Studies on the Metabolism of Adipose Tissue. XVI. Inhibition by Phlorizin and Phloretin of the Insulin-stimulated Uptake of Glucose*

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Net gas exchange, lactic acid production, and substrate consumption were employed to measure the increased utilization of glucose, mannose, or fructose that results when insulin is added to rat adipose tissue *in vitro*. Phlorizin and phloretin inhibit all these processes and phloretin is some fifty times as effective as phlorizin. The per cent inhibition obtained at a given concentration of phlorizin varies inversely with the glucose concentration but is independent of the insulin concentration. Inhibition by phlorizin occurs within minutes after its addition to the tissue and its effect is readily reversible by brief washing of the tissue and its transfer to new medium. The action of phloretin differs in certain respects from that of phlorizin. At the concentrations employed, phlorizin (5 mM) and phloretin (0.1 mM) have no inhibitory effects upon either the activity of hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, or upon the over-all conversion of glucose-6-phosphate to triose phosphate as measured in adipose tissue homogenate. Also no inhibition by these compounds on the conversion of glycogen to lactic acid by intact adipose tissue under anaerobic conditions could be observed. The ability of insulin to initiate pinocytosis is not blocked by phlorizin or phloretin. It is concluded that these inhibitors do not exert their effects by interference with the action of insulin, but rather by blocking the pathways made available by insulin in the cell membrane for entrance of sugars into the cell.

The glucoside, phlorizin, has long been known to inhibit glucose transport across the kidney tubule and the intestinal mucosa (Lotspeich and Wheeler, (1962; Crane, 1960). More recently it has been shown that phlorizin inhibits the insulin-stimulated uptake of glucose by muscle (Battaglia *et al*, 1960), and that it blocks the ability of insulin to enhance the permeation of galactose into muscle both *in vivo* (Keller and Lotspeich, 1959) and *in vitro* (Lotspeich and Wheeler, 1962). Since insulin markedly increases the rate of glucose utilization by adipose tissue (Krahl, 1951–52; Winegrad and Renold, 1958), it seemed of interest to investigate the action of phlorizin and its aglucone phloretin upon this process. Some of the results of such a study are presented here.

METHODS

The epididymal fat body of rats weighing between 160 and 210 g and purchased from the Holtzman Co. has been used exclusively in these studies. The general care and precautions employed in handling of the animals as well as the procedure for removal of adipose tissue have been described previously (Ball and Merrill, 1961). Glycogen-rich adipose tissue was obtained from rats fasted and re-fed as described by Frerichs and Ball (1962). Incubations were performed in Warburg vessels of approximately 15-ml capacity with a center well and a side arm with a vented stopper. The center well was not used. Tissue samples that weighed in the neighborhood of 100 mg were employed. The main compartment of each vessel contained 3.0 ml of bicarbonate-buffered medium (Krebs and Henseleit, 1932) but with half the recommended calcium. The side arm contained 0.1 ml of the incubation medium in which was dissolved insulin or other materials, as stated. The gas phase was 95% air–5% CO_2 or 95% nitrogen–5% CO_2 and the temperature was 37.2°.

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Determination of net gas exchange was made by the procedure described by Ball *et al.* (1959); glucose and mannose were determined by the methods of Nelson (1944) and Somogyi (1945), fructose according to Roe *et al.* (1949), and lactate by the procedure of Barker and Summerson (1941) or enzymatically by the method introduced by Scholz *et al.* (1959) with reagents supplied by Boehringer und Soehne.

The phlorizin, phloretin, amygdalin, arbutin, and salizin used were commercial samples. Before use, the phlorizin was recrystallized from distilled water and the phloretin from ethanol. The purity of the recrystallized phlorizin and phloretin was checked by chromatography, using the procedure described by Braun *et al.* (1957). If the phlorizin contained phloretin, then the amount was less than 0.1%. The glucosides were dissolved in the incubation medium that was placed in the main compartment of the vessel, or in a few cases phlorizin was added as the solid from the side arm. Phloretin was dissolved in 100% ethanol and added in this form to the vessels. The final ethanol concentration in the vessel never exceeded 1% by volume and in most cases was around 0.3%. It was found that no effect on the *in vitro* metabolism of adipose tissue was exerted by ethanol if its concentration did not exceed 2% by volume. Fructose and mannose were samples used in a previous study and which had been shown to contain no detectable glucose (Ball and Cooper, 1960).

