Mediated (Nonactive) Transport of Glucose in Mammalian Cells and its Regulation

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ABSTRACT Mediated (nonactive) transport of glucose in mammalian cells is characterized by saturation kinetics, stereospecificity, sensitivity to inhibition by phlorizin and certain sulfhydryl-blocking agents, a temperature coefficient of about 2, an inability to utilize metabolic energy, and countertransport. Countertransport can be explained by the development of carrier gradients in the cell membrane and provides the best evidence for carrier mobility. Efforts to identify and isolate chemical components of the transport system have not been successful. Transport among different types of mammalian cells shows a wide range of activities (V_{max} values differ by three or more orders of magnitude) and different sensitivities to hormones. Glucose enters the liver cell by mediated transport, as shown by a difference in the penetration rates of D- and L-glucose and sensitivity to phlorizin. The activity of the system is one of the highest known. Transport in muscle is the most important rate-controlling step for glucose utilization and is strongly accelerated by hypoxia, work, and insulin. The effect of work or insulin is strongly inhibited by metabolism of fatty acids. Insulin also stimulates glucose transport in adipose tissue. Using isolated fat cells, it could be shown that insulin is rapidly bound to sites on the cell surface. The effect is lost within a few minutes after the exogenous hormone is removed. The bound insulin is not released as such, but is metabolized to unknown products. Binding is prevented by preexposure of cells to maleimide, which presumably blocks certain sulfhydryl groups at or near the insulin-binding site. Pretreatment with insulin protects against maleimide. Digestion of the cell with trypsin eliminates the acceleration of glucose transport and the inhibition of lipolysis by insulin. The glucose transport and adenyl cyclase systems are not grossly affected by trypsin, indicating that the insulin effector system is a separate entity.

In mammalian tissues, glucose passes across the cell membranes by a process which has been called mediated transport, facilitated diffusion, or nonactive transport. There are objections to all of these terms, but they serve to distinguish the process from active transport. The most important difference

between mediated and active transport is that the nonactive process cannot use metabolic energy to move glucose against a concentration gradient. Mediated transport occurs in most, if not all, mammalian cells, whereas active transport is seen prominently only in the kidney and gut.

This paper will review the basic concepts of mediated transport in mammalian cells and will mention some recent efforts to determine the components of the system. It will discuss some aspects of the regulation of the process, and will describe some recent observations on the mode and site of insulin action.

The basic concepts of nonactive transport were developed in the late 1940s by LeFevre in this country, Widdas in England, and Wilbrandt and Rosenberg in Switzerland (see references 1 and 2 for review). Their studies, carried out with the human erythrocyte, assume great significance today when it is realized that this was one of the first clear expositions of membrane transport as we now understand it. Fig. 1 shows the simplest representation of the carrier system which is in accord with the experimental findings. Glucose on the outside, G_o, complexes with some membrane component, X, to form a complex, GX, which moves across the membrane to discharge the glucose, on the inside, G_i. The system is freely reversible at all points. The six most important observations which establish this concept of transport are as follows.

Saturation Kinetics It has been observed that the initial rate of entry or exit of glucose approaches a maximum above a certain concentration of sugar. This phenomenon, termed saturation, argues strongly that glucose must complex with some membrane component, analogous to X in Fig. 1, during the permeation process. The combining site will be continuously occupied from a statistical view when bombarded by a sufficiently high concentration of glucose molecules. Permeation at this point becomes maximal and is limited by the number of carriers and their motility.

Stereospecificity The selectivity of the transport process for certain sugars greatly strengthens the idea of a combining site. It suggests further that the site is in a protein, since only proteins have the necessary complexity of structure to discriminate among closely related small molecules such as the sugars. The stereospecificity of the system has been studied particularly by LeFevre and Marshall (3), who concluded that the three-dimensional conformation of the sugar was the most important factor determining affinity. D-Glucose, the pyranose sugar which exists to the greatest extent in a chair conformation of the so-called C-1 type, has the highest affinity. Other hexoses and pentoses can be arranged in a descending order of affinity which correlates with the decline in the stability of the C-1 conformation in water. L-Glucose, which is extremely close to D-glucose in most of its chemical and physical properties, does not exist at all in the C-1 conformation, but is

largely in the mirror image conformation, 1-C. L-Glucose has a very poor affinity indeed for the transport site and hardly penetrates most cells.

An important consequence of stereospecificity, which is very useful in distinguishing transport from subsequent metabolic steps, is the characteristic pattern of competition for transport among the common pentoses and hexoses.

Inhibition by Certain Agents Inhibition by certain compounds is a third characteristic of the system. Phloretin, phlorizin, and related compounds have long been known as moderately specific inhibitors (1, 2). Transport in many cells is also very sensitive to low levels of sulfhydryl-blocking agents (4),

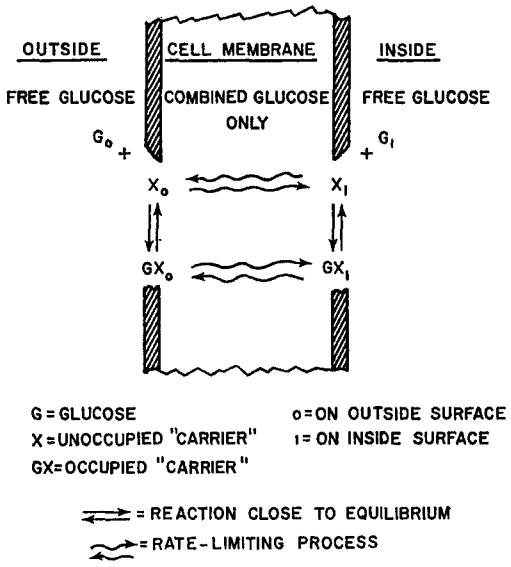

FIGURE 1. Schematic representation of the nonactive transport system for glucose. See the text for description.

a point we will mention again later, and to dinitrofluorobenzene (5). Inhibition by these substances also suggests participation of protein components.

Temperature Coefficient The coefficient is about 2 for the temperature range of 27–37°C. This value is well above that for simple aqueous diffusion and suggests, but by no means proves, that transport involves the formation and breaking of chemical bonds.

Energy Requirements Nonactive transport cannot utilize metabolic energy. This means, of course, that no transport against a concentration gradient is possible, although a special case, which looks superficially like active transport, will be discussed in the next paragraph. Mediated transport is freely reversible and tends simply to equilibrate sugar concentrations across the cell membranes; phosphorylation is not involved, and free sugar is the product of transport in either direction.

