase activity (munits/10^8 cells) a	Pyruvate dehydrogenase activity (munits/10^8 cells) a+b	Pyruvate dehydrogenase a (% of total)
Starved	None		173±28	1030±153 (4)	16.6±1.4
	Lactate	10	197±32	1066±155 (4)	18.5±2.4
Streptozotocin-diabetic (non-ketotic)	None		183±43	969± 33 (4)	18.6±4.0
	Fructose	12	403±49	1077± 40 (3)	37.2±3.6
	Glycerol	12	223±78	1074± 55 (3)	20.4±6.3
	Albumin–NaCl		264±62	1036± 24 (4)	25.0±5.4
	Albumin–oleate	1.26	202±50	1002± 28 (4)	19.3±4.6
Alloxan-diabetic (ketotic)	None		117±18	861± 93 (5)	14.2±2.9
	Fructose	12	220±37	943± 79 (5)	23.1±3.2
	Sorbitol	12	111±15	863± 82 (5)	13.5±2.6
	Glycerol	12	124±20	914± 89 (5)	14.0±2.6
	Albumin–NaCl		142±21	908± 84 (5)	16.1±2.9
	Albumin–oleate	1.26	118±20	868± 93 (5)	14.1±2.9

Table 9. *Effect of NH_4Cl on pyruvate dehydrogenase a and adenine nucleotide concentrations in the pellet fraction of hepatocytes from normal fed rats incubated for 5 min*

Mean values ± S.E.M. are given, for the numbers of different cell preparations in parentheses.

Additions	Content (μmol/10^8 cells) ATP	ADP	ATP/ADP	Pyruvate dehydrogenase a (% of total)
None	0.80 ± 0.09 (5)	0.44 ± 0.06 (5)	1.88 ± 0.20	30.6 ± 3.2 (4)
2.5 mM-NH_4Cl	0.63 ± 0.10 (4)	0.32 ± 0.04 (4)	1.98 ± 0.22	35.9 ± 4.3 (3)
5 mM-NH_4Cl	0.67 ± 0.13 (4)	0.33 ± 0.04 (4)	2.09 ± 0.39	43.6 ± 1.8 (3)

Table 10. *Ketone-body production, long-chain acyl-CoA concentration and pyruvate dehydrogenase activity of hepatocytes isolated from normal and diabetic rats*

Mean values ± S.E.M. are given for the numbers of different cell preparations in parentheses.

Experimental group	Additions	Ketone-body production (μmol/10 min per 10^8 cells) 3-Hydroxybutyrate	Acetoacetate	3-Hydroxybutyrate / Acetoacetate	Long-chain acyl-CoA (nmol/10^8 cells)	Pyruvate dehydrogenase a (% of total)
Normal fed	Albumin–NaCl	0.66 ± 0.16 (7)	3.37 ± 0.43 (7)	0.19	40.4 ± 5.3 (4)	31.0 ± 1.9 (4)
	Albumin–oleate (1.26 mM)	10.45 ± 1.36 (7)	8.29 ± 0.66 (7)	1.26	67.2 ± 4.7 (4)	22.2 ± 2.1 (4)
Alloxan-diabetic	Albumin–NaCl	0.56 ± 0.10 (5)	9.84 ± 1.24 (5)	0.057	33.6 ± 3.6 (5)	16.1 ± 2.9 (5)
	Albumin–oleate (1.26 mM)	15.33 ± 2.05 (5)	9.82 ± 0.31 (5)	1.56	69.1 ± 10.6 (5)	14.1 ± 2.9 (5)

Table 11. *Ketone-body production and long-chain acyl-CoA concentration of hepatocytes isolated from alloxan-diabetic rats in the presence of various substrates*