RESULTS

A typical experiment illustrating the inhibitory action of the compounds under study upon the net gas exchange of adipose tissue is portrayed in Figure 1. Here tissue samples from a single rat were incubated in the presence of 20 mM glucose. The tissue in flask D received no additions and showed the characteristic negative net gas exchange throughout the incubation period. At 60 minutes insulin was added to the other three flasks so as to yield a final concentration of 100 μunits/ml. The tissue in the control flask A showed the usual prompt response to insulin with a marked positive net gas exchange which ensues when glucose is converted

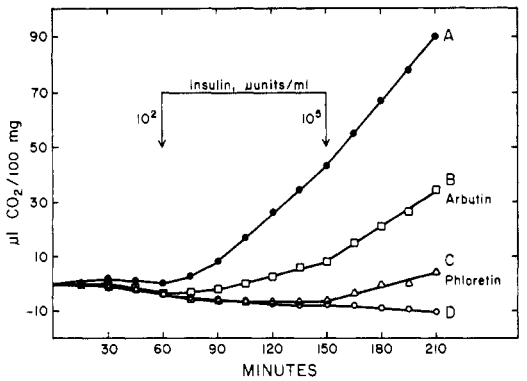

FIG. 1.—Inhibition by arbutin and phloretin of the insulin-stimulated net gas exchange of adipose tissue. The final concentration of arbutin was 20 mM and of phloretin 0.075 mM.

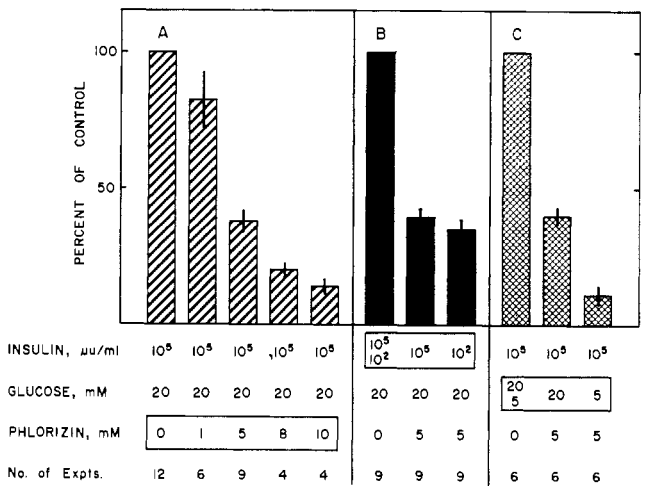

FIG. 2.—Inhibition of net gas exchange by phlorizin as influenced by alterations in the concentrations of phlorizin, glucose and insulin. Inhibition is expressed as the percentage of the values observed in each case in the absence of phlorizin. It should be noted that the absolute value observed for the control in the experiments where the insulin concentration is 10^2 μunits/ml is on the average only 56% of that at the higher insulin concentration. Likewise, the absolute value observed for the control in the case where the glucose concentration is 5 mM is on the average about 75% of that observed at the higher glucose concentration. The vertical lines at the top of the bars represent the standard error of the mean.

to fatty acids (cf. Flatt and Ball, 1964). The tissue in flask B containing 20 mM arbutin responded at a lesser rate, while the response seen in the presence of 0.075 mM phloretin in flask C was just detectable. At 150 minutes a further addition of insulin was made to flasks A, B, and C in an amount to give a concentration of 0.1 unit/ml. An increase in the rate of the net gas exchange was seen in all three flasks. Thus the magnitude of the response to be seen is a function of the insulin concentration even in the presence of the inhibitors. The response of tissue in the presence of 5 mM phlorizin resembles that shown in Figure 1 for 0.075 mM phloretin, but differs in certain respects to be presented here. Amygdalin and salizin resemble arbutin in their action and both are also less effective than phlorizin and phloretin. Further studies were therefore limited to the action of phlorizin and its aglucone.

