THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 250, No. 22, Issue of November 25, pp. 8856–8862, 1975
Printed in U.S.A.

Respiratory Properties of Rat Liver Mitochondria Immobilized on an Alkylsilylated Glass Surface*

(Received for publication, July 16, 1975)

BARRY ARKLES AND WILLIAM S. BRINIGAR

From the Department of Chemistry, Temple University, Philadelphia, Pennsylvania 19122

Rat liver mitochondria are shown to adhere to the alkylsilylated glass beads in essentially a monolayer. The amount of mitochondria bound to the beads reaches a maximum where the length of the alkyl groups covalently linked to the beads exceeds eight carbons. Mitochondria immobilized on the beads and placed in a flow system exhibit normal: (*a*) respiratory control, (*b*) phosphate to oxygen ratio, (*c*) uncoupling by 2,4-dinitrophenol and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, and (*d*) inhibition by cyanide, azide, rotenone, oligomycin, and antimycin. Reversibility of the effects of 2,4-dinitrophenol, cyanide, and azide was rapid and complete. Inhibition by rotenone, oligomycin, and antimycin was essentially irreversible. Mitochondria have been maintained in a viable state on the beads at 27° for periods up to 4 hours. The use of immobilized organelles appears to offer a new technique for the study of membrane-bound particles whereby substances can be rapidly added and removed while monitoring the composition of solution flowing over the particles.

Mitochondria exist *in vivo* in a dynamic chemical environment where the concentrations of substrates, metal ions, and other factors influencing oxidative phosphorylation are continuously changing. Classical techniques for investigating mitochondrial properties do not approximate this dynamic situation. Additions made to mitochondrial suspensions are cumulative since one substance cannot be removed from the system as another is added, and decreasing concentrations of substances cannot be easily effected.

Techniques which could be used to simulate the *in vivo* situation require that the mitochondria be stationary in respect to a continuously replenished medium. To achieve this purpose we have attached mitochondria in a viable condition to a finely divided solid support. Solutions may be passed through the mitochondrial bed while monitoring changes in the effluent composition.

EXPERIMENTAL PROCEDURE

Materials—All chemicals were obtained from commercial sources and used without further purification. Rotenone, oligomycin, dinitrophenol, monopotassium α-ketoglutarate, monopotassium succinate, and firefly extract were obtained from Sigma Chemical Co. Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)[1] was obtained from Pierce Chemical Company. ADP, trisodium salt, was obtained from Miles Laboratories. Ultrapure sucrose was obtained from Schwarz/Mann. Porasil C (porous silica beads, 50 to 100 m^2/g, 200 to 400 A pores) and Porasil B (porous silica beads, 200 m^2/g, 100 to 200 A pores) were obtained from Water Associates. Water was double-distilled in glass (specific resistivity, 2 Mohms/cm). Silanes were obtained from Petrarch Systems, Levittown, Pa. Adult male Sprague-Dawley albino rats were obtained from Charles River Breeding Laboratories.

Mitochondria—Rat liver mitochondria were isolated in 0.25 M sucrose, essentially as described by Schneider and Hogeboom (1). In later experiments the procedure of Johnson and Lardy (2) was used.

Preparation of Beads—All glassware used for silylation was pretreated by refluxing for 2 hours a solution of hexamethyldisilazane in tetrahydrofuran containing 1 drop of sulfuric acid. For silylation of the beads, the trichlorosilane was added to 50 ml of anhydrous ethanol in a 100-ml beaker. The mixture was stirred until formation of the corresponding triethoxy derivative was complete (5 to 10 min) as observed by the termination of HCl evolution and complete solution of the silane. It should be noted that the beads as received normally contain 0.5 to 1% water which is necessary for the silylation reaction. Five grams of Porasil were then added with constant stirring. After 5 min ethanol was decanted, and the beads were washed three times with anhydrous ethanol. The residual ethanol was allowed to evaporate and the beads were desiccated *in vacuo*. The beads were then washed twice with water and again dried *in vacuo*. The amount of hydrocarbon attached to the beads was determined by loss in weight after burnout in an open crucible.

Binding of Mitochondria to Beads—The dry weight of mitochondria adhering to the beads was determined by washing the mixture of mitochondria and beads twice with fresh sucrose and then with distilled water, drying *in vacuo*, and finally analyzing for total nitrogen. The nitrogen content of mitochondria alone, determined from samples of several mitochondrial preparations which had been washed with distilled water and dried *in vacuo*, was found to be 3.7 ± 0.1%. This value was used to determine the amount of mitochondria bound to the beads in per cent by weight. Analyses were performed by Galbraith Laboratories. In later experiments the amount of protein nitrogen bound to the beads was determined by the biuret method (3) and found to be 7.0 ± 0.3 mg of protein/g of octadecylsilylated Porasil C.