Countertransport This phenomenon provides the best evidence that the sugar complex has mobility across the membrane, an essential feature in the concept of a carrier. Countertransport was predicted on kinetic grounds by Widdas (6); it was first demonstrated in our laboratory (7) and has subsequently become a common test for a mobile carrier.

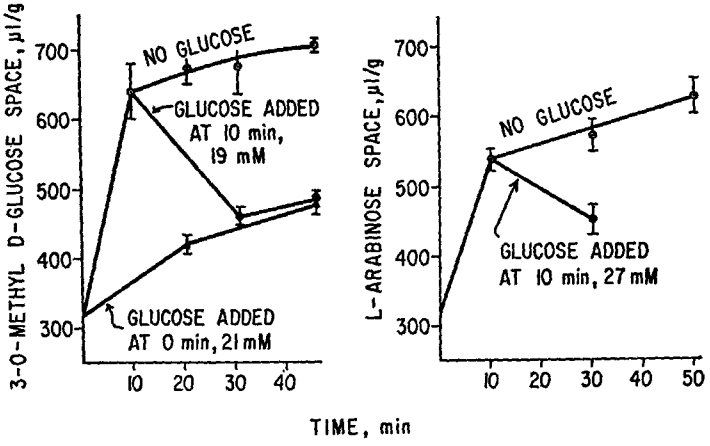

FIGURE 2. Countertransport of 3-*O*-methyl D-glucose and L-arabinose in the isolated, perfused rat heart on addition of D-glucose to the perfusate. Hearts were perfused for the times indicated with oxygenated buffer containing 3-*O*-methyl D-glucose-^{14}C (0.75 mM) or L-arabinose (13 mM) (○). In two groups of hearts perfused with 3-*O*-methyl glucose, perfusion was switched after 10 min to buffer containing 21 mM D-glucose in addition, and perfusion was continued for either 20 or 35 min (●). Additional groups were perfused with 0.75 mM 3-*O*-methyl glucose and 21 mM glucose from zero time (▲). One group of hearts, perfused with L-arabinose for 10 min, was perfused for an additional 20 min with buffer containing L-arabinose (13 mM) and D-glucose (27 mM) (●). At least six hearts were perfused for each point. The vertical line through each point indicates two standard errors of the mean. *Figure reprinted by permission from the Journal of Biological Chemistry, 1964, 239: 369.*

Fig. 2 demonstrates countertransport in studies using the isolated rat heart preparation (8, 9). In these experiments, the tissue was perfused with the nonmetabolized sugar 3-*O*-methyl glucose (left panel) until a substantial rise in the concentration of intracellular sugar had occurred, as reflected by the increase in sugar space above the extracellular space (about 325 µl/g of tissue). At this point, a high concentration of glucose was added to the medium, causing a rapid drop in the intracellular 3-*O*-methyl glucose concentration. Since the latter sugar could not be metabolized, it must have been transported out of the cell, although the extracellular concentration was higher than the intracellular concentration. Similar results were obtained using the nonmetabolized pentose, L-arabinose, as shown in the panel on the right.

The explanation for this phenomenon can be understood by reference to Fig. 3. (The following discussion applies equally to the case of 3-*O*-methyl glucose.)

The external medium, containing glucose and L-arabinose molecules, is shown on the left. In the center, the cell membrane is represented with mobile carriers, most of which have sugar molecules attached. On the right is the intracellular water, which contains L-arabinose almost exclusively, since glucose, on entering the cell, has been rapidly transformed to glucose-6-P and other metabolic products. As shown, glucose competes favorably for the carrier at the external surface of the cell and cuts off arabinose entrance, whereas arabinose meets no competition at the inside surface and continues

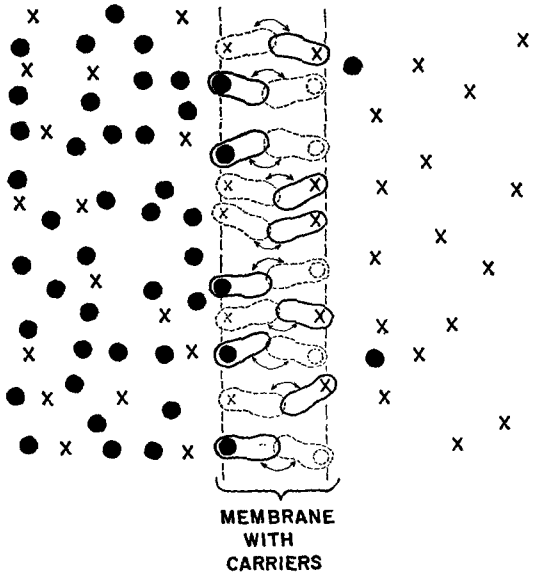

FIGURE 3. Schematic representation of sugar-carrier complexes in a cell membrane during counterflow of L-arabinose (×) induced by D-glucose (●). The figure is explained in the text.

to be transported out. Thus, the net flow of arabinose is outward. Under these conditions, a gradient for glucose-loaded carrier is established within the membrane from outside to inside. As glucose is moved into the cell, empty carriers become available at the inner side of the membrane for arabinose and create a gradient of arabinose-loaded carriers from inside to outside. The latter is the immediate source of energy to move arabinose out of the cell into a higher external concentration. The ultimate source of energy, however, is in the movement of glucose down its concentration gradient with the carrier gradients mediating the energy transfer. The conclusion that the carriers must be mobile derives from this concept of changing concentration gradients of the carrier in the membrane. Movement is also implicit in the conclusion that the carrier can be accessible to sugars only at one side of the membrane

at a given time. If the carrier were bombarded simultaneously from both sides, glucose on the outside would compete with arabinose on the inside for the carrier. As a consequence, glucose would inhibit rather than enhance the flow of arabinose outward.

As regards the composition of the transport system, no components have been isolated and identified to date. The reason for this failure lies in our inability to assay for transport activity after a cell is broken. Nevertheless, Bobinski and Stein (10, 11) and Langdon and Sloan (12) have recently searched for transport components by looking for membrane proteins which would combine with monosaccharides in a manner corresponding to the characteristic stereospecificity and kinetics of the transport system. To date these efforts have not yielded convincing results (13), presumably because glucose can react reversibly with many proteins through their free amino groups. This huge background of nonspecific reactivity makes it very difficult to isolate a specific reaction, particularly when the component involved may be present in an extremely small amount. Another effort has gone into investigating the long-held proposition that glucose might cross the membrane as a complex with some lipoid substance, possibly even a phospholipid. Several years ago we noted that erythrocyte membrane phospholipids would carry glucose from an aqueous into a lipid phase, and that phlorizin inhibited this transport (14). This phenomenon has been studied in more detail by LeFevre et al. (15). Recent experiments by Wood (16), however, with bilayer membrane of the Rudin-Mueller type (see reference 16 *a*) made with the phospholipid from ghosts of human erythrocytes, suggest that phospholipids in this geometrical arrangement cannot transport glucose. The possibility remains, nevertheless, that lipid components in conjunction with specific protein(s) may be part of the transport system.