In Figure 2 results are shown which illustrate the inhibition of the insulin-stimulated net gas exchange by phlorizin as the concentrations of phlorizin, insulin, and glucose are varied independently. When the glucose concentration is held at 20 mM and insulin is present in excess, 10^5 μunits/ml an 18% inhibition of the net gas exchange is seen with phlorizin at 1 mM and 86% inhibition at 10 mM. If the concentration of phlorizin is held at 5 mM and glucose at 20 mM, the percentage inhibition is about the same whether the insulin concentration is 10^2 or 10^5 μunits/ml. It should be noted that the average response of adipose tissue to a concentration of 10^2 μunits of insulin per ml is only about 56% of that seen in the presence of excess insulin, 10^5 μunits/ml (Ball and Merrill, 1961). Thus, as the insulin concentration is increased and more pathways or carriers are made available for the entry of glucose into the cell, phlorizin would appear to block a constant percentage of these pathways or carriers. A competition between phlorizin and glucose is again indicated by the experiments where the insulin concentration is maintained at 10^5 μunits and phlorizin at 5 mM but the glucose concentration is altered. Phlorizin produces 89% inhibition at a glucose concentration of 5 mM but only 60% at 20 mM.

The action of phloretin is similar to that of phlorizin in some respects but different in others. The inhibition by phloretin, like that of phlorizin, can be diminished by raising the glucose concentration. For example, at a concentration of 0.05 mM, phloretin produces about a 60% inhibition of the net gas exchange induced by 0.1 unit of insulin per ml with a glucose concentration of 5 mM. If the concentration of glucose is raised to 20 mM then the inhibition drops to around 10%. Unlike phlorizin the percentage of inhibition by phloretin varies with the insulin concentration. For example, at a glucose concentration of 5 mM and 10^2 μunits of insulin per ml, a 40% inhibition of the net gas exchange results at a concentration of 0.01 mM phloretin. When the insulin concentration is raised to 10^3 μunits/ml, a concentration just sufficient to yield a maximum response, then this concentration of phloretin is without inhibitory action. The phloretin concentration has to be increased to around 0.025 mM to achieve a 40% inhibition at this higher insulin concentration. However, it is important to note that if the insulin concentration is further increased to 10^5 μunits, the inhibitory action of phloretin remains unchanged from that seen with 10^3 μunits/ml. This aspect of the action of phloretin deserves further investigation.

The effect of both phlorizin and phloretin on net gas exchange, hexose uptake, and lactate production by tissue incubated in the presence of glucose, mannose, or fructose is shown in Table I. Insulin, as previously shown by Ball and Cooper (1960) stimulates the net gas exchange of the tissue in the presence of all three of these hexoses (cf. also Hernandez and Sols, 1963). The data presented here confirm these findings and show in addition that concomitantly with the positive net gas exchange there occurs an increased uptake of hexose and lactate production. Since the amount of lactic acid formed can account for but a fraction of the increased CO_2 output, conversion of all three hexoses to fatty acid must be occurring. Wood *et al.* (1961) have shown that labeled fatty acids are produced from [^{14}C]mannose in rat adipose tissue stimulated with insulin. The data in Table I show that in the presence of phlorizin and phloretin the effect of insulin on all three hexoses is inhibited. Moreover, the degree of inhibition exerted is about the same whether it is measured by net gas exchange, hexose uptake, or lactate production. The values found in the presence of the inhibitors drop to about those measurable in the absence of insulin. These results suggest that if phlorizin or phloretin is acting to inhibit some enzymatic step in the metabolism of glucose, then it must be on some step prior to the formation of pyruvate.

