Accumulation of Sulfate by Mitochondria of Rat Kidney Cortex

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ABSTRACT Twice washed mitochondria from rat kidney cortex can accumulate sulfate ions from low (10^{-7} M) ambient concentrations to create virtual gradients of several hundred to one. This sulfate is subsequently released. The activation energy for the uptake is 12,000 calories per mole; for release it is about 30,000 calories per mole. Variations in the sulfate concentration of the medium show that there is a straight line Freundlich adsorption isotherm over a million-fold range of concentration of sulfate in the medium. There are 9×10^4 sites at 10^{-5} M and 9×10^5 sites at 10^{-3} M sulfate per average single mitochondrion. Preincubation at 30°C rapidly destroys the ability to accumulate sulfate. Partial protection occurs if oxidative phosphorylation is proceeding during the preincubation. The concentration of the endogenous inorganic sulfate of twice washed mitochondria is 4.2×10^{-4} moles per liter of mitochondrial pellet water; 99.85 per cent of this endogenous sulfate is inexchangeable with external sulfate *in vitro*. It is all exchangeable *in vivo*. The pH optimum for accumulation of radiosulfate from dilute external sulfate concentrations is 5.5. These observations show that there is a delicate and specific mechanism in mitochondria from kidney cortex which accumulates sulfate. The chemical nature of the accumulated sulfate is unknown.

INTRODUCTION

Slices of mammalian kidney cortex can accumulate sulfate (1, 2) *in vitro*, and it seemed possible that the mitochondria might be involved in this process. Preliminary studies showed that such localization of radiosulfate could be demonstrated readily in mitochondria isolated from kidney cortex tissue by conventional procedures (3), although the properties of the mitochondrial system involved in uptake of radiosulfate differed in several respects from those of the accumulative system of kidney cortex slices incubated at 38°C. The experiments described in the present paper represent a detailed study of the

characteristics of mitochondrial uptake of radiosulfate. Preliminary results of some of these studies have been published (4-7).

METHODS AND MATERIALS

Preparation of Mitochondria All operations involved in the preparation of mitochondria were carried out at 0°C. A male albino rat was stunned by a blow on the head. The kidneys were rapidly removed, cooled in 15 ml of cold 0.25 M sucrose, and split along the longitudinal axis into halves. The medullary tissue was removed by dissection. The remaining cortical tissue was then minced with scissors and homogenized by hand in 4 ml of cold 0.25 M sucrose in a 10 ml Potter-Elvehjem homogenizer by two full strokes with a teflon pestle. The initial centrifugation was carried out at 600 *g* for 10 minutes, following which the supernatant solution was recentrifuged at 8500 *g* for 10 minutes. The sedimented mitochondria were then washed with 3 ml of cold 0.25 M sucrose and recentrifuged for 10 minutes at 8500 *g*. This washing was repeated twice. Following the final centrifugation, the pellet was resuspended in from 1 to 3 ml of cold 0.25 M sucrose depending upon the concentration of mitochondria desired.

Assay of Uptake of S^{35} $O_4^=$ The test system generally employed to study the uptake of S^{35} $O_4^=$ consisted of 0.25 M sucrose in 20 mM KCl in polyethylene tubes to which varying amounts of K_2SO_4 and "carrier-free" radiosulfate were added. The radiosulfate was received from Oak Ridge in the form of H_2SO_4 in dilute HCl. The radioactivity was greater than 10 millicuries per ml in from 10^{-3} to 10^{-5} M sulfate. Approximately 0.2 microcurie was used in each tube.

In order to measure the uptake of S^{35} $O_4^=$, 0.1 ml of a well mixed mitochondrial suspension (in 0.25 M sucrose) was pipetted into 1.9 ml of the above mixture. In experiments where the ambient sulfate concentration did not exceed 10^{-4} M, approximately 0.5 mg dry weight of mitochondria was used per tube; with higher ambient sulfate concentrations, suspensions five to eight times more concentrated were employed. It was shown that the pH of the medium during uptake remained nearly constant and at a value close to that for optimal uptake (see Fig. 6).

The mitochondria were allowed to incubate in the test medium for various periods of time and at various temperatures and then rapidly separated by high speed centrifugation at 0°. Following the method for determination of $\int \omega^2 dt$ where ω is the angular velocity of the centrifuge head (see Bartley and Davies (8) and Price, Fonnesu, and Davies (9)), it was determined that with the media used 50 per cent of the mitochondria were sedimented in 50 seconds in head No. 295 of the International refrigerated centrifuge (model PR-1) when accelerated to a maximum velocity equivalent to 22,000 *g*. Therefore, 50 seconds was allowed for in all measurements of the time of incubation.

Following centrifugation, the clear supernatant solution was decanted as completely as possible, the tubes inverted, and allowed to drain. The small amount of fluid remaining on the walls of the tube was removed by carefully wiping with a piece of absorbent tissue paper, a method which left only approximately 0.3 per cent of the total radioactivity adhering to the tubes. The mitochondrial pellet was then dissolved

by addition of 1.0 ml of a solution of 0.02 per cent sterox SK (Monsanto) in 0.2 per cent NH_4OH. Samples of this solution and of dilutions of the supernatant solution were perpared and counted according to the technique outlined by Deyrup and Ussing (1). S^{35} was counted with a gas-flow Geiger tube. The counting error was usually ± 4 per cent.

The water content of the mitochondrial pellet was determined by the difference between wet and dry weights obtained by centrifuging a known volume of suspension, decanting the supernatant solution, removing the last traces of this by wiping with absorbent tissue paper, weighing, drying at 100°C overnight, and reweighing.

Mitochondrial Counts Mitochondrial suspensions were first examined microscopically to insure the absence of non-mitochondrial material. Particle counts were then made with a phase contrast microscope and hemocytometer after suitable dilution of the suspension with 0.25 M sucrose.

