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Reevaluation of Use of Retardation Chromatography to Demonstrate Selective Monosaccharide "Binding" by Erythrocyte Membranes

PAUL G. LEFEVRE and STANLEY J. MASIAK

Department of Physiology and Biophysics, Health Sciences Center, State University of New York at Stony Brook, Stony Brook, New York 11790*

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Summary. Retardation chromatography has been reported to show stereoselective binding of the preferred sugar-transport substrate, D-glucose, by human red cell "ghosts" or by certain protein extracts of such membranes. However, no detectable differential elution of glucose (as compared with the poorly transported analogue, L-sorbose) was observed with columns of diethylaminoethanol-cellulose impregnated with ghost proteins prepared by either of the recommended procedures (using Triton X-100, or NaI followed by extensive dialysis). Celite columns bearing intact ghosts did show marked initial relative delay in glucose emergence, but this persisted only partially through the elution peak, to be followed by a nearly equivalent and much more protracted retardation of the sorbose. Moreover, this differentiation required that the columns be handled sufficiently gently as to leave intact whole-cell membranes or fairly gross vesicles; freeze-thaw treatment or abrasive stirring abolished both the early glucose retardation and the subsequent sorbose retention. Although conflicting seriously with the simple selective-binding interpretation, these phenomena accord with the suggestion that the sugar-complexing entities on the columns continue to operate as "carriers" mediating sugar access into membrane-enclosed compartments. Effects of experimental manipulation of elution rates and of applied sugar levels lend further support to this interpretation. The capacity of the vesicular spaces so vastly exceeds that of the binding itself that resolution of the latter cannot be achieved through this approach.

Extensive kinetic studies (reviewed by Bowyer, 1957, and Miller, 1969) have led to the postulation of a facilitated-diffusion system as the basis for the translocation of simple sugars between the interior and exterior of human erythrocytes. For some years, it has been generally agreed that further significant understanding of the mechanisms involved in the operation of this system must await the isolation, or at least partial chemical identification, of the stereoselective membrane component. Phospholipids

* Initial studies were carried out at Department of Pharmacology, University of Louisville School of Medicine, Louisville, Ky.

from human red cell ghosts have shown a distinctly carrier-like capacity *in vitro*, greatly enhancing the transfer of glucose and other monosaccharides into a chloroform layer (Jung, Chaney & LeFevre, 1968) and thence into a second aqueous compartment (LeFevre, Jung & Chaney, 1968). However, the lack of appropriate selectivity among the several sugars and the absence of saturation kinetics at reasonable substrate levels rendered this system quite inadequate as a model for the sugar-carrier apparatus of the intact cells. A more promising selectivity has been offered in several reports from the University of Manchester (Bobinski & Stein, 1966; Bonsall & Hunt, 1966; Levine & Stein, 1967), indicating a preferential binding of D-glucose (as compared with the poorly transported analogues, L-sorbose or L-glucose) by human red cell membranes or by protein extracts from such "ghosts", as revealed by the technique of retardation chromatography.

We now find, however, that a number of the characteristics of this selective retardation phenomenon, observed on inert columns impregnated with relatively crude (pink) ghosts, are quite incompatible with the simple binding interpretation, and can best be explained by the presumption that the sugar-transport system continues to operate on the columns, so that the D-glucose passes rather readily into (and out of) a compartment not readily accessible to those analogues with much lower affinity for the transport sites. Moreover, in our hands, more adequately "cleaned" membranes (white ghosts) retain very little of this behavior; and neither the Triton X-100 ghost extracts (Bonsall & Hunt, 1966) nor the dialyzed NaI extracts (Bobinski & Stein, 1966; Levine & Stein, 1967) have exhibited any definite capacity at all to retard D-glucose passage through diethylaminoethanol (DEAE)-cellulose columns, in spite of a considered effort to follow precisely the Manchester recipes and procedures.

A preliminary report of portions of this work was presented to the Biophysical Society (LeFevre & Masiak, 1968).