TABLE I
INHIBITION BY PHLORIZIN AND PHLORETIN OF INSULIN-STIMULATED NET GAS EXCHANGE, HEXOSE UPTAKE, AND LACTATE PRODUCTION IN THE PRESENCE OF GLUCOSE, MANNOSE, OR FRUCTOSE[a]

Measurement	Glucose 5 mM	Mannose 5 mM	Fructose 20 mM
	(μmoles/100 mg/hr)		
Net gas exchange			
No additions	−0.20	−0.43	−0.15
Insulin	1.53 ± 0.18	1.40	0.88
Insulin + phloretin	0.24 ± 0.05	0.08	0.00
Insulin + phlorizin	0.16 ± 0.07	0.05	0.11
Hexose uptake			
No additions	0.15	0.05	0.18
Insulin	1.34 ± 0.13	1.48	0.60
Insulin + phloretin	0.18 ± 0.07	0.17	0.04
Insulin + phlorizin	0.20 ± 0.07	0.18	0.03
Lactate production			
No additions	0.15	0.09	0.13
Insulin	0.41 ± 0.05	0.35	0.31
Insulin + phloretin	0.15 ± 0.03	0.13	0.21
Insulin + phlorizin	0.12 ± 0.03	0.04	0.06

[a] When present, the concentration of insulin was 0.1 unit/ml and of phlorizin was 5.0 mM in all cases. The phloretin concentration was 0.075 mM for the glucose experiments and 0.1 mM in the mannose and fructose experiments. The results of the glucose experiments are the mean of ten experiments plus or minus the standard error. The mannose and fructose results are the average of two experiments. Different rats were used for each of the hexose experiments so results are not directly comparable from one hexose to another.

In order to determine whether phlorizin and phloretin were acting by inhibiting some enzymatic step rather than by interference with the transport of hexose across the cell membrane several types of experiments were performed. First the effect of these inhibitors upon the conversion of glycogen to lactic acid was studied. This was done by employing the procedure described by Frerichs and Ball (1962) in which glycogen-rich tissue obtained from fasted-refed rats is incubated in Krebs-Ringer bicarbonate medium with a gas phase of 95% N_2–5% CO_2. Lactic acid production was measured by following manometrically the CO_2 released from the medium throughout the experiment and by chemical determination of the product at the end of the incubation. In a series of experiments performed in this manner, both the CO_2 release and lactic acid production by the control tissue were 8.7 ± 0.7 μmoles/100 mg per 2 hours. In the presence of 5.0 mM phlorizin or 0.1 mM phloretin the corresponding values were 11.7 ± 0.6 and 10.9 ± 1.5 μmoles, respectively. Thus, at concentrations of phlorizin and phloretin which effectively block the insulin-stimulated conversion of extracellular glucose to lactic acid, these compounds have no inhibitory effect upon the anaerobic conversion of glycogen to lactic acid which is occurring inside the cell. Indeed it appears as if the rate of glycogenolysis may be enhanced in the presence of these compounds.

The effect of phlorizin and phloretin on the hexokinase activity of adipose tissue homogenates was also investigated since this enzyme is involved when glucose, but not glycogen, is converted to lactic acid. At the same time, the activity of these inhibitors upon glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and the overall conversion of glucose-6-phosphate to triose phosphates was investigated. The procedures employed for the assay of these enzymes in adipose tissue homogenates were the same as

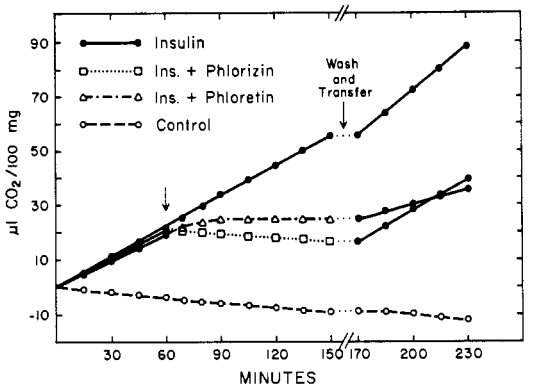

FIG. 3.—The reversal of phlorizin and phloretin inhibition by washing and transfer of the poisoned tissue.