Preincubation Experiments Mitochondria were preincubated in various media at 30°C in Warburg vessels. In those experiments in which oxidative phosphorylation was allowed to occur, a medium modified from Lardy and Wellman (10) was employed. This contained 96 mM KCl, 15 mM potassium phosphate buffer at pH of 7.4, 7.5 mM $MgCl_2$, 1.75 mM adenosinetriphosphate, 10.5 mM substrate, 17.5 mM glucose, and 5 mg (per 2 ml) of hexokinase (Pabst). The final volume was 2.0 ml in all cases. In six experiments with several substrates, measurement of the amount of inorganic phosphate esterified compared with the amount of oxygen utilized (P:O ratio) showed good agreement with the values obtained by Lardy and Wellman (10), 2.7 for α-ketoglutarate, 2.8 for glutamate, and 1.7 for succinate. Following this preincubation, the flasks were cooled to 0°C and the contents of the main compartments quantitatively transferred with the aid of cold 0.25 M sucrose to chilled centrifuge tubes. The mitochondria were then centrifuged at 0°C, resuspended in cold sucrose, and their ability to take up $S^{35}O_4^{=}$ determined as outlined above.

Measurement of Mitochondrial Sulfate Rats were given a water load by stomach tube in the amount of 5 per cent of their body weight to insure an adequate urine flow and 30 minutes later, they were injected with 50 to 100 microcuries of carrier-free $S^{35}O_4^{=}$ in 1.0 ml of 5 per cent glucose in water. The animals were induced to void 90 minutes after injection by brief inhalation of ether and were then placed in metabolism cages. Urine was collected for a 30 minute period at the end of which the animals were again induced to void. The rats were then promptly anesthetized, exsanguinated from the aorta, and mitochondria prepared from the kidney cortex as described above. Samples of the washed mitochondria were then dissolved as outlined above and the radioactivity measured before and after precipitation with $BaCl_2$. Precipitation was carried out in the presence of added carrier K_2SO_4 (10 mM) after acidification. The mitochondrial extracts were repeatedly precipitated (two to four times) until the non-precipitable counts remained constant.

Samples of the serum and urine were analyzed for inorganic sulfate following the method of Jones and Letham (11) in which 4-amino-4′-chlorodiphenyl is used as the precipitating reagent. The serum proteins were precipitated by 2M perchloric acid.

After centrifugation the excess perchlorate was precipitated by addition of saturated KOH until the pH was 7.0.

Measurement of pH Optimum for Uptake of $S^{35}O_4^=$ These experiments were carried out by allowing mitochondria to incubate in 0.25 M sucrose containing 10^{-2} M KCl and 10^{-7} M $K_2S^{35}O_4$ in the absence of added buffers. The incubation was carried out in a 10 ml beaker mounted in a small water bath (26°C), the whole assembly being fixed on the top of the head of a Clay-Adams angle centrifuge. The contents of the beaker were mixed by turning the head of the centrifuge with an external motor to prevent heating of the reaction vessel. The pH was continuously monitored by small electrodes. Two micrometer syringes were used to deliver 0.01 N HCl and 0.01 N KOH into the reaction vessel to keep the pH constant. The temperature of the incubation medium was monitored by a thermistor. The sucrose-KCl solution was pipetted into the beaker followed by a sample of the cold mitochondrial suspension. A period of 30 seconds was allowed for thermal equilibrium and for adjustment of pH to the desired value. At the end of that period, radiosulfate was added to the incubation mixture and uptake allowed to proceed for exactly 2 minutes with the pH being continuously adjusted as required. This time interval was chosen because it represents the peak of uptake at this temperature (see below). At the end of this time, the contents of the beaker were transferred to a chilled centrifuge tube, and the mitochondria were rapidly separated by brief high speed centrifugation at 0°C. The radioactivities of the supernatant and the mitochondria were then determined as outlined above. At least two such experiments were performed at each pH studied.

RESULTS

Effects of Temperature Fig. 1 shows the influence of temperature upon the rate and extent of uptake and loss of radioactive sulfate by mitochondria from rat kidney cortex and gives the relative virtual concentration gradients for $S^{35}O_4^=$ between the total mitochondrial pellet water and the supernatant solution. The concentration of sulfate in the medium in these experiments was 10^{-7} M, and the absolute values for the maximal gradients achieved at 0°C varied from 56 to 256. Since the volume of the extraparticulate fluid is 19 to 22 per cent of the total water of the pellet for mitochondria isolated from 0.25 M sucrose at 0°C (*cf.* Werkheiser and Bartley (12)), the values for gradients between the mitochondrial water and the supernatant fluid would be correspondingly higher.

Temperature exerts three distinct effects. First, the rate of uptake of radiosulfate is enhanced as temperature rises. Second, the extent of uptake is somewhat greater with increasing temperature. Third, the rate of loss is quite different at different temperatures; at 0°C there is a relatively well maintained plateau up to at least 50 minutes, whereas at 26°C the rate of loss of isotope is quite rapid. At the intermediate temperature of 16°C, the curve has a shape similar to that at 26°C, but the rate of loss is less than at the higher temperatures.

A few experiments were carried out at 38°C, and it was found that at this temperature the uptake and subsequent output were so rapid that accurate results could not be obtained since the time needed to develop the maximum gradient was less than the time required to sediment the mitochondria even at maximum acceleration of the centrifuge.

Activation Energy for Uptake and Loss Fig. 2 shows an Arrhenius plot from which the activation energy for uptake of radiosulfate can be calculated. The initial rates of uptake are the rates observed over the initial 2 or 2½ minute

