B. C. Arkles, A. S. Miller, W. S. Brinigar, Whole cell and cell organelle immobilization on siliceous surfaces," in "Silylated Surfaces", D.E. Leyden, W. T. Collins, eds. Gordon and Breach, New York, 1980, 363-375.

Whole cell and cell organelle immobilization on siliceous surfaces

BARRY C. ARKLES[†], ANDREA S. MILLER, AND WILLIAM S. BRINIGAR

† *Research Laboratories, Petrarch Systems, Inc., Levittown, PA 19059*
Dept. of Chemistry, Temple University, Philadelphia, PA 19122

Normal and sickle erythrocytes, mitochondria, chloroplasts and microsomes were bound in a physiologically active state to silylated siliceous surfaces. In order to optimize binding the following parameters were studied: silane surface treatment, cell and organelle preparation, temperature and time of contact of the cells and organelles with the substrate needed to achieve immobilization. Long chain alkyl silylated surfaces demonstrated good affinity for cell organelles, but only fair affinity for erythrocytes. Optimum surface treatments for binding erythrocytes are not simply described, but apparently mixed hydrophobic and polar functionality is required. While chloroplasts and microsomes bind readily at 10-30°, both erythrocytes and mitochondria requires somewhat higher temperatures for optimum binding. Once immobilized the erythrocytes remain bound at low temperatures. The binding of mitochondria is labile at low temperatures.

1 INTRODUCTION

The immobilization of whole cells and cell organelles has demonstrated greatest utility in elucidating their own biochemistry. Traditional suspension studies allowed convenient addition of various substances, but not their removal. Immobilization of whole cells and cell organelles allows study under conditions of changing cellular environment which more closely corresponds to the *in vivo* situation. The immobilization of enzymes, in contrast, has demonstrated greatest utility in bioengineering applications for the production of foodstuffs and biologically active molecules. In the long term, the utility of immobilized cells and organelles will be similar to that of the enzymes. The emergence of genetic engineering via recombinant DNA allows the production of whole cells containing desired enzymes. Rather than isolating the enzymes from the cells, it may well be more convenient to use the cell or a portion of it. The same advantages that favor immobilized enzyme technology over fermentation technology are still present: the need for process rather than batch chemistry, the expense of producing "engineered" cells, the need to produce a biologically clean product.

Whole cells and cell organelles are sensitive to most reagents required for covalent bonding. Consequently, immobilization is effected by non-covalent methods. Methods of physical entrapment involving starch and polyarcylamide gels, or collodion and polypeptide matricies are possible approaches. Whole cells have been immobilized in cross-linked collagen and shown to produce desirable metabolic products.[1] While in some instances these techniques may be applied satisfactorily, the diffusion of substrates and products is seriously hindered and precludes their broad application of entrapment techniques. Adsorption of cells and organelles through hydrophobic or ionic interaction is both mild and has little effect on substrate or metabolite diffusion.

A priori reservations concerning the effectiveness of adsorption immobilization techniques generally arise from the parsity of data available for applying physio-chemical effects to macrostructural cells or cell components. Enough data is available on mitochondria to satisfy these reservations. It has been shown that at the slightly alkaline conditions of mitochondrial media the majority of proteins tested bind non-specifically to n-alkylated agarose. A rat liver mitochondrion has a projected planar contact area of $0.45\mu^2$. A typical E type borosilicate glass[2] substrate has 6-15 silanol groups per $m\mu^2$ which can be converted to sites of hydrophobic interaction by alkylsilylation. This is the equivalent of 30,000 sites of hydrophobic interaction per mitochondrion. Taking the free energy of solution of butane − 5800 cal/mole,[3] it can be noted that approximately 20 hydrophobic interactions are equivalent to one covalent bond. The hydrophobic interaction between a mitochondrion and an alkylsilylated surface is, therefore, equivalent to 1000 covalent bonds. Silica surfaces have greater concentrations of silanol groups than glass and oligomerization of trialkoxy or trihalo silanes results in an even greater concentration of sites for hydrophobic interaction.

Earlier work has reported physiological properties of immobilized mitochondria in detail,[4] and the immobilization of microsomes and chloroplastes.[5] While the immobilization of erythrocytes has been reported,[6] an examination of modified siliceous substrates has not been conducted. In this report emphasis has been placed on immobilization methods for both cell organelles and whole cells.