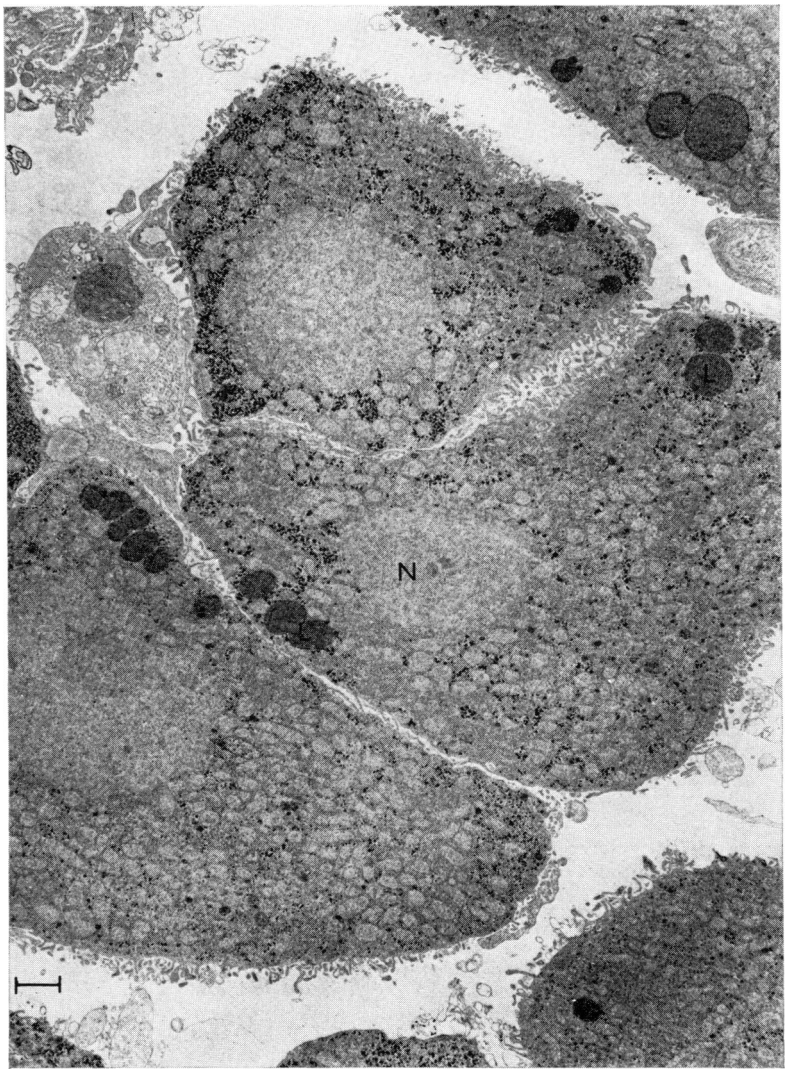
Mean values ± S.E.M. of five experiments are given. Statistical significance was measured by Student's paired-data *t* test: $*P<0.05$, $**P=0.01$, $***P<0.005$.

Additions (mM)	Ketone-body formation (μmol/10 min per 10^8 cells) Acetoacetate	3-Hydroxybutyrate	Long-chain acyl-CoA (nmol/10^8 cells)
None	9.33 ± 0.91	0.60 ± 0.24	49.70 ± 5.59
Glycerol (12)	5.88 ± 0.45***	1.00 ± 0.50	34.12 ± 1.93*
Fructose (12)	5.24 ± 0.51**	0.98 ± 0.19	37.86 ± 3.24***
Sorbitol (12)	5.71 ± 0.32***	0.70 ± 0.30	38.40 ± 1.80*

result in a marked elevation of pyruvate dehydrogenase a (Häussinger *et al.*, 1975). As indicated in Table 9, this is not observed when isolated hepatocytes were exposed to comparable concentrations of NH_4Cl. Only at 5 mM-NH_4Cl was there a significant effect. Table 9 further shows that NH_4Cl up to 5 mM caused no detectable change in the mitochondrial phosphorylation state, and the sum of ATP+ADP was somewhat decreased.

Long-chain acyl-CoA content and 3-hydroxybutyrate/acetoacetate ratio in hepatocytes as related to the amount of pyruvate dehydrogenase a

