Disequilibrium between Steady-State Ca^{2+} Accumulation Ratio and Membrane Potential in Mitochondria. Pathway and Role of Ca^{2+} Efflux†

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ABSTRACT: Ruthenium Red inhibits cation^{2+} influx in mitochondria whether driven by respiration or by ion gradients, whether in the absence or in the presence of uncouplers. On the other hand, Ruthenium Red inhibits cation^{2+} efflux from steady-state mitochondria when induced by EDTA and not when induced by respiratory inhibitors or by uncouplers. Active extrusion of cation^{2+} driven by respiration is also sensitive to Ruthenium Red. The sensitivity of cation^{2+} efflux to Ruthenium Red is markedly increased by inorganic anions. It is suggested that the binding of Ruthenium Red at the divalent cation carrier site depends on an electrical field, negative inside. The rate of cation^{2+} efflux induced by Ruthenium Red in steady-state mitochondria increases with ageing and decreases with an increase in bovine serum albumin concentration. Furthermore the rate of cation^{2+} release correlates with the magnitude of respiratory control ratio. This indicates that the Ruthenium Red induced cation^{2+} efflux depends on a H^+ leak rather than on a native H^+/cation^{2+} antiporter. The dimension of the accumulation ratio for cation^{2+} depends on the rates of cation^{2+} influx and efflux. Restriction of the rate of influx by Ruthenium Red, and enhancement of the rate of efflux by A23187, cause a decrease of the cation^{2+} accumulation ratio. The accumulation ratios of divalent cations measured simultaneously correlate with the relative influx rates. It is suggested that the maintenance of a Ca^{2+} accumulation ratio in a range of 10^3 under physiological conditions is obtained by (a) a slow rate of H^+ leak coupled to Ca^{2+} efflux; (b) restriction of the rate of Ca^{2+} influx through a cooperative effect.

Since a number of enzymatic reactions in the cell are Ca^{2+} activated (Rasmussen, 1970; Gompers, 1976), a major problem in cell physiology concerns the regulation of cytoplasmic Ca^{2+} concentration. Mitochondria might play a role since these organelles transport Ca^{2+} in large amounts and with a powerful driving force. Application of the Nernst equation to the distribution of K^+, in valinomycin-treated mitochondria, leads to a $\Delta\psi$ in steady state of 180–200 mV (Rossi and Azzone, 1969; Mitchell and Moyle, 1969; Mitchell, 1976). A distribution of Ca^{2+} at electrochemical equilibrium with a $\Delta\psi$ of 200 mV is incompatible with the maintenance of a physiological Ca^{2+} concentration in the cytoplasm (Azzone et al., 1975). There are two alternatives. Either the driving force is lower than 200 mV, or the Ca^{2+} distribution in steady state is the result of two processes, one of influx and another of efflux (pump and leak). This prompts interest in the mechanism of Ca^{2+} efflux and its role in affecting the Ca^{2+} distribution. Both questions have remained neglected for a period of 16 years since most investigations have been concentrating on the process of Ca^{2+} influx.

Transport of divalent cations through the mitochondrial membrane occurs via a native carrier which catalyzes an electrical flux and is inhibited by La^{3+} (Mela, 1968) and Ruthenium Red (Moore, 1971). The question arises as to whether the pathways for Ca^{2+} influx and efflux are identical. Two cases of cation efflux may be identified: (1) the efflux following the addition of respiratory inhibitors or uncouplers; (2) the efflux following the addition of La^{3+} or Ruthenium Red to steady-state mitochondria (Sordahl, 1974; Stucki and Ineichen, 1974; Pozzan and Azzone, 1976; Puskin et al., 1976). The former process is Ruthenium Red insensitive (Vasington et al., 1972). That the uncoupler-induced cation efflux goes through the native carrier is not questioned. However, uncouplers or respiratory inhibitors do not induce per se insensitivity of cation transport to Ruthenium Red. The latter process is Ruthenium Red insensitive by definition and this may be in accord with the existence of an alternative pathway, i.e., a H^+/cation^{2+} antiporter. However, the Ruthenium Red induced efflux correlates with the electrical H^+ leak (Pozzan and Azzone, 1976).

The present paper analyzes two problems: (a) the pathway for cation efflux induced either by respiratory inhibitors or by Ruthenium Red under steady-state conditions; and (b) the role of cation efflux on the cation distribution. Evidence is presented suggesting that Ca^{2+} efflux occurs always through the native carrier. The sensitivity to Ruthenium Red cannot be taken as a criterion for the existence of an alternative pathway because the interaction of Ruthenium Red seems to vary according to the transmembrane electrical field. Furthermore it will be shown that the cation^{2+} distribution depends on the difference between rates of cation^{2+} influx and efflux. The view is discussed that the disequilibrium between $\Delta\psi$ and Ca^{2+} distribution in vivo is achieved by restricting the rate of Ca^{2+} influx, through a cooperative effect (Bygrave et al., 1971; Vinogradov and Scarpa 1973), in the presence of a small rate of H^+ leak coupled with Ca^{2+} efflux.

Experimental Section

Rat liver mitochondria were prepared as described previously (Massari et al., 1972a). The last washing was carried out in an EDTA-free medium. Mitochondrial protein was determined with the Biuret method. Incubation of mitochondria was carried out in a medium of the following composition: 0.2 M sucrose, 20 mM Hepes (pH 7.2), 2.5 mM succinate–

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Tris, 10 mM acetate–Tris. Changes to this medium were described in the figures.

The medium was extensively bubbled with oxygen before use. Changes of the Ca^{2+} and Mn^{2+} concentrations were followed kinetically, on the basis of the Murexide absorbance at 534–492 nm, in a dual wavelength spectrophotometer made in the workshop of the Physics Institute of the University of Padova. The Murexide absorbance change overlapped with the absorbance change due to swelling of the mitochondrial matrix. Graphic correction was applied for absorbance change, due to swelling, in the murexide unsupplemented sample.

When Ca^{2+} efflux was induced by the addition of chelating agents, the rate of Ca^{2+} efflux was followed, as indicated by Massari et al. (1972b), through the absorbance change at 546 nm in an Eppendorf photometer equipped with a stirring device. The procedure permits transformation of the absorbance changes into rates of ion transport. The validity of the photometric procedure was ascertained by measuring in parallel experiments Ca^{2+} efflux with radioisotopes.