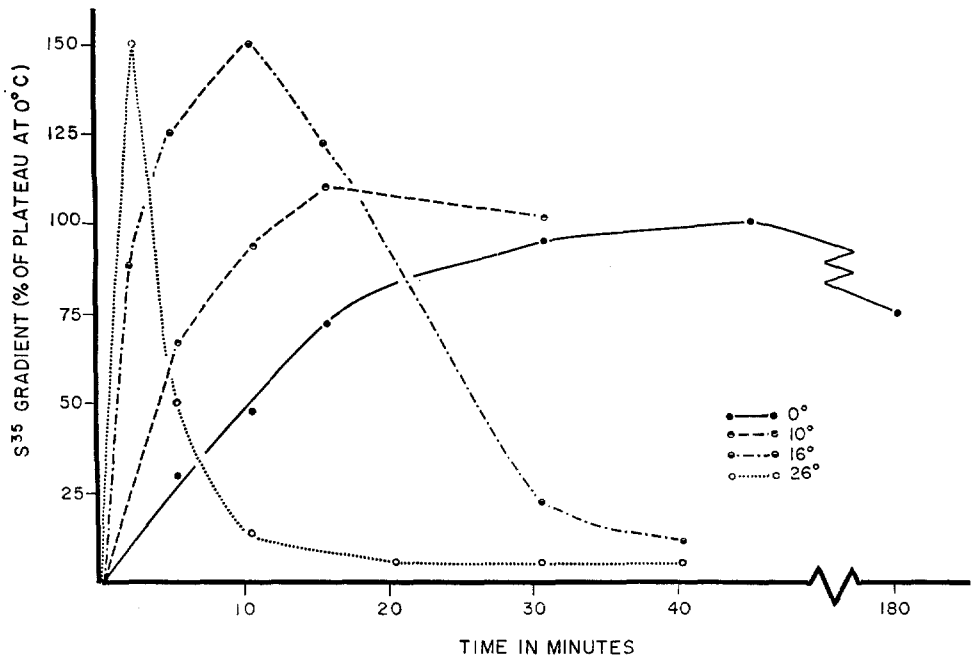

FIGURE 1. The influence of temperature upon the uptake and loss of radiosulfate by mitochondria. The data shown on the graph represent mean values of five experiments. All data have been normalized against the plateau value at 0°C taken as 100 per cent.

period (corrected for the time of centrifugation); these have been normalized against the initial rate observed at 0°C. The activation energy for uptake is approximately 12,000 ± 1,000 calories. The rates of loss have not been as accurately timed as the rates of uptake and therefore the calculation of the activation energy for the loss process is somewhat less accurate. However, calculations based upon the data available give a figure of roughly 30,000 calories for the activation energy of the loss process.

Effects of Sodium, Potassium, and Osmolarity upon Uptake of $S^{35}O_4^=$ The effects of either sodium or potassium were examined over a wide range of concentra-

tions. Data from four such experiments are summarized in Table I and represent the uptakes of radiosulfate by mitochondria at 45 minutes at 0°C expressed as per cent of the value observed in 20 mM KCl and 0.25 M sucrose, the latter being the concentration used for most of the other experiments reported in this paper. In the absence of either cation, uptake was only about 40 per cent of the value observed at 20 mM KCl. The optimal concentration for either sodium or potassium appeared to be about 10 to 20 mM. At any given concentration, potassium appeared to be more effective in supporting uptake than did sodium.

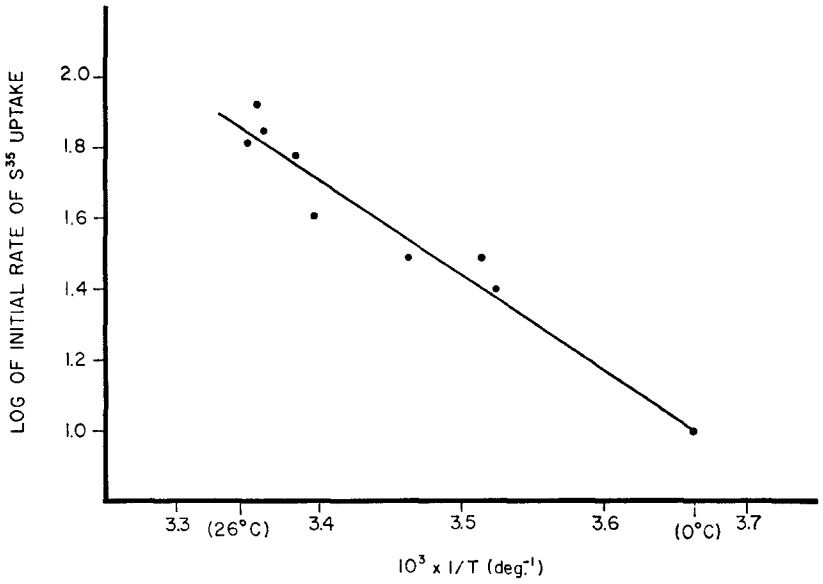

FIGURE 2. Arrhenius plot for calculation of the activation energy of uptake of radiosulfate by mitochondria. The initial rates of uptake have been normalized against the rate observed at 0° for all experiments taken to be 100 per cent.

The influence of varying the osmolarity of the medium with sucrose at a constant concentration of potassium chloride (20 mM) was also studied, and the results are shown in Table II. These data have been normalized to the value observed under usual conditions (290 mOsM/liter) taken as 100 per cent. Maximal uptake occurred when osmolarity was 175 to 290 mOsM/liter; variation of osmolarity below or above this range was associated with decreasing uptake.

Although all the above results were obtained at 0°C, comparable results were obtained when uptake was studied at 2½ minutes at 26°C.

Effect of "Fluffy" Layer on Uptake of $S^{35}O_4^{=}$ In the preparation of mitochondria by the methods outlined above, a fluffy layer is observed to deposit

on the mitochondrial pellet. This material was eluted separately from the packed mitochondria, and its activity with respect to uptake of $S^{35}O_4^=$ determined. The results demonstrated that the fluffy layer doesn't take up radiosulfate under conditions in which the mitochondria are active. The inclusion

TABLE I

EFFECTS OF SODIUM AND POTASSIUM UPON UPTAKE OF $S^{35}O_4^=$ BY MITOCHONDRIA AT 0°C

Concentration of cation	Uptake of $S^{35}O_4^=$ (% of control)	
	Potassium	Sodium
mM	(sodium = 0)	(potassium = 0)
0	40	40
10	109	77
20 (control)	100	71
35	82	60
50	59	47
75	40	29
100	31	19
135	29	12

All incubations were carried out at 0°C for 45 minutes; concentration of sulfate in the medium was 10^{-7} M. Osmolarity held constant by addition of appropriate volumes of 0.25 M sucrose.