Table 10 illustrates the effect on the amount of

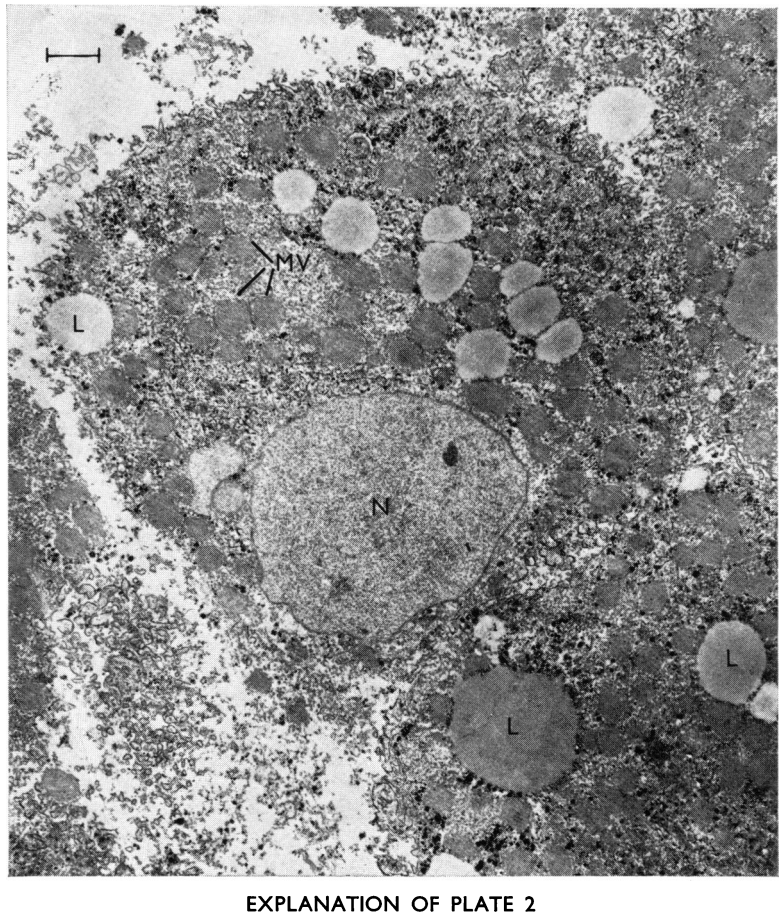


EXPLANATION OF PLATE 1

Electron micrograph of a section of a pellet fraction of isolated hepatocytes treated with a digitonin-free medium after incubation for 10 *min at* 37°*C with endogenous substrate*

The cell surface is covered with microvilli. N, nucleus; L, lipid droplet. The bar represents 1 μm.

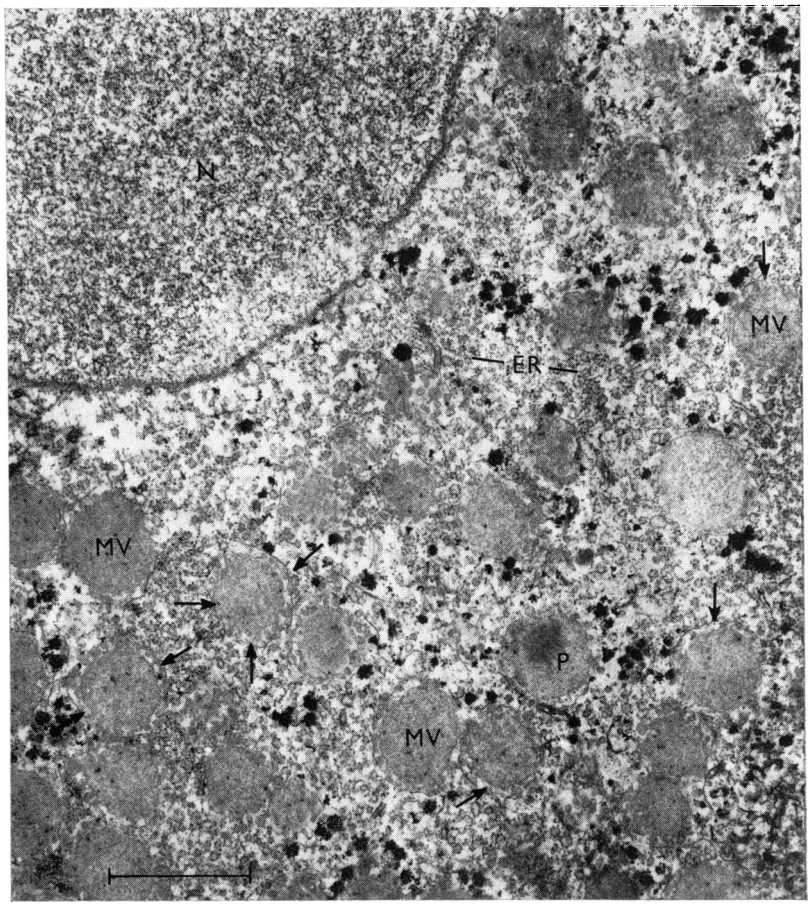
(*Facing p.* 98)



