Sodium-dependent calcium efflux from rat liver mitochondria has been studied as a function of mitochondrial calcium loads (2 to 40 nmol/mg) and extramitochondrial sodium concentrations (5 to 40 mM). The resulting data can be fit to a terreactant model which exhibits simultaneous kinetics (i.e. both sodium and calcium must be bound simultaneously for transport to occur). The Hill coefficients for the calcium and sodium dependences were 1.0 ± 0.1 and 2.0 ± 0.2, respectively. The cooperativity of the sodium dependence allows the terreactant model to be reduced to a bireactant model in which the sodium concentration only appears mathematically as the square of the sodium concentration. The data then fit the relationship

\[ V = V_{\text{max}} \frac{[\text{Na}]^2}{K_{\text{Na}} + [\text{Na}^+]} \frac{[\text{Ca}]}{K_{\text{Ca}} + [\text{Ca}]} \]

The experimentally determined value of \( V_{\text{max}} \) is found to be 2.6 ± 0.5 nmol/mg/min, and the load of calcium (\( K_{\text{Ca}} \)) and concentration of sodium (\( K_{\text{Na}} \)) necessary to stimulate the efflux to half its maximal sodium-dependent activity and sodium-dependent activity, respectively, were 8.1 ± 1.4 nmol of \( \text{Ca}^{2+} \)/mg and 9.4 ± 0.6 mM Na⁺. This sodium-dependent calcium efflux from liver mitochondria was inhibited by magnesium, by ruthenium red, and by tetraphenylphosphonium. Fifty percent inhibition was obtained at 1.0–1.5 mM magnesium, at 12 nmol of ruthenium red/mg of protein, and at 0.2 µM tetraphenylphosphonium.

Addition of sodium at concentrations of 10 mM or greater to a suspension of calcium-loaded mitochondria from heart, brain, or skeletal muscle is known to stimulate calcium efflux at rates of 10 nmol/mg/min or more (1–3). Until recently, calcium efflux from liver mitochondria has been considered to be insensitive to sodium addition (4–6). Recently, a low activity of sodium-dependent calcium efflux has been reported in liver mitochondria (7, 8). This sodium-dependent efflux has been found to show selectivity for sodium or lithium (9) and to show a sigmoidal dependence on sodium concentration (10).

External sodium has been shown to alter the set-point of calcium distribution across the inner membrane of liver mitochondria, i.e. addition of sodium to the suspension causes the steady state to shift to a higher extramitochondrial calcium concentration (11, 12). It has been proposed that the sodium-dependent calcium efflux system is hormonally sensitive and this plays an important role in cytosolic calcium regulation. There is evidence that this process is activated by both β-adrenergic agents and by glucagon (13, 14).

Almost no information on the kinetics of sodium-dependent calcium efflux from liver mitochondria is available in the literature. More is known, however, about the characteristics of the sodium-dependent calcium efflux mechanism of heart mitochondria, which may or may not be a similar mechanism. In heart mitochondria, lithium can elicit calcium efflux although it is much less effective than sodium (1). This mechanism can exchange intramitochondrial \( \text{Ca}^{2+} \) for extramitochondrial calcium or strontium (2). Both this exchange process and sodium-dependent calcium efflux can be inhibited by lanthanum but not by ruthenium red. The heart mechanism was originally reported to depend upon the cube of the external sodium concentration (1) and later reported to depend upon the square of the sodium concentration (2).

Based on observations of \( \text{TPP}^+ \) distribution, sodium-dependent calcium efflux of heart mitochondria has been reported to be electroneutral (15). However, it has also been suggested (16) that, regardless of the calcium/sodium stoichiometry, membrane potential would not be expected to vary, due to charge compensation from the electron transport chain. Other properties include inhibition by magnesium (17), diltiazem, amiloride, and amiloride analogs (18, 19).

Although the sodium-dependent efflux mechanism has been widely described as a sodium-calcium exchange mechanism, no compelling evidence exists to support this contention. It has also been proposed that the mechanism may be an exchange of calcium for protons, in which case sodium acts as an activator and is not stoichiometrically transported (3). Heart mitochondria possess a very active sodium/proton exchanger and thus the sodium and hydrogen ion gradients would equilibrate by either type of mechanism.

Some groups have observed sodium-dependent calcium efflux from liver mitochondria (7–9), while others have not (4–6). This suggests that commonly used components of mitochondrial suspending media might inhibit this mechanism. In the present study, the kinetics of this sodium-dependent mechanism were studied and the effects of several types of inhibitors, some of which are common components of the suspending media in mitochondrial experiments, were investigated.