described by Ball and Jungas (1963). We are indebted to Dr. Jungas for assistance with the measurements. None of these enzymatic functions was inhibited by 5 mM phlorizin or 0.1 mM phloretin. Hernandez and Sols (1963) have reported that 2 mM phlorizin does not inhibit the hexokinase activity of adipose tissue homogenates. They also reported that at this concentration it inhibited by 72% the insulin-stimulated utilization of fructose by the intact tissue.

An attempt was next made to obtain an insight into the rapidity with which phlorizin and phloretin exerted their inhibitory effect and whether once established it could be readily reversed. The experimental approach employed is illustrated by the data presented in Figure 3. Here the net gas exchange of the tissue was measured. In three flasks insulin (10^5 μunits/ml) was present from the start of the experiment. No additions were made to the fourth flask, which served as a control. At 60 minutes, phlorizin was added as the solid from the side arm of one flask containing insulin, to give a final concentration of 5.0 mM. A prompt inhibition of the insulin-stimulated positive net gas exchange ensued. At the same time, phloretin dissolved in alcohol was added from the side arm of another flask containing insulin to yield a final concentration of 0.1 mM. The inhibitory effect of this compound was not as prompt as with phlorizin. After 150 minutes, each piece of tissue was removed from its flask, blotted carefully with filter paper, and washed by gentle shaking at 37° for 5 minutes in 10 ml of Krebs-Ringer bicarbonate equilibrated with 5% CO_2–95% air. Each tissue was then placed in a new flask containing medium identical to that present at the beginning of the experiment. When readings were begun again it can be seen that the phlorizin-treated tissue had regained its initial activity. Washing of the phloretin-treated tissue also restored some of its activity but the process was not as effective as with phlorizin. In five experiments performed in this manner, it was found that the phlorizin-treated tissue showed an activity after washing that averaged 90% of that displayed by the tissue sample incubated with insulin and no inhibitors. Washing restored the activity of the phloretin-treated tissue to only 39% on the average. It should be noted that the activity of the tissue from flask A increased after the washing process so that the net gas exchange of this tissue as well as that of the phlorizin-treated tissue were both higher than the values recorded at the outset of the experiment. This apparent increase in sensitivity of tissue to insulin after a period of incubation, washing, and transfer to fresh medium has been repeatedly observed in our laboratories (cf. Ball and Jungas, 1964). It is as if some native inhibitor present in or on the tissue was released during incubation and removed by

the washing procedure. Other experiments of the type shown in Figure 3 were performed in which the washing procedure was shortened to 1 minute or prolonged to 15 minutes. The outcome of the experiment was not appreciably affected. It would appear from these experiments that phlorizin exerts its inhibitory effects promptly and that its action is readily reversed by washing the tissue. Phloretin acts more slowly and its effect is not as readily reversed by washing as is that of phlorizin.

Finally, it was deemed of interest to see whether phlorizin or phloretin had any effect upon the morphologic changes that can be induced by insulin in adipose tissue. As shown by Barrnett and Ball (1960), the addition of insulin to this tissue either in the presence or absence of glucose produces changes in the plasma membrane of the adipose tissue cells which suggest that insulin initiates pinocytosis. If phlorizin and phloretin reacted with the plasma membrane then the possibility existed that these compounds might compete with insulin for similar sites on this membrane. Tissue was therefore incubated in the presence of 5 mM glucose in a manner similar to that used in the experiments illustrated in Figure 1. Tissue to which phlorizin, 5 mM, and phloretin, 0.1 mM, had been added was compared with tissue incubated in their absence. Insulin to give a final concentration of 10^5 μunits/ml was added after 30 minutes of incubation and the reaction was then monitored for an additional 30 minutes. The tissue was then removed and fixed as described by Barrnett and Ball (1960). We are indebted to Dr. Barrnett for examination of the tissue with the electron microscope. Both the inhibitor-treated tissue and the control revealed the typical changes induced by insulin in the plasma membrane and vesicle formation as described by Barrnett and Ball (1960). The tissue treated with phlorizin or phloretin did not show the diminution in granularity and density nor the formation of small lipid droplets to be seen in control tissue, changes which presumably reflect the stimulation of glucose metabolism. Thus the ability of insulin to induce pinocytosis did not appear to be blocked by the presence of these inhibitors of glucose utilization.