TABLE II

EFFECT OF OSMOLARITY OF MEDIUM UPON UPTAKE OF $S^{35}O_4^=$ BY MITOCHONDRIA AT 0°C

Osmolarity	Uptake of $S^{35}O_4^=$
mOs M/l	*per cent of control*
54	57
100	84
175	116
290 (control)	100
375	53
500	52
750	32

All incubations were carried out at 0°C for 45 minutes; concentration of sulfate in the medium was 10^{-7} M; potassium concentration held constant at 20 mM.

of the fluffy layer in the final mitochondrial suspension thus contributes inert material, and this may account for some of the variability in the observed gradients in earlier experiments before this observation was made.

Influence of Ambient Concentration of Sulfate upon Uptake and Release of $S^{35}O_4^=$ at 26°C The influence of the concentration of sulfate in the medium upon

the uptake and loss of $S^{35}O_4^=$ by mitochondria was studied at 26°C. The data, expressed as virtual gradients between the mitochondrial pellet water and the supernatant at various times of incubation, are shown in Table III. At low concentrations of sulfate (less than 10^{-4} M), high gradients were observed at 2½ minutes but fell abruptly to low values thereafter. On the other hand, when the concentration of sulfate exceeded 10^{-4} M, the gradients achieved were lower, but they were well maintained over the entire course of the experiment (30 minutes).

Although the gradients observed in these experiments vary considerably and inversely with concentration of sulfate in the medium, the absolute amounts of sulfate taken up by the mitochondria (calculated as the product of

TABLE III

INFLUENCE OF VARYING SULFATE CONCENTRATION OF THE MEDIUM UPON UPTAKE AND RELEASE OF $S^{35}O_4^=$ BY MITOCHONDRIA AT 26°C

Concentration of sulfate in medium	0 min.	2½ min.	5 min.	15 min.	30 min.	Concentration of sulfate in mitochondria at 2½ min.
moles/liter						*moles/liter of mitochondrial water*
10^{-7}	0	62	16	5		6.2×10^{-6}
10^{-5}	0	47	12	4		4.7×10^{-4}
10^{-4}	0	30	6	4	4	3×10^{-3}
10^{-3}	0	6	5	4	4	6×10^{-3}
10^{-2}	0	4	4	4	3	4×10^{-2}

All incubations carried out in 0.25 M sucrose in which potassium concentration was held constant at 20mM by appropriate additions of KCl. Observed gradient represents the concentration of $S^{35}O_4^=$ in the total mitochondrial pellet water divided by the concentration in the supernatant.

the gradient and the ambient sulfate concentration) increased with increase in the external sulfate concentration (see *e.g.* last column in Table III). The results suggest that when small absolute amounts of sulfate are taken up, the sites responsible for uptake become inactivated at 26°C, whereas when large amounts of sulfate are taken up, the sites seem to be relatively well protected against inactivation. In other words, sulfate itself appears to protect the sites against inactivation at 26°C.

Virtual $S^{35}O_4^=$ Concentration Gradients As indicated above, maximal concentration gradients for $S^{35}O_4^=$ between the mitochondrial pellet water and the medium of several hundred to one were often observed at 0°C when the ambient sulfate concentration was 10^{-7} M. The relationship between the maximal gradient achieved and the external concentration of sulfate when the latter was varied from 10^{-9} to 10^{-1} M is shown in Fig. 3. It is evident from this that a linear relationship exists between the logarithms of these two varia-

bles over a large span of concentration of sulfate. A departure from linearity occurs, however, when the concentration of sulfate is less than about 10^{-7} M. At the other extreme when the gradient is down to approximately 1.0, the intercept on the abscissa occurs at a sulfate concentration of about 10^{-1} M; no further change occurred in the gradient at 2×10^{-1} M.

Over the linear portion the relationship is mathematically similar to the classical (Freundlich) adsorption isotherm, *viz.* $\log C_i = k_1 \log C_o + k_2$ where

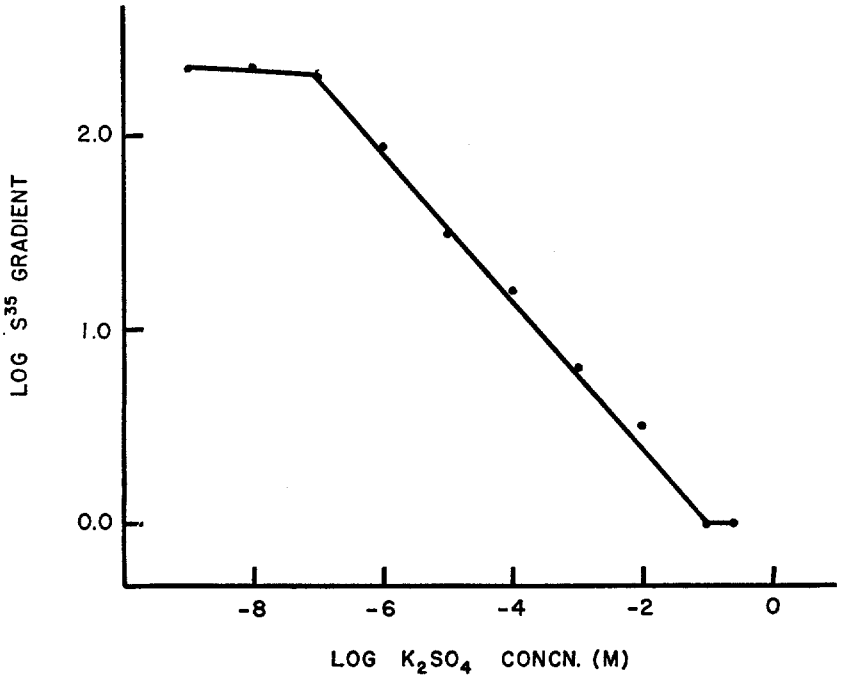

FIGURE 3. The relationship between the maximum gradient achieved by mitochondria and the concentration of sulfate in medium at 0°C. The points represent mean values of eight experiments.

C_i and C_o are concentrations of sulfate per liter of the mitochondrial pellet water and of the supernatant solution respectively, and k_1 and k_2 are constants. This result strongly suggests that an adsorptive process may be involved in the initial uptake of the sulfate ions by the mitochondria. It is unusual that the linear relationship holds over a millionfold change in the concentration of sulfate (13).

