SUGAR TRANSPORT IN THE RED BLOOD CELL: STRUCTURE-ACTIVITY RELATIONSHIPS IN SUBSTRATES AND ANTAGONISTS[1]

PAUL G. LEFEVRE[2]

Brookhaven National Laboratory, Upton, New York, and University of Louisville School of Medicine, Louisville, Kentucky

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I. NATURE OF THE TRANSPORT SYSTEM

A. Development of the concept

The importance of the "physiological permeability" of biological barriers, as distinguished from their passive "physical permeability," was noted by the earliest physiologists of modern times; but until recent years, application of this concept was limited essentially to those special multicellular sheets which separate fluid compartments within, or immediately adjacent to, the animal body. Thus, the intestinal and renal tubular epithelia, the gill membranes of fishes, amphibian skin, and many specifically differentiated glandular structures were recognized as displaying "active transfer" phenomena, the metabolic activity of the cells providing the energy for (or at least taking some causal role in) the translocation of materials from one side of the sheet to the other side. In contrast to such *cellular* membranes, the *cell* (or *plasma*) membrane (separating the individual cell's protoplasm from the interstitial fluid, plasma, or other external bathing medium) was until about twenty years ago seldom credited with secretory

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[2] Present address: University of Louisville School of Medicine, Louisville, Kentucky.

activity. The permeability properties of living cells were classically interpreted almost exclusively in terms of the plasma membrane's simply presenting a structural or physicochemical resistance to diffusion. The readiness with which water or solute molecules might pass through the cell surface was generally taken as a reflection of the relative ease with which these molecules could dissolve in the substance of the membrane, or fit into aqueous channels passing through it. The energy for any fluxes through the membrane derived from the thermal agitation of the particles concerned, so that any net migration required a corresponding activity gradient across the membrane. Although it was recognized that cells often displayed a highly refined selectivity in their permeability properties, the cell membrane was rarely considered to partake directly in the mechanics or the energetics of the translocations.

But during the 1940's, reports of the apparent occurrence of transport phenomena at cell surfaces began to appear with steadily increasing frequency; in the last decade this has become one of the most active fields in physiological research. It is primarily with regard to the amino acids and certain biologically prominent inorganic ions that the most thorough cases have now been made for the operation at cell surfaces of a "pump," or *active transport*; that is, that net movement has been shown to occur against a chemical or electrochemical activity gradient. The monosaccharide transport mechanism with which this review is to be concerned does *not* have this characteristic; in fact it has become the prime example of another variety of "mediated transfer" which is often designated by the rather misleading term, *facilitated diffusion*. This type of transfer operates apparently by way of a transient stoichiometric association of the sugar (or whatever molecular species is being carried) with a limited number of reactive sites which are accessible at the cell surface. Typically the penetrant as such is completely excluded from the cell membrane, by reason of its dimensions and other physical properties; but it becomes in effect solubilized in the membrane by forming a complex at one of these special reactive sites. In the case of the red blood cell's sugar transport system, certain experimental facts (78, 93, 107) suggest that the reactive sites and the complexes formed are mobile (free to diffuse) within the substance of the membrane, so that the particles bearing the reactive sites may legitimately be called *carriers*. Other interpretations of the facts are, however, tenable (122), and for the present it should perhaps be said only that the process by which the complex moves or becomes reoriented with respect to the inner and outer interfaces shows diffusion-like kinetics and appears to require no metabolic energy investment. In such a system the transfer will proceed toward a steady state in which the penetrant will be homogeneously distributed throughout the aqueous compartments, just as with uncomplicated diffusion. However, a facilitated-diffusion system is in other respects likely to behave quite differently from an untrammeled diffusion: there may be "saturation kinetics," competition for transport when two or more species of passengers are present, high steric specificity, and peculiar sensitivities to small amounts of special blocking or stimulating agents which may show no generalized action on non-specific cell permeability.

B. Occurrence of mediated transfer of monosaccharides

In connection with movements of simple sugars, some or all of these criteria for a carrier-mediated transfer have been noted in skeletal muscle (81, 93), cardiac muscle (26, 88, 89, 93), erythrocytes (9, 73, 93, 125, 132), the placenta (124), and several types of ascites tumor cells (17, 41, 90, 91); also, there is peripheral evidence of similar processes in adipose tissue (135), the crystalline lens (110), the blood-brain barrier (29), and the blood-aqueous barrier in the eye (20, 109). A matter of great interest among cell physiologists and biochemists in recent years has been the fact that in the muscle, adipose, and ocular tissues, this system apparently requires insulin (or some humoral factor from contracting muscle) for full activation (41, 81, 93, 109, 110). In contrast, this hormone does not detectably affect the sugar movements in the other types of cells listed (17, 36, 93, 97). As yet, however, there is no further basis for drawing a fundamental distinction between the insulin-dependent and the insulin-indifferent sugar transfers, and it is commonly supposed that the former will prove to be a special complication of the latter.

Special handling of monosaccharides at the yeast cell surface is also well established (111); but this appears to entail the actual introduction of the sugars into metabolic reactions at that point, rather than the delivery of the sugars, as such, into the cell's interior. Rothstein noted (112) that this membrane-fixed "uptake" process mimics the enzyme hexokinase in several details of its behavior, most notably in its substrate specificity. On the other hand, the priority of some transport step to the fermentative events is suggested by the finding (7) of a competitive inhibition of fermentation by 6-deoxy-6-fluoro-D-glucose in intact yeast cells, which was absent in fermenting extracts from the same cells, and also by the demonstration of competitive phenomena in the movements of sugars in both directions through the membranes of yeast cells in which sugar utilization had been blocked by iodoacetate (13). Bacteria of various species are known to have the capacity for a decided *accumulation* of specific sugars and glycosides, up to many times the level presented in the medium (16); but as noted above, such *active transport* is not characteristic of the sugar-transport system which is our special concern here.

Among the variety of mammalian cells in which this system appears to operate, it has been (primarily for technical reasons) the erythrocyte, and especially the human erythrocyte, that has provided the most detailed analysis of the system's behavior. The peculiar ease of sugar penetration into human and macaque red blood cells, as compared with those of a variety of rodents, carnivores, and ungulates, was first emphasized by Kozawa (63). The non-primate mammalian red cells, though not absolutely monosaccharide-impermeable as is sometimes stated, are generally so nearly so that it is only fairly recently that they have been seriously studied in this connection (42, 70, 93), while the same process in the human erythrocyte has been the subject of recurrent inquiry. Widdas (126) made the interesting discovery that in this respect the erythrocytes from *fetal* blood of the pig, rabbit, guinea-pig, sheep, and deer resemble those from adult

human blood, displaying a comparably high glucose permeability which begins to diminish approximately at the time of birth. Thus, the peculiarity in the primates appears to be the persistence of a general fetal condition into the adult stage, rather than the introduction of a generically unique mechanism.

C. The model system

In this review the primary emphasis is on the specificity characteristics (in the red cell) of this monosaccharide facilitated-diffusion transport system, rather than on its kinetic peculiarities. But the significance of the "affinities" to be discussed will be greatly clarified by a brief preliminary consideration of the picture of the carrier system which has emerged as probably the simplest model consonant with the experimental kinetic information (78, 106, 124, 125). In this model, it is presumed that the penetrant or passenger molecule, P, enters into a reversible association with the carrier entity, C, at either interface:

$$P + C \underset{k_2}{\overset{k_1}{\rightleftharpoons}} PC.$$

The free P exists only in the aqueous phases, inside or outside the cell, while C (either free, or as the complex PC) is retained entirely within the barrier which separates the two aqueous compartments. Evidently the above association-dissociation reaction at the interfaces is so rapid, relative to the other steps in the process, that it is always close to the equilibrium point; that is, at either interface, $k_1[P][C] = k_2[PC]$. This is conveniently expressed in terms of a conventional dissociation constant, K: $K = [P][C]/[PC] = k_2/k_1$. The total amount of C available in the membrane is fixed, so that the proportion of C which is combined as PC at each interface will be $\frac{[P]}{[P] + K}$, where $[P]$ refers to the adjacent aqueous compartment. Then, if it is assumed (in line with the relative thinness of the membrane) that there is within it a nearly linear gradient for PC (*i.e.*, that a steady state is approached), the rate of transfer from compartment 1 to compartment 2 ($_1R_2$) at any moment will be proportional to the difference in relative concentration of PC at the two interfaces:

$$_1R_2 = a\left(\frac{[P]_1}{[P]_1 + K} - \frac{[P]_2}{[P]_2 + K}\right),$$

where a is a constant defined by the particular membrane characteristics and the density of the special reactive sites. As can be seen by inspection of this equation, the form of the temporal course of the transfer, and the nature of the relation between the transfer rate and the sugar concentrations on the two sides of the membrane, depend entirely on the magnitude of these concentrations, $[P]_1$ and $[P]_2$, relative to the dissociation constant, K. If the sugar-carrier association is a tight one (small K), the transfer will show saturation at correspondingly low sugar levels; further increase in the gradient will not lead to an appreciably greater rate of sugar migration. But if K is large (low "affinity" between sugar and carrier), the kinetics will not differ detectably from those of free diffusion. Details

of the various sorts of kinetic behavior that may be anticipated have been elaborated elsewhere (125).