Scanning Electron Microscopy—Freshly isolated mitochondria were mixed with coated glass beads at 27° and washed with sucrose solution. A sample of the beads was transferred to a cover slip affixed to a

* This work is taken from the dissertation of B.A. to be submitted to the Graduate School of Temple University in partial fulfillment of the requirements for a Ph.D. degree.

[1] The abbreviation used is: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

scanning electron microscopy mount and excess solution removed by touching the corner of a Kimwipe to the mount. Two to three drops of sucrose solution containing 0.2% glutaraldehyde were added and the beads gently agitated with a pipette. After standing for 20 min at room temperature, the solution was drawn off and replaced with distilled water. The beads were gently agitated and the water drawn off. The beads were washed once again in the same manner. The samples were then placed in the deposition chamber and immediately evacuated. A heavy coat of carbon (200 to 400 A) was applied followed by a layer of gold. During microscopy the beads tend to charge easily and it is essential for a thick conductive coat to be applied. Photographs were taken with a JEOL JSM V-3 scanning electron microscope in the secondary emission mode. The beam voltage was usually 25 kv and beam current was 10^{-4} μa. Representative photographs are shown in Fig. 1. The mitochondria are similar in appearance and size to those previously reported by Kurahasi *et al.* (4). We are grateful to Dr. Charles Garber and Thomas Nightingale at Structure Probe Inc., West Chester, Pa., for taking the pictures.

Flow Experiments—All flow experiments were performed in the jacketed apparatus diagrammed in Fig. 2. A YSI model 5331 Clark type polarographic electrode was inserted through the 13-mm opening placing the tip within 1 mm of the fritted disc. The electrode was held in place with a rubber sleeve. Measurements were made with the YSI oxygen monitor and recorded on a Bausch & Lomb VOM5 recorder. The total volume of the empty apparatus from point of entry to level of contact with the electrode is 0.6 ml. Between 0.35 and 0.45 g of treated glass beads was placed in the inner compartment above the fritted glass disc. Flow of solution to the apparatus was supplied by a Sage variable speed syringe pump with a 50-ml syringe. Before reaching the apparatus, the solution passed through a heat exchanger of 7-ml volume packed with broken fritted glass to remove supersaturated air. A 4-inch gum rubber tube connected the heat exchanger to sample chamber inlet. Both the heat exchanger and sample apparatus were maintained at 27.0°.

The flow solution for these experiments was 0.25 M sucrose containing 25 mM KCl and 5 mM potassium phosphate, pH 7.4.

ATP was measured using firefly luminescence as described previously (5). For this assay aliquots of effluent fractions were diluted appropriately and mixed with firefly lantern extract in 0.02 M Gly-Gly buffer containing 0.5 mM $MgCl_2$.

Total O_2 consumption was determined by measuring the area of the oxygen electrode traces and comparing their areas with rectangular areas for which the corresponding amount of O_2 could be calculated from the electrode calibration.

RESULTS

Optimum Conditions for Binding Mitochondria to Beads—Relative amounts of mitochondria bound to the beads as a function of the length of the silane hydrocarbon chains and the amount of silane attached to the beads is shown in Tables I and II. Five grams of silylated Porasil B were first washed with 0.25 M sucrose and then resuspended in 25 ml of sucrose. Three milliliters of cold mitochondrial suspension were added and the resulting suspension was gently mixed at room temperature. The solution was decanted and the beads were washed twice with fresh sucrose solution, then with distilled water, and finally analyzed for total nitrogen as described above. Because of the necessity to remove sucrose by washing with distilled water, the mitochondrial membranes were doubtlessly ruptured and some of the contents were lost. Therefore, the values recorded in Tables I and II should not be taken as the actual dry weight of mitochondria bound to the beads. However, all samples were treated in the same manner and therefore the values represent a relative measure of the amount of mitochondria bound.

Maximum mitochondrial binding occurred where the beads were coated with at least 3% alkyl silane and the length of the alkyl group exceeded eight carbons. In all subsequent experiments the beads used in the flow experiments were reacted with octadecyltrichlorosilane to an extent of approximately 3% hydrocarbon.

The binding of mitochondria to the beads is a dramatic function of temperature. At 27° mitochondria bind tightly and are not washed off the beads even after passage of sucrose solution through the beads at 1 to 3 ml/min for several hours. However, at 0° the mitochondria immediately dissociate from the beads. Scanning electron microscopy photographs of beads which had been coated with mitochondria at room temperature and then placed in an ice bucket showed a total absence of bound mitochondria. This property can be used to advantage in flow experiments since lowering the temperature permits the mitochondria to be washed off and the beads can be recoated with fresh mitochondria after raising the temperature.