Respiratory rates and respiratory control ratios were routinely assayed on the mitochondrial suspensions by means of a Clark oxygen electrode. In the experiment where the respiratory control ratio was compared with the rate of Ca^{2+} efflux, variations of the respiratory control ratio were obtained either by adding varying amounts of BSA[1] or by letting the mitochondria undergo varying degrees of ageing.

The cation accumulation ratio was measured essentially as described by Azzone et al. (1977). The reaction was initiated through the addition of mitochondria to the complete medium and was terminated by centrifugation in the Rotor S12 of the Sorvall RC2B centrifuge at 20 000 rpm for 5 min. Alternatively the reaction was stopped with a rapid filtration technique through Millipore filters. The rapid filtration method was used to measure the kinetics of KCN induced Ca^{2+} release. $[Cation^{2+}]_o$ was determined on a 100-μL aliquot of the supernatant dissolved in the Bray liquid scintillation medium. $[Cation^{2+}]_i$ was determined on the increase of matrix volume. The matrix volume was determined with a double-labeling technique where the total water was determined with ^3H_2O and the extramitochondrial water with [^{14}C]sucrose. In some experiments the total water was measured gravimetrically. The pellet was dissolved in a liquid scintillation medium containing Triton. $^{45}Ca^{2+}$, ^3H_2O, and [^{14}C]sucrose were measured in a Packard TriCarb 2455 liquid scintillation spectrometer. In the case of simultaneous measurements of Ca^{2+} and Mn^{2+} accumulation ratios, Ca^{2+} was measured isotopically and Mn^{2+} with an absorption spectrophotometer. The measurements were kindly performed in the laboratory of General Physiology of Professor G. Ghiretti. No correction for internal binding was applied in the case of the simultaneous uptake of Ca^{2+} and Mn^{2+}.

The initial rate of Ca^{2+} influx was measured in a dual wavelength spectrophotometer with a stopped-flow apparatus made in the workshop of the Johnson Foundation. The equipment was made available by A. Azzi. The initial rate of Ca^{2+} efflux was measured after addition of Ruthenium Red to steady-state mitochondria. This implies that the rate of Ca^{2+} efflux is not affected by Ruthenium Red (see below).

As discussed previously (Bragadin et al., 1975; Pozzan et al., 1976; Azzone et al., 1977), ESR measurements were carried out with a continuous-flow apparatus which permits good reproducibility and avoids anaerobiosis. The process of active Mn^{2+} uptake leads, in the absence of acetate, to a quenching of the sextet signal of $Mn(H_2O)_6^{2+}$ due to binding to the matrix phospholipids. The extent of binding is about 50 nmol (mg of protein^{-1}). In the absence of acetate, the amount of free Mn^{2+} in the matrix is below 1 nmol (mg of protein^{-1}), which can be neglected (Bragadin et al., 1975). Furthermore the signal amplitude may be so adjusted to avoid any interference by the spin-exchange spectrum. The process of Mn^{2+} uptake and release can be followed, therefore, simply by measuring the height of sextet signal of Mn^{2+} in the external medium which is proportional to the concentration of Mn^{2+} (Cohn and Townsend, 1954). The process of influx leads to a decrease and the process of efflux to an increase of the ESR signal. The kinetics of Mn^{2+} influx and efflux has been measured in the following manner: the field range is shifted and brought to the peak of the first line of absorption of $Mn(H_2O)_6^{2+}$. The "field range" is then switched off so that only the variation of intensity of the Mn^{2+} line is registered as a function of the scanning velocity. The kinetics obtained by this procedure is identical with that obtained by repetitive scanning on the whole Mn^{2+} spectrum at various times.

ESR spectra were carried out with a E-4 Varian spectrometer at X band (9100 mHz). The microwave frequency was 9.5 GHz. The modulation amplitude was 16 G and the recorder time constant was 1 s.

In previous studies, Ruthenium Red has been used either as a crude preparation (Moore, 1971; Vasington et al., 1972; Rossi et al., 1973; Sordahl, 1974; Reed and Bygrave, 1974b; Stucki and Ineichen, 1974; Puskin et al., 1976) or purified according to Luft (1971; Sordahl, 1974; Ackerman et al., 1977) or purified according to Fletcher (1961; Reed and Bygrave, 1974a; Heaton and Nicholls, 1976). Although no basic differences have been reported with respect to the inhibitory effect on divalent cation transport, the specific activity of the purified Ruthenium Red may vary depending on the purification procedure. In our case the compound purified according to Luft (1971) showed a maximum at 533 nm corresponding to Ruthenium Red (Luft, 1971; Fletcher, 1961) and no contamination by Ruthenium Brown and Ruthenium Violet. An additional peak was present in the ultraviolet region at 250 nm corresponding to Nitrosyl Ruthenium. This amounted to about 30% (w/w) of the total. In the present study, the concentration of Ruthenium Red was recalculated on the basis of an ϵ_{max}^{533} of 68 000 (Luft, 1971). Ruthenium Red was obtained from Sigma.

A 23187 was kindly provided by Dr. R. J. Hosley, Lilly Research Laboratories. All chemicals were of analytical grade. All experiments were carried out at room temperature.

Results

The Sensitivity of Cation Efflux to Ruthenium Red. The sensitivity of cation^{2+} efflux to Ruthenium Red is ambiguous. Vasington et al. (1972) observed insensitivity in the case of respiratory inhibitors and uncoupler-induced efflux. Puskin et al. (1976) observed in the case of the uncoupler-induced efflux sensitivity with Mn^{2+} and not with Ca^{2+}. Reed and Bygrave (1974b) and Pozzan and Azzone (1977) observed sensitivity to Ruthenium Red of the EGTA-induced efflux. Pozzan and Azzone (1977) have reported sensitivity of cation efflux to Ruthenium Red during respiration induced extrusion of $Sr(NO_3)_2$. Table I reports a summary of a variety of experimental conditions where influx and efflux of divalent cations were analyzed with respect to their sensitivity to Ruthenium Red. It is seen that the influx of divalent cations was always sensitive to Ruthenium Red, either driven directly by

[1] Abbreviations used: BSA, bovine serum albumin, Tris, tris(hydroxymethyl)aminomethane; ESR, electron spin resonance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone.