2 EXPERIMENTAL

2.1 Materials

Porasil B (porous silica beands $200m^2/g$, 100-200 A) pores and Porasil C ($50\text{-}100m^2/g$, 200-400 A pores) were obtained from Waters Associates, Borosilicate glass beads (80-100 mesh) were obtained from Potters Brothers, Inc.,

rescreened, then washed with nitric acid and distilled water before use. Silanes were obtained from Petrarch Systems, Fine Chemicals Division. Monopotassium ketoglutarate, monopotassium succinate and firefly extract were obtained from Sigma Chemical. Ultrapure sucrose was obtained from Schwarz-Mann. ADP, trisodium salt was obtained from Miles Laboratories. Inorganic salts were reagent grade materials obtained from J.T. Baker or Fisher. All chemicals were used without further purification. Water was double distilled in glass (specific resistivity 3MΩ).

2.2 Preparation of supports

Porous silica and glass beads were allowed to condition at ambient humidity. For silica this resulted in 0.5-1.0% water content. For glass this resulted in 0.1-2.0% water content. For silylation of supports with chlorosilanes, approximately 1g of silane was added to 50 ml of anhydrous ethanol in a 100 ml beaker. The mixture was stirred until the formation of the corresponding ethoxy derivative was complete (5-10 minutes) as observed by the termination of HC1 evolution and complete solution of the silane. The solution was warmed to just below reflux on a hot plate and 5g of the support was added while stirring with a glass rod. After five minutes the ethanol was decanted, and the support washed three times with anhydrous ethanol. The residual ethanol was allowed to evaporate and the beads were dessicated *in vacuo*. The same procedure was followed for alkoxy silanes, except that 95% ethanol was used and one drop of acetic acid was added before warming the solution and treating the support. The amount of organic coupled to the beads was determined by loss in weight after burn out in an open crucible.

2.3 Scanning electron microscopy

Since they were not stable to usual preparative procedures,[7 8] a simplified procedure was developed for preparing cell and organelle composites for microscopy. A cover slip was affixed to an SEM mount with epoxy cement. A Pasteur pipette was utilized to transfer the mitochondria bead composite to the mount while in the sucrose solution. Excess solution was removed by touching the corner of a kimwipe to the mount and drawing off the liquid. Two-three drops of appropriate medium containing 0.2% gluraraldehyde were then added. The beads were gently agitated with a pipette and the mixture was allowed to stand 20 minutes at ambient temperature. The solution was drawn off and replaced with distilled water. The beads were gently agitated and the water drawn off. Erythrocytes were dehydrated with successively more concentrated ethanol. The samples were then placed in the deposition chamber and immediately evaluated. As soon as an acceptable vacuum was achieved a heavy coat of carbon

(200-400 A) was applied. The sample was then coated with a layer of gold. During spectroscopy the beads tend to charge easily, and it is essential that a thick conductive coat be applied. Photographs were taken with JEOL JSM V-3 in the secondary emission mode. The beam voltage was 25 KV for most of the samples tested. In preliminary work in which samples started to become charged, voltage was dropped to 15 KV. Beam current was 10^{-4} microamps.

2.4 Flow experiments

Two types of apparatus have been used to house the cells and cell organelles for the flow experiments. A simple all glass cell is shown in Figure 1, and a more elaborate Plexiglass cell in Figure 2. In both cases flow is maintained through the cell by a syringe pump, or more conveniently, by a peristaltic pump. The inner chamber of both cells accommodated to 0.3–0.4 g of octadecylsilylated beads. Flow rates of 3.0 ± 0.5 ml $\min^{-1}$ have been routinely used. A Yellow Springs Instrument (YSI) model 5331 Clark polarographic electrode with a YSI oxygen monitor was employed with both cells. Monitoring the O_2 concentration requires a recorder with sufficient sensitivity to accurately measure changes of a few mV. An Esterline Angus Speed Servo Series S recorder with an adjustable range from 0 to 100 mV and low noise, was found to provide convenient flexibility, although a Bausch and Lomb VOM5 also has been used.

2.4.1 Preparation of mitochondria. Rat liver mitochondria were prepared essentially by the method preciously described,[9] with the added precaution of removing as much excess lipuid as possible with cotton swabs after each centrifugation step. A final washing of the mitochondria with 1% fat free bovine serum albumin (Miles Laboratories) was found to give better reproducibility of mitochondrial binding to the beads. It also appears to be advantageous to allow the animals to feed freely prior to preparing the mitochondria, rather than starve them for 24 hours as is routinely done in some laboratories. Even with these precautions, considerable variability has been experienced in the number of mitochondria from different preparations which bind to the beads.

