In Vivo Effect of Uncoupling Agents on the Incorporation of Calcium and Strontium into Mitochondria and Other Subcellular Fractions of Rat Liver

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ABSTRACT After injection of $^{45}Ca^{++}$ or $^{89}Sr^{++}$ into rats, the largest part of the radioactivity in the liver cell is associated with the subcellular structures, only negligible amounts of it being found in the soluble hyaloplasm. 50% or more of the $^{45}Ca^{++}$ and $^{89}Sr^{++}$ in the liver cell is recovered in the mitochondrial fraction. The specific activity of Ca^{++} after injection of $^{45}Ca^{++}$ is far greater in mitochondria than in microsomes. Pretreatment of the rats with uncouplers of oxidative phosphorylation markedly decreases the amount of radioactivity associated with the mitochondrial fraction. The amount of radioactivity recovered in the microsomes and in the final supernatant on the contrary increases. These effects are present only when mitochondrial oxidative phosphorylation is completely uncoupled. The Ca^{++} content of mitochondria from the livers of rats pretreated with uncouplers is sharply decreased with respect to the controls. It is concluded that in the liver cells of the intact animal energy-linked movements of Ca^{++} and Sr^{++} take place in mitochondria.

INTRODUCTION

The present investigation was prompted by recent studies of the active uptake of Ca^{++} and other divalent cations by mitochondria isolated from liver and other tissues (1–6). It was known from the studies of Maynard and Cotzias (7) on the distribution of the intraperitoneally injected radioactive Mn^{++} among rat liver subcellular fractions that the isotope was specifically concentrated in mitochondria. A recent study by Cosmos (8) has shown that mitochondria in the muscle cell demonstrate a greater affinity for Ca^{++} than do other subcellular structures. Thiers and Vallee (9) found some years ago that Mn^{++} and Ca^{++} are more concentrated in mitochondria than in microsomes and in the soluble supernatant, and a recent study of beef heart has also demonstrated a high concentration of Ca^{++} in the mitochondrial fraction (10).

All these studies indicate that mitochondria as they exist in the living cell are probably capable of active uptake of Ca^{++} and other divalent cations. The experiments reported in this paper have been designed to demonstrate that such an active uptake does actually take place in mitochondria in vivo, and to shed light on the possible mechanism of the uptake. It is now firmly established that the accumulation of Ca^{++} and of other divalent cations such as Sr^{++} by mitochondria in vitro is dependent on metabolic energy, derived from either electron transport or the hydrolysis of ATP (1–6, 11–12). Both the electron transport and the ATP-supported accumulation are inhibited by uncouplers of oxidative phosphorylation; uncouplers have also been shown to promote the rapid discharge of the accumulated Ca^{++} from mitochondria (13, 14).

In the present paper, the effect of parenterally administered uncouplers of oxidative phosphorylation on the in vivo distribution of injected radioactive Ca^{++} and Sr^{++} among subcellular fractions of rat liver has been studied. The uncouplers of oxidative phosphorylation have a marked inhibitory effect on the uptake of Ca^{++} and Sr^{++} by mitochondria in vivo. It is concluded that energy-dependent transfer of Ca^{++} and Sr^{++} across the mitochondrial membrane takes place in the liver cells of the intact animal.

MATERIALS AND METHODS

Young male Wistar strain rats were used, averaging 200 g in body weight. They were kept on a standard commercial diet, and fasted 16 hr before they were killed. Approximately 10 μc of $^{45}CaCl_2$ were injected intraperitoneally. The total amount of Ca^{++} injected was only 1.4 μg per 100 g body weight; therefore, no changes in the total Ca^{++} of the animal occurred. In the experiments with Sr^{++}, approximately 20 μc of carrier-free $^{89}SrCl_2$ were injected, corresponding to about 1.9 μg of Sr per 100 g of body weight. The stock solutions of $^{45}Ca^{++}$ and $^{89}Sr^{++}$ were diluted with distilled water to contain 40 μc $^{45}CaCl_2$ or $^{89}SrCl_2$ per ml. Pentachlorophenol (PCP) was injected intraperitoneally as a mildly alkaline aqueous solution (15) and dinitrophenol (15) as the sodium salt. The controls received an equal volume of 0.9% NaCl.