Respiratory Behavior—A 0.35-g sample of Porasil C treated with octadecyltrichlorosilane to yield a 3.2% hydrocarbon content was placed in the sample chamber of the apparatus. After uniform flow of the sucrose P_i solution was established, 0.4 ml of mitochondrial suspension was injected. Within approximately 30 s a large drop in oxygen concentration was observed (Fig. 3). The oxygen concentration returned to a steady state after 2 to 3 min consuming O_2 at a rate lower than the detectable limit, *i.e.* less than 0.06 μl/min. After 5 min the solution passing over the electrode became completely clear and the beads had assumed a light yellow cast. Three injections of α-ketoglutarate made 2 min apart produced no observable effect on oxygen concentration. A simultaneous injection of ADP and α-ketoglutarate resulted in an increase in oxygen consumption to a maximum of 0.4 μl/min.

Fig. 3 was one of our early successful experiments at a time when we were not aware that mitochondria do not bind to the beads at low temperature. In this experiment mitochondria were injected as an ice-cold suspension and the amount of mitochondria bound to the beads was less than in later experiments where the mitochondrial suspension was brought to room temperature before it was injected into the stream directly above the beads. The amount of mitochondria bound to 0.4 g of beads was found to be 2.1 ± 0.1 mg of protein based on biuret analysis of several samples. Oxygen uptake was always less when substrate and ADP were added together than in experiments where substrate was a component of the sucrose P_i flow solution and the ADP solution was injected (see Fig. 4). Moreover, the rate of O_2 uptake under the latter conditions was always less than the rate when both substrate and ADP were present in the flow solution (see Fig. 5). A typical rate of O_2 uptake when both α-ketoglutarate and ADP were in the flow solution is 6.5 μl/min giving a standard O_2 uptake of approximately 200 μl/hour/mg of protein. The value compares favorably with values obtained from mitochondria in suspension (2).

Oxygen uptake in the absence of ADP was so small it could not be measured accurately and a quantitative estimate of the respiratory control ratio cannot be made from these data. In later experiments (*e.g.* Fig. 4) where a measurable O_2 uptake did occur in the absence of ADP, the respiratory control ratio is approximately 4.

In order to determine the lifetime of mitochondria bound to the beads, experiments similar to the one shown in Fig. 3 were carried out adding α-ketoglutarate and ADP at 10-min intervals. Oxygen consumption of 0.6 to 0.9 μl/min occurred for about 1 hour, gradually diminishing to less than 0.06 μl/min during the next 2 hours. In one experiment with succinate as substrate, O_2 consumption was still observable after 4 hours.