Mediated transport of glucose has been seen in all mammalian cells that have been studied, and it is probably an essential feature of the cell. The process shows a number of variations among different cells, some of which are noted below.

There is a remarkable range of transport activity among cell types, among species, and at different ages within a given species. Transport is so fast in the human red blood cell that it is best measured by optical systems which can follow the very rapid osmotic shrinkage or swelling of a cell as sugar enters or leaves. By contrast, nonprimate erythrocytes, such as those of the rabbit, were for many years considered to be impermeable to glucose because very little or no sugar could be found inside the cell no matter how high the external concentration might be. These erythrocytes are not impermeable, in fact, but transport is relatively very slow, as shown in Table I. The V_{max} for the rabbit cell is only about 0.4% of that for the human cell. As a consequence, transport is so slow that the sugar is phosphorylated as quickly as it enters and intra-

cellular free glucose remains nearly undetectable. The K_m values of the human cells and rabbit systems are about the same, but human cells are very sensitive to certain sulfhydryl-blocking agents whereas rabbit cells are not. The stereospecificities appear to be similar, except that fructose apparently does not employ the glucose system in the rabbit cell, as judged from the absence of competition with glucose. The system in the rabbit cell shows countertransport between sugars, as in the human cell. It would thus appear that the rabbit cell has the basic elements of the carrier system but is deficient in some element(s) which confers a high rate, a degree of specificity, and sensitivity to sulfhydryl-blocking agents. A related observation is that of Widdas (17), who found some years ago that fetal erythrocytes of nonprimates have a very fast rate of glucose transport, like that in human cells. About the time of birth, synthesis of the component that confers this high rate apparently ceases. Thus nature has provided in these nonprimate fetal and adult erythrocytes, cells which are analogous to transport mutants in bacteria and which might be profitably studied to dissect out components of the transport system.

TABLE I
SOME PROPERTIES OF GLUCOSE TRANSPORT IN HUMAN AND RABBIT ERYTHROCYTES

Property	Human	Rabbit
V_{max}	600*	0.15*
K_m	8 mM	6 mM
Inhibition by SH blockers	Yes	No
Competition: glucose vs. fructose	Yes	No

* Millimoles per liter of cell water per minute.

The liver has long been known to be very permeable to glucose, since the concentration of the sugar has been found to be almost the same in the tissue water and blood under various conditions. Cahill and associates (18), who studied hepatic permeability to monosaccharides in some detail a number of years ago, concluded that glucose entered by free diffusion. However, recent work of Williams et al. (19) indicates that this is not the case and that glucose penetrates by an extremely fast transport system. The following two experiments support this conclusion. In the first, an isolated liver preparation was perfused with medium without glucose for about 15 min to establish steady state conditions (Fig. 4). At this point, the perfusion was switched to another reservoir of medium of the same composition, except that it contained D-glucose and L-glucose. At intervals, samples of the liver were taken to determine its content of these two sugars. As can be seen, the concentration of D-glucose in the liver was approaching that in the perfusate at 2 min, whereas the concentration of L-glucose was much less than in the medium. Since

D-glucose and L-glucose are almost identical in size, solubility, and other physical properties, the difference in permeability indicates very strongly that a process other than simple aqueous diffusion is involved. The second experiment to suggest transport was the finding that phlorizin inhibits permeation (Fig. 5). Phlorizin is a well-known inhibitor of the monosaccharide transport system in other cells. As can be seen, it inhibited D-glucose and, more strongly, the entry of L-glucose. While the effect of phlorizin on the D-isomer was small, kinetic analysis from other, more extensive experiments

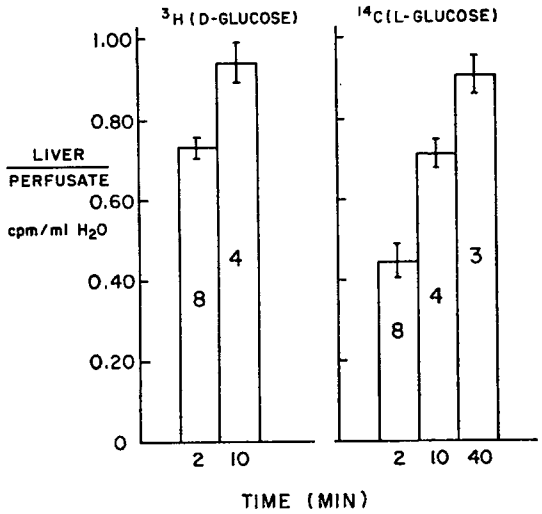

FIGURE 4. Penetration of D-glucose and L-glucose into the cells of the perfused rat liver The experiment is described in the text, and details will be published (19). D-Glucose labeled with tritium and L-glucose labeled with ^{14}C were used to facilitate analysis in the tissue and the perfusion medium. Both sugars were added to the medium at zero time in tracer concentration. Livers were fixed by freeze-clamping, followed by cold perchlorate extraction. Distribution of the sugars in extracellular space only would give a ratio of about 0.25.

made it apparent that transport is so fast that the major limiting step for D-glucose permeation in liver tissue is actually extracellular transfer, a process that is not affected by phlorizin. Since transport of L-glucose is much slower, it is more limiting for permeation and the phlorizin effect is therefore larger. In contrast to muscle and fat cells, to be discussed below, transport in liver is not sensitive to insulin, although the hormone affects other parameters of liver function.