**MATERIALS AND METHODS**

Procedures and media for the isolation of mitochondria, depletion of calcium, measurement of endogenous calcium, and measurement

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The abbreviation used is: TPP, tetraphenylphosphonium.
Sodium-dependent Mitochondrial Calcium Efflux

The rate of sodium-dependent calcium efflux was studied as a function of the calcium load at a number of different external sodium concentrations. A Hill plot of efflux as a function of sodium concentration for a fixed calcium load (data not shown) yields a slope of 2.0 ± 0.2 suggesting that efflux depends on the square of the sodium concentration. This is similar to the more recent reports on the sodium-dependent calcium efflux mechanism in brain and heart mitochondria (2, 4). A Hill plot for data at a fixed sodium concentration, while varying the calcium load, yields a slope of 1.0 ± 0.1 suggesting simple Michaelis-Menten behavior for the calcium dependence (data not shown).

As suggested by the Hill plots, the plot of efflux versus (efflux/calcium load) (Fig. 1a) forms a straight line for any fixed concentration of sodium and a plot of efflux versus (efflux/sodium squared) forms a straight line for any fixed calcium concentration (Fig. 1b). Note that each set of points at a given sodium concentration in Fig. 1a is nearly parallel to the lines formed by points at other sodium concentrations.

**RESULTS**

**Observation of Sodium-induced Calcium Efflux**

Fig. 1 of the accompanying paper (20) shows a typical efflux trace in which sodium causes a clear enhancement of efflux. The same sample was used for measurement of efflux, first in the absence and then in the presence of sodium. In that way any abnormally high sodium-independent rate could be discovered and that sample discarded. The efflux values obtained by this method agree with the values obtained when sodium-independent and sodium-dependent rates are measured in separate cuvettes, i.e., the same total efflux rate is achieved if the sodium-dependent cuvette has NaCl added prior to the addition of the mitochondria. In agreement with Heffron and Harris (9), the increase in efflux is relatively specific for sodium. Lithium will elicit some increase in efflux rate while potassium and tetramethylammonium are ineffective (data not shown).

As with the study of sodium-independent efflux, if accurate rates of sodium-dependent efflux are to be obtained, the calcium-induced membrane transition must be avoided. If the sodium-independent efflux rate is approximately 2 nmol/mg-min or higher, the increase in efflux following sodium addition was not as pronounced. At a sodium-independent efflux rate of above approximately 3 nmol/mg/min, the sodium-induced increase in efflux was difficult to observe (data not shown). Apparently, either the transformed mitochondria lost their ability to couple efflux of calcium to sodium movements or this sodium-dependent efflux was masked by a much greater transition-induced efflux. Consequently, the calcium depletion procedures and medium described in the previous study were employed in order to keep transition-induced efflux (see Refs. 20 and 22) at a negligible rate. Under these conditions for measurement of efflux, the membrane potential remained at 150 mV or higher.
bound, transported, and released, and the second substrate is
external sodium concentration in mM. The value of the pa-
taneous mechanism yields nonparallel lines. In this case the
perform this test is to display the data in a Lineweaver-Burk
plot since for this type of plot the slope of the line through
the data points represent lines of best fit to all the data points simul-
taneously using Equation 1.

**Evidence for Simultaneous Kinetics**

There are two fundamentally different models that can be
used to describe how a two-substrate transport mechanism
operates. The first of these models is known as a simultaneous
model, in which both substrates must be bound simultane-
ously to the transporter in order for transport to occur. The second is the ping-pong model, in which one substrate is
bound, transported, and released (23, 24).

As a test to see which of these mechanisms applies, the
ratio of apparent $K_a$ to apparent $V_{max}$ can be calculated for
the dependence on the first substrate while temporarily hold-
ing the second substrate constant. For a ping-pong model this
ratio will be independent of the concentration of the second
substrate. The ratio will vary with concentration of the second
substrate for a simultaneous model (25). The easiest way to
perform this test is to display the data in a Lineweaver-Burk
plot since for this type of plot the slope of the line through
each set of points is equal to the ratio of: $V_{max(apparent)}/
V_{max(apparent)}$. Such a plot is shown in Fig. 2. A ping-pong
mechanism would imply parallel lines in Fig. 2 while a simulta-
neous mechanism yields nonparallel lines. In this case the
lines converge to a point on or nearly on the negative abscissa.