Thus, a rough estimate of the apparent dissociation constant, K, can be obtained by suitable analysis of experiments recording the transfer rate for a sugar as a function of its concentrations in the two compartments, provided that K does not fall too far outside the range of sugar concentration which it is feasible to use. With current methods, K's between about 2 mM and 3 M can thus be defined, not with great precision, but with sufficient resolution that the familiar hexoses and pentoses (among which the affinities differ rather widely) may be readily distinguished. Moreover, the sequence of K's thus calculated is in good agreement with the relative affinity sequence provided by competition experiments, in which the effect of one sugar on the transport of a second sugar is observed (76). It is this consistency which provides the principal argument for adoption of the particular assumptions in the above model, as adequately defining the formal operation of this mediated-transfer system. Several additional points of unusual behavior, predicted from this model, have also been established experimentally (78, 93, 107). This quantitative adequacy in fitting the differing kinetic behavior observed in a variety of monosaccharides suggests that if any additional parallel process contributes to the passage of sugar through these cell membranes, it must operate at a rate so much slower that it is completely obscured by this primary mechanism. This is in keeping with the fact that these cells are typically quite impermeable to other molecules similar in size, structure, and physical properties to the monosaccharides, such as pentahydric and hexahydric alcohols (63, 76), including even the cyclic inositol [*meso*-inositol, *i*-inositol, 1,2,3,4,5,6-cyclohexanehexol] (76, 125). Thus, the "physical permeability" of the cells to the sugars is probably nearly nil.

II. METHODS OF STUDY

The methods used in these transfer studies have been for the most part optical, involving the recording of light transmission through very dilute suspensions of erythrocytes. The principal such method is that of Ørskov (87, 92); this is based on an empirical relation between the average red cell volume in the sample and the fraction of light which is scattered in passing through the suspension. The nature of this relation depends on the concentration of the cells in the suspension, and on various purely instrumental factors; but conditions can readily be chosen so that the recorded deflections are virtually linear with the changes in cell volume. For many years this has been the most widely used method for study of erythrocyte permeability in general. In the specific application to sugar movements, analysis with the Ørskov method is simplified by the fact that the equilibration of water through the membrane is so very much more rapid than the specific solute movements that no appreciable error is introduced by treating the osmotic water migration as instantaneous, and employing a correspondingly slowly responding recording device. In other words, the cells may be considered to be in osmotic equilibrium with the suspension medium at all times, so that the volume changes with time serve as an index of migration of the sugar in osmotically

equivalent amounts. Generally, the suspension medium used is an isotonic saline or balanced salt mixture, buffered at physiological pH, and the sugars and other test materials are made up in this stock. Initially the cells are centrifugally washed free of sugar, or are equilibrated with a known concentration; then, in the optical cuvette, this initial equilibrium is abruptly upset either by addition of a higher concentration of sugar to the suspension, or by switching of pre-equilibrated cells into a medium of lower sugar concentration. The "instantaneous" osmotic cell volume adjustment, and the subsequent gradual return to the original isotonic volume (as the sugar either enters or leaves the cells in accordance with the concentration gradient), are then recorded in terms of changes in direct light transmission.

This means of following the solute movements is so obviously indirect that the method would find little use were it not for its great practical convenience, in providing immediately at the time of each experiment a continuous, complete record of the sequence of events, with a very satisfactory resolution and reproducibility. The systematic (linear) correlation of the recorded deflections with osmotic responses in the cell volume in these sugar-salt mixtures has been repeatedly established (77, 87, 125), and the sugar movements thus reflected agree well with those shown by direct chemical analysis whenever the appropriate comparisons have been made (78, 104). Nevertheless, it is essential in critical applications of the Ørskov method always to take account of factors other than osmotic volume changes which may also be modifying the opacity of the suspensions. Changes in the refractive index of the medium upon addition of sugars may introduce minor but appreciable disturbances, which have been accounted for or circumvented in the more quantitative studies (77, 87, 125). Also, various responses of the cells to added reagents (partial hemolysis or agglutination, discsphere shape transformations, chemical alteration in the hemoglobin, and other more obscure disturbances) are often encountered; usually these interfere so grossly and characteristically with the optical recording that they are readily recognized.

A different sort of photometric method, based on Parpart's (95) continuous recording of the increase in optical transmission accompanying hemolysis, has been used extensively by Wilbrandt and his colleagues (36, 127, 130, 131). This involves following either (a) the course of hemolysis ensuing as sugar enters the cells from a medium in which the salt concentration alone is insufficient to maintain cellular integrity, or (b) the progressive changes in the cells' osmotic resistance curve after addition of sugar to an isotonic medium. Both these procedures depend on the assumption that the distribution of critical hemolytic volumes in the cell population is independent of the concentrations of the sugars present in the cells or the medium, and are therefore subject to all the uncertainties surrounding the fixity of this critical volume (98). However, many of the results obtained in Wilbrandt's laboratory by the use of this lytic index have been borne out in considerable quantitative detail in other laboratories by different methods.

Various conventional chemical and radiochemical analytic methods have also

been applied (42, 63, 78, 93, 107) in elucidating the kinetics of this system (in much thicker cell suspensions than used in the optical methods). Such procedures, though far more tedious than the optical methods, obviously provide a more direct measure of the process in question, and have often been appended as a basis for validation and calibration of the more routinely used techniques. One difficulty with the chemical analytic approach is in getting adequate temporal resolution in the separation of cells and medium, especially for short time intervals; this usually requires rapid cooling or special transfer-halting procedures (78), followed by high-speed centrifugation in the cold.

Whatever the analytic method involved, the study of sugar transfer in human erythrocytes is generally simplified in comparison with similar studies in muscles, ascites tumor cells, and the more slowly transporting species of red cells, by the fact that the amounts of sugar handled by the transfer system in the typical experimental situation are enormously greater than the quantities that the cells can metabolize (37). Thus, with human erythrocytes it is seldom necessary to take any account of the loss in total sugar in the course of the experiments.

III. SUBSTRATE SPECIFICITY

Kozawa's original description (63) of the hexose and pentose permeability of human red blood cells showed a wide range of rates of penetration, suggesting a rather refined structural specificity. Wilbrandt provided further comparative information of this type (128), and later showed that even direct enantiomorphs are distinguished in this system, D-xylose and L-arabinose entering readily while L-xylose and D-arabinose were said to be completely excluded (129). Moreover, D-xylose and L-arabinose interfered with each other during simultaneous entry. It was on the basis of such comparative prowess in competition experiments that LeFevre and Davies (76) first presented a sequence of apparent affinities for the carrier, among the commonly available monosaccharides. As mentioned earlier, this ranking of the affinities was reflected also in the systematically varying kinetic pattern (the concentration-dependency of the transfer) among the several sugars. Dissociation constants consistent with this affinity sequence were subsequently estimated by kinetic analysis of the competitive inhibition by phloretin [phloroglucinol monophloretate, β-(p-hydroxyphenyl)-phlorpropiophenone] (73). Such studies clearly showed a highly refined recognition by the transport system of the steric distinctions between the several hexose or pentose isomers, but did not suggest any specific configurational requirement. Various attempts to fix on some correlation of configuration with affinity for the carrier system all ran into difficulties with one or more sugars obviously out of line. For the aldoses, however, LeFevre and Marshall (79) have called attention to a systematic correlation with molecular shape; this correlation suggests that reaction with the specific membrane sites requires not a certain sugar configuration *per se*, but rather that the configuration be such as to favor the assumption of a particular one of the relatively strainless conformations which are recognized for the pyranose ring structure. The nature of this specificity will now be examined in some detail.

A. The conformational correlation

A variety of physicochemical measurements indicates that free hexoses and pentoses, after completion of mutarotational equilibrium in aqueous solution, are preponderantly in the form of α- and β-pyranoses (six-membered rings) in an anomeric equilibrium mixture; among the aldoses, only a few show appreciable fractions of open-chain or the five-membered ring furanose forms (51, 96). Thus, the sugar molecules with which the cells are presented in these experiments are primarily in the form of rings made up of one oxygen atom and five carbon atoms. Since the valence angle at the oxygen corner of the ring differs very little from the tetrahedral angle approached at the carbons, and since the C—O bond length is not much less than the C—C bond lengths in the ring, this structure differs geometrically only slightly from the cyclohexane ring, for which the two specific strainless conformations were defined over seventy years ago by Sachse (115). These strainless shapes are now generally designated as the "chair" and "boat" conformations, because of the obvious gross analogies in form. In the case of a pyranose, the identifiability of the separate ring atoms leads to a distinction between two kinds of chair shapes, and six kinds of boat shapes; in Figure 1, these are illustrated for the particular configuration, α-D-mannose. The six boat forms are readily interconvertible with little ring-strain, and may be considered as a single flexible cycle, while the chairs are relatively rigid, large deformation of the valence angles being required to twist a molecule from either chair form to any other conformation, or *vice versa* (40). At each carbon atom in the ring, there are two extracyclic valences; in the boat shapes, some of the adjacent extracyclic bonds are aligned in a true *cis* relationship (directly eclipsing each other in sighting along the adjacent C—C link in the ring). Repulsion between such directly apposed groups encourages rotation of the bonds in the ring so as to move these groups as far away from each other as possible, by staggering the orientation of the valences on the adjacent carbon atoms (4, 39, 99, 100). In the chair forms, *all* adjacent valences are staggered in this way. This factor renders the chairs fundamentally more stable energetically than the boats (31, 39, 99, 100, 117) or any of the other shapes in the flexible cycle (40), so that the chairs are theoretically to be expected at ordinary temperatures, unless the particular structure of the rest of the molecule makes the chairs geometrically impossible. Reeves (99, 100) has summarized much experimental verification of this expected great predominance of the chair forms of pyranoses.