In muscle cells, a striking feature of mediated transport is its regulation by factors such as hypoxia, muscular work, fatty acids, and certain hormones, notably insulin. These controls have been elucidated in a number of laboratories, including those of Randle, Kipnis, Cori, and Narahara as well as

our own. The data chosen in this paper to illustrate some of these controls have been collected by Morgan and associates in our laboratory using the isolated, perfused rat heart as the test object. An experiment demonstrating the effects of insulin and muscle work on transport is shown in Table II. Transport was evaluated by determining the accumulation of L-arabinose within the cell when the heart was perfused for 10 min with medium containing the pentose. The sugar enters cardiac cells by the glucose transport system, as shown by competition studies, but is not metabolized (22). At the end of the perfusion, the tissue content of arabinose was determined, and, after correcting for sugar in the extracellular space, the intracellular con-

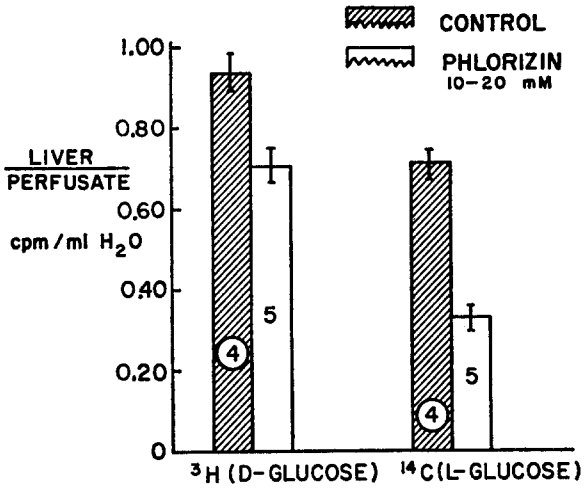

FIGURE 5. Effect of phlorizin on the distribution of labeled D- and L-glucose in the perfused rat liver. Livers were exposed to phlorizin (10 mM) by perfusion for 15 min before the sugars were added in tracer concentrations. The tissue was taken for analysis 10 min later. Details will be published (19).

centration could be calculated (9). The term "Equilibration (%)" indicates the extent to which the intracellular concentration reached the extracellular or perfusate concentration. In the aerobic heart, the transport of L-arabinose was very slow in the absence of insulin and was stimulated strongly by insulin addition. When the tissue was made severely hypoxic, transport was greatly accelerated, even in the absence of insulin, and could be increased further to a modest extent on addition of the hormone. Similarly, transport was strongly stimulated by muscular work with or without insulin. The work effect did not appear to be due to hypoxia, since measurements of oxygen tension and lactate production in other experiments indicated that oxygenation was adequate (23). It was also clear that work (or hypoxia) increased the sensitivity of transport to suboptimal concentrations of insulin. The

combination of transport acceleration and increased insulin sensitivity may explain the improved glucose tolerance of diabetic individuals with exercise.

As would be expected from the above, muscular work can cause a substantial increase in glucose uptake, as shown in Table III. In these experiments, work was increased by raising the perfusion pressure in the aorta of a

TABLE II

EFFECT OF INSULIN, HYPOXIA, AND WORK ON THE TRANSPORT OF L-ARABINOSE IN THE PERFUSED RAT HEART

After a preliminary 10 min perfusion with arabinose-free buffer, perfusion was switched to buffer containing pentose (13 mM) and sorbitol-^{3}H. This buffer was recirculated for an additional 10 min. Insulin was added only to the recirculating buffer. The aerobic or anaerobic nonworking heart was a Langendorff preparation perfused retrogradely via the aorta. It was not a completely "nonworking" preparation, since contractions occurred against fluid contained in the left ventricle at the aortic pressure (about 60 mm Hg). The working heart was perfused by introducing the perfusion medium into the left auricle at the left auricular pressure indicated. The left ventricle pumped out the fluid (about 130 ml g^{-1} min^{-1}), developing a peak pressure of about 95 mm Hg. The oxygen consumption was approximately doubled by the work load (20). The data shown are from Morgan et al. (21).

Perfusion conditions	Insulin added	Equilibrium
	(*units/ml*) × 10^6	%
Aerobic	0	4±4*
	26	0±4
	100	4±3
	300	20±5
	900	29±4
Anaerobic	0	28±3
	26	41±3
	100	41±5
	300	40±4
	900	46±3
Aerobic-working, 10 mm Hg atrial pressure	0	23±3
	100	48±5
	300	62±5
	900	61±7

Langendorff isolated heart preparation, as a consequence of which the pressure against which the left ventricle contracted was proportionately increased (see legend to Table II). On going from 60 to 100 mg Hg perfusion pressure, no free intracellular glucose could be detected at any time. The absence of free glucose indicates, as discussed in detail elsewhere (7, 9), that phosphorylation kept pace with sugar entry and that transport was the rate-limiting step for the uptake process. Table III also shows an important effect of fatty

acid on transport. At a low level of cardiac work (60 mm Hg perfusion pressure) addition of palmitate to the medium caused a modest reduction in glucose uptake, but at the higher work load the fatty acid suppressed the transport acceleration completely. Fatty acid had a similar inhibitory effect on the stimulation of glucose transport by insulin, as shown by Randle et al. (25, 26) and recently confirmed by Neely, Bowman, and Morgan (24). Evidence to date suggests that the inhibition is not a direct effect of the fatty acid on the transport system, but occurs indirectly as a consequence of fatty acid oxidation (24, 26).

There are a number of other physiological agents concerned with transport control in addition to those mentioned above. In most instances, how-

TABLE III

EFFECTS OF WORK AND FATTY ACID ON GLUCOSE UPTAKE AND INTRACELLULAR FREE GLUCOSE IN THE PERFUSED RAT HEART

The hearts of 18-hr fasted rats were perfused with Krebs bicarbonate buffer containing 3% bovine albumin with or without bound palmitate for 1 hr at 37°C. The work load was varied by changing the perfusion pressure (see legend to Table II) in a Langendorff perfusion apparatus. The additional work load increased oxygen consumption by 50%. Oxygen consumption was not affected by the addition of the fatty acid. The data shown are from Neely et al. (24).

Perfusion pressure	Glucose, 15 mM	Palmitate, 1.6 mM	Glucose uptake	Free intracellular glucose detected
mm Hg			*μmoles g⁻¹ hr⁻¹*	*mM*
60	+	0	87±14	0
	+	+	58±10	0
100	+	0	232±8	0
	+	+	35±7	0

ever, the mechanisms involved are probably the same. Epinephrine, for example, stimulates glucose uptake (27) and, by inference, transport in the heart, but this may be largely secondary to its inotropic and chronotropic effects. In fat tissue, where mechanical activity is not involved, the stimulation of sugar transport by epinephrine is very small. The sensitivity of transport to insulin in muscle is reduced by growth hormone and/or the glucocorticoids, and in certain forms of diabetes. This reduction may be secondary, however, to the increased availability and metabolism of fat, as suggested originally by Randle et al. (25) as a part of the "glucose–fatty acid cycle." In all cases of transport control, with the possible exception of that by insulin, acceleration or inhibition is coordinated with regulation of glycolytic enzyme activities, particularly phosphorylase, hexokinase, and

phosphofructokinase. The most critical control, however, remains that exerted on transport, since this is the predominately rate-limited step for glucose utilization by muscle under virtually all circumstances.