The data can be fit very closely with the following formula:

$$V = V_{max} \frac{[Ca]}{K_{Ca} + [Ca]} \frac{[Na]^2}{K_{Na} + [Na]}$$

(1)

where $V$ is the sodium-dependent efflux rate in nmol/mg/
min; $[Ca]$ is the calcium load in nmol/mg; and $[Na]$ is the
external sodium concentration in mM. The value of the pa-
rameters averaged over three experiments is: $V_{max} = 2.6 \pm 0.5$
nmol/mg/min; $K_{Ca} = 8.1 \pm 1.4$ nmol/mg; and $K_{Na} = 9.4 \pm 0.6$
mM.

According to the results of Coll et al. (21), this value of $K_{Ca}$
would correspond to an intramitochondrial free calcium con-
centration of 8.5 $\mu$M. Consistent with the interpretation of
this data as initial slope data, medium calcium concentrations
at the beginning of each experiment are far below this value of
$K_{Ca}$. These results should then be considered essentially
independent of the exact level of internal sodium and external
calcium.

**Inhibition of Sodium-dependent Calcium Efflux**

**Magnesium Inhibition**—A number of laboratories, includ-
ing this one, have had difficulty observing the sodium dependence of efflux in liver mitochondria. This sug-
gested that one or more of the substances commonly added
to mitochondrial suspensions may inhibit this mechanism. Magnesium ion inhibits the sodium-dependent calcium efflux
mechanism in brain mitochondria (17) and, as Fig. 3 shows,
magnesium also inhibits this mechanism in liver mitochon-
dria. Magnesium affects the sensitivity of the arsenazo III
dye. Thus, to avoid the necessity of recalibrating the absorb-
ance of the dye, efflux rates were measured using calcium-45
and filtration techniques. Fifty percent inhibition of sodium-
dependent calcium efflux occurred at a magnesium concentra-
tion of 1.0–1.5 mM. The sodium-independent efflux rate was
not significantly affected over this range of magnesium con-
centrations.

**Ruthenium Red Inhibition**—Another commonly added sub-
stance that inhibits the sodium-dependent calcium mecha-
nism is ruthenium red (which inhibits the uniporter), as
shown in Fig. 4. This inhibitor is usually used in the concen-
tration range of 2–5 $\mu$mol/mg mitochondrial protein (i.e. 2–5
$\mu$M). In this range ruthenium red completely blocks uptake
but has a relatively small effect on sodium-dependent or
sodium-independent efflux. As the ruthenium red concentra-
tion is raised above 5 nmol/mg, it increasingly inhibits so-
dium-dependent efflux. Fifty percent inhibition occurs at
approximately 12 nmol/mg ruthenium red.
has little effect on the sodium-independent efflux mechanism over the concentration range tested (0.5–20 nmol/mg). The apparent inhibition of both types of efflux at the lowest concentration of ruthenium red, 0.5 nmol/mg, is possibly an artifact resulting from incomplete inhibition of the uniport mechanism and consequent reuptake of calcium.

The nature of the ruthenium red inhibition was investigated. In one set of experiments the sodium concentration was held constant while the calcium load was varied at two different concentrations of ruthenium red. The results suggested uncompetitive inhibition with calcium (data not shown). In another set of experiments, the calcium load was held constant while varying the sodium concentration at three different ruthenium red concentrations. The results of this experiment suggest noncompetitive inhibition with sodium. Thus, ruthenium red inhibition of sodium-dependent calcium efflux is not competitive with either sodium or calcium but appears to be a mixed type inhibition.

**Inhibition of TPP and TPP Analogs**—Early efforts to measure sodium-induced efflux while simultaneously measuring mitochondrial membrane potential using a TPP electrode were unsuccessful. Whenever this was attempted, the stimulation of calcium efflux by sodium failed to occur. The reason for this became clear when the sodium-dependent efflux rate was studied as a function of TPP concentration (Fig. 5). TPP is an extremely potent inhibitor of sodium-dependent calcium efflux. Fifty percent inhibition occurs at a TPP concentration of approximately 0.2 μM. Inhibition is essentially complete at TPP concentrations above approximately 2–3 μM. Sodium-independent efflux is also inhibited by TPP, although to a much lesser extent. Fifty percent inhibition of this mechanism occurs at a TPP concentration of approximately 10 μM. Two other compounds closely related to TPP were also tested for their ability to inhibit sodium-dependent efflux: triphenylmethylphosphonium and tetraphenylarsonium (data not shown). Tetraphenylarsonium has approximately the same inhibitory effects as TPP, while triphenylmethylphosphonium requires a concentration roughly 10 times higher to achieve equal inhibition.