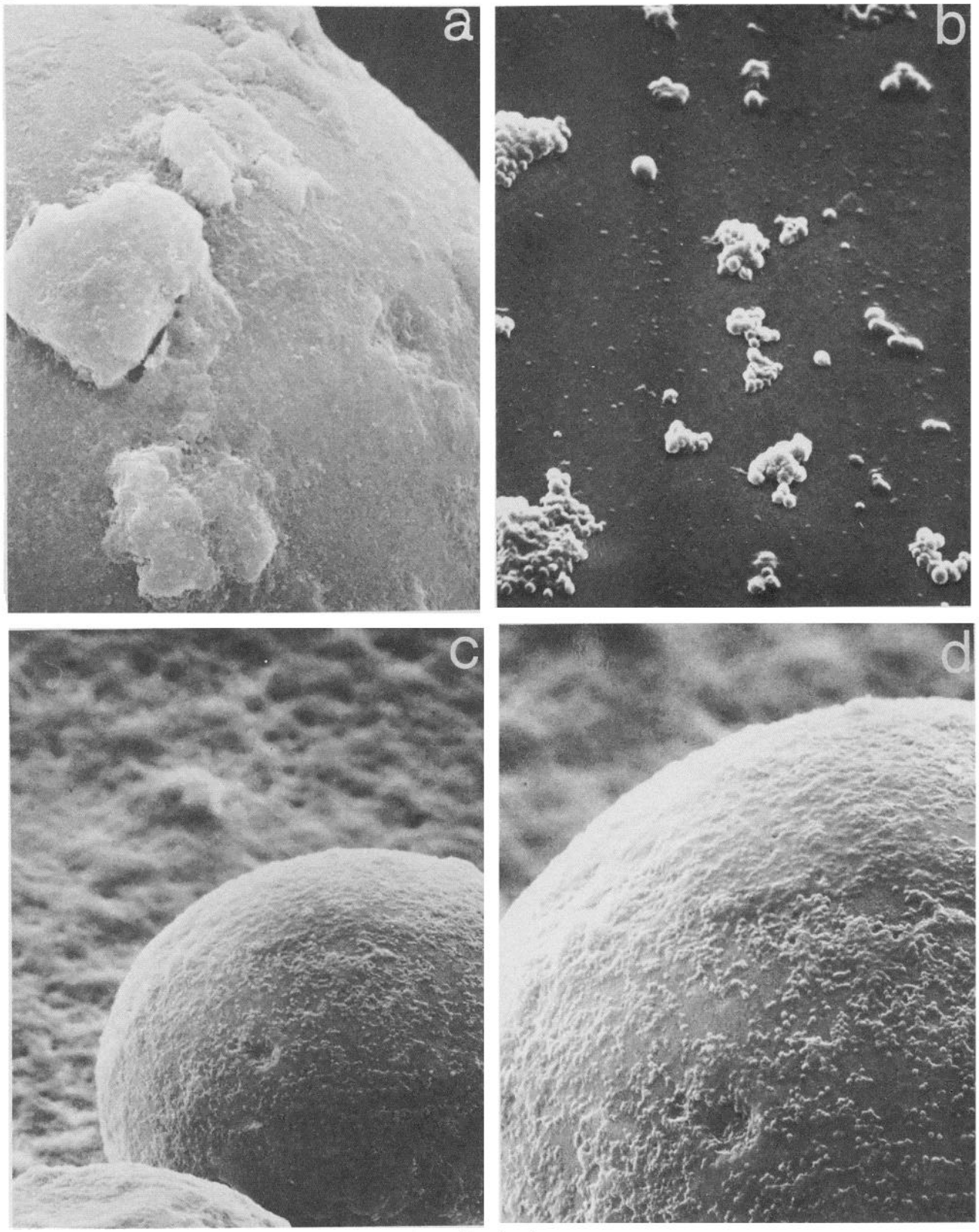

FIG. 1 *a–d*

FIG. 1. Scanning electron micrographs. *a*, surface of a bead after reaction with octadecyltrichlorosilane, × 1000; *b*, rat liver mitochondria fixed with 0.2% glutaraldehyde, × 3000; *c*, *d*, *e*, and *f*, mitochondria bound to a bead surface, fixed with 0.2% glutaraldehyde, × 500, × 1000, × 3000, and × 10,000, respectively.