2.4.2 Binding of mitochondria. Binding of mitochondria was determined by first washing 5 g of the treated beads twice with 0.25 M sucrose solution buffered with 5 mM phosphare to pH 7.4 and placing them in 50 ml polyethylene test tubes in 25 mls of the sucrose solution. Three milliliters of mitochondrial suspension were added and the mixture was agitated mildly for 5 minutes. The solution was decanted and the beads washed twice with 30 mls of sucrose and then 3 times with glass distilled water. The beads were dessicated in vacio. The entire sample was submitted for nitrogen analysis by Galbraith Laboratories. The nitrogen content of mitochondria alone, determined from

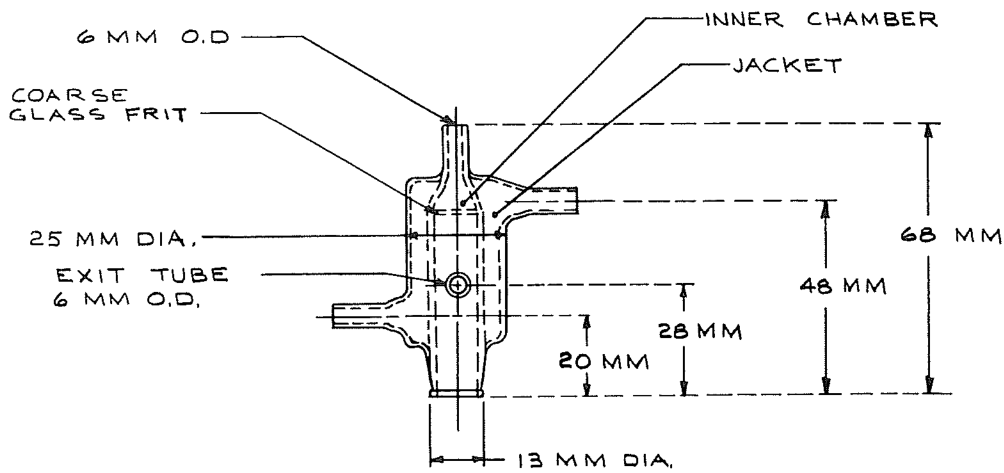

FIGURE 1. Glass cell for flow experiments. Cells and organelles are bound to silylated beads in the upper inside chamber and a Clark oxygen electrode is fitted into the 13 mm opening to within 1 mm of the fritted glass disc.

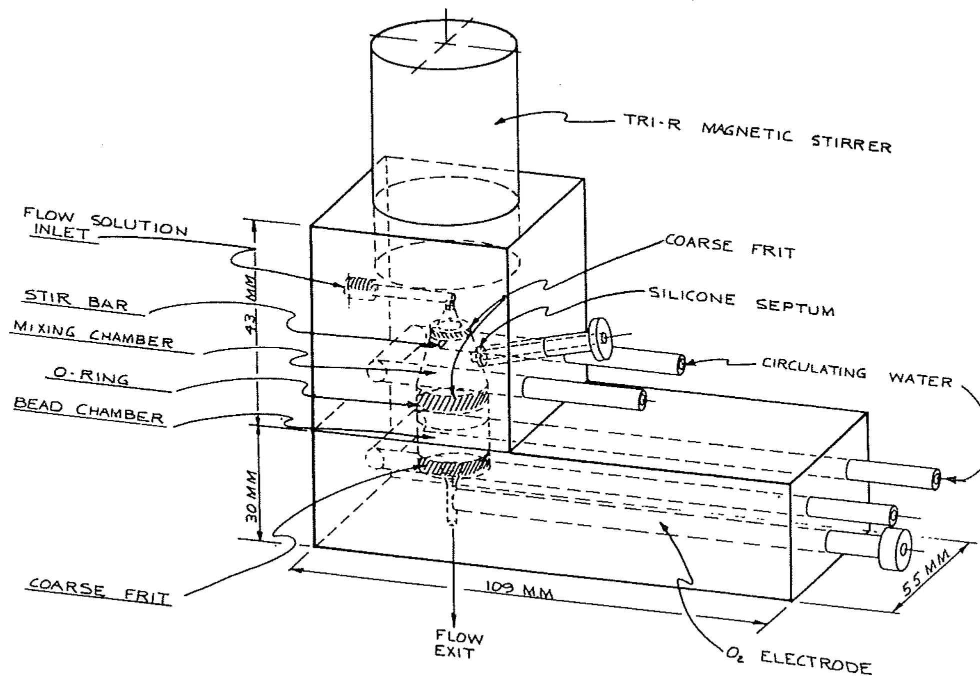

FIGURE 2. Plexiglass cell for flow experiments. Immobilized cells and organelles are contained between the two fritted glass discs. Additions are made into the mixing chamber by syringe through the septum.