The animals were killed by decapitation, the liver quickly excised, cut into small pieces, squeezed repeatedly between two layers of filter paper to remove as much blood as possible, and chilled for about 2 min in 100 ml ice-cold 0.25 M sucrose. The pieces of liver were then squeezed again between two layers of filter paper and transferred to a new beaker containing 100 ml fresh ice-cold 0.25 M sucrose. It was assumed that this procedure effectively removed most of the blood, thereby minimizing contamination by highly labeled blood Ca^{++}. All the subsequent operations were carried out at 0–3°C. 10% homogenates were prepared in a Potter homogenizer with a Teflon pestle. Small aliquots of the homogenate were thoroughly rehomogenized in a tight, all-glass homogenizer to yield a homogeneous suspension that could be carefully pipetted for Ca^{++}, ^{45}Ca, ^{89}Sr, and nitrogen analysis. The remainder of the original homogenate was centrifuged at 800 *g* for 10 min. The sediment, which constitutes the "residue" or nuclear fraction, was resuspended in 0.25 M sucrose and

thoroughly rehomogenized in a tight, all-glass homogenizer (as was the original homogenate) to yield a homogeneous suspension. The supernatant fraction was centrifuged at 14,000 *g* for 7 min to sediment the mitochondria. "Heavy" microsomes were sedimented from the mitochondrial supernatant at 30,000 *g* for 20 min, and the normal microsomes were sedimented from the heavy microsome supernatant at 150,000 *g* for 40 min. Mitochondria were resuspended in 0.25 M sucrose with the aid of a glass rod, while the resuspension of the heavy microsomes and of the microsomes required the use of a Teflon pestle driven by a motor. Exactly 0.25 ml of each fraction was then added to 1.0 ml of 0.2% Na lauryl sulfate to "solubilize" the suspensions. Aliquots of the clear solutions were put on stainless steel planchets, dried carefully at 70°C in an oven, and counted in a thin mica window Geiger counter with a low

TABLE I

$^{45}Ca^{++}$ AND TOTAL Ca^{++} IN SUBCELLULAR FRACTIONS OF RAT LIVER

Fraction	Distribution of Ca^{++}	nMoles Ca^{++} per mg protein	Distribution of $^{45}Ca^{++}$	Relative content of $^{45}Ca^{++}$ (Activity in homogenate = 1.00)	Specific activity (cpm per nmole Ca^{++})
	%		%		
Residue	23.9±2.41 (7)	16.5±3.19 (7)	22.6±1.12 (9)	0.92±0.150 (9)	=
Mitochondria	30.9±1.86 (7)	19.5±3.04 (7)	55.5±1.84 (9)	2.28±0.115 (9)	15.3±2.62 (4)
Heavy microsomes	6.2±1.58 (7)	8.5±1.71 (7)	4.3±0.28 (9)	0.98±0.015 (9)	4.15 (2)
Microsomes	26.7±4.41 (7)	21.6±4.91 (7)	15.3±2.90 (9)	0.87±0.060 (9)	2.20±0.39 (4)
Supernatant	11.2±1.82 (7)	9.3±2.25 (7)	2.5±0.38 (9)	0.07±0.005 (9)	0.90±0.11 (3)

$^{45}Ca^{++}$ was injected 6 min before the death of the animals, except for the experiments on specific activity (4 min). Other experimental details are given in the Methods section. Results are given ± standard error. The number of experiments is given in parentheses.

background scaler. At least 1000 counts above the background were taken for each planchet

For the chemical determination of total Ca^{++}, the pellets were resuspended in 0.1 M Na succinate buffer, pH 5.5 (approximately 1 mg protein per ml buffer) and boiled 5 min (16) to precipitate the denatured proteins and to extract Ca^{++} quantitatively. The denatured protein was discarded after centrifugation and aliquots of the clear supernatant were used to determine Ca^{++} according to the method of Walser (16), scaled down to permit the determination of microquantities. The limit of sensitivity of the method was 0.01 μmole Ca^{++}. Occasionally, Ca^{++} was determined on TCA extracts of the various fractions by flame photometry, with a Beckman model B spectrophotometer and a Beckman flame attachment. Using flame photometry, corrections for Na^{+} interference had to be applied to the values obtained in the homogenate, in the residue, and in the final supernatant; interference by phosphates in all fractions was eliminated with $LaCl_3$. In addition, in three of the experiments reported in Table I, Ca^{++} was determined on TCA-$LaCl_3$ extracts of the subcellular fractions by atomic absorption spectrophotometry, using a Perkin-Elmer model 303 atomic absorption spectrophotometer. The results of the flame and of the atomic

absorption spectrophotometry were in good agreement with those obtained with the chemical titration.

The acceptor control ratios and ADP:O ratios of isolated mitochondria were measured with a Clark oxygen electrode according to Kielley and Bronk (17). Total cytochrome oxidase activity in the isolated subcellular fractions was measured polarographically with a Clark oxygen electrode, in a medium of the following composition: 0.04 M phosphate buffer, pH 7.4, 0.0004 M $ALCl_3$, 0.0001 M cytochrome *c*, 0.015 M Na ascorbate, and 0.15–0.6 mg protein; temperature, 25°C volume, 1.9 ml.