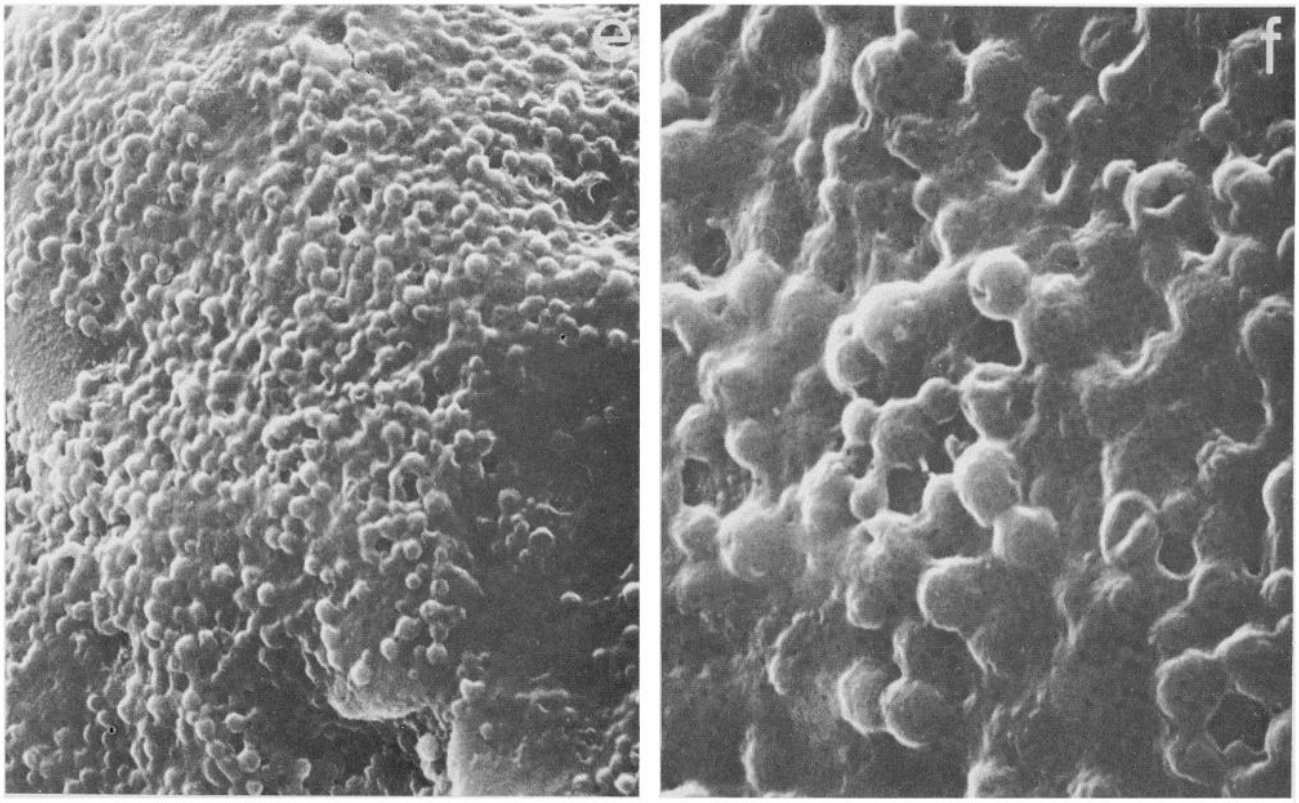

FIG. 1 *e–f*

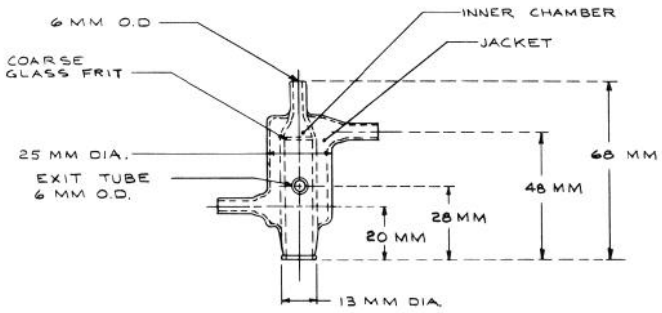

FIG. 2. Glass cell used for flow experiments. Mitochondria bound to coated beads were placed in the inner chamber and a Clark oxygen electrode fitted into the 13-mm opening to within 1 mm of the fritted glass disc.

TABLE I

Amount of mitrochondria bound as function of length of hydrocarbon chain

The indicated amount of alkyltrichlorosilane in 50 ml of ethanol was allowed to react with 5 g of Porasil B. The coated beads were mixed with an excess of mitochondrial suspension and the washed beads were analyzed for bound mitochondria as described under "Experimental Procedure."

Silane	Solution in ethanol	Hydrocarbon on beads	Mitochondria dry weight on beads
	g/100 ml	*g/100 g*	*g/100 g*
Ethyltrichlorosilane	9	3.1	0.4
Butyltrichlorosilane	8	2.7	0.4
Octyltrichlorosilane	6	3.5	0.9
Tetradecyltrichlorosilane	5	3.2	1.4
Octadecyltrichlorosilane	4	3.0	1.3

TABLE II

Amount of mitochondria bound as function of amount of hydrocarbon on beads

Porasil B was reacted with varying amounts of octadecyltrichlorosilane and the washed beads were analyzed for hydrocarbon content. The maximum amount of mitochondria adhering to each bead preparation was determined as described under "Experimental Procedure."

Hydrocarbon g/100 g	0.002	0.29	1.1	3.9	7.4
Bound mitochondria, dry weight, g/100 g	0.0	0.2	0.8	1.2	1.6

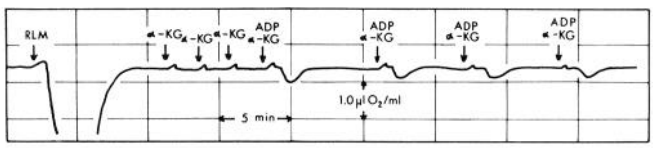

FIG. 3. Respiratory control in immobilized mitochondria. Sucrose P_i solution was passed through the mitochondrial bed at 1.3 ml/min. Five microliters of 1 M α-ketoglutarate (α-*KG*) was added at times indicated. At times indicated by ADP α-KG, 5 μl of 30 mM ADP and 5 μl of 1 M α-ketoglutarate were added simultaneously. *RLM*, rat liver mitochondria.

The slow decline in the observed rate of O_2 consumption may be due to irreversible loss of respiration in the immobilized mitochondria, to a gradual loss of mitochondria from the beads, or both.

Phosphate to Oxygen Ratio—Sucrose P_i solution containing 5 mM α-ketoglutarate and 3 mM Mg^{2+} was used in this

experiment with a flow rate of 3.0 ml/min. Mitochondrial suspension was injected into a 0.45-g portion of Porasil C beads containing 2.9% C_{18} hydrocarbon. Several preliminary additions of ADP were made in order to determine the amount of ADP required to give a maximal rate of O_2 uptake. By adding ADP in an amount less than that required to give the optimum O_2 uptake, we could ensure that most of the added ADP would be converted to ATP. This precaution eliminated errors in the firefly ATP analysis arising from the presence of relatively large amounts of ADP.