samples of several mitochondrial preparations which had been washed with distilled water and dried in vacio was found to be 3.7 ± 0.2%. This value was used to estimate the amount of mitochrondria bound to the beads by weight, i.e. percent nitrogen found for mitochondrial bead composite × 27 equals the percent mitochondria bound. Biuret analysis of protein was conducted according to the method of Gornall et al.[10]

2.5 Respiratory properties

A charge of 0.35 gms of octadecyl treated silica beads was placed in a closed sample chamber. A uniform flow of 0.25 M sucrose solution containing 5 mM potassium monobasic phosphate and 25 mM potassium chloride adjusted to pH 7.4 was established at 1.3 ml/minute. Approximately 0.4 ml of mitochondrial suspension was equilibrated at 27.0°C and then injected. In approximately 30 seconds a large drop in oxygen concentration was observed. The silica beads assumed a light yellow cast indicative of the mitochondria bound on the bead surface. After several minutes the oxygen concentration returned to a steady state. A simultaneous injection of ADP and α-ketoglutarate resulted in an increase on oxygen consumption to a maximum of 0.4 ml/min. The course of the experiment is shown in Figure 3. Analysis of the effluent by firefly luminescence indicated presence of ATP.

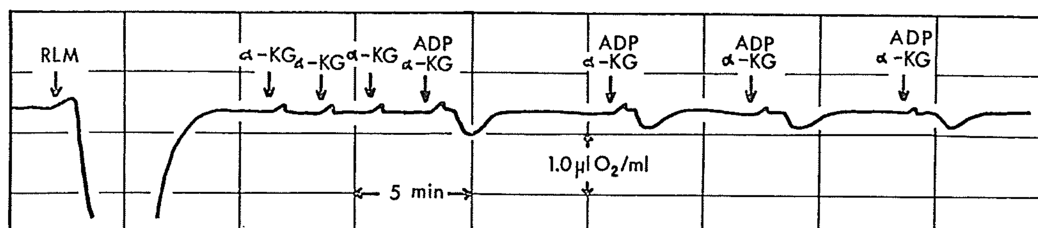

FIGURE 3. Respiratory response of mitochondria. After addition of rat liver mitochondria (RLM) and binding to octadecylsilylated silica, additions of α-ketoglutaric acid (αKG) alone, or in combination with ADP are made. The oxygen uptake in the presence of ADP indicates coupled oxidative phosphorylation.

3 RESULTS

3.1 Substrate optimization for binding mitochondria

The relative amounts of mitochondria bound to the beads as a function of the length of silane hydrocarbon chains and the amount of silane attached to the beads is shown in Tables 1 and 2. Because it is necessary to remove sucrose by washing with distilled water, the mitochondria were doubtlessly ruptured and

TABLE 1
Optimum hydrocarbon length

Silane	% weight solution in	% hydrocarbon on porasil B	% mitochondria, dry weight on porasil B
Ethyltrichlorosilane M.W. 163.5	9	3.1	0.4
Butyltrichlorosilane M.W. 191.5	8	2.7	0.4
Octyltrichlorosilane M.W. 242.7	6	3.5	0.9
Tetradecyltrichlorosilane M.W. 331.8	5	3.2	1.4
Octadecyltrichlorosilane M.W. 388.0	4	3.0	1.3
Octadecyldimethyl 3-(trimethoxysilyl)-propyl ammonium chloride M.W. 496.3	3	1.7	0.9

TABLE 2
Binding of RLM as a function of hydrocarbon concentration
(Hydrocarbon from octoadecyltrichlorosilane treatment of porasil B)

% Hydrocarbon	0.02	0.29	1.1	3.9	7.4
% Mitochondria, dry weight	0.0	0.2	0.8	1.2	1.6

TABLE 3
Binding of RLM as a function of temperature on 2.7% octadecyl silylated porous silica