Discussion

The literature contains numerous reports on the relative inhibitory action of phlorizin and phloretin on the permeability of a variety of tissues to sugars. As first shown by Lambrechts (1937), the kidney is less sensitive to phloretin than to phlorizin. The same is true of the small intestine (Jervis *et al.*, 1956). Alvarado and Crane (1962) report a K_i for phlorizin of 4.5×10^{-7} M and 8×10^{-7} M, respectively, for the entry of 1.5 anhydro-D-glucitol and 6-deoxy-D-glucose into intestinal epithelial cells. Phloretin is reported by these workers to be less than 1% as active as the glycoside. On the other hand phloretin has been found to be more effective than phlorizin in blocking the permeability of the red cell to sugars. Le Fevre (1954) reports K_i values of 4.5×10^{-6} M for phloretin as compared to 1×10^{-4} M for phlorizin. The relative response of adipose tissue to these inhibitors thus resembles that shown by the erythrocyte rather than that of the kidney or intestine. At glucose concentrations of 20 mM, a 50% inhibition of the positive net gas exchange induced by insulin is produced by phlorizin at a concentration of 2×10^{-3} M. This value diminishes as the glucose concentration is lowered. The concentration of phloretin needed to produce comparable effects is about 2% of that required for phlorizin. In studies on the rat diaphragm muscle *in vitro*, Lotspeich and Wheeler (1962) have reported that phlorizin at a concentration of 1×10^{-3} M causes about a 50% inhibition of the insulin-induced uptake of galactose (concentration 5.6 mM) though no effect of phlorizin was observed on the appreciable uptake of galactose that occurred in the absence of insulin. Battaglia *et al.* (1960) have reported that phlorizin at a concentration of 3×10^{-3} M produces a 60% inhibition of the glucose (concentration 14 mM) uptake induced by insulin in isolated rat diaphragm muscle. In a few exploratory experiments with rat hemi-diaphrams we have obtained similar results and have also found that phloretin is a much more effective inhibitor than phlorizin. Thus the concentrations of phlorizin and phloretin needed to inhibit the insulin-induced uptake of hexoses by diaphragm muscle and adipose tissue are very similar.

At the concentrations employed we could find no evidence to indicate that phlorizin or phloretin exert their action upon adipose tissue by interfering with the metabolism of glucose. All the data obtained suggest that the action of these compounds is by interference with the transport of hexoses across the cell membrane. That phlorizin acts at the cell membrane is suggested by the fact that inhibition sets in promptly and to its full extent within minutes after its addition to the tissue. Also this inhibition is rapidly and nearly completely reversed by a brief washing of the tissue followed by its transfer to fresh medium. In the case of phloretin the picture is not as clear in this regard. However, none of the evidence contradicts the conclusion drawn by other workers (cf. Lotspeich, 1961, Crane, 1960) that the action of these compounds is to be accounted for by inhibition of sugar entry into the cell.