Characterization of the Binding Sites Further treatment of the above data was undertaken in an attempt to characterize more fully the properties of the binding sites. For this purpose, an analogy was made to the formal theory of ion-binding by macromolecules as developed by Klotz (14). The theory shows

that if the binding sites are equivalent and independent, an equation can be derived from the Law of mass action which predicts that a linear relationship exists between r/A and r, where r is the amount of ion bound per unit of macromolecule and A is the concentration of the ion in the solution. On a graph of this relationship, it is possible to determine the number of binding sites (n).

Such a plot was made with the data obtained with the uptake of sulfate by mitochondria. It was found that over the intermediate concentrations of ambient sulfate studied (*i.e.*, from 10^{-6} to 10^{-3} M), the curve showed a marked deviation from linearity, this deviation being a measure of heterogeneity of the binding sites. By extrapolation from the curve obtained, the approximate

TABLE IV

EFFECT OF PREINCUBATION ON RADIOSULFATE UPTAKE BY KIDNEY CORTEX MITOCHONDRIA

Conditions of preincubation	Uptake of $S^{35}O_4^=$
	Percentage of the control
0.25 M sucrose at 0°C (control)	100
0.25 M sucrose at 30°C	20
Medium without substrate at 30°C	27
Medium with succinate at 30°C	68
Medium with α-ketoglutarate at 30°C	65
Medium with glutamate at 30°C	60
Medium with α-ketoglutarate and 10^{-4} M 2:4 dinitrophenol at 30°C	25
Medium with α-ketoglutarate and 10^{-4} M CN^- at 30°C	28
Medium with α-ketoglutarate, anaerobic, at 30°C	27

Preincubation was carried out in Warburg flasks at 30°C for 15 minutes; the control was preincubated in 0.25 M sucrose at 0°C (for further details see Methods and Materials). The uptake was measured after 45 minutes' incubation at 0°C in a medium containing 10^{-7} M $SO_4^=$, 0.25 M sucrose, and 20 mM KCl.

maximal number of binding sites per average mitochondrial particle could be estimated. Because of the non-linear nature of the curve, this number varied with external concentration of sulfate, being about 9×10^5 sites per particle at 10^{-3} M sulfate concentration and about 9×10^4 sites at 10^{-5} sulfate concentration.

Thermolability of Binding Sites and Oxidative Phosphorylation The rapid rate of loss of sulfate from mitochondria observed at 26°C (see Fig. 1) suggested that the binding sites might be inactivated at these temperatures, whereas at 0°C they might be more stable.

This hypothesis was tested in a series of experiments in which mitochondria were preincubated in media of varying composition, separated by centrifugation at 0°C, and their ability to accumulate sulfate at 0°C assayed. The results are shown in Table IV.

Preincubation at 0°C in 0.25 M sucrose for as long as 2 hours caused no significant loss of ability to accumulate sulfate when compared with non-preincubated mitochondria at 0°C in 0.25 M sucrose serving as a control, and data from all other conditions of preincubation have been normalized against this control taken as 100 per cent.

Preincubation for 15 minutes at 30°C in 0.25 M sucrose was found to cause a major inactivation of the mechanism of uptake. Similar results were obtained when preincubation was carried out in a medium lacking an oxidizable substrate but otherwise adequate to support oxidative phosphorylation. However, when succinate, α-ketoglutarate, or glutamate were provided and oxidative phosphorylation allowed to occur, about 60 to 65 per cent of the original capacity of the mitochondria to accumulate sulfate was retained. Incubation in the absence of oxygen or with addition of either 2:4-dinitrophenol or cyanide to the actively phosphorylating mitochondria prevented subsequent uptake of sulfate.

These results are of interest in showing the extreme lability of the ability to accumulate sulfate, which falls off during incubation at 30°C even though oxidative phosphorylation can continue unchanged. However, energy metabolism, though not oxidation alone, is able to preserve partially whatever structure or mechanism is responsible for creating the high gradients of sulfate between the mitochondria and the ambient fluid. Thus, this process is not just a binding to an inert positively charged site on the particles.

Exchange versus Net Accumulation of Sulfate The occurrence of significant gradients of radioactive sulfate between the mitochondrial pellet water and the supernatant solution could be caused by one or both of the following types of mechanisms: (*a*) an exchange of isotope occurring between the medium and a pool of sulfate already existing within the mitochondria and (*b*) an accumulation of new sulfate from the medium by the mitochondria.

The relative contributions of each of these two processes would be a simple matter to determine if there were an accurate chemical method available which was sensitive enough to detect the exceedingly small amounts of sulfate present in the milligram quantities of mitochondria that were available. The lack of such a chemical method necessitated an indirect approach.

It was first necessary to measure the amount of sulfate present in mitochondria which were isolated and twice washed in 0.25 M sucrose at 0°C by the procedures uniformly used in all these experiments. This was carried out in a series of experiments based upon the principle of isotope dilution. Rats were injected with carrier-free $K_2S^{35}O_4$ and killed 2 hours later, a time at which the specific activity of the inorganic sulfate of the serum and that of the urine were shown to be approximately equal (within 10 per cent of each other). Mitochondria were prepared from the kidney cortex of such animals,

and, after treatment with a non-ionic detergent (sterox SK) at pH 2, the radioactivity of the barium-precipitable fraction was determined. This was shown by chromatography and electrophoresis to be pure $BaSO_4$. On the reasonable assumption that the specific activity of the mitochondrial sulfate would be equal to that of the two fluids (plasma and urine) to which the kidney is exposed *in vivo*, the mitochondrial sulfate content was calculated. The mean value for the endogenous inorganic sulfate in twice washed mitochondria was found by this method to be 4.2×10^{-4} moles per liter of mitochondrial pellet water.

This value defines the maximal gradients of isotope which could occur on the basis of isotope exchange alone. For example, if the mitochondria were suspended in a fluid with a sulfate concentration of 4.2×10^{-4} M, the maximum gradient of isotope which could develop if isotope exchange were the sole mechanism would be 1.0. Fig. 3 shows that the observed gradients were actually considerably greater at this external concentration of sulfate, thereby proving that almost all of the observed uptake of isotope must represent a real net accumulation of sulfate from the medium by the mitochondria. Furthermore, the lack of any sharp break in the curve in this region suggested that net accumulation of sulfate might also be the major process in accounting for the observed isotopic gradients occurring at sulfate concentrations less than 10^{-4} M. This would be the case if only a small fraction of the total mitochondrial sulfate were readily exchangeable under the *in vitro* conditions used to study uptake.