Electron microscope examinations were carried out on pellets fixed in 2.5% glutaraldehyde in 0.13 M phosphate, pH 7.4. Fixed pellets were dehydrated in increasing concentrations of acetone, stained in absolute acetone with 0.3% uranyl acetate, and embedded in a 1:1 mixture of Epon and Araldite. Sections were cut with a Porter-Blum ultramicrotome, collected on grids, stained with lead citrate, and observed in an Akashi-Tronscope EI TRS electron microscope.

Protein determinations were carried out by a micro-Kjeldahl procedure: a factor of 6.25 was used to convert nitrogen to protein.

All reagents used were analytical grade. Water distilled twice from glass was used throughout the entire investigation. High specific activity $^{45}CaCl_2$ was purchased from Nuclear Science and Engineering Co., Pittsburgh, Pa., and carrier-free $^{89}SrCl_2$ from Oak Ridge National Laboratory, Tenn.

RESULTS

Total Ca^{++} Content and $^{45}Ca^{++}$ Distribution in the Normal Subcellular Fractions of Liver Table I shows the distribution of Ca^{++} in liver subcellular fractions: about 31% of the total Ca^{++} of the cell is found in mitochondria. On a protein basis, this amounted to between 14 and 33 nmoles of Ca^{++} per mg of mitochondrial protein; these values are in fair agreement with those generally reported in the literature (1, 9, 10). The value for the total Ca^{++} content of mitochondria is, however, most probably underestimated, since a large number of mitochondria remain associated with the residue. Actually, the total Ca^{++} content of the mitochondrial fraction could be increased to over 50% of the total Ca^{++} of the liver cell when the residue was resuspended and rehomogenized to extract the mitochondria. Figures generally greater than those reported by Thiers and Vallee (9) have been found for the total Ca^{++} associated with the microsomes; moreover, the microsomal Ca^{++} "pool" was rather variable in size from animal to animal: 19 to 45% of the total Ca^{++} of the cell was recovered in the microsomes in the different experiments. As a consequence, the microsomal pool has been found to be smaller, about equal to, or greater than the mitochondrial pool in different experiments. When the residue was resuspended and rehomogenized to extract mitochondria, the total microsomal pool became markedly smaller than the mitochondrial one. Only a smaller fraction of the Ca^{++} of the cell was found associated with the final supernatant and with the heavy microsomes.

Fig. 1 shows the total radioactivity of liver homogenates as a function of the time after the injection of $^{45}Ca^{++}$. The radioactivity increases rapidly between 0 and 3 min, to a plateau which is maintained for about 10 min. Then, the radioactivity decreases.[1] Except for the experiments described in Fig. 3, the results reported in this paper were all obtained from animals killed 4–6 min after the administration of the isotope; i.e., at a time when the radioactivity in the liver cell was at its maximum.

The distribution of the injected $^{45}Ca^{++}$ among liver subcellular fractions is shown in Table I. More than half the isotope is found in the mitochondrial fraction, about 25% in the microsomes, and rather negligible amounts in

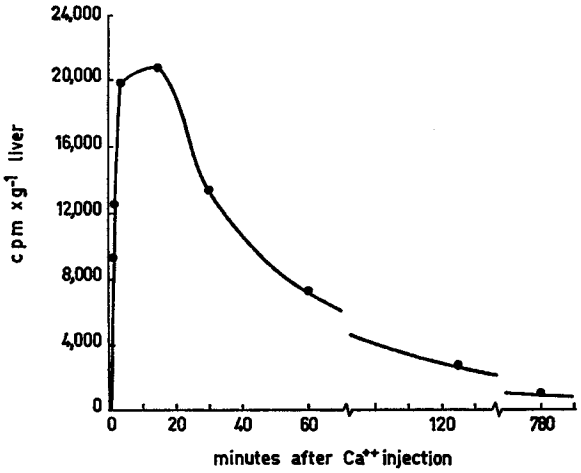

FIGURE 1. Radioactivity in liver homogenates at various times after the injection o $^{45}Ca^{++}$. Technical details in the Methods section.

the heavy microsomes and in the final supernatant. The heavy microsomes fraction is composed mainly of vesicles of the endoplasmic reticulum, and it can therefore be considered together with the normal microsomes. About 25% of the total radioactivity was recovered in the residue fraction. The residue contains not only nuclei, but also broken and unbroken cells, mitochondria, and lysosomes, as well as some microsomes, and it is therefore not useful to discuss it as an individual fraction. Resuspension, rehomogenization, and washing of the residue decreased the radioactivity considerably, presumably because of the removal of highly labeled mitochondria. Since the residue was not routinely resuspended, rehomogenized, and washed, it is probable that the total radioactivity figures for the mitochondrial fraction were somewhat underestimated under these experimental conditions. Mito-

[1] Similar results were obtained using $^{89}Sr^{++}$.

chondria concentrate the radioactivity, on a protein basis, to values that may be more than twice those of the unfractionated homogenate.