When the O_2 concentration again became constant after a previous ADP addition, 10 μl of 6.0 mM ADP were injected into the stream. The resultant change in O_2 concentration was monitored and effluent fractions of 0.5 ml were collected over a period of 3 min. Oxygen uptake, 0.26 μl (0.11 μmol), was complete within 1 min. Analysis of each of the fractions gave a total ATP content of 0.06 μmol, *i.e.* essentially all the ADP was converted to ATP. Therefore, the phosphate to oxygen ratio was 0.06/2(0.011) = 2.7, essentially the same as the traditional value obtained for mitochondria in suspension (6). Although a satisfactory phosphate to oxygen ratio is not a sensitive indicator of mitochondrial integrity, the result clearly demonstrates that the coupling mechanism is functional.

Uncouplers—Experimental conditions were the same as previously described except that the sucrose P_i solution contained Mg^{2+} and succinate. The course of typical experiments is shown in Fig. 4. The addition of amounts of 2,4-dinitrophenol to give a final concentration of approximately 30 μM (Fig. 4) produced primarily uncoupling which was completely reversed within 5 min. Larger amounts (Fig. 4*b*) produced a more rapid uncoupling, followed by inhibition and then uncoupling again prior to recovery. 2,4-Dinitrophenol could be added repeatedly; however, eventually, usually the fourth or fifth time, O_2 uptake diminished to zero with irreversible loss of respiration. ADP added between additions of 2,4-dinitrophenol gave the same response as when it was added before the mitochondria had been exposed to 2,4-dinitrophenol.

Under the same conditions, addition of 10 μl of 100 μM FCCP also induced an increase in O_2 consumption (Fig. 4*c*). However, in this case, uncoupling reversed much more slowly, and additional FCCP failed to elicit any further O_2 uptake. Two microliters of FCCP solution caused an increased O_2 uptake approximately 50% of the maximum achieved with 2,4-dinitrophenol. Reversal again was slow, 9 to 10 min, but the addition of 2 μl of FCCP could usually be repeated three times with resultant increase in O_2 consumption before total loss of respiration occurred.

Cytochrome Oxidase Inhibitors—The effect of both cyanide and azide on O_2 uptake of bound mitochondria is shown in Fig. 5. Addition of cyanide (Fig. 5*a*) reduced the oxygen uptake from 6.5 μl/min to a minimum of 1.0 μl/min. Oxygen consumption slowly returned to 6.1 μl/min over a period of 6 to 7 min. Cyanide was added again with similar response; however, the O_2 uptake recovered only partially, and addition of cyanide a third time usually resulted in irreversible loss of respiration.

With azide (Fig. 5*b*) the inhibition reversed much more rapidly, 2 to 3 min, and azide could be added successively as many as six times before all respiration ceased.

Rotenone Inhibition—Using sucrose P_i solution supplemented with 5 mM α-ketoglutarate, 0.5 mM ADP, and 3 mM Mg^{2+}, the addition of 10 μl of 0.3 mM rotenone resulted in a decrease in O_2 consumption from 4.3 μl/min to 0.5 μl/min within 5 min. The mitochondria did not recover the ability to oxidize α-ketoglutarate, but rather O_2 uptake decreased slowly to zero over the next 30 min. Response to less rotenone (10 μl of a 10 μM solution) was slightly slower but, again, all respiration had ceased after 1 hour. When 10 μl of 1 M succinate was added approximately 5 min after the rotenone addition (Fig. 6*a*), O_2 uptake increased to a maximum of 2.2 μl/min before decreasing again to zero. Repeated additions of succinate elicited similar responses of diminishing magnitude for almost 1 hour.

When the sucrose P_i solution contained 5 mM succinate in place of α-ketoglutarate, no change in O_2 uptake was observed after the addition of 10 μl of 30 μM rotenone.

Oligomycin Inhibition—Oxygen uptake decreased to zero within 3 min after adding 10 μl of 1.0 mM oligomycin to the sucrose P_i stream containing substrate and ADP. The oxygen electrode recordings appeared similar to those observed after rotenone addition in that the onset of inhibition was rapid and recovery of respiration was not observed. Oligomycin inhibition of O_2 uptake could be overcome temporarily with 2,4-dinitrophenol (Fig. 6), showing that the electron transport chain remains functional and that oligomycin is not washed out of the mitochondria.