The true size of the readily exchangeable fraction of the total mitochondrial sulfate was determined by experiments based upon the following considerations. If samples of mitochondria were exposed to different size pools of tagged sulfate of constant concentration in the medium, then measurement of the distribution of isotope between mitochondria and supernatant fluid after centrifugation would allow calculation of the size of the mitochondrial pool. For example, if the amount of exchangeable sulfate in the mitochondria were always large compared with the sulfate pool in the medium, increasing the size of the latter would be accompanied by only a slight fall in the percentage of total isotope appearing in the mitochondria. On the other hand, if the mitochondrial pool were about the same as the medium pool, a similar plot would reveal a more abrupt initial fall in the percentage of radioactivity in the mitochondria as the size of the sulfate pool in the medium was increased.

A series of experiments based upon this design was carried out using a concentration of 10^{-8} M K_2SO_4 in the medium. Variations of the pool size were effected by variation of volume. Fig. 4 shows the observed results and demonstrates their close agreement with the results expected if only 0.15 per cent of the total endogenous mitochondrial sulfate were exchangeable. On the basis

of these experiments, the endogenous mitochondrial sulfate which readily exchanges under these circumstance is thus 6.3×10^{-8} moles per liter of mitochondrial pellet water.

This result shows that the uptake of radioactivity which has been studied represented a real net increase in the sulfate content of the mitochondria, and that exchange with sulfate in the medium is insignificant.

Exchangeability of Newly Accumulated Sulfate Since 99.85 per cent of the endogenous, apparently inorganic, sulfate of the mitochondria does not ex-

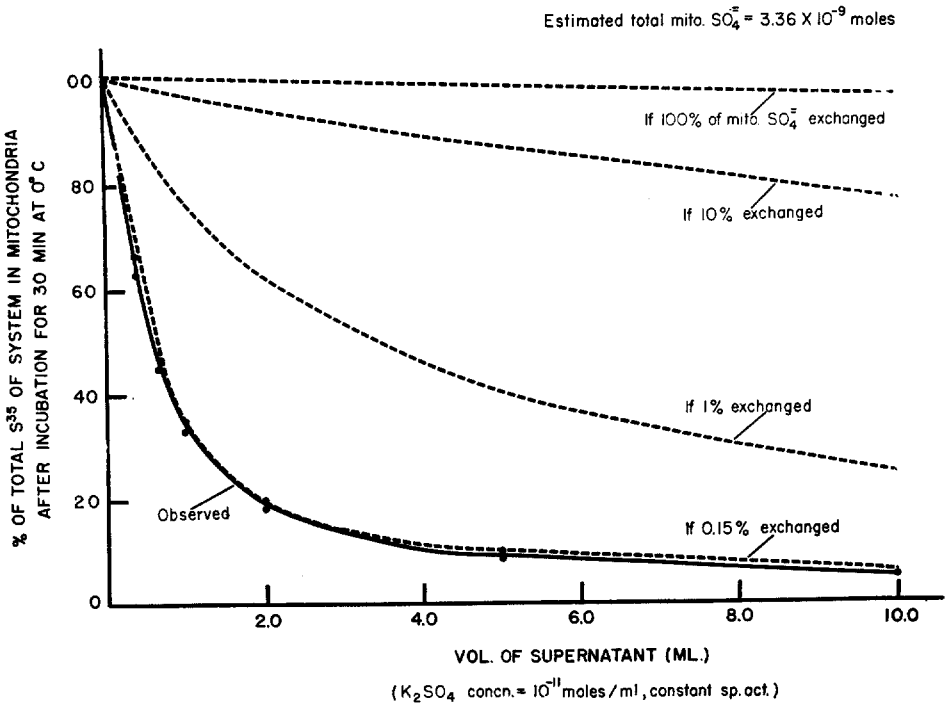

FIGURE 4. Measurement of exchangeable sulfate of mitochondria.

change under *in vitro* conditions (although it does under *in vivo* conditions) it was important to find out the behavior of sulfate accumulated *in vitro*. Fig. 5 shows the results of an experiment designed to determine whether the newly accumulated sulfate itself exchanges with sulfate in the medium. Samples of mitochondria were incubated in 10^{-7} M $K_2S^{35}O_4$ for 30 minutes at 0°C. They were then centrifuged, one sample being suspended in the same supernatant while the other were suspended in 10^{-7} M $K_2S^{32}O_4$. The radioactivity of the mitochondria and the supernatants was then measured during a subsequent 30 minute incubation period at 0°C. The data obtained demonstrate that

despite the large loss of isotope in the sample incubated in non-radioactive sulfate, the gradients of the isotope remaining in these mitochondria were not significantly different from those of the control mitochondria. These results are expected if all of the newly accumulated sulfate were readily exchangeable.

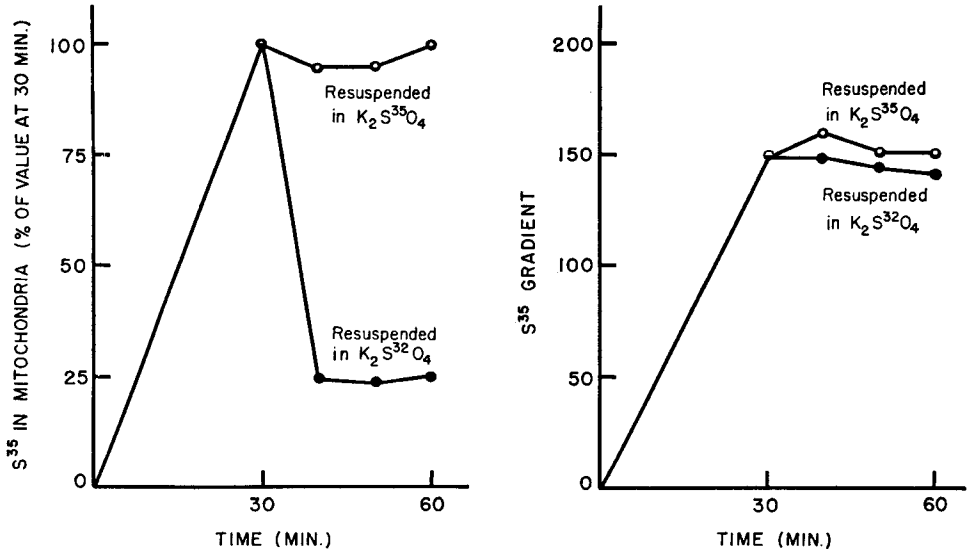

FIGURE 5. Exchangeability of radiosulfate accumulated by mitochondria.