Data on the specific activity of the total Ca^{++} in the different subcellular fractions are also presented in Table I. The specific activity of Ca^{++} in the mitochondrial fraction was 4 to 20 times higher than that of microsomes, but that of the final supernatant was very low. The specific activity of the Ca^{++} associated with the heavy microsomes was also found to be much lower than that of mitochondria.

A preliminary investigation of the distribution of the radioactivity among the different cellular subfractions at various times after the injection of the isotope has indicated that the distribution of $^{45}Ca^{++}$ is probably independent

TABLE II

DISTRIBUTION OF $^{45}Ca^{++}$ AMONG SUBCELLULAR FRACTIONS FROM THE LIVERS OF PCP-TREATED RATS

Fraction	Distribution	Relative content of $^{45}Ca^{++}$ (Activity in homogenate = 1.00)
	%	
Residue	21.5±4.33 (3)	0.93±0.17 (3)
Mitochondria	22.2±2.40 (3)	0.81±0.02 (3)
Heavy microsomes	12.8±1.65 (3)	2.12±0.05 (3)
Microsomes	30.3±0.46 (3)	2.13±0.32 (3)
Supernatant	13.2±0.52 (3)	0.41±0.03 (3)

$^{45}Ca^{++}$ injected 3 min after the injection of PCP, and 6 min before the rat was killed. Other technical details in the Methods section. Results are given ± standard error. The number of experiments is given in parentheses.

of time within a 60 min period, with the possible exception of a rise in the microsomal fraction during the first 5 min after the injection.

$^{45}Ca^{++}$ in the Subcellular Fractions from the Livers of Rats Injected with PCP and DNP It has been shown by Buffa et al. (18) that mitochondria isolated from the livers of rats injected intraperitoneally with 20–60 mg PCP per kg body weight showed lowered P:O ratios in in vitro tests of the efficiency of oxidative phosphorylation. Uncoupling was found to be complete 15 sec after the injection; however, P:O ratios and the acceptor control ratios began to recover 7–10 min after the injection. 2–3 hr after the injection, the uncoupling had declined to about 30%. The administration of DNP did not cause decline of the P:O ratios (15), probably because DNP was "washed out" of the mitochondria during the isolation. It is likely, however, that uncoupling took place in the intact liver, since there was a marked drop in the content of the high energy phosphate compounds of liver (15). In the experiments reported here, a standard dose of 60 mg of PCP per kg body weight

has been used in order to ensure that uncoupling of rat liver mitochondria was complete for at least 8–10 min after the injection.

As shown in Table II, in the PCP-treated animals the distribution of the radioactivity was markedly altered with respect to the controls. Only about 25% of the total $^{45}Ca^{++}$ was recovered in the mitochondria, while significantly more isotope was found in the total microsome fraction and in the heavy microsomes. However, before it can be concluded that PCP causes uncoupling of energy-linked Ca^{++} accumulation, it is necessary to prove

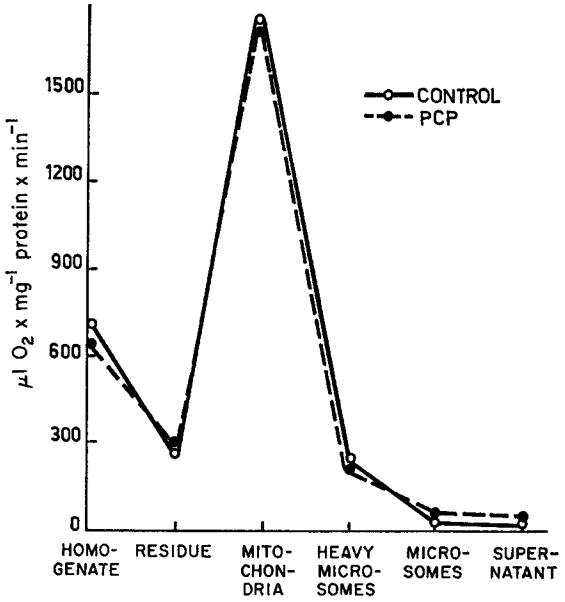

FIGURE 2. Cytochrome oxidase activity in the subcellular fractions of livers from normal and PCP-treated rats. PCP was injected intraperitoneally, and the rats were killed 6 min thereafter. Other technical details are described in the Methods section.

that the results are not due to an alteration in the cell fractionation scheme induced by the presence of PCP. This possibility was tested in three independent ways. The total yield of protein in each of the various subcellular fractions from PCP-treated rats was found not to differ from that observed in the control fractions isolated in the absence of PCP. In particular, approximately the same amount of total mitochondrial and microsomal protein was obtained in the two cases. A large number of experiments gave the following averages: for the controls, 45.8 = 0.07[2] mg of mitochondrial protein and 28.1 ± 2.90 mg of microsomal protein per g of liver; for the PCP-treated rats, 49.5 ± 1.32 mg of mitochondrial protein and 27.3 ± 2.55 mg of microsomal protein. Fig. 2 gives additional evidence that no change in the frac-