In the fat cell, transport of glucose is also strongly stimulated by insulin (28). An opportunity to examine some of the initial steps in this action of the hormone has been provided by Rodbell's (29) development of an isolated cell preparation of adipose tissue. In this connection, Crofford (30), using the Rodbell cell preparation, has reexamined the question of how insulin binds to the cell. He could thus avoid a major difficulty in earlier studies employing intact tissues, in which it was not possible to distinguish satisfactorily between binding to the cell and trapping in the interstitial spaces. Crofford also employed native rather than labeled insulin, since the biological activity and binding qualities of labeled insulin are probably altered. In Fig. 6 is shown the time course of binding by the fat cells. Two separate experiments are shown in each panel. The cells were placed in medium containing a physiological level of insulin, and the fall in concentration was followed. There was an immediate uptake of insulin, presumably reflecting binding to the cell surface, followed by a relatively very slow, progressive uptake, reflecting utilization of the hormone (Fig. 6 *A*). This interpretation was supported by showing that the initial uptake was not reduced when the incubation temperature was dropped from 37°C to 17°C (Fig. 6 *B*), but that the utilization was greatly slowed. As shown by the experiment of Fig. 6 *C*, the utilization of insulin required the presence of the cells. Fig. 6 *D* shows that the initial binding could be prevented by a short prior exposure of the cells to maleimide, although this agent did not suppress the subsequent rate of insulin destruction. The effects of maleimide in the fat cell[1] are similar to those observed earlier in heart muscle (31, 32) and may be summarized as follows. A brief exposure to maleimide (or *N*-ethylmaleimide), which is a sulfhydryl-blocking agent with rather high specificity under the conditions employed, can suppress the stimulatory effect of insulin on glucose transport without destroying the activity of the transport system itself, and without poisoning intracellular glycolytic enzymes, such as 3-phosphoglyceraldehyde dehydrogenase (21). The effect of the blocking agent is prevented, however, if the cells are exposed first to insulin. The simplest interpretation of these observations is that the binding and/or action of insulin requires the integrity of a sulfhydryl group which is at or near the cell surface and is closely associated with the hormone-binding site.

Crofford (30) has studied the quantitative relationship between the initial uptake and the insulin concentration of the medium (Table IV). The "binding" remained linearly related to concentration up to about 10 milliunits/

[1] O. B. Crofford. Unpublished observations.

ml, a concentration considerably in excess of the concentration giving a maximal stimulation of transport, at which point binding appeared to be approaching a maximum. The fact that the transport effect reached a

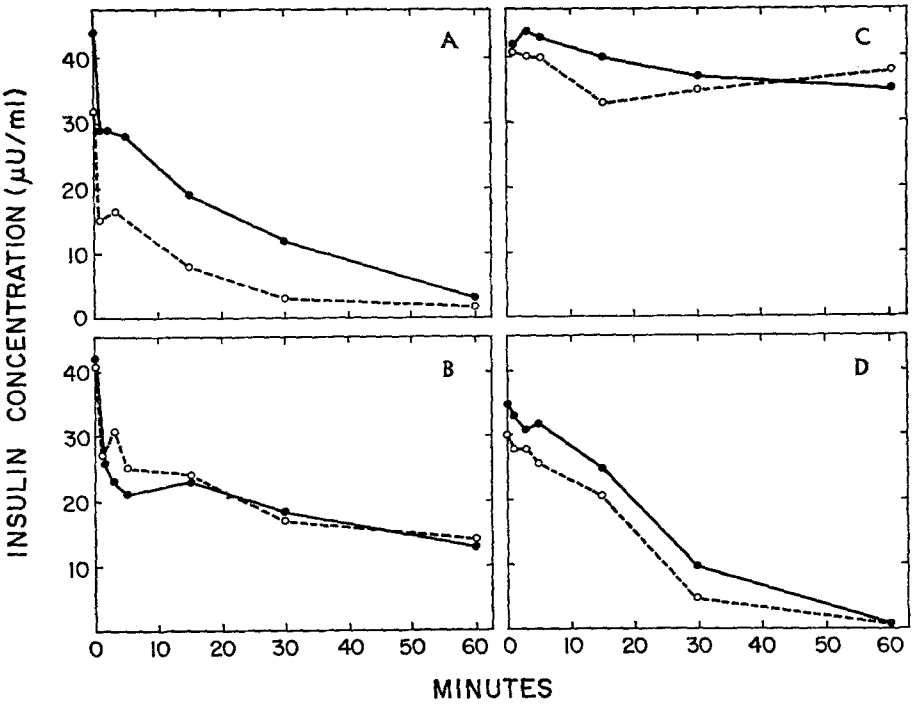

FIGURE 6. Time course of disappearance of insulin from the incubation medium. In each of the experiments, approximately 1200 mg of isolated fat cells were incubated in 4 ml of medium containing between 30 and 45 microunits (μU) of insulin per ml and 1 mM glucose. 30 sec before each sampling time, the incubation mixture was centrifuged so that approximately 0.3 ml of cell-free incubation medium could be aspirated from the tube and used for the assay of immunoreactive insulin. In each panel, the data from two experiments are shown; one by the solid curve and a duplicate by the dashed curve. *A*. Results of experiments performed at 37°C. *B*. at 17.5°C. *C*. Cells were incubated at 37°C for 60 min, removed from the incubation medium, and discarded. At zero time, insulin was added to the medium in which the fat cells had been incubated, the incubation was continued for 60 min, and the cell-free medium was sampled at the times indicated. *D*. The fat cells were given a 30 sec exposure to 10^{-3} M maleimide ($C_4H_3NO_2$) immediately after collagenase treatment. The maleimide was then removed by washing the cell suspension four times in fresh buffer, and the cells were used for experiments identical with those of *A*. *Figure reprinted by permission from The Journal of Biological Chemistry, 1968, 243: 362.*

plateau before the binding reached saturation does not mean necessarily that the binding was nonspecific. Transport acceleration is not the primary or only response to insulin (see below), and other actions of insulin may re-

quire much higher concentrations. Furthermore, a substantial factor of safety may be built into the hormone-binding system. Specificity is also suggested by the fact that 10^{-11} M insulin competed successfully in the above experiments with 5×10^{-4} M bovine albumin.

Crofford has also noted (30) that a brief period of washing (less than 5 min) eliminated the insulin effect and left the cell capable of binding another aliquot of hormone with concomitant restoration of the transport effect.

TABLE IV

RELATIONSHIP BETWEEN UPTAKE OF INSULIN BY ISOLATED FAT CELLS AND INSULIN CONCENTRATION IN INCUBATION MEDIUM

A suspension of fat cells containing approximately 300 mg of cells was incubated for 5 min at 37°C in 5 ml of medium containing no insulin. The cell suspension was then centrifuged, and the cells were removed and transferred to 1 ml of fresh medium containing insulin in the concentration shown. The suspension was incubated at 37°C for 5 min; the cells were removed by centrifugation, and the insulin uptake was determined as the change in insulin concentration in the incubation medium. Each value given in the table represents the mean of six observations ± the standard error of the mean. The data shown are from Crofford (30).