TABLE I: Sensitivity of Divalent Cation Transport to Ruthenium Red.

Type of flux	Driving force	Conditions	Ruthenium Red sensitivity
$Cation^{2+}$ influx	Proton pump	Uptake driven by respiration	High[a,b]
$Cation^{2+}$ influx	$[K^+]_i/[K^+]_o$ gradient	Uptake coupled to K^+ efflux	High[a]
$Cation^{2+}$ influx	$[SCN^-]_o/[SCN^-]_i$ gradient	Uptake coupled to SCN^- influx (± uncoupler)	High[c]
$Cation^{2+}$ influx	$[H^+]_i/[H^+]_o$ gradient	Uptake coupled to weak acid influx (+ uncoupler)	High[c]
$Cation^{2+}$ efflux	$[H^+]_o/[H^+]_i$ gradient	Extrusion driven by respiration	High[c]
$Cation^{2+}$ efflux	$[cation^{2+}]_i/[cation^{2+}]_o + [H^+]_o/[H^+]_i$ gradients	EGTA added in steady state	High[c,d] Low[e]
$Cation^{2+}$ efflux	$[cation^{2+}]_i/[cation^{2+}]_o + [H^+]_o/[H^+]_i$ gradients	Uncoupler added in steady state	Low (Ca^{2+})[a,c,e] High (Mn^{2+})[e]
$Cation^{2+}$ efflux	$[cation^{2+}]_i/[cation^{2+}]_o + [H^+]_o/[H^+]_i$ gradients	Respiratory inhibitors added in steady state	Low[a]

[a] Vasington et al. (1972). [b] Moore (1971). [c] Pozzan and Azzone (1977). [d] Reed and Bygrave (1974b). [e] Puskin et al. (1977).

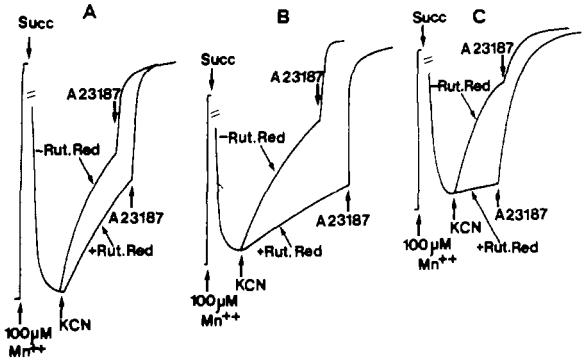

FIGURE 1: Effect of Ruthenium Red on the KCN-induced Mn^{2+} release. (A) The medium contained: 200 mM sucrose, 20 mM Hepes (pH 7), 3 μM Rotenone, 100 μM Murexide, 2 mg/mL mitochondrial protein. (B) As in A except that sucrose was replaced with 100 mM LiCl and 5 mM LiSCN. (C) As in A except that sucrose was replaced with 100 mM $LiNO_3$. Where indicated, the following were added: 100 μM $MnCl_2$, 2.5 mM succinate-Tris, 2.5 mM KCN, 200 pmol/mg protein Ruthenium Red, 1 μg/mg protein A23187. Mn^{2+} concentration was determined with a dual wavelength spectrophotometer as described in the Experimental Section.

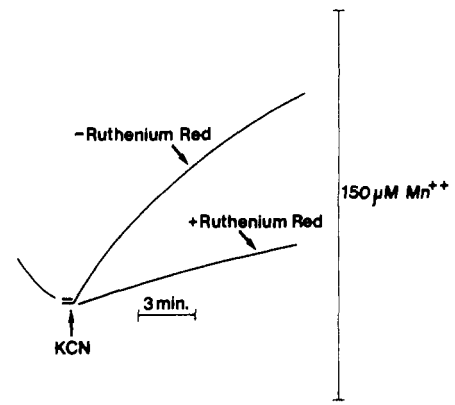

FIGURE 2: Effect of Ruthenium Red on the KCN-induced Mn^{2+} release. Medium was the same as in Figure 1C: 2.5 mM succinate; 300 μM $MnCl_2$; 3 mg/mL mitochondrial protein. Where indicated, 2.5 mM KCN was added. Mn^{2+} concentration was determined with ESR as described in the Experimental Section. Lowering and increasing of the sextet signal of $Mn(H_2O)_6^{2+}$ indicate influx and efflux of Mn^{2+}, respectively. Ruthenium Red was 200 pmol (mg of $protein^{-1}$).

the proton pump or driven by concentration gradients of other ions such as the $[K^+]_i/[K^+]_o$ gradient, the $[SCN^-]_o/[SCN^-]_i$ gradient or the $[H^+]_i/[H^+]_o$ gradient. The $[SCN^-]_o/[SCN^-]_i$ gradient was obtained by incubating rotenone treated mitochondria in LiSCN. The $[H^+]_i/[H^+]_o$ gradient was obtained by incubating mitochondria in $Ca(acetate)_2$ and initiating the Ca^{2+} penetration with uncouplers. Table I also shows two conditions where the divalent cation efflux was found to be sensitive to Ruthenium Red: the energy-linked extrusion of Sr^{2+} and the EGTA-induced efflux of Ca^{2+}. The insensitivity to Ruthenium Red of the uncoupler induced efflux of Mn^{2+} observed by Puskin et al. (1976) has not been confirmed in our laboratory. In order to explain that uncouplers and respiratory inhibitors induce an insensitivity of Ca^{2+} efflux to Ruthenium Red, Pozzan and Azzone (1977) suggested that the inhibitory effect of Ruthenium Red is dependent on the presence of an electrical field, negative inside, on the membrane. In the experiment of Figure 1A-C, mitochondria were left to accumulate Mn^{2+} either in a sucrose medium (A) or in the presence of LiCl + LiSCN (B) or $LiNO_3$ (C). After completion of uptake, Mn^{2+} efflux was initiated by addition of KCN. The KCN-induced Mn^{2+} efflux was partially sensitive to Ruthenium Red in the sucrose medium (A) and considerably more sensitive to Ruthenium Red in the LiCl + LiSCN (B) or in $LiNO_3$ medium (C).