Studies concerning the nature of the TPP inhibition were conducted in the same manner as the ruthenium red inhibition study. Efflux rates in the absence of TPP were compared with those at the 50% inhibitory concentration of 0.2 μM (data not shown). As with ruthenium red the inhibition was not a simple competitive one. It appeared to be a mixed type inhibition with a dominant uncompetitive nature.

**DISCUSSION**

The data were consistent with a simultaneous model and were inconsistent with a ping-pong model. Consequently, only models in which both calcium and sodium must be bound simultaneously for transport to occur will be considered. Given that sodium-dependent calcium efflux is usually thought of as an n-sodium/lcalcium exchange mechanism, a ping-pong mechanism might at first have seemed more likely. Ping-pong kinetics are consistent with diffusible carriers and "flip-flop" mechanisms of gated pores. However, the existence of simultaneous kinetics is neither inconsistent with an exchange mechanism nor without precedent. The sodium-potassium pump of the plasma membrane, for example, has been shown to exhibit simultaneous kinetics with respect to sodium and potassium transport (24). Perhaps a better example would be the adenine nucleotide exchanger of the mitochondrial inner membrane, which has been shown by Barbour and Chan (26) to function as a simultaneous mechanism.

The simplest model to consider is a random order, rapid equilibrium model in which the transport step is rate-limiting. This model also ignores trans effects, i.e. it assumes a given substrate is on only one side of the membrane. The absence of trans effects is probably a condition that is met, for the most part, in this study. The mitochondria were washed thoroughly in a sodium-free medium and were stored in sodium-free media until the initiation of sodium-dependent calcium efflux.

Also, prior to the initiation of efflux, the mitochondria were always allowed to come to a steady state calcium distribution in which the external calcium concentration was typically well below 0.5 μM (as low as 0.2 μM). Even at the lowest calcium load used, the internal free calcium concentration at the lowest calcium loads was greater than 2 μM (as determined by the coefficients of Coll et al. (21)).

The data would suggest that transport involves the binding of 2 sodium and 1 calcium ions. This system would have three possible binding events and thus could be considered as a...
random terreactant system (25). In the case of extremely high cooperativity between two of the substrates, as seen in the data reported here for the two sodium-binding events, this terreactant system simplifies to a random bireactant system in which the sodium concentration enters only as the square. This equation is as follows (24):

\[
V = \frac{V_{\text{max}} [\text{Na}]^{a} [\text{Ca}]}{aK_{\text{Na}}[\text{Ca}] + aK_{\text{Ca}}[\text{Na}] + K_{\text{Ca}}[\text{Na}]^{2} + [\text{Na}]^{a}[\text{Ca}]}
\]

where \( V \), \( V_{\text{max}} \), \([\text{Ca}]\), and \([\text{Na}]\) as are defined before, and \( K_{\text{Na}} \) and \( K_{\text{Ca}} \) represent the dissociation constants for the binding of sodium or calcium, respectively, to the transporter. The factor \( a \) is the cooperativity coefficient, i.e., the factor by which the binding of one substrate changes the dissociation constant for the other substrate. If \( a = 1 \), this equation simplifies to the equation that is shown to fit the data, given under “Results.” While the random order, rapid equilibrium model is certainly not the only model possible, it does fit the data quite well and is a simple, convenient way to describe the data. This model and the resulting equation are consistent with a simultaneous exchange mechanism which transports 2 sodium ions inward for 1 calcium ion outward. It should be mentioned, however, that kinetic data alone are not usually sufficient for a conclusive evaluation of the stoichiometry (27), which should be confirmed through other, more direct methods. Our efforts to measure stoichiometry directly have failed, largely due to a high sodium influx that is not coupled to calcium movements. The possibility then cannot be ruled out that sodium is not transported at all but simply acts as an activator of calcium efflux. However, if this were the case, the energy utilized for calcium transport could not come from the sodium gradient.

The development above leads to a number of possible ways in which the sodium-dependent efflux mechanism could be stimulated as in the case of the \( \beta \)-adrenergic stimulation reported by Crompton et al. (28). Hormone treatment does not appear to cause an increase in \( V_{\text{max}} \), according to the data presented (28). Hormone treatment, however, could change the affinity for the carrier for either sodium or calcium, i.e., cause a decrease in \( K_{\text{Na}} \) or \( K_{\text{Ca}} \). Hormone treatment could also lead to an increase in cooperativity between the binding of sodium and the binding of calcium and thus decrease \( a \).