TABLE V

EFFECT OF WASHING KIDNEY CORTEX MITOCHONDRIA ON $S^{35}O_4^=$ PREVIOUSLY ACCUMULATED AT 0°

Conditions	Mitochondria
	counts/min.
Mitochondria incubated with 10^{-7} M $K_2S^{35}O_4$	4190
After washing three times in 10^{-7}M $K_2S^{32}O_4$ in 0.25 sucrose	10
After washing 3 times in 0.25 M sucrose	20
Mitochondria incubated with 10^{-3}M $K_2S^{35}O_4$	1380
After washing three times in 10^{-3}M $K_2S^{32}O_4$ in 0.25 M sucrose	5
After washing three times in 0.25 M sucrose	40

Initial incubation was carried out for 30 minutes at 0° in 2 ml of 0.25 M sucrose–10 mM KCl in presence of $K_2S^{35}O_4$ in the concentrations shown above.

The effect of multiple washings with non-radioactive media upon the newly accumulated $S^{35}O_4^=$ was also determined (Table V). In these experiments, mitochondria were allowed to accumulate $S^{35}O_4^=$ from either 10^{-7} M or 10^{-3} M $K_2S^{35}O_4$ and then subjected to three washings in sucrose–$K_2S^{32}O_4$ solutions or in sucrose alone. Under any of these conditions, the initial mitochondrial radioactivity was virtually completely removed.

Effect of pH on the Uptake of Sulfate There was the possibility that the binding of sulfate by the mitochondria was simply a non-specific ionic binding of the sulfate of the type that occurs for example during protein precipitation by tungstate. One test of this hypothesis was to find out whether the sulfate uptake increased with decreasing pH.

The results of a series of experiments are given in Fig. 6. Preliminary observations had shown that the presence of different buffer solutions (acetate, citrate, and phosphate) caused confusing effects independent of the pH

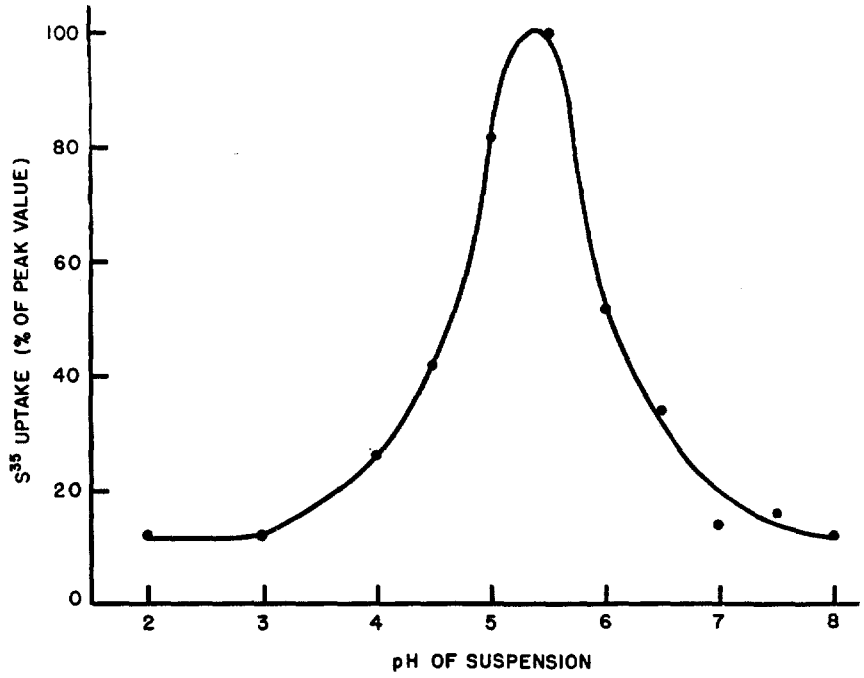

FIGURE 6. The effect of pH of the medium upon the 2 minute uptake of radiosulfate by mitochondria at 26°.

changes. These experiments were therefore carried out in the absence of all ions other than potassium, hydrogen, sulfate, chloride, and hydroxyl (see Methods and Materials). There is a very clean sharp optimum at pH 5.5 and only small uptakes occur at both high and low pH values.

Chemical Nature of Newly Accumulated Sulfate in Mitochondria As shown earlier all the newly accumulated sulfate in mitochondria was rapidly exchangeable with the inorganic sulfate of the medium. Both sterox SK preparations or water suspensions of mitochondria isolated at the peak of sulfate ($S^{35}O_4^=$) accumulation were chromatographed in the following three solvent mixtures: (*a*) butanol 125 vol.:glacial acetic acid 30 vol.:H_2O 125 vol., (*b*)

95 per cent ethanol 65 vol.:0.1 M K_2CO_3 35 vol., (*c*) absolute ethanol 70 vol.:concentrated NH_3 1 vol.:H_2O 29 vol. In all these chromatograms the distribution of radioactivity was identical to that of control chromatograms prepared by adding inorganic radiosulfate to non-radioactive mitochondrial extracts. Appearance of extra radioactive spots was found to be caused by salt formation with the various cations present (Na^+, K^+, NH_4^+, and protein) and not by the formation in the mitochondria of organically bound sulfate. This was confirmed by experiments with paper electrophoresis. Under all conditions, a single discrete spot, which ran with added carrier inorganic sulfate, was obtained. The buffer systems used were 0.1 M barbiturate, pH 8.6, and 0.1 M acetate, pH 5.0.