Figure 2 shows an experiment where the sensitivity of KCN-induced Mn^{2+} efflux to Ruthenium Red was followed with the ESR technique. It is seen that in the presence of $LiNO_3$ there was a marked inhibition by Ruthenium Red. This is in accord with the result obtained in Figure 1 with the spectrophotometric technique.

The spectrophotometric technique could not be used to test the sensitivity of KCN-induced Ca^{2+} efflux to Ruthenium Red, because of a large decrease of absorbance in the presence of KCN, Ruthenium Red, and $LiNO_3$. This may be due to penetration of HNO_3. Instead, the sensitivity of Ca^{2+} efflux to Ruthenium Red was determined with a rapid filtration technique following uptake of $^{45}Ca^{2+}$. Figure 3 (A and B) shows such an experiment carried out in sucrose and $LiNO_3$ media. It is seen that Ruthenium Red did not inhibit (or slightly enhanced) Ca^{2+} efflux in sucrose (A) while it markedly depressed Ca^{2+} efflux in $LiNO_3$ (B). Replacement of Li^+ with other cations such as K^+ or Na^+, addition of Mg^{2+}, or replacement of NO_3^- with other permeant anions resulted in a similar picture. Since in anaerobic mitochondria, permeant anions generate a diffusion potential, the experiments of Figures 1-3 are in accord with the suggestion (Pozzan and Azzone, 1977) of a dependence of the Ruthenium Red inhibition on an electrical field.

Figure 4A shows that, after completion of Ca^{2+} uptake, addition of EGTA initiated a Ca^{2+} efflux which was sensitive

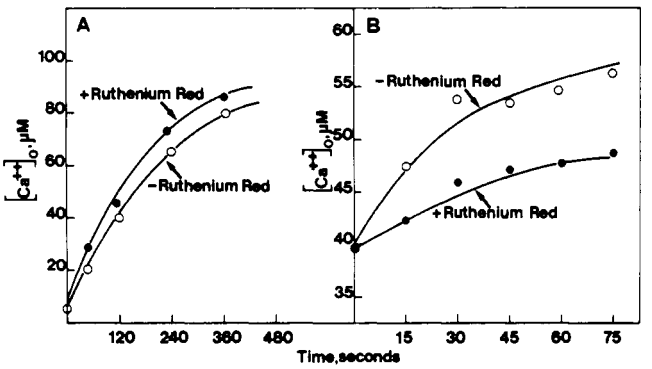

FIGURE 3: Effect of Ruthenium Red on the KCN-induced Ca^{2+} release. (A) Medium was the same as in Figure 1A + 2.5 mM succinate-Tris. (B) Medium was the same as in Figure 1C + 2.5 mM succinate-Tris. The release was started with addition of 2.5 mM KCN. Where indicated, 200 pmol/mg protein Ruthenium Red was added before KCN. Mitochondrial protein, 1 mg/mL. Ca^{2+} concentration in the supernatant was determined after rapid filtration as described in the Experimental Section.
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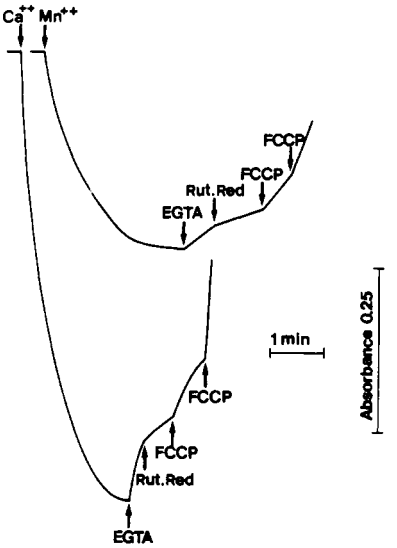

FIGURE 4: Ruthenium Red inhibition of EGTA-induced Ca^{2+} and Mn^{2+} release and effect of FCCP. The medium contained: 200 mM sucrose, 20 mM Hepes (pH 7), 2.5 mM succinate-Tris, 10 mM acetate-Tris, 1 mg/mL mitochondrial protein. Where indicated, the following were added: $CaCl_2$, 150 nmol/mg protein or $MnCl_2$, 200 nmol/mg protein, EGTA, 1.5 mM, Ruthenium Red, 100 pmol/mg protein. FCCP was 1.6×10^{-8} M at the first addition and 3.2×10^{-8} M at the second addition, respectively.

to Ruthenium Red. The fact that Ruthenium Red inhibited about 90% the rate of EGTA induced cation efflux (Pozzan and Azzone, 1977) indicates that cation transport was rate limiting. However, addition of FCCP in increasing concentrations caused an enhancement of the rate of cation efflux. Similar results were obtained with Mn^{2+}, Figure 4B. Thus the increase of FCCP induced H^+ leak caused a corresponding decrease of the extent of Ruthenium Red inhibition. Puskin et al. (1976) reported that in the presence of Mg^{2+} the effect of Ruthenium Red on the EGTA-induced efflux was negligible. We have confirmed this observation but have no explanation for it. However, the observation has an important implication. In fact, when Ruthenium Red is added in steady state, the rate of Ruthenium Red induced cation^{2+} efflux may be partly diminished by an inhibition by Ruthenium Red also of the efflux rate. If, on the other hand, it is assumed that, in the presence of Mg^{2+}, cation^{2+} efflux is Ruthenium Red in-