Insulin concentration in incubation medium	Insulin uptake per 100 mg of fat cells	
	Amount	Percentage of initial content of incubaton tube
(units/ml) × 10^6	*units* × 10^6	%
4	0.3±0.1	7.5
6	0.6±0.1	10.0
16	1.7±0.1	10.6
37	3.5±0.3	9.5
72	8.4±1.3	11.7
157	13±1.6	8.3
833	78±4.4	9.4
8,715	652±53	7.5
86,800	874±659	1.0
896,000	900±8,640	0.1

No insulin could be found in the wash fluid by immunological or biological assay, although it would have been detected easily if the binding were reversible. The concept thus emerges that insulin must be continuously present for its effect to be manifest; it is not a key that turns a lock and then can be removed. Furthermore, the insulin bound is metabolized to as yet unknown products.

Another study of the initial interaction of insulin with the fat cell has been carried out by Kono.[2] As shown in Table V, preliminary treatment of adipose

[2] T. Kono. Unpublished data.

tissue with trypsin under appropriate conditions virtually eliminated the acceleratory effect of insulin on glucose utilization. Appropriate controls showed that this was not due simply to destruction of insulin by the enzyme, but appeared rather to involve a modification of the cell surface. The ability

TABLE V

LOSS OF INSULIN EFFECT ON GLUCOSE UTILIZATION BY ADIPOSE TISSUE AFTER TREATMENT WITH TRYPSIN

Rat epididymal fat pads were first incubated in Krebs bicarbonate buffer at 37°C with 1 mg/ml crystalline trypsin without albumin. The fat pads were then washed extensively with two changes of buffer containing albumin (2%) and trypsin inhibitor (0.1 mg/ml). A third washing was carried out without the inhibitor. The second incubation was carried out for 30 min at 37°C in Krebs bicarbonate buffer with 2% albumin. The medium contained uniformly labeled glucose-^{14}C (0.3 mM) and insulin (0.1 unit/ml) and phloretin (1 mM) where indicated. The data shown are from unpublished experiments of T. Kono.[2]

Treatment	Glucose in incubation	Glucose: CO_2 ratio	
		Control	Insulin
	mM	*mμmoles g⁻¹ hr⁻¹*	
None	0.3	46	644
Trypsin	0.3	38	56
None	3	540	2680
Trypsin	3	360	468
Trypsin	3 plus phloretin	54	

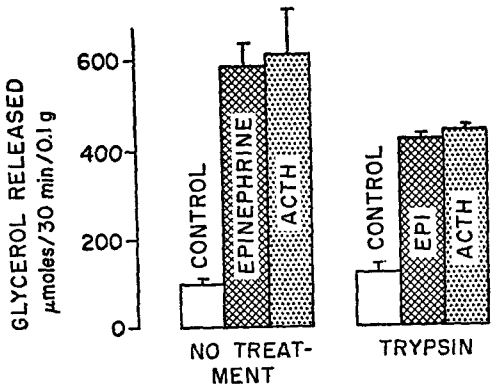

FIGURE 7. Persistence of response to epinephrine and ACTH in adipose tissue after treatment with trypsin. Rat epididymal fat pads were treated with trypsin and washed as described in Table V. They were then incubated in Krebs bicarbonate buffer with 2% albumin for 30 min at 37°C. Epinephrine (EPI) was added in a concentration of 1 μg/ml with 1 mM caffeine, and ACTH was added in a concentration of 1 μg/ml with 1 mM caffeine. From unpublished results of T. Kono.[2]

of the cell to transport and metabolize glucose was not destroyed by trypsin treatment, however, since these functions could be strongly stimulated simply by raising the concentration of glucose in the medium. Glucose utilization, furthermore, could be markedly inhibited by phloretin, indicating that the cell membrane was not made "leaky" by the enzyme treatment.

As shown in Fig. 7, the same trypsin treatment did not destroy the lipolytic response of the cell to epinephrine or to ACTH. It did, however, eliminate the antilipolytic response of the cell to insulin, as shown in Fig. 8. This antilipolytic action has been recently explained by the observation (33) that insulin lowers the tissue level of 3′,5′-adenosine monophosphate (cyclic adenylate), the compound which mediates the action of ACTH and epinephrine on lipolysis (34). The effect of insulin on transport, however, does not

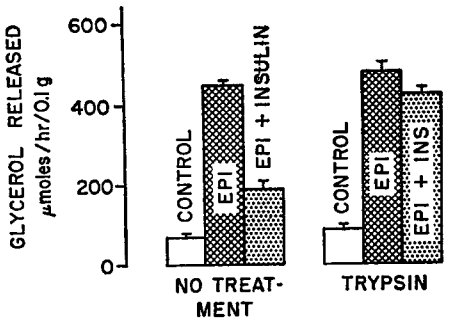

FIGURE 8. Loss of insulin effect on lipolysis in adipose tissue after treatment with trypsin. Rat epididymal fat pads were treated with trypsin and washed as described in Table V. They were then incubated in Krebs bicarbonate buffer containing 2% albumin for 1 hr at 37°C with the additions shown, but no glucose. The concentrations employed were as follows: epinephrine (EPI), 0.1 μg/ml; insulin (INS), 1 milliunit/ml. From unpublished results of T. Kono.[2]

appear to be altered by a change in cyclic adenylate levels (35, 36). It seems likely, from Kono's results,[2] that a part of the insulin effector system is at the cell surface and contains peptide elements; it is apparently distinct from the glucose transport and the adenyl cyclase systems. How interaction of insulin with this system accelerates transport and reduces levels of cyclic adenylate in the cell remains unknown.

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Discussion

Dr. Kennedy: I should like to call upon Dr. Crane to begin a discussion of Dr. Park's presentation.

Dr. Robert K. Crane: I would like to show you three by now rather ancient slides to illustrate the concept of gradient coupling in membrane transport, which I think has some relevance to what Dr. Park has been talking about. Fig. 1, Discussion, simply shows the mobile carrier as we draw it. It does not differ at all from Dr. Park's concept. It has all the features that he listed as characteristic of mobile carrier transports systems. A carrier such as shown in Fig. 2, Discussion is a useful description of the kinetic properties of the transport system in the intestine for sugars, amino acids, bile salts, and several other compounds, and, in most animal cells at least, for amino acids. Again, this is a mobile carrier with properties just like those that Dr. Park has described, but with the one additional property that the carrier appears to associate not only with substrate but also with sodium ion, and transports both across the membrane at the same time.