EXPLANATION OF PLATE 2

Electron micrograph of a section of a pellet fraction of isolated hepatocytes treated with digitonin after incubation for 10 *min at* 37°*C with endogenous substrate*

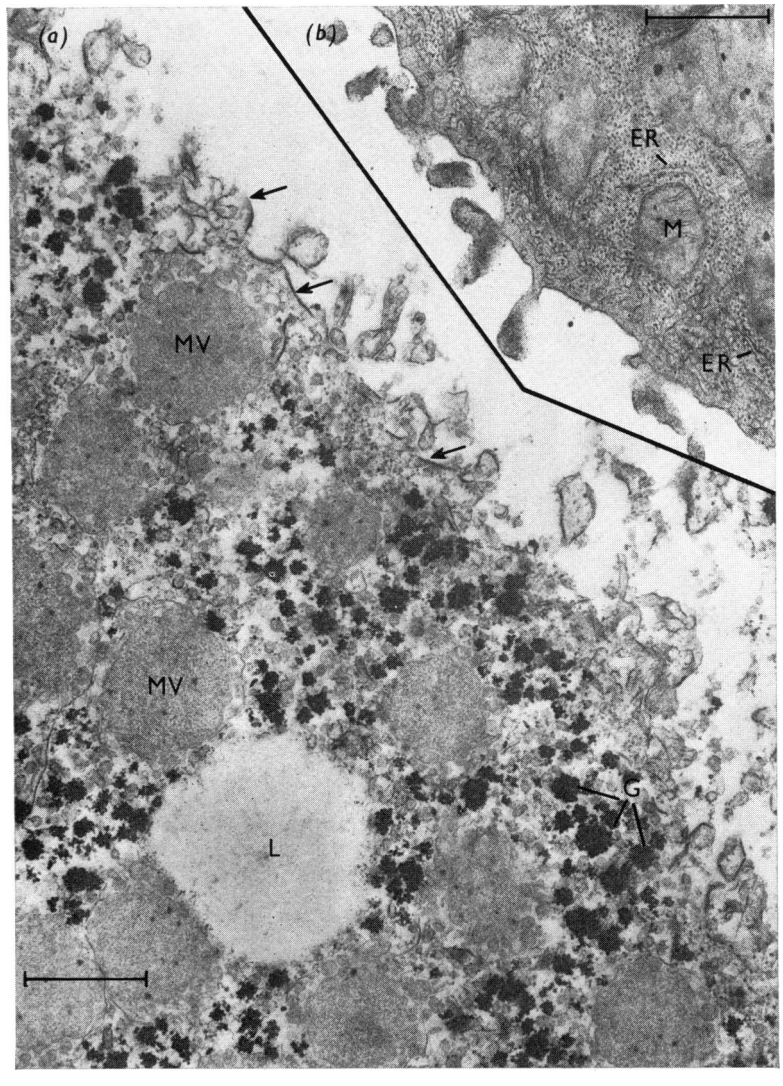
Microvilli are absent. N, nucleus; L, lipid droplet; MV, inner-membrane-matrix vesicles. The bar represents 1 μm.



EXPLANATION OF PLATE 3

Electron micrograph of a portion of a digitonin-treated hepatocyte

N, nucleus; P, peroxisome; MV, inner-membrane-matrix vesicle surrounded by the outer mitochondrial membrane (arrow); ER, granular endoplasmic reticulum. The bar represents 1 μm.



EXPLANATION OF PLATE 4

Electron-microscopic appearance of the cell surface of hepatocytes treated with (*a*) *or without* (*b*) *digitonin*

In (*a*) only fragments of the plasma membrane are visible (arrows). L, lipid droplet; G, glycogen; MV, inner-membrane-matrix vesicle; ER, granular endoplasmic reticulum; M, mitochondrion. The bar represents 0.5 μm.

Table 12. *Long-chain acyl-CoA in unfractionated liver cells and in the supernatant and the pellet after digitonin fractionation*

For details see the Materials and Methods section. Percentage values are given in parentheses.