[2] Standard error.

tionation was induced by PCP. It shows a profile of the total cytochrome oxidase activity, taken as a mitochondrial marker, in the subcellular fractions from the livers of normal and PCP-treated rats; the profile is identical in the two cases. Finally, electron microscopic examination of the various pellets

TABLE III

$^{45}Ca^{++}$ IN SUBCELLULAR FRACTIONS FROM THE LIVER OF A DNP-TREATED RAT

Fraction	Distribution		Relative content of $^{45}Ca^{++}$ (Activity in homogenate = 1.00)	
	Control	DNP	Control	DNP
	%	%		
Residue	17.9	23.6	0.75	0.73
Mitochondria	57.8	24.3	2.16	0.85
Heavy microsomes	3.7	10.6	0.74	1.70
Microsomes	18.9	30.0	0.91	1.94
Supernatant	1.7	11.4	0.04	0.26

Technical details are given in the Methods section. DNP (60 mg per kg body weight) injected 9 min before the rat was killed. $^{45}Ca^{++}$ was injected 6 min before the animal was killed.

obtained from the livers of normal and PCP-treated rats also showed that the gross morphological appearance of the pellets from the livers of the control and of the treated animals was the same.

It appears therefore permissible to conclude that the observed alterations in the $^{45}Ca^{++}$ distribution pattern following injection of PCP are not a re-

TABLE IV

TOTAL Ca^{++} IN MITOCHONDRIA FROM THE LIVERS OF DNP-TREATED RATS

Experiment	Mitochondrial Ca^{++}	
	Control	DNP
	nmoles/mg protein	
49	31.6	9.9
50	33.8	9.3

Technical details are given in the Methods section. DNP (60 mg/kg body weight) was iniected intraperitoneally 6 min before the animal was killed.

flection of artifactual changes in the mitochondrial fraction, but rather that they show that the mitochondria from livers from PCP-treated rats have failed to accumulate Ca^{++}.

Similar results were obtained when DNP, instead of PCP, was injected, as shown in Table III. A sharp diminution of the total mitochondrial Ca^{++} was also observed in the livers of DNP-treated rats (Table IV).

TABLE V

$^{89}Sr^{++}$ IN SUBCELLULAR FRACTIONS FROM THE LIVER OF A PCP-TREATED RAT

Fraction	Distribution		Relative content of $^{89}Sr^{++}$ (Activity in homogenate = 1.00)	
	Control	PCP	Control	PCP
	%	%		
Residue	23.3	18.8	0.84	0.89
Mitochondria	45.5	25.2	1.95	0.87
Heavy microsomes	7.8	8.1	1.41	1.89
Microsomes	19.4	30.5	1.45	2.32
Supernatant	4.0	16.1	0.18	0.59

Technical details given in the Methods section. The rat was killed 5 min after the injection of $^{89}Sr^{++}$. PCP was injected 3.5 min before the injection of $^{89}Sr^{++}$.

Distribution of $^{89}Sr^{++}$ among Liver Subcellular Fractions: Effect of PCP Data presented in Table V show that the distribution of injected $^{89}Sr^{++}$ among liver subcellular fractions is very similar to that of $^{45}Ca^{++}$. The treatment with PCP causes a very marked diminution in the amount of radioactive strontium which is recovered in the mitochondria, and a concomitant increase in the heavy microsomes, in the microsomes, and in the final supernatant. Thus the effect of PCP on the distribution of $^{89}Sr^{++}$ is practically the same as in the case of $^{45}Ca^{++}$. Preliminary experiments on five rats shown in Fig. 3 indicate that the distribution of $^{89}Sr^{++}$ among the subcellular frac-

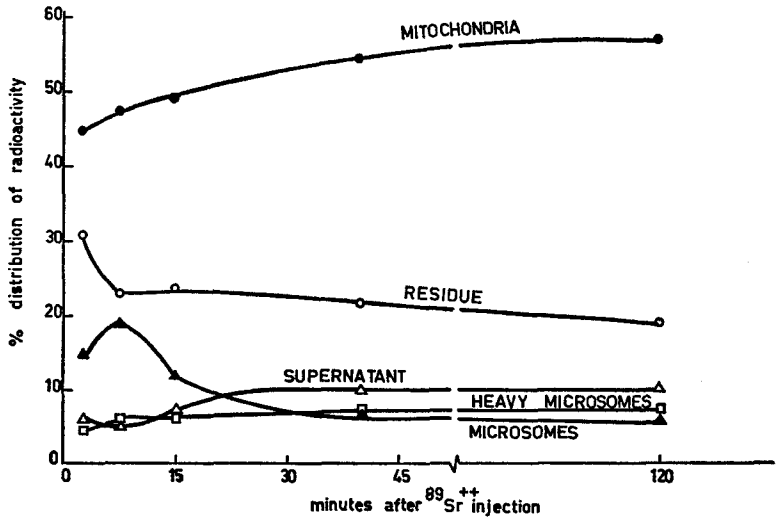

FIGURE 3. Distribution of radioactivity among liver subcellular fractions at various times after injection of $^{89}Sr^{++}$. Technical details in the Methods section.

tions does not show any important variation in the period between 15 and 120 min after the injection. During the first 15 min, however, the distribution pattern shows variations, particularly in the residue and in the microsomes. A more extensive investigation of the intracellular distribution pattern of $^{89}Sr^{++}$ in the minutes immediately following injection is presently being carried out in this laboratory.