All the complete aldoses (*e.g.*, mannose in Fig. 1) carry one hydroxyl substituent at each of carbon atoms 1, 2, 3, and 4, and, in the case of hexoses, a carbinol substituent at carbon 5; at each point, the second extracyclic bond connects with a hydrogen atom. In any of the conformations in Figure 1, at each carbon atom one or the other of these valences, represented by a broken line, extends radially outward from the ring only slightly out of the general ring plane; this orientation is designated as "equatorial" (2). The other, "axial" group, represented by an unbroken line, is directed approximately perpendicularly to the ring. The equatorially placed groups are thus well out of each other's way, while the axial sub-

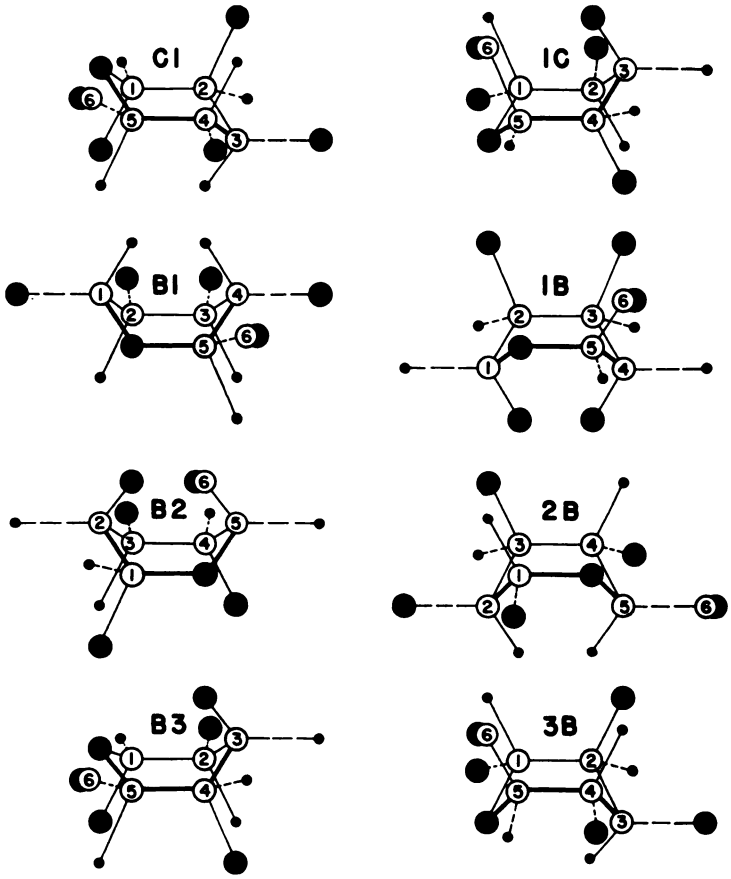

FIG. 1. Chair and boat conformations of α-D-mannose.

Solid circle in ring represents the O-atom; the numbered circles, the conventionally numbered carbon atoms. Larger solid circles outside ring represent —OH groups; smaller solid circles, —H. Heavy lines in ring mark the side facing the reader. Equatorial valences are shown by broken lines; axial valences by continuous lines.

stituents on the same side of the ring are more crowded together. Hence, the erection in an axial position of any substituent larger than hydrogen constitutes an unstabilizing influence in that conformation: *i.e.*, a lower energy state can be achieved by twisting of the ring into the opposite conformation, in which the larger group will be switched from the axial to the equatorial position, rotating the small H-atom into the axial spot. It is this sort of consideration which determines the equilibrium proportions between the two chairs, C1 and 1C, in a pyranose solution. But switching between these two forms entails reversal of *all* the extracyclic valences, so that removal of an instability factor at one carbon may be offset by introduction of a new instability at other parts of the ring. Thus, the entire molecular configuration must be taken into account in judging the relative stabilities of the alternative chair forms. Physical chemists have characterized

certain juxtapositions as particularly unstabilizing (39, 99), and Reeves (100) has given a simple set of empirical rules for approximate weighting of the instability factors in the aldopyranosides. More recently, Reeves has in fact suggested (101, 102) that for those pyranoses in which both chairs are distinctly unstable, this sort of consideration may even override the basically greater instability in the shapes of the flexible cycle, particularly if (as in the B1 form of α-D-mannose in Fig. 1) a boat would allow *all* of the substituents larger than the hydrogen atom to assume equatorial positions.

It is in such terms that for the aldoses the correlation with affinity for the red blood cell's monosaccharide transfer system may be drawn (79). This is shown

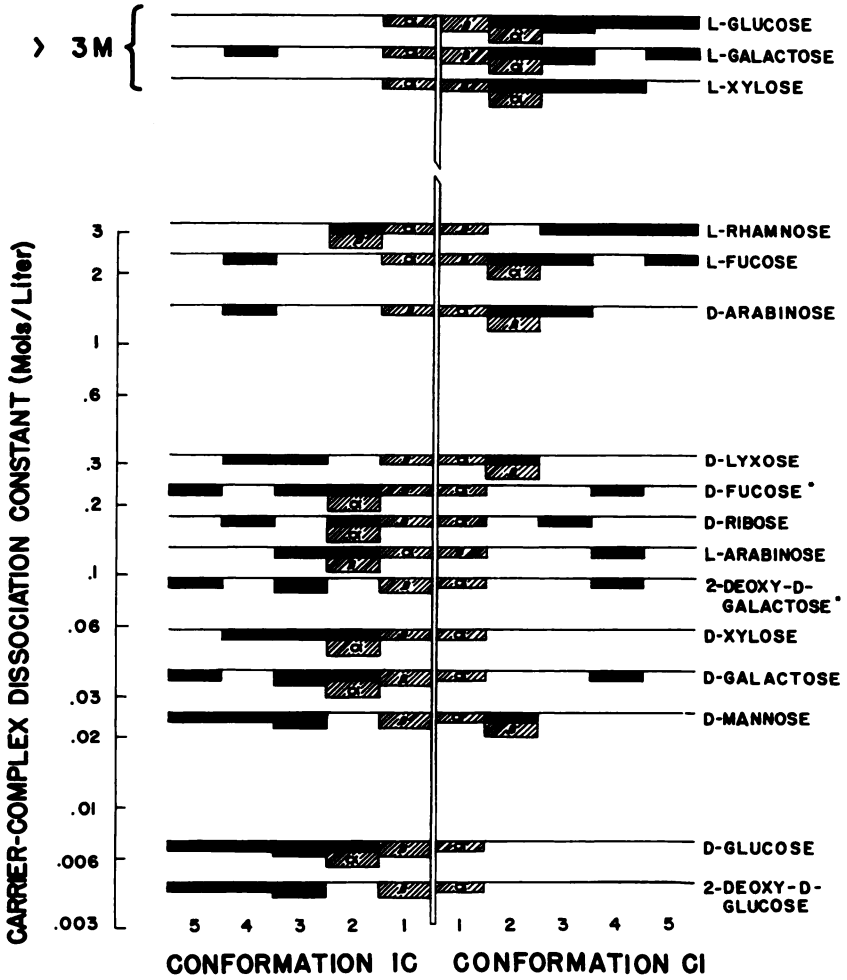

FIG. 2. Conformational stabilities of aldoses in relation to affinity for human red blood cell sugar-transport system.

Blocks represent "instability factors," as weighted by Reeves (100), at each ring carbon, as numbered at bottom, in each of the alternative chair conformations. Cross-hatching marks instability factors shifting with mutarotation between pyranose anomers. Asterisk (*) denotes sugars not previously included in relevant published reports.

in Figure 2; here the instability factors at each of the five ring carbons in the alternative chair conformations are designated by the dark blocks; the cross-hatched blocks mark those factors which differ in the α- and β-anomers. The vertical thickness of the blocks reflects Reeves's weightings of the several instabilities. The biological parameter is given on the logarithmic scale at the left, in terms of the apparent dissociation constant, K, for the several sugars. The arrangement in Figure 2 is that of increasing affinity from top to bottom. Because of practical limitations on the experimental sugar concentrations, K's greater than about 3 M could not be adequately measured, so that these three sugars can only be classed together as having very low (if any) affinity for the system. The correlation of increasing affinity with increasing relative stability in the C1 conformation is obvious in Figure 2. Of the unmodified hexoses, the ordinary blood sugar D-glucose [dextrose] shows the highest affinity; and its homomorph, D-xylose, is similarly the most favored pentose. These are correspondingly the sugars which (as the β-anomers) are completely free of axial substituents in the C1 conformation. Their mirror-image enantiomorphs, L-glucose and L-xylose, are of course comparably stable in the opposite (1C) form, and these show such extremely low affinity that it is not feasible to estimate the magnitude of their K's. The comparison between D- and L-galactose is similar. On the other hand, arabinose, which has a less one-sided distribution of instability factors, can make use of the system in both enantiomorphs, but more readily so in the L- than in the D-configuration, in line with the comparative distribution of axial substituents in the C1 conformation. The situation is similar for the two kinds of the deoxyhexose (or methylpentose), fucose [galactomethylose, 6-deoxygalactose].

This correlation clearly suggests that affinity for the carrier system not only demands the C1 conformation, but is modified by the same factors which determine conformational stability; *i.e.*, increasing numbers of axial substituents reduce the ability of the C1-aldopyranoses to react with the cell surface component. This is consistent with observations on the affinities of methyl-substituted aldoses; thus, α-methyl-D-glucoside (63, 75), α-methyl-D-mannoside (63, 75), 5-methyl-D-arabinose [L-fucose] (79), and 5-methyl-L-lyxose [L-rhamnose, L-mannomethylose, 6-deoxy-L-mannose] (79), in which the methyl is axially located in the C1 conformation, can scarcely enter the cells at all; while β-methyl-D-xyloside (75), 3-methyl-D-glucose (94, 104), and 5-methyl-L-arabinose [D-fucose] (75), which in the C1 form have the methyl groups in equatorial positions, show reasonably high affinities for the transport system. The issue in respect to α-methyl-D-glucoside is somewhat confused by Bowyer's statement (9) that, in spite of its extremely low rate of penetration, its K is only about 40 mM. But this figure does not seem compatible with the fact that this glucoside does not interfere with the entry of the low-affinity ketose, D-fructose [levulose] (75), and the basis on which the K of 40 mM was assigned is not clear.