	Long-chain acyl-CoA (nmol/10^8 cells)					
	Normal rats			Alloxan-diabetic rats		
	No addition	NaCl–albumin	Oleate (1.26mM)–albumin	No addition	NaCl–albumin	Oleate (1.26mM)–albumin
Unfractionated cells	48.3 (100)	47.4 (100)	75.5 (100)	41.0 (100)	40.8 (100)	92.1 (100)
Pellet	33.2 (68.7)	31.3 (66.0)	52.5 (69.5)	31.4 (76.6)	37.2 (91.2)	62.9 (68.3)
Supernatant	6.5 (13.5)	6.8 (14.3)	6.5 (8.6)	8.6 (20.9)	5.7 (14.0)	7.4 (8.0)

pyruvate dehydrogenase a when the 3-hydroxybutyrate/acetoacetate ratio and long-chain acyl-CoA were increased by incubation with oleate. Oleate in both the diabetic and the control group increased acyl-CoA contents and ketone-body production to almost the same extent. Pyruvate dehydrogenase a, however, was diminished by oleate only in the cells from normal rats. On the other hand, the concomitant decrease in acyl-CoA and ketone-body formation caused in hepatocytes from diabetic rats by sorbitol or glycerol as shown in Table 11 was not paralleled by a change in the amount of active form of pyruvate dehydrogenase (see Table 8). In agreement with Akerboom & Zuurendonk (1974), the bulk of the acyl-CoA was recovered from the pellet fraction independent of the metabolic state of the donor animal (Table 12). No attempt, however, was made to differentiate further between the proportions of acyl-CoA bound to the various types of proteins or membranes contained in the pellet (i.e. plasma membrane, microsomal, mitochondrial, nuclear membranes etc.).

Ultrastructure of the pellet fraction

Thin sections from pellets of hepatocytes exposed to digitonin, and from pellets of hepatocytes treated in parallel with a digitonin-free medium, were examined in the electron microscope. In contrast with the controls (Plates 1 and 4*b*), the digitonin-treated liver cells (Plates 2, 3 and 4*a*) did not display the typical hepatocytic architecture. The most prominent alterations were the loss of the intact plasma membrane covered with microvilli (Plates 2 and 4*a*), the appearance of the mitochondria (Plate 4*b*) as inner-membrane-matrix vesicles (Plates 3 and 4*a*), and the transformation of the endoplasmic reticulum (Plate 4*b*) into small vesicles (Plates 3 and 4*a*). These morphological features were found in all parts of the pellets studied and were the same in hepatocytes isolated from normal fed or starved rats. No intact hepatocytes were observed after digitonin treatment.

Discussion

A study on the regulation of pyruvate dehydrogenase interconversion by the phosphorylation state of the mitochondrial adenine nucleotides in intact cells requires the separation of cytosolic and mitochondrial adenine nucleotides and the conservation of a given metabolic state during the separation procedure. Evidence that the digitonin method meets these requirements has been presented (Siess & Wieland, 1975). This is further supported in this study by the fact that adenosine caused a shift in the ATP content in opposite directions in the supernatant and the pellet fraction, as shown in Table 3. Moreover, the data in Table 1 indicate that the adenine nucleotide contents and distribution do not change during the time required for fractionation, provided that the temperature is kept below 6°C.

Total amounts of ATP and ADP reported herein agree well with those found by Krebs *et al.* (1974) in unfractionated isolated hepatocytes. Our values for the cytosolic and mitochondrial ATP/ADP ratios confirm the results of Zuurendonk & Tager (1974), and are comparable with those reported by Elbers *et al.* (1974), using a different approach. In addition, the close agreement of our cytosolic ATP/ADP ratio with that of isolated rat liver nuclei (ATP/ADP = 7.3) (Siebert, 1972) is notable, as nuclei, lacking permeability barriers for adenine nucleotides (Siebert, 1972), probably reflect the adenine nucleotide concentration of the surrounding cytoplasm.