Antimycin Inhibition—The addition of 5 μl of a 100 μM antimycin solution under the conditions of Fig. 6*c*, produced no observable effect on O_2 uptake. A 5-fold higher concentration of antimycin (Fig. 6*c*) reduced O_2 uptake to about 20% of its original value within 6 to 7 min. Respiration then diminished

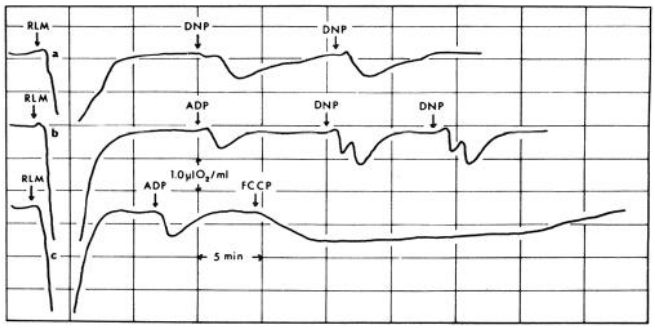

FIG. 4. The effect of uncouplers on O_2 uptake of immobilized mitochondria. The sucrose P_i solution contained 10 mM Mg^{2+} and 5 mM succinate; flow rate was 3.0 ml/min. Additions were made at times indicated by *arrows: a*, 10 μl of 3 mM 2,4-dinitrophenol (*DNP*); *b*, 10 μl of 30 mM ADP and 5 μl of 30 mM 2,4-dinitrophenol; *c*, 10 μl of 30 mM ADP and 10 μl of 100 μM FCCP (60% ethanol). *RLM*, rat liver mitochondria.

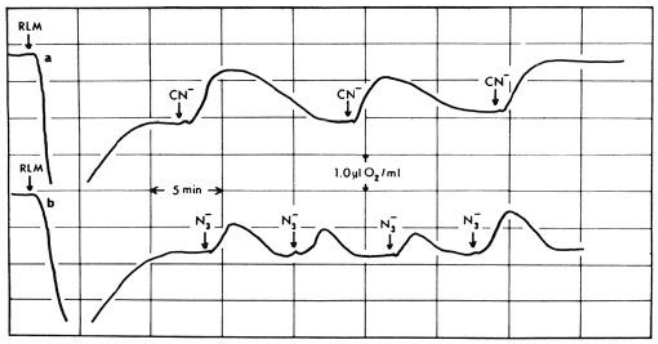

FIG. 5. The effect of cyanide and azide on O_2 uptake of immobilized mitochondria. The sucrose P_i solution contained 3 mM Mg^{2+}, 5 mM succinate, and 0.5 mM ADP; flow rate, 3.4 ml/min. Additions were made at times indicated by *arrows: a*, 10 μl of 0.1 M KCN in 0.25 M sucrose, pH 7.0; *b*, 10 μl of 0.1 M KN_3 in 0.25 M sucrose, pH 7.0. *RLM*, rat liver mitochondria.

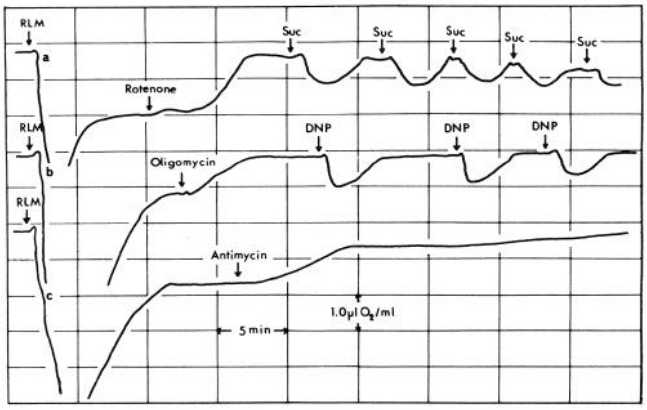

FIG. 6. Effects of rotenone (*a*), oligomycin (*b*), and antimycin (*c*) on O_2 uptake of immobilized mitochondria. *a*, sucrose P_i solution was supplemented with 5 mM α-ketoglutarate, 0.5 mM ADP, and 3 mM Mg^{2+}; flow rate was 2.5 ml/min. Ten microliters of 30 μM rotenone and 10 μl of 1 M succinate (*Suc*) were added at the times indicated. *b*, the composition of the sucrose P_i solution was the same as that used in *a* with a flow rate of 3.4 ml/min. Ten microliters of 1.0 mM oligomycin (50% ethanol) and 10 μl of 3 mM 2,4-dinitrophenol (*DNP*) were added as indicated. *c*, sucrose P_i solution contained 5 mM succinate, 0.5 mM ADP, and 3 mM Mg^{2+}; flow rate was 3.1 ml/min. Twenty-five microliters of 100 μM antimycin (95% ethanol) was added as indicated. *RLM*, rat liver mitochondria.

gradually to zero during the following 20 min. Reversal of antimycin inhibition was never observed.

DISCUSSION

The results clearly demonstrate that mitochondria can be attached to a solid support in essentially a monolayer and can be maintained in a viable condition for substantial periods of time. The machinery of oxidative phosphorylation in the immobilized mitochondria responds to substrates, ADP, uncouplers, and inhibitors in precisely the same way as when the mitochondria are in suspension. Thus, the binding process does not appear to have resulted in any functional changes in the organelle.