Adsorption temp., °	Wash temp., °	% mitochondria dry weight
30	30	1.2
27	27	1.2
27	7	0.3
7	7	0.0

some of the contents lost. The reported result of 3.7% nitrogen is quite low when compared to 12.8% reported for intact mitochondria.[11] Since over 55% of mitochondrial protein can be solubilized[12] along with some nucleic acid, the largest remaining source of nitrogen is probably membrane-bound protein for which 3.7% is a reasonable number. The value is reported here only for correlating the amount of bound mitochondria. In later experiments, the amount of mitochrondria bound to the beads was determined by the Biuret method and found to be 7.0 ± 0.3 mg protein/gm of octadecylsilylated porasil C.

Adhesion of mitochondria achieved a maximum when carbon chain length exceeded eight and percent hydrocarbon loading exceeded 1%. Throughout the experiments octadecylsilylated silica beads with a concentration between 2% and 3.5% were utilized unless otherwise noted. Variation of the silane within this range did not appear to affect binding or respiratory behavior of the mitochondria.

Binding of mitochondria is extremely temperature sensitive, apparently requiring a minimum temperature of 25°. Apart from the results reported, mitochondria were observed to lose adhesion at ambient temperature. The physiological response profile indicates utilization of substrate and oxygen with a requirement for ADP. The implied coupling of oxidation with phosphorylation is validated by the positive assay for ATP.

3.2 Microsomes

Microsomes were prepared from rat liver following the procedure for mitochondria. The supernatant, after the 9000 xg sedimentation of mitochondria, was recentrifuged at 24,000 xg and the supernatant consisting of crude microsomes was retained. The apparatus contained porous silica beads treated with octadecyltriethoxysilane to yield a hydrocarbon content of 2.7%. Upon injection of the microsonal suspension, the beads assumed a pale pinkish cast. Analysis indicated 0.7% bound microsomes.

3.3 Chloroplasts

Active phosphorylating chloroplasts were isolated from spinach leaves by an abbreviation of previously reported procedure[13] by grinding 50 g of leaves in a pre-cooled mortar with 100 ml of 0.35 M sodium acetate, 10 ml of 0.2 M Tris buffer at pH 8, and 50 g of cold sand. The slurry was squeezed through cheese cloth and centrifuged at 0°C for 1 minute at 200 xg. The supernatant was retained and centrifuged for 7 minutes at 1000 xg. The supernatant was discarded and the pellet of chloroplasts resuspended in about 2 ml of 0.35 M sodium acetate.

Chloroplasts were added to octadecylsilylated silic beads. The beads

immediately assumed a green cast. Oxygen evolution was observed with the bound chloroplasts were exposed to intense light, indicating that the Hill reaction portion of the photosynthetic apparatus remained intact. No indication of any reduction in adhesion was observed over a temperature range of 0-30°C for a period of two weeks.

3.4 Erythrocytes

3.4.1 Preparation. Erythrocytes[14] (1-21 days old) were washed twice with 0.85% saline – pH 7.6 phosphate buffer. The cells were centrifuged at 600-700 g. The supernatant was removed and the cells were resuspended in either 310 mosM pH 7.6 phosphate buffer or 310 mosM sucrose-phosphate (7:3). They were centrifuged again at 600-700 g and the supernatant removed from the packed cells.

3.4.2 Binding. After washing silylated supports with either phosphate or sucrose-phosphate buffer, 0.1-0.2 g in buffer was transferred to a 15 ml centrifuge tube. The support and the erythrocytes were allowed to equilibrate separately at the temperature at which binding was to be investigated. A 9.5 ml aliquot of a 50% suspension of the packed red blood cells was added to the support. The tube was gently agitated and then allowed to remain at the test temperature. In later experiments a temperature of 37° for 90 min. was used. The supernatant was removed and the composite washed twice with 5 ml of

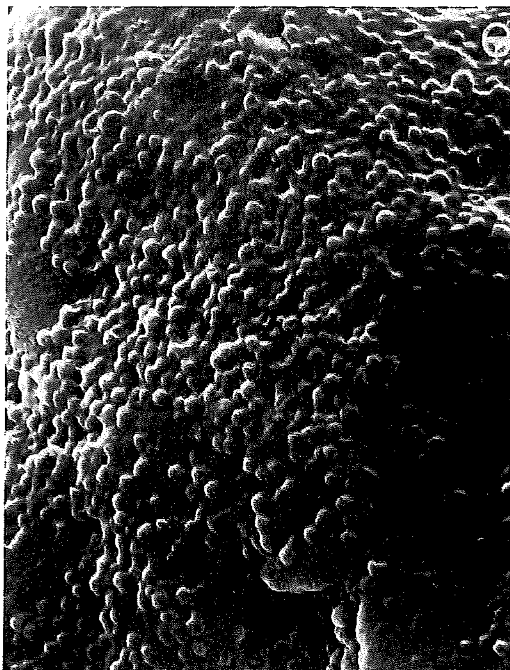

FIGURE 4. Rat liver mitrochondria on octadecylsilylated silica x 3000.