B. Implications

In the sequence of Figure 2, the hexoses tend to be more extreme in their affinities than the homomorphous pentoses; this is in keeping with the special weight which Hassel and Ottar (39) have assigned to the hexoses' extracyclic

carbinol group as an unstabilizing influence when it assumes an axial position along with other substituents on the same side of the ring (as, for instance, in conformation 1C in Fig. 1). However, a more significant factor in the comparison of hexose with pentose may be the fact that the latter is more likely to include an appreciable component of furanose forms in the equilibrium mixture (96). That this consideration may modify the affinity sequence is suggested by the fact that D-galactose has an appreciably lower affinity than D-mannose, since the former is unusual among the aldohexoses in the appreciable degree of conversion to furanose which is suggested by its mutarotational characteristics (51). The reverse order for galactose and mannose in Fig. 2 would be expected on the basis of either the general correlation with instability factors, or the comparative positions of the corresponding pair of pentoses (L-arabinose and D-lyxose). Galactose also behaves peculiarly in the rat diaphragm sugar transport system (3). Further suspicion of the significance of furanose components as complicating factors arises from the failure of the *ketoses* to fit into the pattern defined by the aldoses, since ketose solutions commonly also contain appreciable furanose components at mutarotational equilibrium (33, 51). The only three ketoses which have been studied in connection with the transfer system (79) show the following approximate *K*'s: D-tagatose, 0.06 M; L-sorbose, 0.6 M; and D-fructose, 1.5 M. This sequence is not readily fitted to any obvious pyranose conformational pattern. It has been established by Gottschalk (32) that yeast hexokinase utilizes fructose specifically only in the furanose configuration, while the aldoses are handled as pyranoses; perhaps a similar complication confuses the ketose pattern in the present instance. Also, D-tagatose uniquely upsets the otherwise consistent specificity pattern of bacterial transglycosidase (34); if the same anomalous behavior for this particular sugar were discounted in connection with the red cell system, the relative affinities of sorbose and fructose could then be harmonized with the aldose pattern.

There is reason to suggest that the fundamental requirement for the C1 form may be quite general for all enzymic reactions with pyranoses, since there does not seem to be any record of biological utilization of any of the sugars for which the 1C conformation is decidedly the more stable of the chairs, in spite of attempts to observe such utilization in various cells (6, 114). But this possible universality in the ring-shape specificity does not extend to the detailed affinity sequence among the C1 aldopyranoses. In the red cell, this sequence appears to be determined simply by the rather non-specific factor of the equatorial or axial distribution of the hydroxyl and carbinol groups, in line with the general observation of the hindrance of chemical reactions produced by moving the reactive group from an equatorial to an axial position (1, 5, 62). In other words, the specificity in the human erythrocyte system seems to be limited to the rather primitive and uncomplicated requirement for the C1 ring shape, beyond which any affinity differences arise only from unspecific hindrances in the details of the assembly of the extracyclic substituents.

The nature of this discrimination between C1 and 1C conformations means that the reactive site on the cell surface must be able to appreciate a purely geometrical distinction which is in the nature of a left- or right-handedness. That

is, given two points of contact, the association must be decided on the basis of the relative alignment of a third point on the molecule; no less than a three-point recognition could draw this type of distinction. Thus, each reactive site presented at the interface, either inside or outside the cell, may be presumed to involve a systematic assembly of at least three contact points within a few Ångström units of each other, in a fairly rigid pattern.

The biological correlation, incidentally, constitutes an argument for the retention of the Reeves approach to classification of the pyranose conformations (99). The nomenclatural systems recently proposed by Guthrie (38) and by Isbell and Tipson (52) are based on the orientation of the *substituents* at carbon atom 1 in the α-anomer, rather than on the arrangement of the atoms of the *ring* itself. This leads to major deviations from the Reeves grouping of the conformations of different sugars, even to the use of the same designation for direct enantiomorphs. With such conformation-categories as these, there would of course be no systematic alignment of conformations with the transport affinities.

C. Transport of other polyhydric compounds

A curious anomaly in the general pattern of the specificity has been brought out by Bowyer and Widdas (10, 11) in connection with the penetration of these cells by *meso*-erythritol [*anti*-1,2,3,4-butanetetrol] and pentaerythritol [tetrakis-(hydroxymethyl)methane, or 2,2-bis(hydroxymethyl)-1,3-propanediol]. Unlike the larger polyols, which fail to penetrate at all, and unlike the smaller homologue, glycerol [glycerin], which appears to use an independent transport system (8, 72), these erythritols apparently enter the cells by means of the monosaccharide-transport apparatus. Among several species of red cells, the comparative erythritol-uptake rates were found to parallel the sorbose-uptake rates. Moreover, although the erythritols showed no competitive interaction with glycerol transfer, their penetration was inhibited in the presence of either glucose or poisons which inactivate the glucose-transport system. In contrast, 1,2,4-trihydroxybutane and dihydroxyacetone do *not* appear to use the hexose transfer system. Bowyer and Widdas (11) therefore suggested that there is a critical transition between the trihydric and the tetrahydric materials with respect to attachment at the special reactive sites.

D. Extension to other cells

So far as the present evidence extends in regard to the specificities of the other non-accumulative, facilitated-diffusion, monosaccharide transfer systems (3, 17, 41, 93), a similarly crude affinity pattern may prevail in cardiac and skeletal muscle, and ascites tumor cells. Moreover, in his comparative studies of various species of fetal erythrocytes, Widdas (126) brought out circumstantial evidence that the interspecific rate differences reflect only differences in the density or mobility of the reactive sites, rather than qualitative differences in the biochemical nature of the sites.

However, the truly *active* sugar transport systems, as in the kidney and gut, are decidedly more discriminatory in their specificities, and some of the bacterial

glycoside-"permeases" may even confine their transport action to glycosides of only one specific configuration (16, 113). Non-transport enzymatic reactions with monosaccharide substrates typically also show fairly high selectivity. These more stringent requirements for particular configurations may be considered to be superimposed on the basic framework of the C1 ring-shape requirement underlying all aldopyranose specificities, which in the human red cell transport system appears in its most primitive form.

The structural requirements in the much slower process in rabbit erythrocytes have been examined by chemical procedures by Hillman, Landau and Ashmore (42). A series of methyl- and deoxy-glucose derivatives, and certain appropriate hexose isomers, were used to test whether alteration at *individual* positions in the D-glucose molecule would modify the ability of the molecule to enter the cells (from an external level of about 20 mM). With regard to position 1, α-methyl-D-glucoside was found to penetrate nearly as readily as D-glucose (in contrast to the situation in the human red cell). Similarly, there were only trivial differences in the uptake rates of molecules differing at the second carbon atom: 2-methyl-D-glucose, 2-deoxy-D-glucose, D-mannose, and D-fructose; or at the sixth carbon: 6 methyl-D-glucose. At the third carbon the largest effect was seen, 3-deoxy-D-glucose entering at only about 60% of the rate of D-glucose; however, 3-methyl-D-glucose was barely distinguishable from D-glucose, as in the human red cell. D-Galactose, representing reversal of the —OH group position at carbon 4, entered at about 70% of the D-glucose rate. Position 5 was not tested. The relative indifference to changes at each of the other five points was taken as ruling out the notion of a three-point attachment, except possibly at the carbon 1-oxygen-carbon 5 segment of the pyranose ring. Had these been the only observations made, in fact, it might have been concluded that the special transfer system previously described in the human red cell had no counterpart in the rabbit erythrocyte, and the slow sugar penetration in the latter cell might have been attributed to simple diffusion. Interpretation in terms of the classical lipoid-solubility theory of cell permeability would also have been consonant with the finding (42) that each increase in the aliphatic chain length of 3-O-substituted D-glucose, in the series from methyl to butyl, resulted in distinctly accelerated entry. However, this simplicity was shattered by the observation that the most rapid penetration rate of all was that of 3-hydroxyethyl-D-glucose, a relatively hydrophilic derivative in the series. Moreover, the uptake of the 6-methyl, 3-methyl, and 2-deoxy-derivatives was inhibited (evidently competitively) when glucose was present. Park and his associates (93) have also observed saturation kinetics and competition phenomena in the rabbit red cell system, but in some details the behavior here was distinctly different from that of the human erythrocyte (94).

IV. INHIBITOR SPECIFICITY

The sensitivity of this system to inhibition by various chemical agents has been investigated extensively. Among the effective inhibitors are mercury bichloride (mercuric chloride), *p*-chloromercuribenzoate, and iodine (72); gold, chloropicrin

(trichlornitromethane), bromoacetophenone, allyl *iso*thiocyanate, and phloretin (131); phlorhizin (phloretin-2-β-D-glucoside) (71, 129), polyphloretin phosphate (134), 2,4-dinitrofluorobenzene (8), dinitrochlorobenzene, dinitrobromobenzene, and urethan (urethane, ethylcarbamate) (11, 12). But no appreciable change from the control rate was seen in the presence of Cu^{++}, Pb^{++}, arsenite, alloxan [2,3,5,6-(1,3)-pyrimidinetetrone], or oxophenarsine hydrochloride (Mapharsen, 2-amino-4-arsenosophenol hydrochloride) (72); fluoride (36, 63), iodoacetate (36, 72), azide, quinacrine hydrochloride [Atabrine, mepacrine hydrochloride, 3-chloro-7-methoxy-9-(1-methyl-4-diethylaminobutylamino)acridine dihydrochloride], thyroxine (3,5,3′,5′-tetraiodothyronine) (132), 2,4-dinitrophenol (8, 132), cyanide, epinephrine (adrenaline), pituitrin, hydrogen peroxide, or carbon dioxide (63); diazonium hydroxide of *p*-nitroaniline, or physostigmine (eserine) (8), or insulin (36, 97, 132). Most of the effective agents listed are known to react with sulfhydryl, amino, and other groups likely to be found at the cell surface, and the general pattern of inhibitor effectiveness has been taken to suggest particularly the involvement of sulfhydryl groups (11, 19, 72). However, this approach has not provided any very specific leads as to the identity of the chemical groups at the reactive sites.