Relationship between the Extent of Uncoupling Induced by PCP and the Concentration of $^{89}Sr^{++}$ in Liver Mitochondria Complete uncoupling of oxidative phosphorylation by injected PCP is required to reduce the level of $^{89}Sr^{++}$ in the mitochondrial fraction to that of the whole homogenate. This is shown

TABLE VI

EFFECT OF VARIOUS DEGREES OF UNCOUPLING OF THE $^{89}Sr^{++}$ DISTRIBUTION IN RAT LIVER MITOCHONDRIA

	Respiratory control index*	% $^{89}Sr^{++}$ in mitochondria (Homogenate = 100%)	Concentration of $^{89}Sr^{++}$ in mitochondria (Homogenate = 1.00)
Control	6.2	48.8	1.87
PCP 30 mg/kg	2.6	50.5	1.84
PCP 60 mg/kg	1.0	25.2	0.87

The animals were killed 8.5 min after the injection of PCP. $^{89}Sr^{++}$ was injected 5 min before the animal was killed.

* Respiratory control was measured polarographically, in a medium containing 10 mM Tris-Cl, pH 7.5, 5 mM Na malate, 5 mM Na glutamate, 80 mM NaCl, 10 mM inorganic phosphate, and 5 mg mitochondrial protein. Final volume, 1.8 ml. Temperature, 25°C. 250 μM ADP was added to initiate state 3 respiration.

by data collected in Table VI. Various amounts of PCP were injected into rats and the degree of uncoupling in the isolated mitochondria was checked polarographically. The data show that, when mitochondria have only partially lost the respiratory control, they are still able to concentrate the isotope to approximately the same values as the controls. However, when respiratory control is completely lost, which occurred at doses of 60 mg PCP per kg, then no accumulation of Ca^{++} occurs in the mitochondrial fraction.

Effect of DNP on the Stability of the Intramitochondrial Ca^{++} Early experiments of Vasington and Murphy (1) demonstrated that DNP specifically discharges endogenous Ca^{++} from isolated rat liver mitochondria. In Fig. 4 it is seen that DNP added in vitro to mitochondria isolated from livers of $^{45}Ca^{++}$-injected rats discharged into the medium about two-thirds of the $^{45}Ca^{++}$ accumulated by liver mitochondria. The discharge was very rapid and approached completion in about 5 min. The remaining one-third of the

mitochondrial $^{45}Ca^{++}$ was not discharged by DNP when the incubation was prolonged. On the other hand, $^{45}Ca^{++}$ in mitochondria from livers of DNP-treated rats (or PCP-treated rats) is much more stable to incubation with DNP than the $^{45}Ca^{++}$ in normal mitochondria; only negligible amounts of radioactivity were discharged during the 60 min incubation period. On the

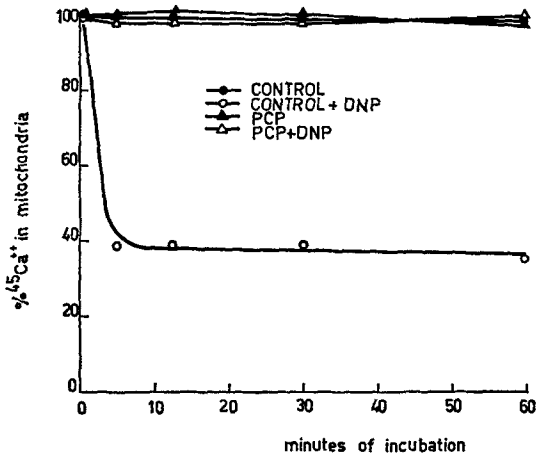

FIGURE 4. DNP-induced discharge of intramitochondrial Ca^{++} in vitro. Mitochondria were isolated from the liver of a normal rat and of a rat killed 6 min after intraperitoneal injection of 60 mg per kg body weight PCP. Both rats received an intraperitoneal injection of 10 μc $^{45}Ca^{++}$ 3 min before death. DNP-induced $^{45}Ca^{++}$ discharge was followed in the following manner: mitochondria from both rats were suspended in 0.25 M sucrose, at a concentration of 2 mg protein per ml sucrose. 30 ml aliquots of the suspensions from each liver were pipetted into 100 ml beakers and placed at 25° C in a Dubnoff shaker. Two beakers were run for each rat. Immediately after the beginning of the incubation, a 5 ml sample was removed from each beaker; immediately thereafter, 10^{-4} M DNP was added to one of the control and one of the PCP beakers. The incubation was carried on, and 5 ml samples were removed at the times indicated in the figure. The samples were immediately transferred to centrifuge tubes placed on ice, and centrifuged at 30,000 *g* for 4 min. Pellets were dissolved in 5 ml 0.2% Na laurylsulfate. Aliquots of the dissolved pellets and of the clear supernatants were analyzed for $^{45}Ca^{++}$ as described in the Methods section.

