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Subcellular Distribution of Enzymes determined by Rapid Digitonin Fractionation of Isolated Hepatocytes

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Conditions were determined for rapid separation of cytosolic and mitochondrial compartments by digitonin fractionation of rat hepatocytes. The minimum time required for separation of mitochondrial and cytosolic enzyme markers decreased rapidly with increasing temperature. Kyro EOB, a non-ionic detergent, increases the release of cytosolic enzymes, particularly at lower temperatures. Experimental procedures are described for greater than 90% release of cytosolic enzymes and less than 2% release of mitochondrial enzymes in 3 s. By using appropriate concentrations of digitonin and Kyro EOB in a fractionation medium maintained at 1°C and a minimum time of exposure to the medium, nearly separate patterns of release were obtained for enzyme markers for the cytosol, mitochondrial matrix and mitochondrial intermembrane space. The distribution of enzymes that exist in more than one of these compartments was quantified by comparing their rates of release with those of marker enzymes. The cytosol/mitochondrial-matrix distributions for such enzymes in hepatocytes from starved rats were 16%/84% for aspartate aminotransferase, 34%/66% for fumarase and 77%/23% for ATP citrate lyase. In hepatocytes from rats that were induced to synthesize ATP citrate lyase by starvation and re-feeding, the ratio had increased to 95%/5%. The maximum cytosol/intermembrane-space ratio for adenylate kinase was 8%/92%. A procedure is also described for treating commercial digitonin that increases its solubility in water from about 1 mg/ml to more than 800 mg/ml.

An understanding of metabolic regulation requires knowledge of how enzymes and metabolites are distributed between various cellular compartments. A widely used procedure (Zuurendonk & Tager, 1974) for obtaining information on metabolite distribution between the cytosol and mitochondria involves the separation of these compartments after exposing isolated hepatocytes to digitonin. In this procedure plasma membranes are disrupted more rapidly than mitochondrial membranes because digitonin acts by complexing with cholesterol, and plasma membranes contain a higher concentration of cholesterol than do mitochondrial membranes (Colbeau *et al.*, 1971). Digitonin has previously been used to determine the location of enzymes in isolated mitochondria (Brdiczka *et al.*, 1968; Schnaitman & Greenawalt, 1968; Wojtczak *et al.*, 1969; Matlib & O'Brien, 1975). Those experiments involved lengthy procedures to isolate mitochondria and long-term exposure of the mitochondria to digitonin, and it remains uncertain whether the mitochondrial location of enzymes is altered by such procedures.

During the course of a critical evaluation and modification of the procedure of Zuurendonk & Tager (1974), it became apparent that digitonin fractionation can be used to quantify the subcellular distribution of enzymes by comparing the rate of release of a given enzyme with the rate of release of marker enzymes for different compartments. A preliminary account of this work has been presented (Janski *et al.*, 1979).

Experimental

Materials

Digitonin was purchased from ICN Pharmaceuticals, Cleveland, OH, U.S.A. Kyro EOB was a gift from Dr. D. H. Hughes (Miami Valley Research Laboratories, Procter and Gamble Co., Cincinnati, OH, U.S.A.). Kyro EOB, a non-ionic detergent, is a secondary alcohol with an average degree of ethoxylation of nine and an average alkyl chain length of fourteen. Bromodecane was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and bromododecane

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was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fat-free diet, catalogue no. TD 70257, was from Teklad Test Diets, Madison, WI, U.S.A. Enzymes were obtained from Boehringer (Mannheim) Corp., Indianapolis, IN, U.S.A., and substrates and cofactors were from Boehringer or from Sigma. Other chemicals were reagent-grade products.

Methods

Preparation of hepatocytes. Isolated hepatocytes were prepared by the procedure of Berry & Friend (1969), modified as described by Cornell *et al.* (1973) and Krebs *et al.* (1974). Animals used for hepatocyte preparation were male Wistar rats (170–210g) that had been starved for 2 days, or rats (200–240g) that were starved for 2 days and then fed on a fat-free diet for 2 days (re-fed rats).