Now, I don't have time to give you any evidence for it and I don't want to get into that area; the consequences of the presence of such a carrier are what I think we are interested in. These are illustrated in Fig. 3, Discussion.

On the one hand, we may consider a red blood cell with its sodium ion pump in the membrane which operates to maintain a low intracellular sodium ion concentration, and a mobile carrier system with a single substrate-specific site. The net result for substrate is what we would call passive transport, where at equilibrium the substrate

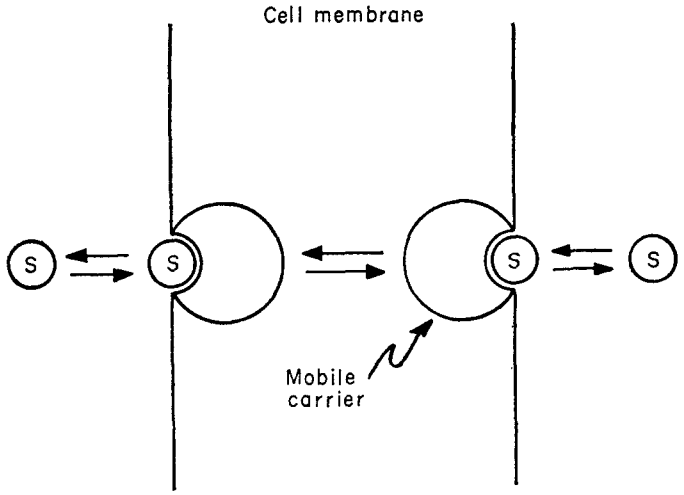

FIGURE 1

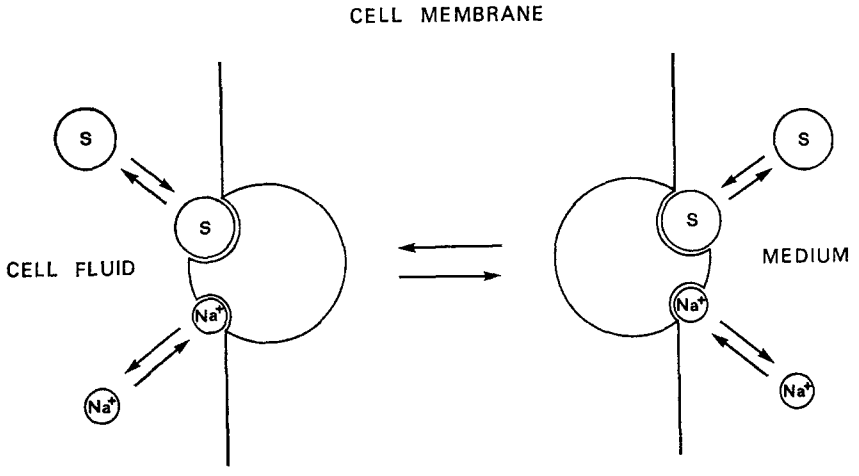

FIGURE 2

concentration inside equals the substrate concentration outside. In the gut epithelial cell, on the contrary, the carrier also associates with sodium ion. Because of the low concentration of sodium ion maintained within the cell, the substrate concentration inside the cell at equilibrium is necessarily going to be higher than the substrate concentration outside the cell. This is what we would nominally call active transport. I remind you that there is no essential difference in the carriers, except that one has

an association with two specific substances, one of which has an asymmetry across the membrane imposed upon it by the action of some other process at a different location within the cell.

The particular relevance of this comparison for the latter part of Dr. Park's talk is that I had the opportunity last May in Bruges, at the 15th Colloquium on the Protides of the Biological Fluids, to hear a paper presented by Dr. Letarte, who had been working with Albert Renold in Geneva. The substance of that paper was that glucose entry into the isolated fat cell in the absence of insulin was associated with ionic effects which were not easily rationalized. In the presence of insulin, however, glucose entry into the isolated fat cell could be analyzed in terms of a sodium-dependent, gradient-coupled system. They saw a specific sodium ion dependence for the entry of the glucose in the presence of insulin.

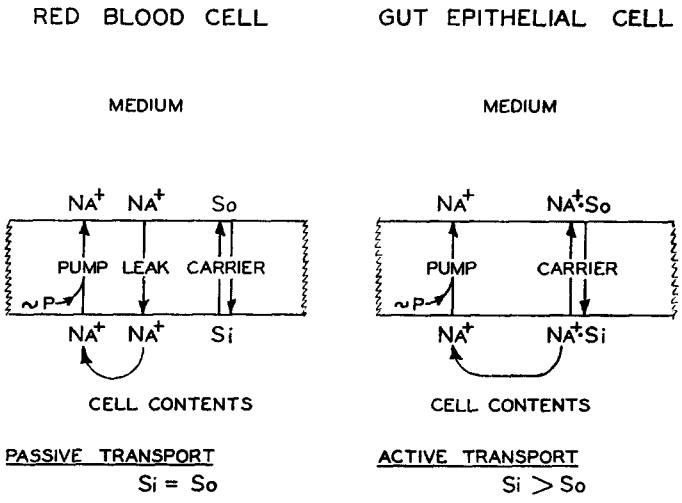

FIGURE 3

Question from the Floor: The insulin effect very clearly activates the glucose system in such a way that it will move glucose out of the cell just to facilitate outward movement as well as inward, and this also, of course, is an immediate effect of insulin. I wonder if this could conceivably fit in with a sodium gradient effect?

Dr. Park: Sodium-dependent glucose transport systems are usually systems of active transport. The transport process in the fat cell is probably simply a "nonactive," mediated transport system. If this is true, and if it is like transport in muscle, insulin would accelerate transport in both directions. This would then seem to exclude the participation of a sodium gradient across the membrane in the insulin effect. I would like to emphasize, however, that the above comments are speculative and experiments should be done along the lines suggested by the question.

Dr. Ivan Bihler: I would like to add to the question of ion effects on insulin-stimulated transport in muscle. We have recently studied the transport of 3-methyl glucose, a nonmetabolized glucose analogue, in the "intact" rat hemidiaphragm and have

found that whenever the sodium pump is inhibited by cardiac glycosides, by low levels of K^+ in the incubation medium, or by other means, sugar transport is increased. This effect is additive to that of a submaximal dose of insulin. In contrast, a high -K^+ medium is known to activate the sodium pump and to reduce its sensitivity to inhibition by ouabain; in such a medium sugar transport is decreased and is less stimulated by ouabain. Furthermore, diphenylhydantoin, a drug supposed to activate the sodium pump, was also able to reduce sugar transport, provided that the sodium pump was not inhibited by other means. It seems as though, in addition to insulin, muscular work, and anoxia, sugar transport in muscle is also regulated by the activity of the sodium pump and/or the related intracellular ionic levels. These results suggest to us that perhaps the effects of anoxia and muscular work are also mediated by alterations in the levels of cations in the cell or in a particular cellular compartment.