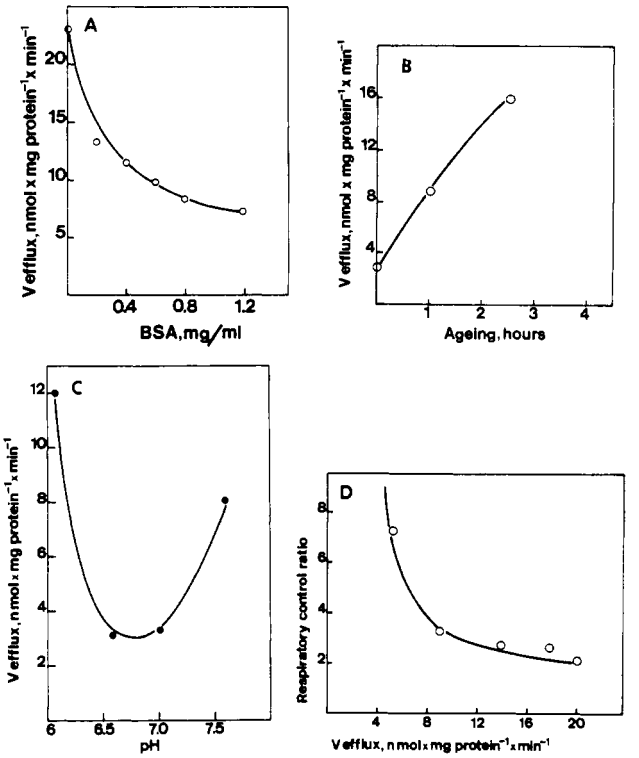

FIGURE 5: Dependence of Ruthenium Red induced Ca^{2+} release on H^+ leak. (A-D) Medium was the same as in Figure 4 + 2.5 mM $MgCl_2$ + 100 μM $CaCl_2$. The release was started by addition of 200 pmol/mg protein Ruthenium Red. BSA, pH, and ageing were varied as indicated. In D, various levels of respiratory control were obtained by varying the time of ageing and the amount of BSA.

sensitive, the Mg^{2+} supplemented system permits to determine the steady-state efflux rate as determined by an effect of Ruthenium Red on the influx and not on the efflux. For this reason in the present work Mg^{2+} was always used to measure the steady-state efflux rate.

The Dependence of Ca^{2+} Efflux on H^+ Leak in Steady State. The Ruthenium Red induced Ca^{2+} efflux in steady state has been shown to be coupled to an influx of H^+ (Pozzan and Azzone, 1976). The pathway for H^+ influx was therefore investigated. Figure 5A shows the effect of BSA on the rate of Ruthenium Red induced Ca^{2+} efflux. It is seen that the Ca^{2+} efflux was about 80% sensitive to the addition of BSA. In Figure 5B it is seen that the rate of Ruthenium Red induced Ca^{2+} efflux increased proportionally to the time of mitochondrial aging. Figure 5C shows the pH dependence of the rate of Ruthenium Red induced Ca^{2+} efflux. The rate was minimal at pH around neutrality and tended to increase both at acidic and alkaline pH. Figure 5D shows a correlation between rate of Ruthenium Red induced Ca^{2+} efflux and respiratory control. Lower degrees of respiratory control were always accompanied by higher efflux rates and the relation between the two parameters was hyperbolic in nature.

Dependence of Cation Accumulation Ratio on Relative Rates of Influx and Efflux. A major question is whether a restriction of the rate of cation influx affects the steady-state accumulation ratio of permeant cations. Puskin et al. (1976) showed a decrease of Mn^{2+} and Ca^{2+} accumulation ratios after addition of Ruthenium Red. The question arises as to whether a steady state can be measured when the rate of cation influx is drastically reduced. This is equivalent to asking whether the conditions considered as steady state are real or apparent. Figures 6A and 6B show that by using either Ca^{2+} or Mn^{2+} as permeant cation, the same extent of uptake is obtained in-

TABLE II: Simultaneous Measurements of Ca^{2+} and Mn^{2+} Accumulation Ratio.[a]

Cation^{2+} added	$[Ca^{2+}]_{in}$ (mM)	$[Mn^{2+}]_{in}$ (mM)	$[Ca^{2+}]_{out}$ (μM)	$[Mn^{2+}]_{out}$ (μM)	$[Ca^{2+}]_{in}/[Ca^{2+}]_{out}$	$[Mn^{2+}]_{in}/[Mn^{2+}]_{out}$
Mn^{2+}, 50 μM						
Ca^{2+}, 50 μM	30.5	24.5	4.14	13.3	7367	1846
Ca^{2+}, 100 μM						
Mn^{2+}, 50 μM	50.6	16.2	12.8	20.9	3953	774
Ca^{2+}, 50 μM						
Mn^{2+}, 100 μM	22.06	32.3	10.3	41.8	2141	772

[a] Experimental conditions: The medium contained 200 mM sucrose, 20 mM Hepes (pH 7), 10 mM Acetate–Tris, 2.5 mM succinate–Tris, 2.5 mM $MgCl_2$, 1 mg/mL mitochondrial protein.

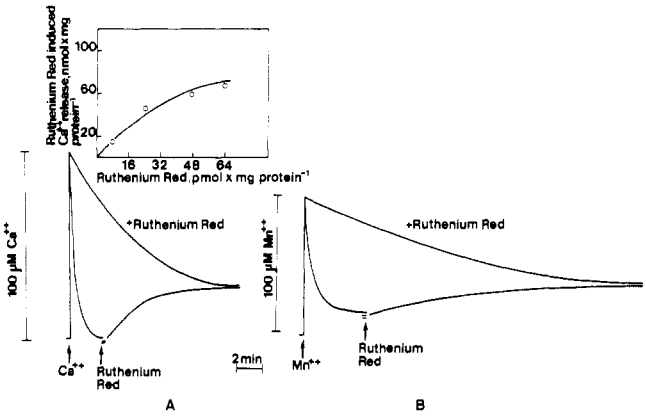

FIGURE 6: Effect of Ruthenium Red on the steady-state accumulation ratio of Ca^{2+} and Mn^{2+}. Medium was the same as in Figure 5. Where indicated, the following were added: $CaCl_2$, 100 nmol/mg protein (A), or $MnCl_2$, 100 nmol/mg protein (B), and Ruthenium Red, 0.32 pmol/mg protein. In the insert, Ruthenium Red concentrations were as indicated.