Treatment of radioactive mitochondrial suspensions with various denaturing agents removed virtually all radioactivity from the particles in the form of inorganic sulfate. These agents were 5 per cent perchloric acid, zinc sulfate and sodium hydroxide, and barium acetate (all used at 0°C).

Boiling a radioactive mitochondrial suspension resulted in material which bound only a small amount of radiosulfate (virtual gradients of 2 to 5 in 10^{-7} M sulfate) comparable in magnitude either to that bound by mitochondria boiled before incubation in the medium normally used, or to that bound by mitochondria which had been preincubated in 0.25 M sucrose at 25°C. The differences in radioactivity of the suspensions of "live" and "dead" mitochondria (obtained as described) were accounted for by a release of sulfate as inorganic sulfate.

DISCUSSION

Mitochondrial Uptake of Sulfate These studies demonstrate that mitochondria prepared from kidney cortex are capable of uptake of $S^{35}O_4^=$ against concentration gradients. The exchange studies also show that this uptake of isotopic sulfate is not a simple exchange process, but rather involves the net gain of sulfate by the mitochondria.

The relatively high values for the activation energies show that for the uptake and for the release of sulfate there is a large effect of temperature similar to that which occurs in the turnover of sodium and potassium in isolated kidney cortex slices (15). This is quite different from the very small effect of temperature on the turnover rate of potassium in a typical cation exchange resin (zeocarb, ten-thirty mesh) (16). It is interesting that the energy barrier to the release of the sulfate ion by the mitochondria is greater than that for the uptake of sulfate onto the available sites on the mitochondria.

The uptake process may be likened to an adsorptive phenomenon similar to the adsorption of a gas by activated charcoal and describable by an equation formally identical to that used for other adsorptive phenomena.

Some of the properties of the sites involved in the binding of sulfate may be inferred from the studies presented. First, the sites are fairly numerous being of the order of 10^5 per average mitochondrial particle. For purposes of reference this figure may be compared to Estabrook and Holowinsky's (17) estimate of about 10^4 molecules of cytochrome *c* and about 10^5 molecules of pyridine nucleotide per average liver mitochondrion. A second property of the sites is their heterogeneity. This may reflect a true qualitative heterogeneity indicating chemically different types of sites for sulfate binding by the mitochondria or it may indicate that the sites are of a single qualitative type but that they show marked interaction. A third attribute of the sites is their apparent thermolability. Despite the fact that the sites are inactivated in 5 to 10 minutes at room temperature it should be noted that the net uptake of radiosulfate is probably somewhat greater (see Fig. 1) than that observed at 0° when inactivation is a much slower process. This apparent discrepancy may be another manifestation of the heterogeneity of the sites. Hence, despite the inactivation of some sites during the uptake, the higher temperature apparently allows more of the sulfate molecules to get over the activation energy barriers and to become adsorbed. An alternative although less likely hypothesis is that at higher temperatures, new sites are being generated or activated concomitantly with the rapid thermal inactivation.

The relationship between the uptake of radiosulfate and the input of energy does not appear to be a direct one in the sense that concomitant oxidative phosphorylation is a necessary requirement for uptake to occur. The need for energy appears rather to be indirect and to be related to prior activation or maintenance of integrity of the binding site. Hence in the preincubation experiments oxidative phosphorylation appears to be a necessary requirement for protection against complete "thermal" inactivation of the sites. Once the sites are activated through the prior input of energy in the intact animal they are capable of uptake of radiosulfate. Although unstable at 26 and 38°C, they are stable at 0°C.

The extreme lability of the binding sites, the partial protection by oxidative phosphorylation against thermal inactivation, and the very sharp pH optimum show that the mechanism of sulfate accumulation is a very delicate and specific process.

The chemical nature of the sulfate which is accumulated by the mitochondria *in vitro* is not known. It is a remarkable fact that sulfate which is accumulated *in vivo* remains in the mitochondria after two washings being virtually non-exchangeable, whereas the sulfate accumulated *in vitro* is virtually completely exchangeable. Presumably the latter fraction also exists in the mitochondria *in vivo* but the method of preparation of the mitochondria is such as to wash it out nearly completely. All of many attempts to find covalently bound organic sulfate in the newly accumulated or released sulfate thus far

have failed in that the sulfate accumulated appears to behave in every way like inorganic sulfate.

Mitochondria are known to contain arylsulfatases and thus must contain sites specific for organic phenols and sulfate. Perhaps the places in the mitochondria which bind sulfate specifically are the "active" sites of these enzymes. This possibility is being investigated.

Relationship of Uptake of Sulfate by Mitochondria to Uptake of Sulfate by Kidney Cortex Slices When kidney cortex slices are incubated at 0°C, radiosulfate from the incubation medium penetrates into the slices slowly, until the virtual concentration of sulfate in slice water is as much as 30 to 40 times that of the ambient medium. Subsequently, such slices may be homogenized in KCl–sucrose containing radiosulfate and the mitochondria separated from the resulting suspension of tissue fragments by differential centrifugation. In these circumstances, the mitochondria show higher virtual gradients of radiosulfate in mitochondrial water than do control mitochondria isolated from slices incubated under conditions identical except for the absence of radiosulfate from the incubation medium. These results (3) indicate that the accumulation of radiosulfate by mitochondria is not an artificial process dependent on conditions incidental to their isolation from the cells. Moreover, the findings offer a reasonable explanation for the similarity between the radiosulfate accumulative processes of isolated mitochondria on the one hand, and, on the other, of kidney cortex slices incubated at 0°C. In both these preparations radiosulfate attains relatively high virtual gradients when the medium contains either Na^+ or K^+ ions, and phlorizin inhibits the process whereas glucose has little effect upon it. In contrast, cortex slices incubated at 38°C show striking depression of sulfate uptake in the presence of Na^+ ions or glucose, and phlorizin enhances sulfate accumulation (3). Thus, it has been suggested that the high sulfate gradients seen in kidney cortex slices incubated at 0°C may be accounted for, at least in part, by intracellular accumulation of the anion at the mitochondrial sites.

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