other hand chemical determinations of total Ca^{++} (see Table IV) have shown that liver mitochondria from PCP- and DNP-treated rats contained only about 30–35% as much Ca^{++} as the controls. These experiments suggest the existence of two pools of Ca^{++} in rat liver mitochondria: a labile pool, sensitive to uncouplers of oxidative phosphorylation, and a stable pool that is insensitive to these agents.

Effect of DNP on the Uptake of $^{45}Ca^{++}$ by Rat Liver Mitochondria and Microsomes In Vitro As seen in Tables II and III, the effect of PCP or DNP in-

toxication is to decrease the amount of $^{45}Ca^{++}$ in mitochondria, and to increase its amount in microsomes and in the heavy microsome fractions. This effect could be explained by assuming a competition between the mitochondria and the endoplasmic reticulum of the liver cell. An uncoupler of oxidative phosphorylation, by putting mitochondria out of play, could permit more Ca^{++} to be accumulated by the endoplasmic reticulum. The experiment reported in Table VII is an attempt to reproduce in vitro the in vivo situation, by studying the uptake of $^{45}Ca^{++}$ in a system containing both isolated mitochondria and microsomes. It is seen that, when no DNP was present, by far the largest proportion of the added $^{45}Ca^{++}$ was recovered in mitochondria, with only negligible amounts of it being found in the microsomes. When DNP was added, the amount of $^{45}Ca^{++}$ in mitochondria dropped markedly, while the amount found in microsomes increased about three

TABLE VII

COMPETITION IN VITRO BETWEEN MITOCHONDRIA AND MICROSOMES FOR Ca^{++} UPTAKE

System	Cpm in mitochondria		% uptake		Cpm in microsomes		% uptake	
	−DNP	+DNP	−DNP	+DNP	−DNP	+DNP	−DNP	+DNP
Mitochondria + microsomes	14,960	8,170	92.0	45.4	218	585	0.10	0.30

The reaction medium contained 10 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 80 mM NaCl, 10 mM Na succinate, 10 mM inorganic phosphate, 3 mM ATP, 10 mg mitochondrial and 10 mg microsomal protein. 0.15 mM $CaCl_2$ labeled with $^{45}Ca^{++}$ was added last. DNP concentration was 0.1 mM. Volume, 10 ml. Temperature, 25°C. After 5 min the tubes were quickly cooled to 0°C, and centrifuged at 15,000 *g*/10 min to separate mitochondria. The tubes were then centrifuged at 150,000 *g*/45 min to collect microsomes. The pellets were dissolved with Na lauryl sulfate and treated as described in the Methods section.

times. The in vitro findings are thus in agreement with the in vivo results, although the absolute amounts of radioactivity recovered in the isolated microsomal fraction are very small, in agreement with the observation (6) that isolated liver microsomes do not actively accumulate appreciable amounts of Ca^{++}.

DISCUSSION

The results reported in the present paper show that the largest part of the Ca^{++} present in the liver cell is associated with the insoluble subcellular components, particularly mitochondria and microsomes. The Ca^{++} concentration in the soluble hyaloplasm is rather low; it appears likely that Ca^{++} in the soluble phase is kept very low by the very high affinity of mitochondria (and, possibly, microsomes) for Ca^{++}. It may be recalled here that studies with isolated mitochondria have shown that their respiration-dependent Ca^{++}

uptake process is capable of keeping the extramitochondrial Ca^{++} concentration below 10^{-6} M (14).

After injection of $^{45}Ca^{++}$, the different pools of Ca^{++} which are present in the subcellular fractions do not become labeled to the same extent. The specific activity of Ca^{++} in mitochondria following injection of $^{45}Ca^{++}$ is several times greater than that of microsomes. These results indicate that in the liver cell mitochondria are much more active than the endoplasmic reticulum in taking up Ca^{++}, in agreement with the finding that liver mitochondria in vitro can accumulate Ca^{++} at a very high rate (1, 6), while isolated liver microsomes lack the capacity to accumulate Ca^{++} (6). In contrast with the high specific activity of mitochondrial Ca^{++}, the specific activity of Ca^{++} in the soluble hyaloplasm was very low. Therefore, $^{45}Ca^{++}$ coming from the extracellular space must enter mitochondria without prior equilibration with the Ca^{++} present in the soluble cytoplasm.