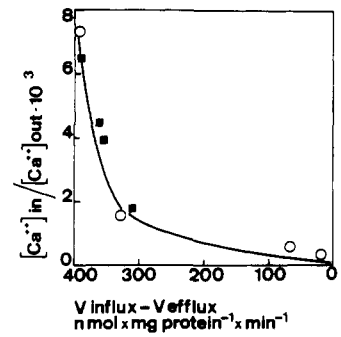

FIGURE 7: Dependence of the steady-state accumulation ratio on the rates of cation influx and efflux. Medium was the same as in Figure 5. (○) In presence of various amounts of Ruthenium Red; (■) in presence of various amounts of A 23187. Both Ruthenium Red and A 23187 were added before Ca^{2+}.

dependently of whether Ruthenium Red was added at the beginning or after reaching the steady state. The attainment of the same cation accumulation ratio when the inhibitor either restricts the influx or induces an efflux strongly supports the view of a real steady state. Similar results were obtained with Sr^{2+} (not shown). In the insert of Figure 6 it is seen that the amount of Ca^{2+} release depends on the Ruthenium Red concentration and, therefore, on the extent of the uptake inhibition. In parallel experiments it was ascertained that the concentrations of Ruthenium Red used did not alter the Rb^+ distribution, the respiratory control, and the *P/O* ratio.

In Figure 7 the Ca^{2+} accumulation ratio is plotted as a function of the difference between the relative maximal rates of influx and efflux. Variations of the rate of influx were obtained by adding variable amounts of Ruthenium Red. Variations of the rate of efflux were obtained by adding increasing amounts of the exogenous divalent cation ionophore A23187 (Reed and Lardy, 1972; Heaton and Nicholls, 1976). It appears that the steady-state accumulation ratio is independent of the procedure used to obtain a variation of the difference between rates of influx and efflux.

If the accumulation ratio is dependent on the relative rates of influx and efflux, it might be expected that permeant cations possessing different rates of influx do not show identical accumulation ratios. This is in contrast to the conclusion of Rottenberg and Scarpa (1974) who observed that permeant cations possess, in steady state, an identical accumulation ratio determined only by the dimension of $\Delta\psi$. Table II shows the accumulation ratio for Ca^{2+} and Mn^{2+} measured simultaneously. It is seen that, whatever the Ca^{2+}/Mn^{2+} ratio, the accumulation ratio for Ca^{2+} was always much higher than for Mn^{2+}. This is in accord with the fact that the rate of Ca^{2+} influx is considerably higher than the rate of Mn^{2+} influx (Vinogradov and Scarpa, 1973).

Discussion

The discovery that the accumulation ratios for divalent are lower than those for univalent cations and different among themselves (Azzone et al., 1975, 1977; Massari and Pozzan, 1976; Puskin et al., 1976) is not in accord with the assumption that all permeant cations are, in steady state, at electrochemical equilibrium. Puskin et al. (1976) have explained the discrepancy by assuming for divalent cation efflux either an electroneutral antiporter or an active pump. The idea of an antiporter is not new. Loyter et al. (1969) found an active Ca^{2+} uptake in submitochondrial particles which may be taken as the expression of a H^+/Ca^{2+} antiporter (Chance and Montal, 1971). The argument in favor of an alternative pathway for Ca^{2+} transport depends largely on the sensitivity of Ca^{2+} efflux to the divalent cation carrier inhibitors, La^{3+} and Ruthenium Red.

Sensitivity of Ca^{2+} Efflux to Ruthenium Red. La^{3+} and Ruthenium Red are powerful inhibitors of energy-driven uptake of divalent cations in mitochondria and are considered to act at the level of the divalent cation carrier. It was soon discovered, however, that the sensitivity of Ca^{2+} efflux to La^{3+} was lower than that of the influx (Scarpa and Azzone, 1970; Lehninger and Carafoli, 1971). The asymmetric sensitivity of Ca^{2+} transport to La^{2+} may be explained on the basis of the competition between La^{3+} and Ca^{2+} for the carrier (Reed and Bygrave, 1974a). However, Ca^{2+} efflux is less sensitive also to Ruthenium Red, which is a noncompetitive inhibitor (Reed and Bygrave, 1974a). Furthermore cation^{2+} efflux possesses a variable sensitivity to Ruthenium Red: (a) it is highly sensitive to Ruthenium Red during active extrusion driven by

metabolism, or after addition of chelating agents in steady state (Pozzan and Azzone, 1977; see also Reed and Bygrave, 1974b); (b) it is almost insensitive to Ruthenium Red after addition of uncouplers, or respiratory inhibitors in sucrose media (Vasington et al., 1972); (c) under conditions of high sensitivity to Ruthenium Red, the rate of efflux increases proportionally to the extent of FCCP-induced electrical H^+ leak (cf. Figure 4). This pattern suggests that the inhibition of divalent cation transport to Ruthenium Red depends on the presence of an electrical field, negative inside. Indeed, replacement of sucrose with Cl^-, SCN^-, and NO_3^- results in a marked enhancement of the Ruthenium Red inhibition. The effect of the lipophilic anions would be that of originating a diffusion potential, negative inside, which favors the interaction of Ruthenium Red with the carrier. The high sensitivity to Ruthenium Red of $cation^{2+}$ influx in the presence of uncouplers (cf. Table I) indicates that uncouplers do not induce per se Ruthenium Red insensitivity. This is not in accord with the view that uncouplers may operate as cation ionophores (Kessler et al., 1976; Green, 1977). On the other hand, uncouplers do induce Ruthenium Red insensitivity when the electrical field is generated by metabolism. Thus, even under conditions where the rate of $cation^{2+}$ efflux is dependent on the rate of operation of the carrier, variations of the extent of H^+ leak (cf. Figure 4) alter the Ruthenium Red sensitivity and thus the rate of $cation^{2+}$ efflux.

The Pathway for Cation Efflux in Steady State. In steady state the rate of Ruthenium Red induced Ca^{2+} efflux increases proportionally to the increase of uncoupler induced electrical H^+ leak (Pozzan and Azzone, 1976). This indicates that, in steady state, at least the increase of efflux induced by uncoupler does not go through a native H^+/Ca^{2+} antiporter. Figure 5 shows that the rate of Ruthenium Red induced Ca^{2+} efflux increases proportionally to mitochondrial ageing, decreases after addition of BSA, increases both at alkaline and acidic pH, and increases proportionally to the release of respiratory control. These observations indicate a dependence of the rate of steady-state Ca^{2+} efflux on the rate of H^+ leak. The proton leak increases with ageing and decreases with BSA. The membrane permeability is modified by alkalinization and acidification, the former increasing permeability to anions (Azzi and Azzone, 1967) and the latter to cations (Carafoli and Rossi, 1967). Release of respiratory control ratio is generally related to increase of H^+ permeability.