Treatment of digitonin. A major problem with commercial digitonin is its very low solubility in water. When heated to 100°C, it is possible to dissolve up to 10mg of digitonin/ml of water, but within 1h of cooling to room temperature it begins to precipitate. Addition of the non-ionic detergent Kyro EOB to unpurified digitonin increased its solubility, without heating, to as much as 50mg/ml at 1.0% (v/v) Kyro EOB. To obtain only the material in commercial digitonin that would complex with cholesterol, digitonin (7mg/ml, 5.7mM) and cholesterol (3.3mg/ml, 8.5mM) were dissolved in methanol, and water was added to a final concentration of 3.3% (v/v). At this concentration of water, uncomplexed digitonin and cholesterol remain soluble, but their digitonide complex is insoluble. The digitonide complex was separated from the soluble material by centrifugation for 10min at 25°C and 10000*g* and was disrupted by dissolving it in dimethyl sulphoxide at 100°C (Issidorides *et al.*, 1962). Cholesterol became insoluble when the dimethyl sulphoxide solution was cooled to room temperature and was removed by centrifugation (as above) or filtration. Digitonin was precipitated by adding, for each volume of dimethyl sulphoxide, 10vol. of diethyl ether followed by 6vol. of chloroform. After 15min, the digitonin precipitate was collected by centrifugation (as above), washed several times with diethyl ether, and dried at 80°C. The purified product was confirmed to be digitonin by its ability to precipitate cholesterol quantitatively and by other analytical tests (N. W. Cornell, A. M. Janski & H. Yeh, unpublished work). The solubility of treated digitonin in water is greater than 800mg/ml (650mM), and it remains in solution at this concentration for at least 1 week at room temperature. Purified digitonin was used in all fractionation experiments described in the present paper.

Fractionation procedure. Hepatocytes (0.2ml, 0.16–0.20g/ml), suspended in saline medium (Krebs & Henseleit, 1932) containing bovine serum albumin (2.5%, w/v) at 38°C, were exposed to 1.0ml of fractionation medium (0.25M-sucrose/20mM-4-morpholinepropanesulphonic acid/3mM-EDTA, pH7.0), containing the additions indicated in each experiment. To obtain good mixing and rapid separation of disrupted cells (which retain the mitochondria) from the fractionation medium (containing the cytosolic fraction), several modifications of the procedure of Zuurendonk & Tager (1974) were employed. A 2.0ml polypropylene centrifuge tube (no. 708; W. Sarstedt Co., Princeton, NJ, U.S.A.) was used, and it contained 0.4ml of a mixture of bromododecane and bromodecane. This brominated-hydrocarbon mixture has a density (ρ) of 1.045g/ml, but is much less viscous than silicone oils that have previously been used for separating cells and medium. The viscosities of bromodecane and bromododecane are 2.35 and 3.45 cP (mPa·s) respectively, and that of a silicone-oil mixture with similar density [1:3 (v/v) mixture of Dow Corning 200 and 500] is 105cP (mPa·s) (N. W. Cornell, unpublished work). Fractionation medium ($\rho = 1.033$g/ml) was layered above the brominated hydrocarbon and the tube was equilibrated at the desired temperature. Just before fractionation the tube was placed in the rotor of an Eppendorf model 5412 centrifuge. Rapid mixing of hepatocytes with the medium was achieved by injecting hepatocytes with an Eppendorf pipette. To prevent disturbance of the brominated-hydrocarbon layer, the pipette tip was bent by heating so that the hepatocytes were forced into the tube at a 90° angle with respect to the length of the tube. A narrow slit was cut in the cap for the centrifuge tube so that the cap could remain on the tube during injection of hepatocytes and removal of the pipette. After the desired period of exposure of hepatocytes to the medium, disrupted cells were separated from the medium in less than 1s by activating the centrifuge. By this procedure it is possible to perform the fractionation in 2s, and good precision can be obtained when performed in 3s. Total activity of enzymes was determined by using 0.2ml of hepatocyte suspension in 1.0ml of fractionation medium and 0.1% (v/v) final concentration of Triton X-100. This mixture was left on ice for 1h. It was then centrifuged for 3min at room temperature and 10000*g*, and the supernatant was saved for enzyme assays. Triton X-100 and sonication were equally effective in releasing the enzymes described in the present paper. After fractionation, Triton X-100 (0.1% final concn.) was added to supernatants that contained the digitonin-released cellular material. The ratio of enzyme activity in these supernatants relative to the total activity in the cell is given as a percentage of the enzyme that was released.