Porous silica beads were chosen as the support since they are commercially available and are of uniform size and composition. Although some of the preliminary experiments were carried out on Porasil B, Porasil C has a slightly larger bead size and was found to permit higher flow rates with less back pressure. Alkylsilylation provided a facile method of providing the beads with a lipophilic surface. Since the quantity of mitochondria bound reaches a maximum where the alkyl chain exceeds eight carbons, it would appear that the mitochondria are attached by penetration of the alkyl chain into the outer mitochondrial membrane. A number of enzymes which utilize long chain fatty acids (7) and phospholipids (8) as substrates are located in the outer membrane and they could also participate in the binding. The observed temperature dependence of the binding is consistent with a hydrophobic interaction between mitochondria and the bead surface.

In many tissues mitochondria are found to be fixed relative to other cellular components. A particularly intriguing example, which may be relevant to the situation here, is the observation of Palade (9, 10) that, in the pancreas of guinea pigs starved for 48 hours, mitochondria were found associated with lipid droplets. In some of the electron micrographs the outer mitochondrial membrane cannot be differentiated from the lipid droplet, the interface appearing to be one continuous phase.

The apparatus currently used for the flow experiments makes no provision for efficient mixing of solutions injected into the stream. Therefore, the actual concentration of substances added is not accurately known. Colored solutions, such as 2,4-dinitrophenol, appeared homogeneous with a volume of approximately 1 ml as they traveled through the mitochondrial bed. Assuming that all injected solutions undergo dilution to 1 ml probably gives a reasonable estimate of actual concentrations. Sophisticated experiments will require a more elaborate flow system whereby the concentration of a number of substances can be accurately known and rapidly changed.

The flow system is well suited for observing the reversibility of uncouplers and inhibitors of oxidative phosphorylation. The customary method of reversing the effects of uncouplers (11, 12) and inhibitors (13) is to add serum albumin to a mitochondrial suspension. However, it has been shown recently (14) that washing mitochondria with bovine serum albumin reduces the rate of phosphorylation by 30 to 40%. Thus serum albumin exerts a direct effect, although perhaps only a minor one, on oxidative phosphorylation in addition to its high affinity for many uncouplers and inhibitors. The use of immobilized mitochondria in a flow system eliminates the necessity of adding serum albumin in the case of some of the widely used inhibitors and uncouplers. On the other hand, some of these agents are not readily washed out of the mitochondria and their action is essentially irreversible.

Low concentrations of 2,4-dinitrophenol elicit only uncoupling whereas higher concentrations initially uncouple, but as absorbed 2,4-dinitrophenol goes through a maximum, a transient inhibition occurs. Both uncoupling and inhibition are rapidly reversed. Such is not the case with FCCP where the initial uncoupling occurs more slowly than with 2,4-dinitrophenol and normal respiration is never recovered. Differences between the reversibility of 2,4-dinitrophenol and FCCP probably can be largely attributed to differences in the partition coefficients of 2,4-dinitrophenol and FCCP between lipid and aqueous regions. The hydrocarbon coating on the beads would be expected to contribute to the slow reversibility of hydrophobic substances by absorbing and then slowly releasing the substances to the mitochondria and the aqueous phase.

The differences in reversal of the inhibition elicited by cyanide and azide cannot be explained on the basis of solubilities. The effect of azide is much more rapidly reversed than that of cyanide. Yonetani and Ray (15) found that the cytochrome oxidase cyanide complex dissociates rapidly. Therefore, this cannot be the only inhibitory reaction occurring. Cyanide is known to react with other mitochondrial components, *e.g.* ketoacids (16), ferricytochrome *c* (16), NAD^+ (17), and these reactions could contribute to the slower reversal of cyanide inhibition.

Inhibition by antimycin, oligomycin, and rotenone was found to be essentially irreversible under the conditions employed here. Therefore, it may be possible to employ this system to advantage in inhibitor binding site titrations (for *e.g.* see Ref. 18).

The use of this technique should facilitate studies of mitochondrial function under a wide variety of experimental conditions heretofore unattainable. Of course, other electrodes can be used to monitor the composition of effluent from the mitochondrial bed, and several electrodes could be used simulataneously. The effluent also could be passed through a spectrophometer flow cell, or mixed with scintillator, or both, and passed through a counter. Immobilized emzymes could be

placed downstream from the mitochondrial bed in order to further facilitate analysis of substances in the effluent. Isotopically labeled compounds could be added and rapidly chased; for example, tritium labeling experiments similar to those reported by Ryrie and Jagendorf (19) could be carried out more readily on immobilized organelles.

Obviously other membrane bound particles should be subject to immobilization by application of the same principles. Studies with chloroplasts and microsomes are in progress in our laboratory. Immobilized cellular organelles appear to offer a promising experimental approach toward further elucidating the intricate chemistry of subcellular particles.

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