Bowyer and Widdas (12) found that the progressive, irreversible inhibition of the system by 2,4-dinitrofluorobenzene developed more rapidly when glucose was present during the incubation of the cells with the inhibitor. Moreover, this effect of glucose showed a saturation relationship in respect to the glucose concentration, the half-maximal acceleration appearing at a level of about 5 mM, suggestively equivalent to the apparent glucose-carrier K in the transport system. Pursuing the possibility that this action of glucose might have its basis in the exposure of active groups at the cell surface by reason of hydrogen bonding (19) between glucose and the presumed reactive sites, Bowyer and Widdas tested guanidine (aminomethanamidine) and urethane (known hydrogen-bonding agents) in the same system, and found that they were nearly as effective as glucose in accelerating the inhibition.

A. Phlorhizin, phloretin, and derivatives

Special attention has been accorded the inhibition by phlorhizin and its congeners because of the classical association of this agent with the experimental induction of glycosuria in mammals by inhibition of sugar reabsorption in the renal tubules. The comparable action of phlorhizin in the red cell system has been taken as a strong argument (131) that similar processes underlie the transport phenomena in these different tissues. More recently, phlorhizin has been found to inhibit also the insulin-activated movement of galactose into rat skeletal muscle (61) and of glucose, xylose, or arabinose into or out of rat heart muscle (88, 89, 94). However, Wilbrandt's discovery (131) that in the red cell the aglucone of phlorhizin, phloretin, is many times more effective than the glucoside throws doubt on the relation of this process to that in the kidney, since the latter scarcely responds to phloretin at all (27, 28, 67); in fact, Lambrechts's comparative study (69) of many homologues in respect to their glucosuric potency

specifically emphasizes the evident necessity for the heteroside linkage. On the other hand, Kalckar (58) reported kidney phosphatase to be three or four times as sensitive to phloretin as to phlorhizin. The corresponding factor in the red cell transport system is at least 50; in fact, the question has even been raised (80) as to whether completely pure phlorhizin would show any activity at all in this system, since only a small degree of phlorhizin hydrolysis (to glucose plus phloretin) would be required to account for the apparent activity of the glucoside. Other processes which are also much more sensitive to the aglucone include phosphorylation in the course of glycogenolysis in rabbit muscle (18), growth and dehydrogenase activity in oat embryos and other higher plant tissues (86), and multiplication of many varieties of bacteria (83).

The inhibitory activities of phlorhizin have not been satisfactorily tied to any particular chemical reaction. Insofar as the drug's action concerns reactions of glucose or other sugars (*e.g.*, phosphorylations), the obvious suggestion has been that its glucose moiety fits into some receptor, displacing the normal sugar substrate, so that the machinery is blocked at that point by reason of the encumbrance of the phloretin moiety of the molecule. However, a number of energy-supplying enzymic reactions which are not directly concerned with sugar are also sensitive to phlorhizin (59, 82, 121), and there is also evidence that this agent may induce structural effects in membranes (60). Moreover, the special effectiveness of the aglucone in some systems, as discussed above, and the inactivity of other glucosides, suggest that the critical structure probably resides in the phloretin part of the phlorhizin molecule (18). The effort to identify this critical structure by pharmacological comparison of a variety of related molecules is the principal topic of the remainder of this review.

The two rings of phloretin are of course aromatic, and hence essentially planar; they cannot assume any of the chair-boat conformations discussed above in connection with the pyranose specificity pattern, so that a quite different factor must be involved in the inhibition of the same system by phloretin. The first obvious focus of attention in the molecule is in the phenolic hydroxyl groups. Thus, Rosenberg and Wilbrandt (108) examined a variety of phlorhizin derivatives in which these hydroxyl groups had been "blocked" by methylation. As a standard test system for comparing the potency of these compounds as inhibitors of the sugar transfer in human erythrocytes, they used the exodus of glucose from cells upon their transfer to solutions of a lower glucose concentration after preequilibration at about 150 mM. This study showed that substitution of a methyl (or glucosido-) group for any one of the four hydroxyl hydrogens in the phloretin molecule reduces the inhibitory potency by at least 95%. The hydroxyl at the simple phenolic end of the molecule appeared to be particularly significant: blocking by a methyl at this point alone reduced the inhibitory potency by about 99.9%.

Methylation at two or three points generally so reduced the activity that no meaningful estimate of the residual potency could be made. The exception to this was 2,4-dimethyl phloretin, which was perhaps even slightly more active than the corresponding monosubstituted derivatives 2-methyl phloretin, 4-β-D-

glucosido-phloretin, or 2-β-D-glucosido-phloretin (which is phlorhizin). This peculiar exception has not been interpreted; but Rosenberg and Wilbrandt suggested that the reason for the much greater depression of activity in all the derivatives having a 2,6-dimethylation might be the resultant impossibility of a ring closure (by hydrogen bonding or perhaps metal chelation) between the carbonyl oxygen and either of the substituted *o*-hydroxyls. There is a special basis for evoking this possibility in that, after such ring closure, the phloretin molecule may be presented in a suggestively steroid-like arrangement. The significance of this lies in the phlorhizin-like glycosuric activity which has been found for desoxycorticosterone glucoside. Pursuing this line of thought, Rosenberg and Wilbrandt found that both desoxycorticosterone [21-hydroxy-4-pregnene-3,20-dione] and its glucoside show approximately equivalent inhibitory action in the red cell glucose transport system; but the potency of these agents is very low, concentrations on the order of a few millimolar being required to match the activity of phloretin at micromolar levels. The observations of Pletscher *et al.* (97) suggest that the potency of cortisone (17-hydroxy-11-dehydrocorticosterone) is somewhat greater than that of the 11-desoxy compounds, but the test system employed in this study was not directly comparable with that of Rosenberg and Wilbrandt.

Wilbrandt (132) has emphasized the significance of the fact that the inhibition by highly polymerized phloretin phosphate is much more marked on the exit of glucose than on its entry. In view of the fact that this agent is unable to enter the cells, this asymmetry in the inhibition led Wilbrandt to conclude that different enzymes were involved in the inward and outward transports, and that the stage at which the phlorhizin group acted was at the disengagement of the complex rather than at its formation. Elsewhere (133), Wilbrandt *et al.* have also brought out certain kinetic evidence of asymmetry in the system. However, Bowyer and Widdas (12) showed that these observations are entirely in accord with the assumption of a completely symmetrical non-enzymatic process, if adequate account is taken of the reactions at both interfaces; a complete kinetic analysis showed that the greater degree of glucose exit inhibition was quantitatively such as would be predicted in such a system for a non-penetrating competitive inhibitor.

B. Other diphenolic agents

A priori, the length of the phloretin molecule raises the likelihood that the inhibitory activity might persist in the absence of one end or the other. But LeFevre (74) found that even rather large fragments from either end of the molecule (*e.g.*, phloretic acid or *p*-alkyl phenols resembling the simple phenolic end, or phlorpropiophenone or phloroglucinaldehyde from the other end) are relatively inactive. Moreover, when a combination of overlapping moieties (phloretic acid and phlorpropiophenone) was used, only a direct additivity of the separate inhibitory effects was observed, with no detectable synergism. The much higher activity of the intact molecule thus focused attention on the orientation and spacing of the terminal groups. Simple α,ω-dihydroxyl derivatives of hydrocarbons of similar length (nonamethylene glycol or decamethylene glycol) were

almost totally ineffective. But several diphenolic agents of physiological interest were found to exert a rather strong inhibition of glucose transfer. The most effective compound of this type previously described (74) is the synthetic estrogen, diethylstilbestrol [stilbestrol, α,α'-diethylstilbenediol, or *trans*-3,4-di(*p*-hydroxyphenyl)-3-hexene]; this was reported to be about 50% more active than phloretin at physiological pH. The saturated homologue of stilbestrol, hexestrol [4,4'-(1,2-diethylethylene)diphenol, or *meso*-3,4-di(*p*-hydroxyphenyl)hexane], proved to be somewhat less potent than phloretin; while the closely related estrogen, dienestrol [4,4'-(diethylideneëthylene)diphenol, or 3,4-di(*p*-hydroxyphenyl)-2,4-hexadiene], was said to be decidedly less active, as was also naringenin (4',5,7-trihydroxyflavanone), which is essentially a di(dehydro)phloretin which has condensed into a flavanone. On the basis of these comparisons, LeFevre suggested a tentative interpretation in terms of a critical spacing of the phenolic hydroxyls at opposite ends of the molecule as a requisite for development of maximal potency. However, the further studies presented below necessitate abandonment of this hypothesis; appreciable activities have now been found among agents in which the two *p*-phenols are separated by only one carbon atom. Moreover, the original argument is undermined by the finding that the relative ineffectiveness of dienestrol was attributable to a solubility difficulty. This, and certain other complicating developments in technique and analysis, must now be noted in order to proceed with exposition of the newer structure-activity studies with the diphenolic compounds.