Methods

Human erythrocytes were taken from out-dated blood in standard acid-citrate-dextrose bags obtained from either the American Red Cross or the Nassau-Suffolk (N.Y.) Inter-County Blood Bank. When NaI extracts were to be used, the ghost preparation and extracts followed the methods given by Levine and Stein (1967); these extracts were dialyzed for 36 to 48 hr against distilled water, and lyophilized. The dried material was resuspended in 2.5 or 3 mM sodium phosphate buffer, at pH 7.0, for mixing with the DEAE-cellulose (Eastman Organic Chemical Co., or Machery, Nagel and Co.). For extracts with Triton X-100 (Rohm & Haas Co.), procedures for both ghost preparation and extraction were taken from Bonsall and Hunt (1966), and DEAE-cellulose mixtures thereof similarly prepared in dilute neutral phosphate buffers.

For the unextracted ghost columns, the support matrix was in almost all instances Celite "L-665-A" (Johns-Manville Co.) which had been treated to remove the finest particles (in order to avoid clogging which would have severely reduced elution rates). Generally, 15 g of Celite was boiled briefly in 200 ml distilled water and poured into an equal volume of 5 mM neutral phosphate buffer in a 500-ml cylinder; this mixture was allowed to settle undisturbed for 50 min, whereupon the supernatant was discarded and the heavier residues resuspended for use as the column matrix. This procedure essentially eliminated the necessity for repeated mechanical agitation during the packing of the ghost columns (a factor which will be seen to bear importantly on the behavior under study). In most instances, similar pretreatment was also given to the DEAE-cellulose used for the protein extract-laden columns, although clogging of the columns was not a major problem with this material. The tops and bottoms of all columns were capped with a 2 to 3-mm layer of untreated L-665-A Celite.

Except as noted otherwise, the ghost columns represented 10 to 15 ml of the packed Levine-Stein ghosts; gently mixed Celite-ghost suspensions were poured into suitably prepared glass tubing of ca. 9 mm I. D., packed under 5 to 10 psi of N_2 pressure to a height of 250 to 300 mm, and the columns thoroughly washed through with dilute neutral sodium phosphate medium. The test bolus was 1.5 or 2.0 ml of a mixture of ^{3}H-D-glucose (Nuclear-Chicago, or Amersham/Searle Corp.) and ^{14}C-L-sorbose (Nuclear-Chicago, or Calbiochem), each at 2 mM except as noted otherwise, applied to the column and eluted with the same neutral phosphate medium. Serial elution samples of three or four drops (typically ca. 30 μliters/drop) were collected automatically on a Fractomat (Buchler Instruments, Inc.), and the recoveries were determined by dual-channel counting in either a Nuclear-Chicago or a Picker Nuclear (Liquimat 330) liquid scintillation spectrometer. The scintillation mixture was 4 g 2,5-diphenyl-oxazole plus 60 g naphthalene per liter of 9:1 dioxane:methanol. Data were analyzed by suitable programming of the DAC-512 computer attachment of the Picker Liquimat 330 instrument.

The radiochemical purity of the ^{3}H-glucose and ^{14}C-sorbose was assayed chromatographically on Whatman 3MM paper, with n-butanol:ethanol:water (52:33:15) as developer. The sugars were detected by the $AgNO_3$ method (Partridge, 1946), and small sections of the chromatograms were counted to ascertain the localization of the radioactivity in the region of the sugar spot. Localization was quite satisfactory except for the one occasion to be cited in Results.

Results

The pattern of elution regularly observed with Celite-supported columns of ghosts of the pink Levine-Stein type is shown in Fig. 1. Plotted in the upper panel is the percentage of the total-bolus sugar load recovered in each four-drop cut; the characteristic differences between the glucose and sorbose elutions are emphasized by the hatching between the two sets of points. The precedent emergence of the sorbose, as described originally by Bobinski and Stein (1966), is quite evident during the rising phase; at the point where the elution curves cross, the integrated sorbose excess (plotted in the lower panel) had attained 7% in this instance. The precedence of the sorbose during the initial part of the descending phase, however, did not merely continue as expected, but became even more pronounced, so that there soon developed a relative *deficit* in the cumulative sorbose recovery –