Puskin et al. (1976) argue that experiments of swelling in acetate salt (Selwyn et al., 1970) do not exclude a slow rate of H^+/Ca^{2+} antiporter. Two observations, however, are not in accord with the view of a minuscule native H^+/Ca^{2+} antiporter. First, following Selwyn et al. (1970), swelling in acetate salts of divalent cation has been obtained by adding variable amounts of X537A; with all cations the titrations provide zero penetration at zero ionophore (Pozzan and Azzone, 1976). Second, the Ruthenium Red induced Ca^{2+} efflux is correlated with the electrical H^+ leak. The concept of native $H^+/cation^+$ antiporter was initially proposed (Mitchell and Moyle, 1969) as a specific mechanism for univalent cation transport, capable of distinguishing Na^+ and K^+ (Douglas and Cockrell, 1974). The existence of a Ruthenium Red insensitive efflux for Ca^{2+}, Mn^{2+}, and Sr^{2+} requires three additional specific carrier systems. This leads to the assumption of a large number of native antiporters. We suggest that also the steady-state, Ruthenium Red insensitive efflux goes through the native carrier. The conditions determining the Ruthenium Red sensitivity do not permit use of this inhibitor as a criterion for the existence of another pathway.

A H^+ coupled $cation^{2+}$ efflux may in principle be due to a fatty acid induced electroneutral $H^+/cation^{2+}$ exchange. Such a mechanism, however, would not explain the enhancement of efflux following addition of FCCP (Pozzan and Azzone, 1976).

We attribute the low Ruthenium Red sensitivity, during steady-state efflux, to the lowering of the electrical field in the region of high H^+ permeability. Transport of divalent cations may therefore be seen as due to a carrier operating in two membrane domains, one, of high electrical field generated by the proton pump and leading to a Ruthenium Red sensitive $cation^{2+}$ uptake, another, of low local electrical field due to a H^+ influx down $\Delta\psi$. In this latter domain the carrier catalyzes a Ruthenium Red insensitive $cation^{2+}$ efflux down the cation concentration gradient. The difference in extent of H^+ leak between various membrane domains may not reflect native structural difference but rather induced dynamic modifications.

Kinetic Dependence of the Steady-State Cation Accumulation Ratio. Consider an electrophoretic $cation^{2+}$ influx driven by an electrical field $\Delta\psi$ (Schlögel, 1954):

$$V_{influx} = K_1 \Delta\psi \frac{[cation^{2+}]_i - [cation^{2+}]_o e^{-nF\Delta\psi/RT}}{1 - e^{-nF\Delta\psi/RT}} \quad (1)$$

where the V_{influx} is the rate of $cation^{2+}$ influx, K_1 is a parameter of the membrane permeability for Ca^{2+}, $\Delta\psi$ is the electrical field, and the other terms have the usual meaning. Under steady-state conditions when $V_{influx} = 0$, also $[cation^{2+}]_i - [cation^{2+}]_o e^{-nF\Delta\psi/RT} = 0$. The accumulation ratio R should correspond to $\Delta\psi$ in accord with the Nernst equation.

Azzone et al. (1975, 1977) and Puskin et al. (1976), however, found that the accumulation ratio is lower for the divalent than for the univalent cations, when divalent and univalent cations are measured simultaneously. Table II shows that the accumulation ratio for Mn^{2+} and Ca^{2+}, measured simultaneously, is lower for the former than for the latter. Furthermore the accumulation ratio for divalent cations depends on the rate of cation influx (cf. Figure 6). These data do not support the prediction of eq 1 that a restriction of the rate of cation influx should affect only the time required to reach the steady-state distribution, but not the magnitude of the accumulation ratio.

Consider now an electrophoretic $cation^{2+}$ influx paralleled by a process of $cation^{2+}$ efflux, V_{efflux}, driven by a force which is $\Delta\psi$ independent. In this case, $V_{net\ influx} = V_{influx} - V_{efflux}$. Furthermore in steady state, net influx = 0 when $V_{influx} = V_{efflux}$. It is predicted that a variation of the rate of influx results in a parallel change of the accumulation ratio and the latter is a function of the difference between rate of $cation^{2+}$ influx and rate of $cation^{2+}$ efflux. This is in accord with what was found (Azzone et al., 1975, 1977; Puskin et al., 1976). The discrepancy will increase or decrease parallel to variations of the cation efflux rates. The dependence of the accumulation ratios on kinetics rather than on thermodynamic parameters explains why conditions may be found under which the accumulation ratios of univalent and divalent cations may be either very different (Azzone et al., 1975, 1977; Puskin et al., 1976) or very close (Rottenberg and Scarpa, 1974). Close ratios are obtained in the presence of large swelling and low $\Delta\psi$ (Azzone et al., 1977).

Mitochondrial Regulation of Ca^{2+} Gradients in Vivo. If $\Delta\psi$ in steady-state mitochondria is 200 mV (Rossi and Azzone, 1969; Mitchell and Moyle, 1969), the accumulation ratio of divalent cation at electrochemical equilibrium should be about 10^7. Since in the mitochondrial matrix Ca^{2+} is not higher than 10^{-3} M, the cytoplasmic Ca^{2+} concentration should be around 10^{-10} M. This is in contrast with the physiological limits of the

operation of the sarcotubular system in the muscle cell and of other Ca^{2+}-dependent enzymatic systems which require a free Ca^{2+} concentration between 10^{-5} and 10^{-8} (Gompers, 1976). Our proposal that the accumulation ratio for divalent cations is dependent on the difference between influx and efflux rates and not on the steady-state $\Delta\psi$ eliminates the discrepancy between $\Delta\psi$ and accumulation ratios in vitro and in vivo.

Crompton et al. (1976) have proposed a regulation of the Ca^{2+} distribution in vivo through a Na^+/Ca^{2+} antiporter. However, a Na^+-induced Ca^{2+} efflux has been observed only in heart mitochondria, while Ca^{2+} must be maintained far from equilibrium in all cells. Another objection against mechanisms based only on Ca^{2+} efflux is that they lead, in the presence of a high rate of Ca^{2+} influx, to a considerable energy drain.