Enzyme assays. All enzyme assays were per-

formed at 38°C. Citrate synthase (EC 4.1.3.7) was assayed by the procedure of Shepherd & Garland (1969), phosphoglycerate kinase (EC 2.7.2.3) by that of Bergmeyer (1974), fumarase (EC 4.2.1.2) by that of Hill & Bradshaw (1969), ATP citrate lyase (EC 4.1.3.8) by that of Cottam & Srere (1969), and glutamate dehydrogenase (EC 1.4.1.3) by that of Schmidt (1974). The aspartate aminotransferase (EC 2.6.1.1) assay mixture contained 20mM-aspartate, but otherwise was as described by Bechtler (1974). For other enzymes, the procedures described by Bergmeyer *et al.* (1974) were modified as follows: the assay for lactate dehydrogenase (EC 1.1.1.27) contained 5.0mM-pyruvate; that for adenylate kinase (EC 2.7.4.3) contained 38mM-Tris/HCl, pH 7.5, 4.5mM-$MgCl_2$, 16mM-KCl, 0.5mM-ATP, 2.0mM-AMP and 0.16mM-NADH; and, because of endogenous 6-phosphogluconate dehydrogenase (EC 1.1.1.43) activity in liver extracts, the glucose 6-phosphate dehydrogenase (EC 1.1.1.49) assay was modified by the addition of 0.03 unit (1 unit $\equiv$ 1 μmol/min) of 6-phosphogluconate dehydrogenase/ml, and the rate of the glucose 6-phosphate dehydrogenase was assumed to be half the rate of NADPH formation.

Results

The data in Table 1 show the effect of temperature on release of the cytosolic marker lactate dehydrogenase, the mitochondrial-matrix marker citrate synthase, and the marker for the mitochondrial intermembrane space, adenylate kinase. In the presence of digitonin, release of lactate dehydrogenase increased rapidly with temperature, particularly in the absence of Kyro EOB. Kyro EOB has previously been shown to be effective in lysing cell membranes while causing little damage to endoplasmic reticulum (Birckbichler & Pryme, 1973). In the absence of digitonin and in the presence of 0.3% Kyro EOB there was little increase in release of lactate dehydrogenase with increasing temperature. At 10mg of digitonin/ml, release of lactate dehydrogenase was amplified most by Kyro EOB at lower temperatures, but at 26°C more than 90% of lactate dehydrogenase was released after 3s exposure of hepatocytes to digitonin medium containing no Kyro EOB. At 36°C, 1.0mg of digitonin/ml in the presence of Kyro EOB was sufficient to cause greater than 90% release of lactate dehydrogenase after 3s exposure. In other experi-

Table 1. *Effect of temperature on release of enzymes*

Hepatocytes were isolated from a rat starved for 2 days. Hepatocytes (0.2ml, 0.18g/ml) at 38°C were exposed for 3s to 1.0ml of fractionation medium, at the indicated temperature and containing the indicated additions, as described in the text. The final temperature was measured with a Yellow Springs Instruments Model 42 SL telethermometer within 5s after adding hepatocytes to fractionation tubes, containing the same contents as those used in measuring enzyme release. Enzyme activities and percentage release were determined as described in the text. Abbreviations: LDH, lactate dehydrogenase; CS, citrate synthase; and AK, adenylate kinase.