The adenine nucleotide contents of the pellet fraction, as listed in Table 3, are possibly somewhat higher than the true intramitochondrial values, because they were neither corrected for the cytosolic adenine nucleotides (Table 2) nor for the nuclear adenine nucleotides present in the pellet. The second source of error is smaller than the first one, since the nuclei, containing about 7% of the cellular ATP (Siebert, 1961), can be expected to release it into the supernatant during digitonin treatment. After cor-

rection for both sources of error, minimal mitochondrial adenine nucleotide contents would amount to 0.45 μmol of ATP/10^8 cells and 0.31 μmol of ADP/10^8 cells. On the basis of 10^8 cells corresponding to 1 g of fresh liver (Zahlten *et al.*, 1973) and of 48 μl of intramitochondrial water space/g of fresh liver (Scholz & Bücher, 1965; Klingenberg & Pfaff, 1966), mitochondrial concentrations of ATP and ADP of 9.4 and 6.5 μmol/ml respectively result. For the cytosol, concentrations of 5.7 μmol of ATP/ml and 1.0 μmol of ADP/ml can be calculated, as 1 g of fresh liver corresponds to about 400 μl of cytoplasmic matrix (Bolender & Weibel, 1973; Weibel *et al.*, 1969). From these data the phosphorylation state, e.g. the $[ATP]/[ADP][HPO_4^{2-}]$ ratio for the whole cell, cytoplasm and mitochondrial matrix can be derived to be $620\,M^{-1}$, $920\,M^{-1}$ and $240\,M^{-1}$ respectively, on the assumption that $[HPO_4^{2-}]$ is approx. 6 mM (Wilson *et al.*, 1974), and is equally distributed in the cell. Thus the value for the cytosolic compartment obtained by digitonin fractionation of cells from normal fed rats compares with that calculated on the basis of mass-action equilibria (Krebs & Veech, 1970; McLean *et al.*, 1971), but the value for the mitochondrial compartment as reported here is several orders of magnitude higher.

In hepatocytes from normal fed rats isolated and incubated without substrate supplementation, pyruvate dehydrogenase a amounted to 33% of total pyruvate dehydrogenase activity, compared with 16% measured *in vivo* (Wieland *et al.*, 1972*a*) and 20% measured in the perfused liver (Patzelt *et al.*, 1973) and in isolated liver mitochondria (Portenhauser & Wieland, 1972). Also somewhat higher pyruvate dehydrogenase a values than *in vivo* (10%) (Wieland *et al.*, 1972*a*) were obtained from cells isolated from starved rats (16%), and total activities of both groups agree well with those determined in freeze-clamped liver tissue (Wieland *et al.*, 1972*a*).

As expected from the results with perfused liver (Patzelt *et al.*, 1973) and isolated liver mitochondria (Portenhauser & Wieland, 1972; Taylor *et al.*, 1975), incubation with pyruvate led to a marked increase of the active form of pyruvate dehydrogenase also in isolated hepatocytes. From the ATP and ADP measurements (see Table 3) it seems clear that this effect is not attributable to a change in the mitochondrial ATP/ADP ratio but rather to the inhibition by pyruvate of pyruvate dehydrogenase kinase (Linn *et al.*, 1969). In contrast, the mechanism by which dihydroxyacetone enhanced pyruvate dehydrogenase dephosphorylation at a high ATP/ADP ratio (see Tables 3 and 7) is not yet understood, as the expected accumulation of pyruvate (Woods & Krebs, 1973) was measurable only in the supernatant but not in the pellet (results not shown).

From experiments with isolated mitochondria it has been concluded (Taylor *et al.*, 1975; Batenburg & Olson, 1975) that a lowering of the mitochondrial $NAD^+/NADH$ ratio leads to increased pyruvate dehydrogenase phosphorylation, and vice versa. Our results do not seem to point in the same direction. First, pyruvate dehydrogenase a was not lowered in cells incubated with ethanol (10 mM) (Table 7), which increases the mitochondrial $NADH/NAD^+$ ratio (Forsander *et al.*, 1965). Secondly, pyruvate dehydrogenase a in cells from normal and diabetic rats amounted to 33 and 16% respectively, although the 3-hydroxybutyrate/acetoacetate ratios were changed in the opposite direction (Table 10). Thirdly, pyruvate dehydrogenase a was not diminished significantly in cells from diabetic rats as the 3-hydroxybutyrate/acetoacetate ratio rose from 0.06 to 1.56 during oleate utilization. From these observations one might infer that the mitochondrial redox state as judged by the 3-hydroxybutyrate/acetoacetate ratio is not directly involved in the regulation of pyruvate dehydrogenase phosphorylation.