1. New technical and formal considerations. Nearly all the diphenolic compounds studied are so insoluble in water that it is not feasible to dissolve them directly in the medium used for the cell suspensions. Since all are ethanol-soluble, and since ethanol itself, even at 4 to 5% (v/v) does not appear to affect the sugar transfer or its response to the other materials, the stock solutions and all dilutions of the diphenolic compounds were prepared in ethanol. In the standard procedure adopted for the newer experiments (75), one part of such an ethanolic solution was mixed with 64 parts of medium in the immediate preparation of each experimental run, as outlined below. Many of the diphenolic compounds showed a considerably higher potency by this procedure than was evident after the agent had stood for several hours in the aqueous medium, allowing gradual aggregation and partial removal from solution. However, of the previously reported potency ratings (74), only that of dienestrol required major revision by reason of this factor. Of greater significance in amending the earlier reports is the following consideration.

In their comparison of the methylated phloretin derivatives, Rosenberg and Wilbrandt (108) expressed the inhibitory potencies on a negative logarithmic scale as a "pA"; the end-point defining the concentration A was a 10% reduction in the rate of sugar transfer. Similarly, LeFevre (74) used a linear potency scale of inverse millimolarity, the end-point defining the critical concentration here being 50% inhibition. In fixing on such single levels of inhibition for comparison of the drugs, these scales imply that all the agents show the same form in the relation between the concentration applied and the degree of inhibition resulting.

Such an assumption is very reasonable in view of the close chemical and physical similarity of the agents concerned, but the new observations (75) show that the actual situation is not so simple as this. Previously, specific consideration of the concentration dependency has been given only in the case of phloretin (73). Inhibition kinetics with this agent suggested a direct competition with the sugars for combination at the same reactive sites on the cell surface. It now appears that phloretin is quite unusual in following this rather simple pattern.

If an inhibitor simply competitively displaces the sugar from its association at the reactive sites at the outer interface of the cell surface (73), its presence at concentration $[I]$ should reduce the fraction of sites occupied by the sugar by the factor

$$\frac{1 + [P]/K}{1 + [P]/K + [I]/K_I},$$

where K_I is the inhibitor-carrier-complex dissociation constant. If only this simple consideration is taken into account, a straight-line relationship is expected in a plot of the reciprocal of the rate of transfer (in some fixed experimental situation) against the inhibitor concentration. Somewhat more complex relations are anticipated (12) in accordance with whether the inhibitor itself is transported (or otherwise enters the cells), and whether a mobile-carrier or a polar-pore "creep" type of transport (11, 122) is assumed. Phloretin inhibition, however, has been found to adhere closely to the simple relationship above, fitting the described linearity and also following the indicated pattern of dependence on the sugar concentration (73). But nearly all the other diphenolic materials that have been examined in this regard deviate systematically from this pattern (75): higher concentrations depress the transfer more profoundly than would be expected by extrapolation from the effects of lower concentrations, *i.e.*, graphic plots of inverse rates against $[I]$, as suggested above, are not rectilinear, but curve decidedly upwards.

This complication can be satisfactorily systematized, however, by recourse to the suggestion of Bowyer and Widdas (11) that the inhibitors may act not by direct displacement of the sugar from the reactive sites, but by attaching to the cell surface at an adjacent point where a steric interference with sugar adsorption may result. (The demonstration by LeFevre and Marshall (80) that overwhelming doses of glucose do not appreciably dislodge phloretin from attachment to the red cell is in keeping with this possibility.) Under such circumstances, there may be other than a 1:1 or other simple integral ratio between the number of molecules of inhibitor acting and the number of glucose sites accordingly inactivated. If m is the average number of inhibitor molecules involved in blocking one reactive site, extension of the relation given above for the univalent situation would suggest the approximation:

$$\frac{\text{Control transfer rate } (R_0)}{\text{Inhibited transfer rate } (R_I)} = 1 + \frac{[I]^m/K_I}{1 + [P]/K}.$$

Thus, in a given test situation in which only $[I]$ is varied, rectilinearity would be

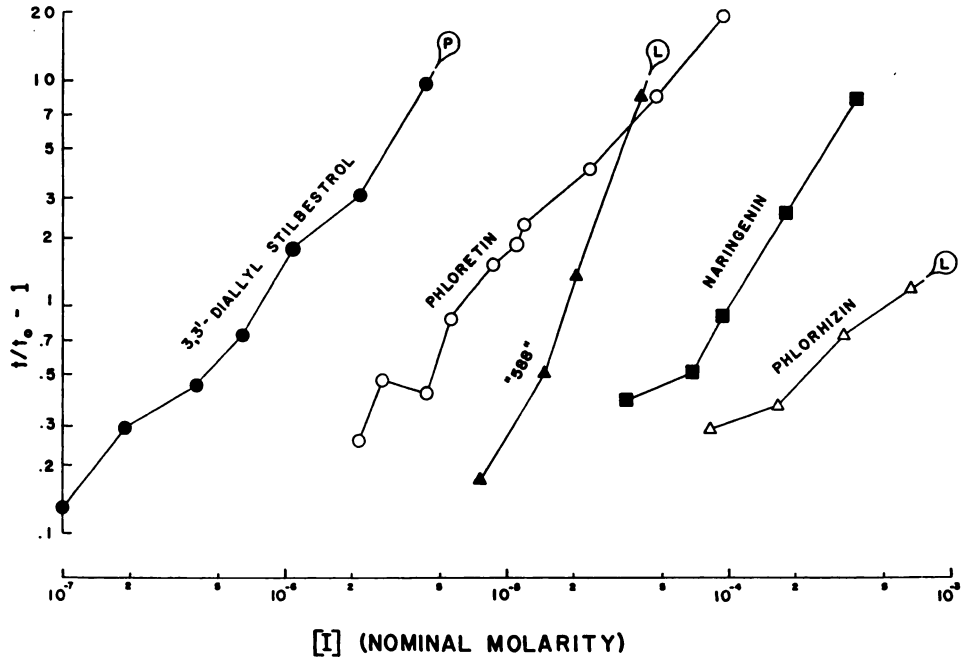

FIG. 3. Inhibition by diphenolic agents as a function of inhibitor concentration.

Contrast of several representative compounds of differing characteristics. Log ($t/t_0 - 1$) is plotted against log of inhibitor concentration, as developed in text. Symbols terminating upper ends of curves mark approximate concentration at which each agent began (L) to lyse the cells, or (P) to precipitate from the aqueous medium.

expected in a log-log plot of the function ($R_0/R_I - 1$) against [I], and the slope of this line should be m. As illustrated in Figures 3 and 4, the experimental data for a wide variety of the diphenolic compounds do fall reasonably close to such straight-line relationships when plotted in this way. The slope m generally lies decidedly above unity, but is only rarely as large as 2; accordingly, by the simple interpretation above, the implication is that an average of typically about 1.5 molecules of inhibitor take part in preventing access of glucose to a single transport site. It must be recognized, however, that this unsophisticated interpretation derives from the above simplifying assumptions which have not been given independent experimental support. More general equations, such as could be derived from those of Bowyer and Widdas (12), would perhaps provide other possible interpretations of the value, m; specifically, difference in the distribution of inhibitor through the thickness of the membrane and in the cell interior might be indicated. But in the absence of independent information on such factors, the wide choice of possible relations that is presented does not allow a meaningful analysis in such terms.

A higher order reaction with the membrane sites has also been reported for 2,4-dinitrofluorobenzene: Bowyer and Widdas (11) found that the time-course of the development of the irreversible inhibition during incubation with this

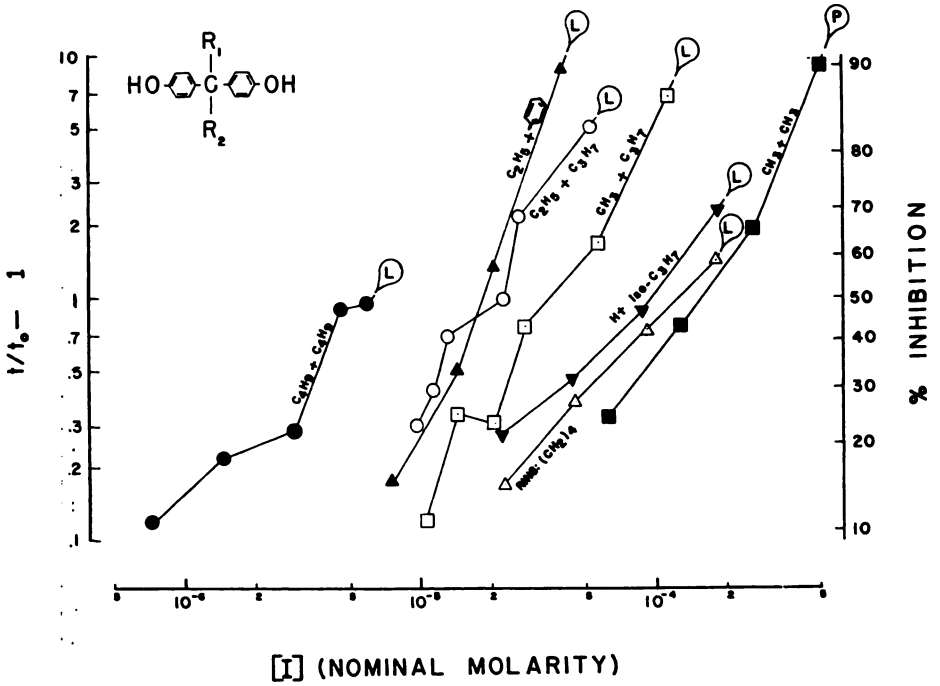

FIG. 4. Inhibition by a series of diphenolic agents having a common skeleton structure.