Our view of a H^+ leak dependent Ca^{2+} efflux coupled with a restriction of Ca^{2+} influx would be very efficient to lower the mitochondrial Ca^{2+} accumulation ratio in vitro as well as in vivo. The physiological mechanism for restricting Ca^{2+} influx might be the cooperativity of the kinetics of Ca^{2+} uptake. Bygrave et al. (1971) Vinogradov and Scarpa (1973) showed that the kinetics of Ca^{2+} uptake is sigmoidal in nature. The apparent K_m reaches values of 50 μM (Scarpa and Graziotti, 1973). Although lower K_ms, have been observed in sucrose media (Carafoli and Azzi, 1971), Hutson et al. (1976) have also shown the existence of cooperativity in media devoid of cations. Presumably high K_m and cooperativity reflect the physiological conditions for Ca^{2+} uptake. Indeed, mitochondria operate in the cytosol in the presence of 150 mM K^+ and about 1 mM Mg^{2+} (Brinley and Scarpa, 1975; Åckerman et al., 1977; Endo, 1975), i.e., close to the conditions used by Vinogradov and Scarpa (1973).

If in steady-state $V_{net\ influx} = 0$, when $V_{influx} = V_{efflux}$, Ca^{2+} influx should be restricted to values similar to Ca^{2+} efflux. In the present study, Ca^{2+} efflux was 1-5 nmol (mg of protein^{-1}) min^{-1} in the presence of 10 mM acetate and 100 nmol of Ca^{2+} (mg of protein^{-1}). Under physiological conditions, Ca^{2+} efflux in steady state should not be higher than 1 nmol (mg of protein^{-1}) min^{-1} as compared with a maximal rate of Ca^{2+} influx around 400 nmol (mg of protein^{-1}) min^{-1}. The reduction of Ca^{2+} influx leads, under conditions of a small H^+ leak, to accumulation ratios of 10^3, i.e., 10^{-3} M in the matrix and 10^{-6} M in the cytosol. The Ca^{2+} accumulation ratio under physiological conditions has then little relation with the force acting on cation and is rather determined by the kinetic parameters based on the affinity of Ca^{2+} with its carrier.

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Mutations in *Escherichia coli* Altering an Apurinic Endonuclease, Endonuclease II, and Exonuclease III and Their Effect on in Vivo Sensitivity to Methylmethanesulfonate†

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ABSTRACT: The levels of endonuclease II, an apurinic endonuclease, and exonuclease III in the parent strain (AB 1157) of *Escherichia coli* and in various mutants were determined by chromatography on DEAE-cellulose. AB 3027 and NH 5016 lacked endonuclease II and exonuclease III. BW 2001 lacked the apurinic endonuclease and exonuclease III while BW 2007, BW 9093, and BW 9059 lacked only exonuclease III. Deletion mutants BW 9101 and BW 9109 lacked all three enzymes. The latter mutants locate the genes for the two endonucleases in the region of exonuclease III (*xth*) of 38.2 min (White et al., 1976). All of the mutants which were sensitive to methylmethanesulfonate in vivo lacked exonuclease III, but not all mutants lacking exonuclease III were MMS sensitive. The deletion mutants and NH 5016 were the exceptions.

Repair of DNA damage in *E. coli* can occur by excision mechanisms involving endonucleases or *N*-glycosidases. Thymine dimers are excised by an endonuclease which recognizes this lesion and other chemical adducts (Grossman et al., 1975). Endonuclease II recognizes alkylated and aralkylated purine bases such as 3-methyladenine, *O*-6-methylguanine (Kirtikar and Goldthwait, 1974), or the dimethylbenz[*a*]anthracene derivatives of adenine and guanine formed by reaction of DNA with 7-bromomethyl-12-methylbenz[*a*]anthracene (Kirtikar et al., 1975a) as well as lesions in DNA produced by γ irradiation (Kirtikar et al., 1975b). Endonuclease II, purified 12 000-fold, has both phosphodiesterase and *N*-glycosidase activities (Kirtikar et al., 1976a). Many alkylated bases of DNA, especially purines, show increased lability of the glycosidic bond and depurinate spontaneously leaving depurinated sites. An endonuclease from *E. coli* which recognizes apurinic sites has been purified approximately 10 000-fold to homogeneity by Verly and Rassart (1976). An enzyme with a similar molecular weight and chromatographic properties has also been purified to homogeneity in this laboratory (Kirtikar et al., 1976a). It recognizes depurinated (apurinic) sites as well as depurinated sites which have been reduced with $NaBH_4$ (Hadi and Goldthwait, 1971). Endonuclease II and the apurinic endonuclease have been separated from each other (Kirtikar et al., 1976a) and from exonuclease III, an enzyme which degrades double-stranded DNA in a 3′ to 5′ direction and which can remove a terminal 3′-phosphate by its 3′ phosphatase activity (Richardson and Kornberg, 1964).

Although there are several enzymes which recognize apurinic sites in DNA, as will be discussed, we shall refer in this paper to the enzyme activity isolated in peak I from DEAE[1]-cellulose as apurinic endonuclease and also as endonuclease VI.

One mutant, AB 3027, isolated in the laboratory of Dr. Howard-Flanders, was reported to be lacking DNA polymerase I and the apurinic endonuclease (Ljungquist et al., 1976). In Dr. Weiss's laboratory, a mutant, BW 2001, was isolated and at the time was thought to be lacking exonuclease III and endonuclease II, the latter defined as an activity on heavily alkylated DNA (Yajko and Weiss, 1975). With a chromatographic procedure developed in this laboratory which could distinguish between the two endonucleases and exonuclease III (Kirtikar et al., 1976a) an examination of these mutants and their derivatives was instigated. An attempt has also been made to relate the specific enzyme complement of each mutant to its in vivo sensitivity to methylmethanesulfonate.

Methods

The parent strain for all the mutants used for this study was *E. coli* AB 1157 (Howard-Flanders and Theriot, 1965) des-

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[1] Abbreviations used: UV, ultraviolet; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MMS, methylmethanesulfonate; apurinic endonuclease is used to designate the enzyme which recognizes depurinated or depurinated reduced sites in DNA and which is present in peak I. This enzyme has the same properties as that isolated by Verly and Rassart (1975) and has been designated endonuclease VI.