Temperature (°C)		Additions to fractionation medium		Release of enzyme activity (%) (3s exposure)		
Fractionation medium	Final	Digitonin (mg/ml)	Kyro EOB (0.3%, v/v)	LDH	CS	AK
1.0	6.5	0	+	7.0	0.41	0.70
1.0	6.5	10	–	19	0.40	1.4
1.0	6.5	10	+	60	0.73	2.6
4.0	9.5	0	+	6.0	0.45	0.60
4.0	9.5	10	–	30	0.45	1.7
4.0	9.5	10	+	72	0.80	3.4
9.0	12	0	+	6.8	0.45	0.90
9.0	12	10	–	51	0.50	3.0
9.0	12	10	+	82	0.98	5.0
14	17	0	+	9.1	0.57	1.1
14	17	10	–	86	0.67	6.2
14	17	10	+	96	1.4	12
26	27	0	+	9.7	0.49	1.1
26	27	10	–	91	0.88	17
26	27	10	+	95	1.7	22
36	36	0.1	–	9.9	0.34	1.1
36	36	0.1	+	21	0.58	1.6
36	36	0.4	–	68	0.44	2.6
36	36	0.4	+	78	0.84	3.0
36	36	1.0	–	86	0.56	3.6
36	36	1.0	+	93	0.96	5.3

ments we have observed that at 38°C greater than 90% release of lactate dehydrogenase can be attained in less than 2s when 10mg of digitonin/ml is used, even in the absence of Kyro EOB. By using conditions that minimize time of exposure of hepatocytes to digitonin, more reliable information on distribution of cellular components, particularly metabolites, can be obtained. Release of citrate synthase was low at all temperatures; the presence of Kyro EOB caused only small increases in release of this matrix enzyme. At 14°C, 96% of lactate dehydrogenase and 12% of adenylate kinase were released in the presence of Kyro EOB and digitonin. Assuming that about 8% of adenylate kinase is in the cytosol and the remainder is in the mitochondrial intermembrane space (see calculations below), the data suggest that there was relatively little damage to the outer mitochondrial membrane, but nearly complete release of cytosolic contents had occurred.

The experiments illustrated by Figs. 1(*a*) and 1(*b*) were conducted to determine the optimum concentration of Kyro EOB. In these tests, the concentrations of digitonin and the times of exposure to the fractionation medium were those that gave 85–90% release of lactate dehydrogenase at a maximally effective concentration of Kyro EOB. That value was 0.3% (v/v) Kyro EOB both when the fractionation medium was at 5°C (Fig. 1*a*) and when it was at 8°C (Fig. 1*b*). Although release of citrate synthase was low, it increased with concentration of Kyro EOB. Therefore, to minimize damage to mitochondria, a minimal concentration of Kyro EOB must be selected. Release of adenylate kinase at both temperatures was less than 7% when Kyro EOB concentration was 0.3% or less. Other experiments were performed to determine the minimum time of exposure to digitonin (10mg/ml) and Kyro EOB (0.3%) required to release more than 90% of lactate dehydrogenase at different temperatures. The minimum exposure times for fractionation medium at 1°C, 5°C, 8°C, 12°C and 26°C were 8.5, 7, 4.5. 3 and less than 2s. The amount of citrate synthase released was 2% or less at the minimum exposure time for each temperature. We have tested other non-ionic detergents and have found that Lubrol PX (Sigma Chemical Co.) may be an effective substitute for Kyro EOB in fractionation experiments.

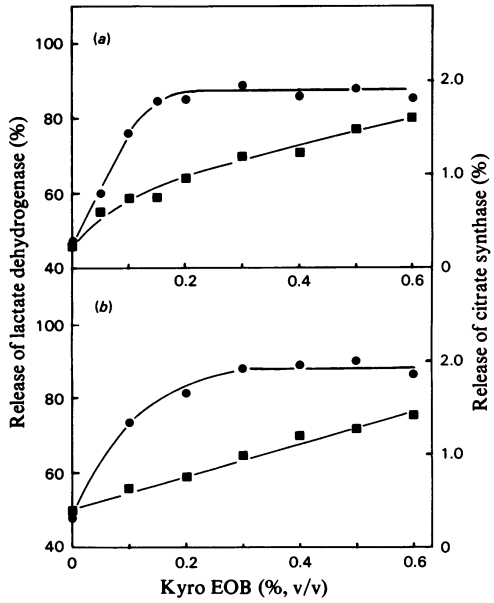

Fig. 1. *Effect of Kyro EOB and temperature on release of lactate dehydrogenase* (●) *and citrate synthase* (■) *from hepatocytes from starved rats*