Plotted as in Figure 3. Additional ordinate scale at right shows percentile inhibition ($100\text{-}100\ t_0/t$) corresponding to the log (t/t_0 - 1) scale at left. Labels on curves denote identity of R_1 and R_2 in the skeleton formula above.

agent, and its dependence on the inhibitor concentration, are such as would be expected if the reaction were second-order not only with respect to the inhibitor, but with respect to the membrane sites as well. Such a fourth-order reaction was deemed unlikely in terms of a simple mass action, and here again the complicating factor of distribution of the reactants about a membrane surface was stressed.

Whatever its physical meaning, some such factor as m needs to be specified, *in addition to* the potency rating expressing some critical concentration, in order to provide an adequate comparison of different inhibitors. This is illustrated in Figure 3, particularly in the comparison of the behavior of phloretin with that of the compound designated as "588," which may be described as 4,4′-(ethyl-phenyl-methylene)diphenol, or 1,1-di(*p*-hydroxyphenyl)-1-phenyl-propane. (This compound, and many of the homologues, were obtained through the courtesy of Prof. Sir Charles Dodds and Mr. Wilfrid Lawson of the Courtauld Institute of Biochemistry, Middlesex Hospital, London.) The m for "588" is about 2.4, while that for phloretin, as noted above, is exceptional in not deviating measurably from 1.0. Thus, although almost three times as high a concentration of "588" is required to effect 50% inhibition as with phloretin, the concentrations needed are identical at about the 87% inhibition level; and at higher concentrations, phloretin is the *weaker* of the two. Therefore, in the comparisons of these agents

TABLE 1

Activity of diphenolic inhibitors of glucose transport in human erythrocyte

Compound			m*	Potency*
Phlorhizin			0.7	1.9
Phloretin			1.0	160
Naringenin			1.4	10
X = Di(p-hydroxyphenyl)		*Dodds-Lawson #*		
2,2-X-propane	(the series of Fig. 4)		1.6	7
1,1-X-2-methylpropane		565	1.0	10
1,1-X-cyclopentane		585	1.0	8
2,2-X-pentane		45	1.7	27
3,3-X-hexane		652	1.7	50
1,1-X-1-phenylpropane		588	2.4	55
5,5-X-nonane		684	1.1	170
X (the plain *bis*-phenol)			1.1	6
1,2-X-ethane		76	1.3	90
3,4-X-hexane-3,4-diol(hexestroldiol)		275	1.2	3
[3,4-Di(*o*-hydroxyphenyl)-hexane-3,4-diol, (*β*-form, M. P. 162°C)]		572	1.4	10
1,5-X-1,4-pentadiene-3-one		115	1.3	110
1,6-X-hexane		459	1.3	160
X = Di(p-hydroxy, m-methylphenyl)		*Dodds-Lawson #*		
1,1-X-cyclopentane		598	1.5	37
3,3-X-pentane		589	1.5	70
2,2-X-pentane		667	1.8	95
3,3-X-hexane		664	1.9	140
1,1-X-hexane		60A	1.6	300
2,2-X-4-methylpentane		643	1.3	160
4,4-X-heptane		650	1.4	230
2,2-X-heptane		687	1.4	200
4,4-X-octane		692	1.3	380
5,5-X-nonane		685	1.9	500
3,3-Di(*p*-hydroxy,*m*-allylphenyl)-4-hexanone			1.3	250
Stilbestrol			1.4	160
Hexestrol			1.6	100
Dienestrol			1.7	100
3,3′-Diallyl stilbestrol			1.1	1300
3,3′-Di(2-methylallyl) stilbestrol			1.8	950
3,3′-Di(2-chloroallyl) stilbestrol			1.4	1600
3,3′-Diallyl hexestrol			1.3	750
3,3′-Dipropyl hexestrol			1.6	750
3,3′-Di(2-methylallyl) hexestrol			1.3	800
3,3′-Di(2-chloroallyl) hexestrol			1.4	1000
3,3′-Diallyl dienestrol			1.6	750

* Expressed as explained in text.

in Table 1 (p. 60), estimates are given of both the standard "potency" (the reciprocal of the millimolarity at the concentration effecting 50% inhibition), and m, provisionally interpreted as the average number of inhibitor molecules required to block attachment of a glucose molecule at a carrier site, under the standard test conditions.

The standard test adopted was as follows: washed human red blood cells suspended at about 4% (v/v) were first equilibrated for about 1 hour at 37°C in the buffered balanced-salt medium (78) containing glucose at 150 mM. Then, to 11.8 ml of the plain medium held at 37°C in the optical cuvette, 0.20 ml of ethanol was added, with or without drug, and rapid stirring begun. About 1 minute later, 1.00 ml of the cell suspension was added abruptly from a tuberculin syringe. Thus, in the final experimental mixtures, the cells made up about $\frac{1}{300}$ of the suspension volume, the alcohol level was about 1.5% (v/v), and the glucose level about 12 mM. Recording was begun at the instant of addition of the cells, and continued until final equilibration, tracing the net exodus of glucose from the preloaded cells into the medium until the cell level had also fallen to about 12 mM. Since typically the rate of sugar exit is quite steady over the major part of the recorded excursion, it is convenient (as recommended by Dr. W. F. Widdas) to extrapolate this rectilinear part of the record to the final equilibrium level, and thus to estimate a time-period (which is virtually equivalent to twice the half-time of equilibration). This time, t, serves for comparison of a series of records at varying $[I]$, and is used in the ordinates of the graphs in Figures 3 and 4, as the function, $\log (t/t_0 - 1)$, where t_0 is the t in the uninhibited control run.

Compounds of the type with which we are dealing here are recognized as potential hemolytic agents (123); generally, the upper limit of concentration feasible in testing the diphenolic materials was set either by the appearance of prelytic disturbances, or by visible precipitation of the drug upon dilution of the ethanolic stock solution into the aqueous medium, as noted in the examples in Figures 3 and 4. Occasionally such limitations made it impossible to secure records of inhibition beyond about 50%, but typically it was feasible to extend measurements to about the 90% inhibition level; for a few especially innocuous and relatively soluble materials, satisfactory records could be obtained at such high concentrations that the limit was set only by the impractically protracted time required for complete equilibration. The limit for meaningful distinction from the control at the lower end of the scale was taken to be an inhibition of approximately 10%. In the application of these procedures, the values of m estimated from the graphs are considered to be uncertain by about 10%; and the "potencies," by about 20%.

2. New structure-activity correlations. In the fairly extensive series of agents examined, only phlorhizin behaved as though a single molecule were capable of inactivating more than one transport site, in that m was less than unity (about 0.7) (Fig. 3). This finding is in keeping with the fact that phlorhizin is the only glycoside in the group, and thus might affix at membrane sites by way of both the sugar and the diphenolic moieties. Only about 60% inhibition at most could be demonstrated with phlorhizin, since (as shown in Fig. 3) its potency was only

about 2, and at about 1 mM it began to induce some lysis; however, having such a low m, it was proportionately more effective at lower concentrations than its potency rating might suggest. (The distinctive m of phlorhizin, incidentally, argues for the reality of its weak inhibitory action as the glycoside, rather than for its acting by way of a small contamination with phloretin.)

In a comparison within any set of the diphenolic agents, all sharing the same skeleton structure in regard to the ring substituents and the carbon chain connecting the two phenols, there is seen a rather consistent increase in potency with increased size of the aliphatic side-groups, so far as the available series extend (75). An illustration of this type of comparison is given in Figure 4, which concerns molecules of the di(p-hydroxyphenyl)-R_1-R_2-methane type. Available in this series were five alkyl derivatives (extending from dimethyl to dibutyl), the ethyl, phenyl-derivative, and also one in which R_1 and R_2 combine in a cyclopentane ring with the central carbon atom. Figure 4 shows the fairly systematic increase of potency with the size of these substituents, over a range of about 20-fold. However, in respect to the slopes, m, the differences among these compounds do not follow any clear pattern. The outstandingly high m of 2.4 was found for the only member of the set having an aromatic side-group (the "588" of Fig. 3), but since no other similarly endowed diphenolic compound was available, the generality of this is uncertain. The remaining six compounds in Figure 4 fall into two classes of three, one showing m near unity, the other in the range of 1.6 to 1.7. No structural correlation with these two classes is obvious with this limited set of homologues; however, there may be some significance to the fact that, like phlorhizin, the three compounds with the lower m in Figure 4 began to hemolyze the cells at concentrations producing only 50 to 60% inhibition in the test system, while those with the higher m did not do so until the concentration was raised to very highly inhibitory levels.

An array similar to that of Figure 4 is also given by a fairly large group of compounds built on the same skeleton, but with the addition of methyl groups *ortho* to the phenolic hydroxyls; the data for these compounds are summarized in the third section of Table 1 (75). The variations in m among these di-*o*-cresols are less extreme than in the plain diphenolic series, but again no orderliness is apparent. However, the pattern of increasing potency with increasing size of side-groups is again clear. The most noteworthy point here is that these 3,3′-dimethylated compounds are decidedly more active than their plain diphenolic counterparts; comparison of directly homologous pairs in the two series shows a 3- to 5-fold increase in potency rating with the 3,3′-dimethyl addition.

An even greater increase of activity over that of the parent compound has been found (75) with somewhat larger 3,3′-substitutions on the rings of the synthetic estrogens, *i.e.*, stilbestrol, hexestrol, and dienestrol. These materials were obtained through the courtesy of Dr. Emil Kaiser and Dr. H. D. Lennon of the Armour Pharmaceutical Co.; preparation of such derivatives by Claisen rearrangement of the corresponding ethers has been described by Kaiser and Svarz (57). These compounds proved to be by far the most potent inhibitors of the sugar transfer system thus far discovered, some effecting 50% inhibition in the test system at

concentrations of 1 μM or less (Fig. 3, and last section of Table 1). The substituents propyl, allyl, or 2-methyl-allyl in the 3,3′-positions are approximately equivalent, generally resulting in an 8-fold increase in potency; while the 2-chloroallyl substitution appears to exert a still larger effect. Throughout, the stilbestrol skeleton seems consistently to carry a distinctly higher potency than the less rigid hexestrol or dienestrol skeleton. (Several derivatives of these compounds, in which the phenolic hydroxyls were blocked by ether or ester formation, were uniformly ineffective, but these materials were so insoluble that interpretation of the inactivity is questionable.)