Ruthenium red has been thought to have no effect on the efflux of calcium, except under conditions in which the mitochondria become de-energized and calcium leaks out through the uniport mechanism. It has been reported that ruthenium red has no effect on the sodium/calcium exchange mechanism of heart mitochondria (2). Previous studies may not have used sufficiently high concentrations of ruthenium red to see inhibition, or the liver mechanism may be different from the sodium/calcium exchange mechanism in heart and brain mitochondria.

Prior to this study, TPP was thought to have little or no effect on mitochondrial transport systems at TPP concentrations below 10 \( \mu \)M. High concentrations have been found to have some adverse effects on the coupling of mitochondria (29), but this is not too surprising given that TPP is a highly lipid permeable cation. With an effective \( K_{\text{i}} \) of 0.2 \( \mu \)M, TPP was an extremely effective inhibitor of sodium-dependent calcium efflux in liver mitochondria. Part of this effectiveness may be due to the ability of mitochondria to concentrate the ion. At a membrane potential of 180 mV, mitochondria will concentrate TPP to an internal concentration 1000 times higher than the external concentration. This implies that, at concentrations yielding 50% inhibition, the internal TPP concentration is roughly 0.1 mM. It would seem likely that the effectiveness of TPP inhibition may be membrane potential-dependent and much higher concentrations of TPP may be required to inhibit this mechanism in de-energized mitochondria.

The inhibition phenomena described in this paper may explain why some studies have failed to observe sodium-dependent calcium efflux in liver mitochondria. Experimental media are often supplemented with 2-5 mM magnesium and this would be sufficient to largely suppress this mechanism. Also, TPP is often used in the range of 5-10 \( \mu \)M in order to measure membrane potential and this is enough to completely inhibit sodium-dependent efflux. While concentrations of ruthenium red that are normally used (1-5 \( \mu \)M) are not very effective at inhibiting efflux, the higher end of this concentration range may have some effect. Triphenylmethylphosphonium is a less effective inhibitor than TPP, but it is often used at concentrations above 2 \( \mu \)M and this can inhibit sodium-dependent efflux by 50% or more. Also, the inhibition by TPP is partially synergistic with the ruthenium red inhibition in that a given concentration of TPP is more inhibitory at 5 nmol/mg ruthenium red than at 2 nmol/mg ruthenium red (data not shown).

A great deal of care should be used when interpreting results whenever TPP or a related compound is present in the medium. Conclusions concerning the quantitative properties of the calcium efflux mechanism of mitochondria cannot be made definitively in the presence of TPP. Furthermore, studies conducted on cellular calcium movements should be interpreted with care if TPP or a related compound is present, since such a study may mask the role that mitochondria play. TPP may interfere with other cellular calcium transport systems, particularly those that involve Na+/Ca\(^{2+}\) exchange. A recent study (2) has found that a concentration of TPP of 1 \( \mu \)M can arrest the growth of up to 80% of HL60 leukemia cells, and 10 \( \mu \)M TPP inhibited the growth of up to 70% percent of V79 Chinese hamster lung fibroblasts. The mitochondria in these cells were found to contain a high number of matrix granules. At least part of this TPP toxicity may be due to interference with mitochondrial calcium efflux.

The properties of mitochondrial calcium efflux described here and in Wingrove and Gunter (20) have interesting implications concerning the distribution and regulation of cellular calcium in liver. The two efflux mechanisms have a combined maximum efflux rate of approximately 4 nmol/min/mg. Data presented earlier (30) show that the uniport mechanism will function at a correspondingly low influx rate only at extramitochondrial calcium concentrations of 0.5 \( \mu \)M or less. Thus, a true steady state of calcium cycling is possible only at or below this concentration. If one assumes a physiological sodium concentration of 10-15 mM and a calcium load of 8-10 nmol/mg, the total calcium efflux would be below 2 nmol/mg/min. At steady state the influx would be equal and this influx corresponds with an extramitochondrial calcium concentration below 0.5 \( \mu \)M (20). These facts, combined with the high capacity of mitochondria to take up calcium and the relatively slow rate of release, make mitochondria an ideal candidate for shaping pulses of intracellular calcium. They would be especially suited for absorbing sudden increases in cytosolic calcium and then releasing this slowly back to the cytosol.

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1 B. D. Jensen, private communication.
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