Hepatocytes were fractionated as described in the text. Data points represent the average of separate experiments with two different hepatocyte preparations. (*a*) Hepatocytes were exposed for 5s to fractionation medium at 5°C, containing 10mg of digitonin/ml. (*b*) Hepatocytes were exposed for 3s to fractionation medium at 8°C containing 5mg of digitonin/ml.

By using hepatocytes from starved rats, release of various enzymes was measured for different times of exposure to digitonin and Kyro EOB (Table 2). The temperature of the fractionation medium and concentrations of digitonin and Kyro EOB were selected to minimize the time of exposure of hepatocytes to the disrupting medium and to cause little overlap in patterns of release for marker enzymes (lactate dehydrogenase, the cytosolic marker; adenylate kinase, marker for mitochondrial intermembrane space; and citrate synthase, mitochondrial-matrix marker). These patterns are shown in Fig. 2(*a*). Phosphoglycerate kinase and glucose 6-phosphate dehydrogenase were released in a pattern almost identical with that for lactate dehydrogenase, and glutamate dehydrogenase was released like citrate synthase (Table 2). ATP citrate lyase, fumarase and aspartate aminotransferase had patterns of release that were partially like that for lactate dehydrogenase and partially like that for citrate synthase (Fig. 2*b*). These data are consistent with the location of these enzymes in both the cytosol and the mitochondrial matrix. With hepatocytes from re-fed rats, the patterns of release of lactate dehydrogenase, citrate synthase, adenylate kinase, fumarase and aspartate aminotransferase were not significantly different from experiments with hepatocytes from starved rats (results not shown). However, in hepatocytes from re-fed rats, ATP citrate lyase activity was 15–20 times higher, and nearly all the enzyme was released like lactate dehydrogenase (Fig. 2*b*). This suggests that ATP citrate lyase activity induced by starvation/re-feeding is pri-

Table 2. *Release of enzymes from hepatocytes isolated from a starved rat*

Hepatocytes were isolated from a rat starved for 2 days. Hepatocytes (0.2ml, 0.18g/ml) at 38°C were exposed to 1.0ml of fractionation medium (1°C), containing 4mg of digitonin/ml and 0.2% Kyro EOB, for the indicated period of time according to the fractionation procedure described in the text. For time points greater than 15s, the fractionation tubes were kept in a 6°C bath until separation by centrifugation. Enzyme activities and percentage release were determined as described in the text. Abbreviations: LDH, lactate dehydrogenase; PGK, phosphoglycerate kinase; G6PDH, glucose 6-phosphate dehydrogenase; CL, ATP citrate lyase; FM, fumarase; AAT, aspartate aminotransferase; AK, adenylate kinase; CS, citrate synthase; GlDH, glutamate dehydrogenase.

Time of exposure of hepatocytes to fractionation medium (s)	Release of enzyme activity (%)								
	LDH	PGK	G6PDH	CL	FM	AAT	AK	CS	ClDH
0	4.5	3.8	2.3	5.6	1.8	0.83	0.50	0.34	0.48
3	38	42	42	34	12	7.6	3.4	0.9	0.87
4	54	58	62	49	18	11	3.0	1.1	0.96
5	71	73	72	53	23	13	4.8	1.2	0.98
6	77	77	80	58	25	14	6.3	1.3	1.1
8	86	81	85	65	29	12	10	1.5	1.3
10	93	91	88	68	32	15	16	1.8	1.5
12	93	90	88	72	33	15	24	2.3	2.0
15	98	91	89	71	35	17	36	2.9	2.4
20	96	93	94	75	37	20	62	4.3	3.4
25	99	96	93	76	38	20	73	5.4	4.1
30	102	95	91	75	40	21	81	6.0	4.7
40	99	96	92	74	41	18	96	7.3	5.7
60	104	98	101	82	43	30	101	8.6	7.0
90	100	96	101	84	45	30	103	10	9.0
600	102	100	104	92	82	76	100	63	52