If one accepts the premise that one of the actions of insulin upon adipose tissue is to increase the number of pathways available for the transport of hexoses across the cell membrane, then one may consider phlorizin and phloretin as competitively inhibiting the use of these pathways by hexose. Now the number of total pathways or carriers for glucose that are made available by insulin in adipose tissue is a function of the insulin concentration and reaches its maximum when the insulin concentration is 10^3 μunits/ml or higher (Ball *et al.*, 1959). The results presented here show that when insulin is present in excess and the glucose concentration is 20 mM, there is a 61% inhibition of net gas exchange by 5 mM phlorizin. Since glucose uptake varies in a linear fashion with net gas exchange (Flatt and Ball, 1964) it may be said that out of every 100 pathways made available to glucose by insulin, 61 are blocked by the phlorizin. Ball and Merrill (1961) found that when the insulin concentration was lowered to 10^2 μunits/ml, on the average the metabolism of glucose was only some 56% of that observed with excess insulin. Now at this insulin level, but with glucose and phlorizin concentrations unchanged, it was observed that the percentage inhibition by phlorizin remained about the same (65 vs. 61) as when insulin was present in excess. Thus out of each potential 100 pathways that insulin can activate the total number blocked by phlorizin drops from 61 to 37 (65×56) as the insulin concentration falls from 10^5 to 10^2 μunits/ml. This would seem to indicate clearly that phlorizin and insulin do not compete for some site on the cell membrane, since the opposite effect would be expected if this were the case.

A similar conclusion concerning the action of phloretin cannot be drawn as readily. The degree of inhibition exerted by phloretin decreases as the insulin concentration is raised. However once the concentration of insulin reaches the value where the maximum me-

tabolism of glucose is achieved, further addition of insulin is without effect on the action of phloretin. This would seem to indicate that insulin and phloretin are not acting competitively. Yet the action of phloretin and phlorizin are obviously different in certain respects. Further evidence for this is furnished by the fact that the inhibitory effect of phloretin can be only partially reversed by washing of the tissue.

The fact that neither phlorizin nor phloretin impairs the ability of insulin to initiate pinocytosis in adipose tissue further suggests that these inhibitors do not act by preventing access of insulin to a cell-membrane site, since the first step in the process of pinocytosis would appear to be the adsorption or attachment of the insulin molecule to the surface of the cell membrane (cf. Barrnett and Ball, 1960; Ball and Jungas, 1964). Unfortunately, the action of these inhibitors does not throw any light upon the question of the relationship that exists between the ability of insulin to induce pinocytosis and its ability to augment glucose uptake. In addition no further insight is provided into the possible role of vesicle formation as a means of transport of glucose into the cell, since if phlorizin or phloretin act by blocking permeability to glucose they could block the permeability not only of the cell membrane but of the membrane surrounding the vesicles.

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New Synthetic Membranes for Dialysis. I. A Copolyether-Ester Membrane System*

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Block copolymers based on polyoxyethylene glycol and polyethylene terephthalate were synthesized, and the effects of varying the molecular weight and the molar composition of the macroglycol on the membrane properties of these copolymers were determined. Membranes from the copolymer containing 0.3 mole % of polyoxyethylene glycol, molecular weight of 1540, dialyzed compounds such as urea, creatinine, and uric acid at a faster rate than did a Cuprophane membrane. However, sugar molecules such as glucose and sucrose showed relatively slower escape rates through the copolyester membrane than through the Cuprophane membrane. A dialysis cell suitable for laboratory studies on new polymeric membranes is described.

The fractionation of solutes by means of their differential rates of diffusion through a membrane is potentially an ideal technique for separating complex mixtures such as blood. However, owing to various shortcomings of existing commercial membrane materials the dialysis technique has been limited in its scientific and clinical applications. For example, gel cellophane is still considered to be the best available material for extracorporeal hemodialysis; yet the slow rate of dialysis of molecules having molecular weights greater than several hundred has limited this technique in treating blood chemistry disorders. Several recent studies (Biget, 1947; Immergut *et al.*, 1954; Craig and Konigsberg, 1961; Michaels *et al.*, 1962) have shown that membranes having improved porosity could be obtained by suitable aftertreatment, such as linear stretching of wet membranes, swelling and annealing of films at elevated temperatures, forming networks in the films by extracting compounds which had been dispersed in the original film, or by chemical reactions on the membrane material. Some of these membranes were reported to be permeable to polymers

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