The results obtained when the rats were intoxicated with uncouplers of oxidative phosphorylation indicate that in the liver cell the uptake of $^{45}Ca^{++}$ by mitochondria is specifically inhibited by uncouplers of oxidative phosphorylation, in agreement with the results obtained with isolated liver mitochondria (1, 2, 6). It can thus be concluded that the mechanisms for Ca^{++} uptake discovered and characterized in mitochondria in vitro are in all likelihood operating in vivo as well. However, the results reported have shown that some Ca^{++} enters mitochondria even when the oxidative phosphorylation is completely uncoupled. Therefore, the uptake of $^{45}Ca^{++}$ by mitochondria in vivo is apparently only partially dependent on oxidative phosphorylation. This finding may be accounted for by the fact that the absolute amounts of Ca^{++} that mitochondria take up in vivo in the experiments described in the present paper are exceedingly small when compared to the preexisting intramitochondrial Ca^{++}. It must be recalled that the total amount of Ca^{++} injected into the rats was less than 0.1 μmole, of which probably only a part entered the liver. Possibly, part of the $^{45}Ca^{++}$ enters the mitochondria in vivo via an exchange reaction with the intramitochondrial Ca^{++}, rather than by way of an active uptake process. Actually an exchange between the intra- and extramitochondrial Ca^{++} pools has been observed to occur in rat liver mitochondria in vitro.[3] Such an exchange would not necessarily be blocked by uncoupling agents.

From a functional standpoint, it appears evident that the different pools of Ca^{++} present in the subcellular organelles are not equivalent. The data reported in Fig. 4, in agreement with the in vivo data of Table IV, further suggest that the mitochondrial pool of Ca^{++} is also not homogeneous. DNP

[3] Carafoli, E., and A. L. Lehninger. Unpublished experiments.

(or PCP) discharges very rapidly about two-thirds of the intramitochondrial Ca^{++}, but the remaining one-third is not readily discharged. It may be pointed out here that chelating agents like EDTA have been found to discharge rapidly only one-half to two-thirds of the endogenous Ca^{++} from rat liver mitochondria in vitro.[3] Perhaps only two-thirds of the mitochondrial Ca^{++} is labile, and thus rapidly available for the metabolic needs of the cell. As for the remaining one-third, it could be located in different compartments in mitochondria, out of reach of the uncoupling agents, but possibly available to other, completely different, discharge-promoting agents. Alternatively, a part of the mitochondrial Ca^{++} could be sequestered as insoluble Ca phosphate, with which free Ca^{++} is in a sluggish equilibrium. Whatever the type of compartmentation, the portion of the mitochondrial Ca^{++} that is not discharged by uncoupling agents does become labeled with $^{45}Ca^{++}$ in vivo.

A competition for free Ca^{++} probably exists in the liver cell between energy-dependent accumulation mechanisms in mitochondria and absorption by the endoplasmic reticulum. Mitochondria under normal conditions accumulate the larger part of the available $^{45}Ca^{++}$ (they are capable of removing Ca^{++} from the medium to concentrations as low as 1 μM). When they are put out of play by an uncoupling agent, endoplasmic reticulum can take up a larger proportion of Ca^{++}. It is suggested here that uptake of Ca^{++} by the endoplasmic reticulum is not energy-dependent, since it is known that isolated liver microsomes do not possess any significant capacity for energy-dependent Ca^{++} accumulation (6). The smaller absolute percentage of radioactivity entering isolated microsomes in the in vitro experiment of Table VII as compared with the much greater percentage of radioactivity that is found associated with the structures of the endoplasmic reticulum in vivo, can possibly be explained on the basis of the severe structural degradation of the endoplasmic reticulum during the isolation procedure. Possibly, liver endoplasmic reticulum can accumulate large amounts of Ca^{++}, but isolated liver microsomes cannot. It is nevertheless unexpected that liver endoplasmic reticulum is so active in taking up Ca^{++} in vivo. This finding is under further examination.

The observations with $^{89}Sr^{++}$ deserve some special comment. The distribution of injected $^{89}Sr^{++}$ in the liver cell is similar to that of $^{45}Ca^{++}$. The effect of uncoupling agents on the distribution of $^{89}Sr^{++}$ is the same as in the case of $^{45}Ca^{++}$. In analogy with the results obtained in vitro (5), the uptake of Sr^{++} by the mitochondria of the intact liver cell is therefore also dependent on oxidative phosphorylation. Thus mitochondria in the liver cell are probable sites for the accumulation and transitory sequestration of Sr^{++}, a possibility already suggested in an earlier study of the energy-dependent uptake of Sr^{++} by isolated rat liver mitochondria (19).

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