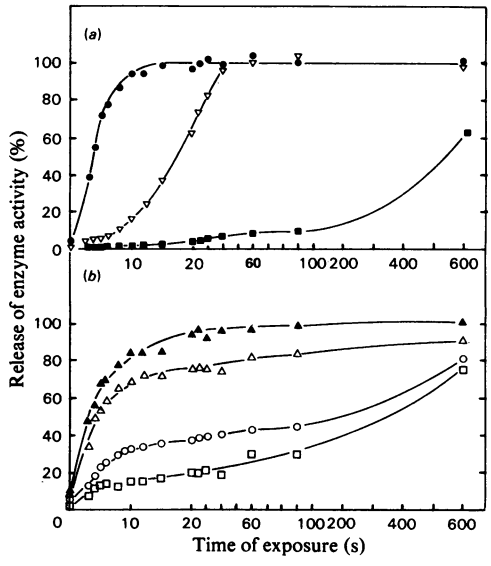

Fig. 2. *Release of cytosolic and mitochondrial enzymes from hepatocytes from starved rats*

Procedure is described in Table 2. (*a*) ●, Lactate dehydrogenase; ▽, adenylate kinase; ■, citrate synthase. (*b*) □, Aspartate aminotransferase; ○, fumarase; △, ATP citrate lyase. ▲, ATP citrate lyase in hepatocytes from re-fed rats.

marily in the cytosol. Although activity of fatty acid synthetase was too low to measure accurately in hepatocytes from starved rats by using the procedure described in Table 2, release of fatty acid synthetase in hepatocytes from re-fed rats also paralleled the release of lactate dehydrogenase (results not shown).

By varying the time of exposure to the disrupting medium and comparing release of a given enzyme with release of lactate dehydrogenase, adenylate kinase and citrate synthase, the distribution of the enzyme between the cytosol, the mitochondrial intermembrane space and the matrix can be quantified. For an enzyme that is released as though it exists in both the cytosol and the mitochondrial matrix, let c represent the percentage released after a given time of exposure to the fractionation medium. If x and y are the percentages of the enzyme in the cytosol and the mitochondrial matrix, respectively, in the untreated hepatocyte, then $x + y = 100\%$. If a and b represent the fractions of lactate dehydrogenase and citrate synthase, respectively, that have been released from the hepatocytes after a given time of exposure, then $ax + by = c$. Since a, b and c are measured and $x = 100 - y$, the percentage of the enzyme in the mitochondria is given by $y = (100a - c)/(a - b)$. This calculation can be per-

formed for each exposure time. If the calculated values for x and y do not vary with the exposure time, it is a good indication that the enzyme is localized in the two compartments. By using this equation, the distribution of enzymes in hepatocytes (starved rats) was calculated at 12 time points (3–40s; Table 2). The cytosol/mitochondrial-matrix distributions (± S.E.M.) for these time points were: ATP citrate lyase, 76.9%/23.1% (± 1.8%); fumarase, 34.1%/65.9% (± 0.8%); aspartate aminotransferase, 15.6%/84.4% (± 0.6%). In hepatocytes from re-fed rats, the distribution of ATP citrate lyase was 95.2%/4.8% (± 1.1%). Because more than 90% of adenylate kinase but only 7.3% of citrate synthase was released after a 40s exposure, it is unlikely that a significant fraction of adenylate kinase exists in the mitochondrial matrix. This observation is consistent with those of Schnaitman & Greenawalt (1968) and Wojtczak *et al.* (1969). By using an analogous derivation and equation, the cytosol/intermembrane-space distribution [± S.E.M. for five time points (3–8s)] for adenylate kinase was 8.2%/91.8% (± 1.0%).

The experiments described here were designed to quantify the distribution of soluble enzymes that may be located in the cytosol and regions of mitochondria. By appropriately adjusting conditions of fractionation and by varying temperature and concentrations of digitonin and Kyro EOB, it may be possible, without the questionable aspects of preliminary isolation of mitochondria, to determine distribution of mitochondrial enzymes between compartments within the mitochondria.