Limitation in the number of congeners available makes it difficult to evaluate the possible significance in the length of the molecular backbone; *i.e.*, of the chain connecting the two phenols. But the few comparisons provided in Table 1 suggest that in this respect again, increasing the size of the molecule results in increased inhibitory potency. For the three compounds of the simple type (*p*-hydroxyphenyl)-$(CH_2)_n$-(*p*-hydroxyphenyl), having no side-groups on the central backbone, the potencies are about 6, 90, and 160, where n = 0, 2, and 6, respectively. For all three of these compounds, *m* proved to be only slightly higher than unity; this may be more than coincidental, since low values of *m* were also given by each of the other agents in which there are no side-groups other than carbonyl oxygen: *viz.*, phloretin, phlorhizin, and the Dodds-Lawson "115" [1,5-di(*p*-hydroxyphenyl)-1,4-pentadiene-3-one]. This is reasonable in the context of the suggested meaning of *m*, since the absence of large side-groups would present less opportunity for mutual potentiation between inhibitor molecules fixed at neighboring points on the membrane.

3. Possible association with estrogenicity. The high inhibitory potency in the sugar-transfer system of some of the well-known synthetic estrogens (21, 22, 23), and the estrogenicity of phloretin itself (24) and some of the more obscure diphenolic agents studied here (14), raise the question of the possible involvement of sugar transfer processes in estrogenic action. In this connection, note may be taken of the fact that definite alterations in blood sugar levels and urinary sugar losses have been observed in animals on various stilbestrol or hexestrol regimes (45–50, 53–56, 105, 118); sugar absorption from the gut may also be affected (35). However, the pattern of these changes is completely altered by differing experimental details, and no simple inhibition by the estrogens of membrane transfer of sugars, in the renal tubule or elsewhere, is adequate to clarify the reported relations. Moreover, the structure-activity studies with respect to estrogenicity and to inhibition of the red cell sugar transfer show no consistent parallelism; *e.g.*, Kaiser's 3,3′-disubstituted stilbestrols and hexestrols, outstandingly active in the red cell system, are much weaker estrogens than the parent compounds (57). Similarly poor correlation with estrogenicity, however, has been noted in connection with the influence of compounds of the stilbestrol-hexestrol-dienestrol type on a variety of enzymatic processes, such as inhibition of succinoxidase and of several dehydrogenases in homogenates of a variety of rat tissues (15, 25, 30, 84, 85) and in yeast (43, 44), and a complex of stimulating and depressant actions on yeast respiration and fermentation

(116, 119, 120). Moreover, in certain of these studies, it was shown that the phenolic groups were not consumed in the process, or that the full original estrogenicity could be recovered from the mixtures at the end of the experiments.

C. Combination of drugs with cells

Since the inhibitory studies implied a rather tight binding of the more active drugs, a direct study of such binding was undertaken by LeFevre and Marshall (80), in the hope of gaining some information regarding the nature of the membrane sites presumably involved. By spectrophotometric analyses of the supernatant media from centrifuged human red cell suspensions, they were able to demonstrate a very rapid, reversible attachment of the several agents studied, except for phlorhizin. The cells, even at only a few per cent (v/v), removed from the medium substantial fractions of the strongest inhibitors known at that time (phloretin, stilbestrol, hexestrol), so that the calculated distribution ratios (cells:medium) were as high as 50 or more. The surprising aspect of this was that these distribution ratios appeared to be essentially independent of the drug concentration in the medium, instead of falling systematically as the concentration was raised. In fact, no evidence of approaching saturation could be demonstrated even at the highest concentrations attainable, although the inhibitory kinetics would imply saturation of the sugar transport sites at much lower levels. It was therefore concluded that the latter specific sites must represent only a small fraction of the potential sites for attachment of the inhibitors, and that the fixation of the drugs at the transport sites is much tighter than at the more numerous non-specific loci. In spite of this apparent basic distinction, both types of sites showed a similar pattern of specificity with respect to the several agents examined; that is, the relative degrees of fixation to the cells paralleled the inhibitors' potency ratings. This implies that the wide range of "potencies" is basically a reflection of the differences among the drugs in the degree to which they become fixed to the cells. Thus, at a given cellular load, all the compounds effect roughly the same degree of transport inhibition. For instance, in the standard test system, 50% inhibition corresponds very roughly to a fixation of 1 millimole of inhibitor per liter of cells, or on the order of fifty million molecules per cell. If these were attached at single sites regularly distributed over the cell surface, the distance between adjacent occupied points would be about 16 Å; such a spacing makes it geometrically reasonable to suppose that the 50% reduction in transport rate might indeed have its basis in a non-specific obstruction of access to sugars in the medium of half of the transport sites.

The same parallelism between the gross attachment and the transport depression was evident in the response to alteration of the hydrogen-ion concentration of the medium. For hexestrol and stilbestrol, change in pH throughout the range tolerated by the erythrocytes did not significantly modify either the attachment or the inhibition. But the other compounds examined in this respect (phloretin, naringenin, phlorpropiophenone, and phloroglucinaldehyde) were dislodged from the cells, and their inhibitory action on the transport correspondingly reduced, by increasing pH; while a shift of one pH unit toward the acid side typically doubled or tripled the activity, by both indices. The pattern of this pH-

dependence was especially suggestive, in that these compounds (but not hexestrol and stilbestrol) all display a keto-enol tautomerism, the equilibrium of which is shifted by pH in this same range (66, 80). The direction of this correlation is such as to suggest that only the ketonic form of these compounds is in direct equilibrium with the cell-fixed material, the enolic form being unable as such to attach to the cells and thus inhibit the sugar transfer. On the other hand, it is plain that the atomic grouping involved in this tautomerism does not in itself provide the essentials for the attachment process, since the rather weak inhibitor, phlorhizin, behaves tautomerically indistinguishably from its aglucone, phloretin (64), but could not be shown to be fixed to the cells at any tolerable pH (80). Lambrechts had previously also concluded that in dog blood, phlorhizin remained completely in the plasma compartment, both *in vivo* (68) and *in vitro* (65).

Other modifications of the medium did not affect the cellular binding of these inhibitors; omission or excess of Ca^{++}, Mg^{++}, or K^{+}, or addition of ethylenediaminetetraacetate [Versene, (ethylenedinitrilo)tetraäcetic acid tetrasodium salt], or replacement of all electrolyte (other than the buffer) by sucrose, or change of temperature over the range 2 to 27°C, led to no detectable change in the partition of the drugs. Furthermore, glucose did not displace phloretin measurably, even at levels so excessive that extrapolation from the kinetic studies would imply essentially complete availability of the transfer sites to the sugar.

These studies (80) did not show whether the cells were actually penetrated by the drugs. The high speed and reversibility suggested a mainly superficial fixation, but the very large quantities which could be attached at high levels of the more soluble and active compounds (such as phloretin) would mean essentially complete coating of the cells with a layer of closely packed molecules.

With such high cell:medium concentration ratios as shown by the most potent inhibitors studied in this respect, it becomes important, in analyzing experiments *in vitro*, to consider whether the amount of fixed drug constitutes a significant fraction of the total quantity present. If such is the case, the effective dose becomes dependent on the relative amounts of tissue used in different runs or samples, and it is insufficient to specify the dose simply in terms of the nominal concentration set up in the medium. Thus, Reilly's finding (103) that a given concentration of stilbestrol produced a more profound depression of dehydrogenase activity in cat heart homogenates, the less tissue used per flask, is almost certainly attributable to this consideration. In the Ørskov densitometric procedure used in the inhibition studies above, however, the extreme dilution of the cell suspensions makes this factor negligible except for the most extremely potent agents at the bottom of Table 1. Direct study of fixation of these agents to the cells has not been reported, but extrapolation from the studies with the somewhat weaker inhibitors in the series (80) would indicate that the nominal potencies for the strongest inhibitors are probably *underestimated* by a factor of three or four.

V. CONCLUDING REMARKS

At this point in virtually any review concerned with biological research, one may expect to encounter the statement that "much more work needs to be done"

before any substantial conclusions can be reached. Though reluctant to contribute to the perpetuation of this hackneyed theme, the author finds little alternative in consideration of the status of the foregoing experimental information. There is perhaps some promise here of the possibility that the type of structure-activity correlation discussed in section III might be extrapolated into other facets of carbohydrate metabolism, and, in fact, to a wide range of problems concerning biochemical and pharmacological specificity. To date, molecular morphological factors of this class have been given only very limited consideration in relation to biological processes; but the fairly extensive systematization which is provided in connection with the present specific system suggests that scrutiny from this point of view might prove rewarding in the study of other systems which are characterized by refined steric discriminations.

Less can be said for the prospects of meaningful correlation in connection with the inhibitor structures discussed in section IV. The progressions in activity among the members of chemically homologous series here are such as to suggest future attention be directed less to the specific chemical groupings, and more toward the comparative physical properties of the molecular assemblies; examination of the differences in the details of electronic organization would appear particularly desirable. A secondary issue brought out by these investigations is the role of differences in local distribution (as opposed to inherent potency differences) in determining the comparative efficacy among a series of compounds. Such factors may well contribute to many pharmacological comparisons in which distribution differences have not been taken into account.

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