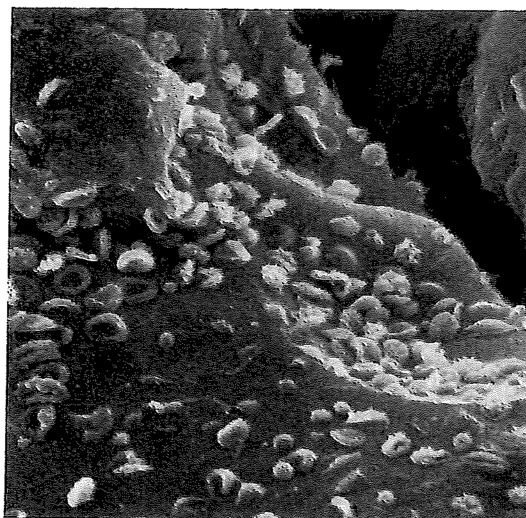

FIGURE 5. Human erythrocytes on ethyltrimethoxysilane treated silica x 1000.

phosphate or sucrose-phosphate buffer.

General visual examination against a white background was used to qualitatively rank adhesion of the red blood cells to the substrate. In order to insure that coloration was due to binding of whole cells and not hemoglobin containing fragments, samples were examined with optical and scanning electron microscopes.

4 CONCLUSIONS

Porous silica beads were selected over borosilicate glass beads throughout most of the experiments. The silica not only demonstrated greater uniformity in composition, but underwent silylation reactions with greater facility. Although no discrete experiments were conducted, it also appeared that the silylated glass surfaces were less stable than the silylated silica surfaces at the mildly basic physiological pHs. Even when both substrates were optimally prepared, greater cell organelle and whole cell affinity was observed for the silica than for the glass. Clearly the cells and organelles are much larger than the silica pores, and binding is confined to the outer surface. Solid glass beads may be advantageous when diffusion of substrates into pores obscures response times.

The experiments clearly demonstrated the affinity of cell organelles for long chain alkyl silylated surfaces. The binding process does not appear to have resulted in any functional changes in the organelles. This is not surprising in view of the fact that organelles are usually found fixed relative to other cell components. A comparison may be drawn between the binding of mitochondria to

TABLE 4
Adhesion of erythrocytes vs time and temperature

Treatment	Organic %	Normal erythrocytes	Sickle erythrocytes
untreated	0.0	+	+
N-(triethoxysilylpropyl) urea	3.7	+++	+++
ethyltrimethoxysilane	1.8	+++	+++
N-(3-triethoxysilylpropyl)-O-cholesteryl urethan	3.5	+++	+++
octadecyltrichlorosilane	3.3	++	++
octadecyldimethylchlorosilane*		0	0
propyldimethylchlorosilane*		0	0
N-trimethoxysilylpropyl (polyethylenimine)		+	+
sulfate quat. of above		+	+
chloride quat. of above		++	++

+++ good adhesion
++ moderate adhesion
+ some adhesion
0 no adhesion
* difficuly in wetting substrate

TABLE 5
Adhesion of erythrocytes cs time and temperature

Time, min	0-5°	25°	37°
0	0	0	0
10	0	+	+
30	0	+	++
45	0	++	+++
60	+	++	+++
90	+	+++	+++
120	+	+++	+++
180	++	+++	+++

Values for ethyltrimethoxysilane treated substrates.

TABLE 6
Comparison of borosilicate glass and silica substrates

cell – organelle	silylation	glass	silica
mitochondria, 27°	octadecyl	++	0
mitochondria, 27°	none	0	0
erythrocytes, 37°	ethyl	+++	+
erythrocytes, 37°	cholesteryl urethan	+++	+
erythrocytes, 37°	none	+	0

alkylsilylated surfaces and their association with lipid droplets within the cell. Hydrophobic interaction is certainly one component contributing to binding. An additional possibility is the existence of specific receptor sites for alkyl groups within the organelle membrane. Apart from the simple experiment depicted in Figure 3, immobilized mitochondria have been shown to undergo reversible DNP uncoupling, cyanide and azide inhibition, and more complex responses to oligomycin, FCCP and rotenone. Immobilized chloroplasts have been shown to produce oxygen when exposed to light.