The stimulatory effect of NH_4Cl on pyruvate dehydrogenase a formation as described for the perfused rat liver (Häussinger *et al.*, 1975) could not be observed with the isolated hepatocytes. Only at unphysiologically high concentrations (5 mM-NH_4Cl) was there a modest increase in the active form of the enzyme at an unchanged mitochondrial ATP/ADP ratio (Table 9). According to studies on the purified pyruvate dehydrogenase complex (Roche & Reed, 1974), NH_4^+ at 1–5 mM increases the affinity of the pyruvate dehydrogenase kinase for ADP, thereby decreasing its activity at a given ATP/ADP ratio. Thus our results with liver cells may be explained by the latter mechanism.

As to the regulation of the pyruvate dehydrogenase system in cells from normal rats by the mitochondrial ATP/ADP ratio (Fig. 1) the question arises whether fluctuations in adenine nucleotide concentrations that are large enough to affect pyruvate dehydrogenase kinase activity occur in liver *in vivo*. The experiments with isolated liver cells kept under conditions of enhanced gluconeogenesis or ureogenesis (see Tables 8 and 9) suggest that this might not be the case. Thus it appears that under physiological conditions, the ATP/ADP ratio in liver mitochondria is maintained high enough to keep pyruvate dehydrogenase a at a constant low activity. This explains the earlier observation that the steady state of pyruvate dehydrogenase a in liver can only be displaced slightly by exposing intact rats to various physiological metabolic conditions, such as starvation or re-feeding with carbohydrate (Wieland *et al.*, 1972*a*). On the other hand, these conditions led to marked changes of pyruvate dehydrogenase activity in heart muscle and kidney (Wieland *et al.*, 1971*a*), suggesting that in these tissues corresponding changes in the mitochondrial ATP/ADP ratio might occur *in vivo*. Observations on pyruvate dehydrogenase interconversion in skeletal

muscle point in the same direction (Hennig *et al.*, 1975).

Hepatocytes from starved or diabetic rats not only displayed lower amounts of pyruvate dehydrogenase a than those from fed rats when incubated without substrate, but also behaved differently on application of substrates. Thus 61, 37 and 23% of total pyruvate dehydrogenase was present in the active form in cells from normal fed, non-ketotic streptozotocin-diabetic and ketotic alloxan-diabetic rats respectively, if the mitochondrial ATP/ADP ratio was lowered to about 0.5 by fructose. The correlation between the amount of active form of pyruvate dehydrogenase and the phosphorylation state of the mitochondrial adenine nucleotides as observed in the isolated cells appears therefore to be dependent on the metabolic state of the donor animal, showing a shift towards lower pyruvate dehydrogenase a values in starvation and diabetes (Table 8). This shift can be ascribed to neither changes in the mitochondrial redox state nor changes in the amount of long-chain acyl-CoA (unless a different distribution of the latter between the various constituents of the pellet fraction is assumed). An elevation of the mitochondrial acetyl-CoA/CoA ratio, as expected to occur in starvation or diabetes would explain the lowered steady-state amount of pyruvate dehydrogenase a taking into account the reported activation of pyruvate dehydrogenase a kinase by acetyl-CoA (Pettit *et al.*, 1975). Unfortunately, we have not been able so far to reproduce this effect with purified pyruvate dehydrogenase preparations from heart muscle or kidney. Another possibility, namely that pyruvate dehydrogenase kinase becomes prevalent because of a decreased pyruvate dehydrogenase phosphatase activity in starvation or diabetes, has been excluded (E. A. Siess, unpublished work). A possible explanation is provided by the finding that the inhibition by ADP of purified pyruvate dehydrogenase kinase is a function of K^+ concentration (Roche & Reed, 1974). This would imply the assumption that a decrease in K^+ occurs in starvation and diabetes at the site of the pyruvate dehydrogenase complex, suggesting a regulatory role of the mitochondrial K^+ content in pyruvate dehydrogenase interconversion in the intact cell.

The skilful technical assistance of Mrs. E. Gundel and Miss A. Schnabl is greatly acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft, Bad Godesberg, Germany.

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