Dr. Park: There is likely to be some common explanation for the effect on transport of muscle work, hypoxia, and other acceleratory agents such as the uncouplers described by Randle and Smith,[1] but I think it is not at all clear what this common mechanism is. It is conceivable that it could be an ionic influence.

Dr. Zierler: One of the things I hoped very much would come out of this symposium was that the physiologists and biophysicists, who largely spoke yesterday morning; the physical chemists, who spoke yesterday afternoon and this morning; and the biochemists, who spoke this afternoon, would find a common understanding and would appreciate one another's data and understand one another's problems, and that somehow some of us would be smart enough to bring them all together. At the moment I feel more confused than able to synthesize what I have learned, and perhaps Dr. Kennedy will bring this all together.

I would like to discuss some of the things that I think may be a little more simple. I don't disagree that one can construct all kinds of plausible models of movement of substances across these interfaces, and as you said, Dr. Kennedy, the question is which of them is really going on. Some of the things that emerge from the study of thin films I think have to be considered in all models, because some of this material is present, and whenever a simple physical chemical phenomenon can be shown we have to assume either that it proceeds in living tissue or that something in nature prevents it. One of the things that impressed me about Dr. Blank's data was the fact that in a purely simple system in which there were no carriers he could demonstrate something which kinetically would have fit the first five constraints that Dr. Park listed as evidence for facilitated diffusion. Everything except countertransport could be demonstrated in Dr. Blank's model. I proposed a few years ago that one could obtain these results in any poorly permeable membrane. If one has a large number of potentially diffusing molecules competing with one another for the opportunity to get through a barrier, where the probability, per molecule, of getting through the barrier is very small, one can mimic such kinetics. One can account for everything except countertransport in this model.

I think, therefore, that the only unique argument that one can make for a carrier from Dr. Park's data lies in countertransport. With so much reservation about the ex-

[1] RANDLE, P. J., and G. H. SMITH. 1958. *Biochem. J.* **70**:490, 501.

planation of competition and saturation, I find myself willing to keep an open mind about the possibility that some day we may be clever enough to find an alternative explanation for countertransport. With respect to Dr. Crane's comments about the link between the sodium and glucose uptake, I doubt that it is a universally operative mechanism. There are many experimental situations in which one can clearly dissociate the action of insulin on glucose uptake from its action on potassium uptake, and on the outward movement of sodium from cells. These seem to me to be two completely independent phenomena, and in living tissues of whole animals, sensitivity to insulin with respect to potassium uptake is an order of magnitude greater than to glucose uptake, which seems to me to argue for their dissociation.

Also, I have a question for Dr. Park which arises from our very crude calculations of the number of molecules of insulin required to exert an effect per cell in intact man. We concluded that as few as 30 molecules/cell were required. In your studies, of course, using Rodbell's preparation, you had an opportunity to make that calculation, and it would be interesting to hear what sort of number you got.

Dr. Park: Dr. O. B. Crofford (personal communication) has calculated that a minimal detectable insulin effect in a fat cell would involve binding of about 100 molecules. This would mean roughly 1 molecule/30 μ^2 of cell surface. Thus the insulin molecules would be very far apart.

Dr. Zierler: The further point being that I doubt it is itself incorporated into the carrier system. An alternative possibility is that it modulates the average configuration of some other membrane components.

Dr. David Satchell: Is it possible that the effects of increased work or anoxia on the uptake of glucose by isolated, perfused hearts might be complicated by the release of endogenous catecholamines? Catecholamines cause marked increases in glucose uptake in this preparation.

Dr. Park: I do not know of any studies of the effects of work or hypoxia on transport that have been carried out with reserpine-treated tissues. In the heart, epinephrine stimulates transport strongly, but this may be secondary to its inotropic and chronotropic effects. In fat tissue, epinephrine has little effect on transport. Furthermore, in heart, neither work nor hypoxia promotes any substantial conversion of phosphorylase *b* to *a* whereas epinephrine does so very strongly. These considerations suggest, but certainly do not prove, that the work and hypoxia effects on transport do not depend on catecholamine release.

Mr. Erold R. Diller: You made your experiment with trypsin, and I think you concluded that this removed some protein which is a receptor for insulin. On the other hand, couldn't the trypsin attack the insulin? Have you studied the effect of trypsin inhibitors?

Dr. Park: Yes, the experiments are carried out with trypsin inhibitor. (Note: The use of inhibitor is now described in the written text although it was not described in the oral presentation.)

Dr. Thomas K. Hathhorn: Perhaps some of the confusion, as previously noted, is associated with the physical evaluation of the membrane being performed in different media. Dr. Ponder demonstrated 20 years ago that there were antisphering agents in

plasma, which included albumin. Recently, in Toronto, cholesterol proved to be another one.

Dr. Rand's evaluation of the red cells' viscoelastic properties was done in a cholesterol-albumin-free medium (saline). This is certainly not an antisphering condition, and thus perhaps might not represent the true physical characteristics of the membrane. I suspect that similar discrepancies are present that distort analysis in other studies of the interface.

Dr. Kennedy: If I may be permitted a few sentences of summary, I should like to suggest that we may be erecting a false dichotomy between alternative models of the membrane. On the one hand, the work of Maddy, Huang, and Thompson[2] and of Mueller, Rudin, Tien, and Wescott[3] shows that artificial lipid bilayer membranes may mimic some of the most striking characteristics of the membranes of living cells. On the other hand, it is a great principle emerging from contemporary biochemistry that highly specific enzymatic and transport functions require highly specific proteins, and here the model suggested by Dr. Korn is much more helpful to the biochemist than the previous model of Davson and Danielli. I know of no evidence, however, that actual membranes in living cells have an entirely uniform, monotonous aspect. It may well be that large areas of the membrane are organized essentially as suggested by the Davson-Danielli model, while other areas contain functional globular proteins intercalated directly into the membrane.

[2] MADDY, A. H., C. HUANG, and T. E. THOMPSON. 1966. Studies on lipid bilayer membranes: a model for the plasma membrane. *Federation Proc.* **25**:933.

[3] MUELLER, P., D. O. RUDIN, G. T. TIEN, and W. C. WESCOTT. 1962. Reconstitution of cell membrane structure *in vitro* and its transformation into an excitable system. *Nature.* **194**:979.