Unlike the cell organelles, erythrocytes are mobile in their physiological environment. Erythrocytes are known to possess anionic protein moieties in their outer membrane. The greater affinity of the erythrocytes for the hydrochloride salt of polyethyleneimine treated substrate than the free base may be explained in terms of electrostatic interaction. The mild affinity of erythrocytes for untreated silica, however, is unexpected since the isoelectric point of silica is 2. The other silylated surfaces indicate the complexity of membrane interaction. Greater binding occurred with cholesteryl urethan and the ethyl silylated silica. Minimal binding occurred with long chain alkyl and dimethylalkyl silylated surfaces. The hydrocarbon loading of the ethyl silylated silica indicates deposition of poly(ethylsilsesquioxane). The polymer would have mixed polar and non-polar functionality which could be compared to the cholesteryl urethan. A combination of functions may be required to adsorb and penetrate the erythrocyte membrane.

References

1. W. R. Veith, Belgium Patent 7,401,542 (1975).
2. J. B. Donnet, R. Battisella, and B. Chatanet, *Glass Technol.* **16**, 139 (1975).
3. C. Tanford, *The Hydrophobic Effect*, Wiley, New York (1973).
4. B. Arkles and W. S. Brinigar, *J. Biol. Chem.* **250**, 8856 (1975).
5. B. Arkles and W. S. Brinigar, U.S. Patent 4078971 (1978).
6. B. S. Jacobson and D. Branton, *Science* **195**, 302 (1977).
7. I. Corvin, *Research/Development* **23** (7), 28 (1972).
8. K. Kurahasi, J. Tokunaga, T. Fujita, M. Miyahara, and M. Arch, *Hist. Jap.* **30**, 217 (1969).
9. D. Johnson and L. Hardy, *Methods in Enzymology*, Vol. X (R. W. Estabrook and M. E. Pullman, eds.), Academic Press, New York (1967), p. 94.
10. A. G. Gornall, C. J. Bardawill, and M. M. David, *J. Biol. Chem.* **177**, 751 (1949).
11. U. Glas and G. F. Bahr, *J. Histochem. Cytochem.* **14**, 40 (1966).
12. W. R. Frissel, M. U. Patwardhan, and C. G. Mackenzie, *J. Biol. Chem.* **24**, 1829 (1965).
13. D. A. Walker, *Methods in Enzymology*, Vol. XXIII (A. San Pietro, ed.), Academic Press, New York (1971), p. 211.
14. E. Antonini and M. Brunori, *Haemoglobin and Myoglobin in their Reactions with Ligands* (A. Neuberger, E. L. Tatum, eds.), American Elsevier, New York (1971), p. 2.

DISCUSSION

Dr. P. K. Smith *(Pierce Chemical Company, Rockford, Ill):* Did you find any difference between cell types?

Dr. B. Arkles: Although we did not examine details in the binding of the various blood types, we did not observe any during our utilization of A and B types. Initially, we thought we observed a greater affinity of sickle cells for substrates, but subsequent experiments did not substantiate these results.

Dr. R. W. Von Korff *(Midland Macromolecular Institute, Midland, MI):* (1) Did you measure the Qo_2 ($\mu g\ O_2$/mg protein/min) of your bound mitochondria to compare them with free isolated mitochondria? (2) Did you try substrates embracing a larger portion of the Krebs cycle, e.g. glutamate and malate or pyrunate and malate?

Dr. B. Arkles: O_2 uptake was comparable to unbound protein, although perhaps 25% lower. In typical experiments bound mitochondria gave a standard O_2 uptake of approximately 200 μl/hour/mg.

Prof. P. W. Carr *(University of Minnesota, Minneapolis, MN):* Does your technique have any significant advantages over entrapment in a gel?

Dr. B. Arkles: While mitochondria can be entrapped, the conditions of entrapment would be anticipated to have a negative offset on the ability of the mitochondria. Moreover, diffusion factors would also be expected to delay and obscure the rapid response we desired in order to observe respiratory properties.