Discussion

The results in Table 1 show that temperature is one of the most important parameters in the digitonin fractionation of hepatocytes. At any given time of exposure to the fractionation medium, temperature must be well controlled in order to obtain reproducible release of cellular materials. Previously, Siess & Wieland (1976) examined the effect of temperature on cellular adenine nucleotides, and they concluded that the distributions of ATP and ADP are altered by varying the temperature of the fractionation medium. Since the procedure used in that study was different from the one described here, the results cannot be strictly compared. However, our data do suggest the possibility (which requires testing) that apparent cytosolic/mitochondrial distributions of metabolites could be influenced by the temperature-dependent rate of release of materials from both compartments.

The mechanisms by which increasing temperature and Kyro EOB accelerate release of cytosolic contents are not known. Since Kyro EOB and higher temperatures both increase the solubility of unpurified digitonin, these agents might have effects on the structure or conformation of digitonin. Alternatively, since Kyro EOB is more effective at lower temperatures, it is conceivable that, at lower temperatures, the interaction between digitonin and membrane cholesterol is impeded, and the detergent in some way diminishes that impediment.

Because ATP citrate lyase is generally considered to be a cytosolic enzyme (Srere, 1975), we were surprised to find that 26% of the activity in hepatocytes from starved rats is released like a mitochondrial enzyme. Previously, DeRosa & Swick (1975), using a lengthy fractionation procedure with livers of rats starved overnight, concluded that about 10% of the ATP citrate lyase activity was mitochondrial. In our experiments with hepatocytes from 48h-starved rats, the ATP citrate lyase which was released like citrate synthase could represent an amount that is associated with the outer mitochondrial membrane. That seems unlikely, however, since adenylate kinase, which is thought to exist primarily in the intermembrane space (Schnaitman & Greenawalt, 1968; Wojtczak *et al.*, 1969), was 96% released after a 40s exposure to digitonin, but ATP citrate lyase was only 74% released (Table 2). By performing fractionations under a variety of conditions and comparing release of ATP citrate lyase with release of citrate synthase, a more definitive statement could be made about the location of the slowly released portion of ATP citrate lyase.

The fraction of aspartate aminotransferase (15.6%) that we calculate to exist in the cytosol is considerably lower than the estimations of 50% (Meijer *et al.*, 1978) for rat hepatocytes and of 29% (Eichel & Bukovsky, 1961) and 59% (DeRosa & Swick, 1975) for rat liver. In these previous studies no quantitative estimation was given for damage to mitochondrial membranes during extraction procedures. The existence of a significant fraction (34%) of the total cellular fumarase in the cytosol suggests that the fumarate generated in the urea cycle can be metabolized (e.g. to glucose) without entering the mitochondria. Tolley & Craig (1975) have reported the existence of two interrelated, yet distinct, forms of fumarase in extracts from cultured mammalian cells; one form was located primarily in mitochondria and the other form was localized in the cytosol. Our calculation of 8.2% adenylate kinase in the cytosol is a maximum value, because adenylate kinase may begin leaking from the intermembrane space before all the lactate dehydrogenase has been released from hepatocytes. Criss (1970) showed that estimates of cytosolic adenylate kinase were very sensitive to conditions of isolation, presumably because of damage to the outer mitochondrial membrane. It should be noted that the minimum value for cytosolic adenylate kinase observed by Criss (1970) was about 20% or about 2.5 times the apparent cytosolic adenylate kinase that we observe by using

the rapid digitonin procedure described here. By modifying conditions used in the fractionations (Table 2) to separate further the release of adenylate kinase from release of lactate dehydrogenase, it may be possible to determine whether any adenylate kinase exists in the cytosol.

Discussions with Dr. Paul A. Srere were important in our decision to study the subcellular location of fumarase and ATP citrate lyase. We thank Dr. Richard L. Veech for his helpful advice and criticisms and Mrs. Mary Leadbetter for excellent technical assistance.

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