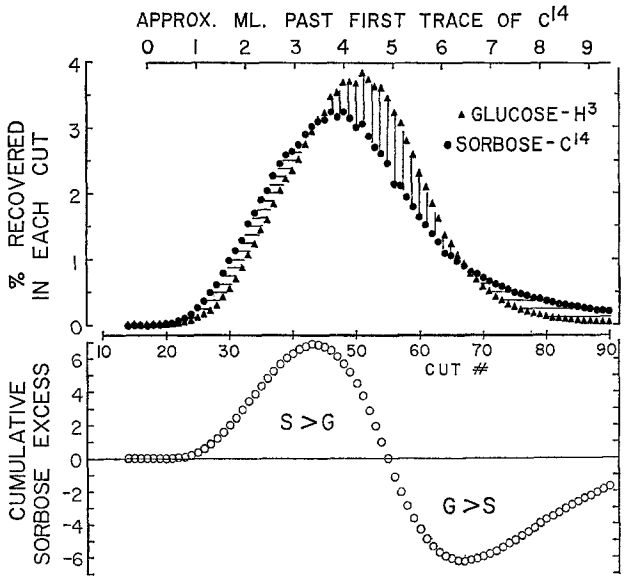

Fig. 1. Differential elution of monosaccharides from inert column loaded with crude erythrocyte "ghosts". Celite column (260 mm × 9 mm) contained 15 ml packed Levine-Stein ghosts. Added bolus was 2 ml of 2.5 mM phosphate buffer, pH 7.0, containing ^{3}H-D-glucose and ^{14}C-L-sorbose, each at 2 mM. Upper panel shows percentage recovery in each 130-μliter cut; lower panel shows cumulative difference between eluted sorbose percentage and eluted glucose percentage (i.e., algebraically added hatched areas between sets of points in upper panel)

a pattern which deviates markedly from that of a simple selective binding. Thus the elution curves necessarily *again* crossed, and subsequent cuts showed an increasingly marked excess of sorbose over glucose. In the example of Fig. 1, this late sorbose retardation approximately equaled the early glucose retardation (but was more commonly somewhat smaller). The terminal elution of the remaining sorbose deficit continued for some time beyond the emergence of virtually *all* the glucose; in fact, the number of cuts and time required to recover the last of the sorbose was generally so protracted as to be technically prohibitive.

Such a picture is obviously not indicative of a simple delay of glucose emergence by reason of its selective reversible binding to some component on the columns: the latter process would generate only a single crossover of the elution curves (near the peaks) and a simple monophasic pattern in the cumulative sorbose excess. We suggest that the observed differential behavior of the two sugars arises from the survival on the columns of the vesicular character of ghosts of this crude type, and the continued operation of the cells' sugar-transport apparatus across these vesicular surfaces. The

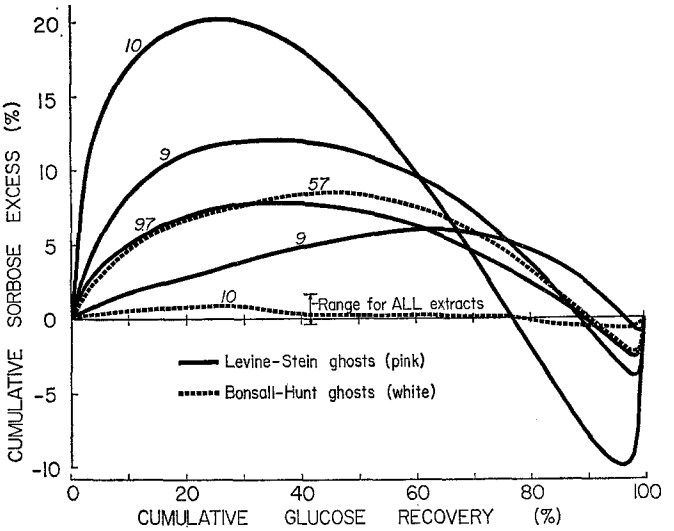

Fig. 2. Differential sugar elution in several representative ghost-laden columns. Experimental arrangement essentially as in Fig. 1 in all cases; column matrix as described in text. Cumulative excess of sorbose over glucose eluted is given as in lower panel of Fig. 1, but it is here normalized by plotting against cumulative glucose elution. Each curve was drawn through numerous points such as illustrated in Fig. 1. Numbers adjacent to curves give milliliters of packed cells from which ghosts on columns were derived. Bracketed double arrow indicates range of most extreme deviations from zero differential elution observed with all protein extracts prepared as recommended by Manchester group

readily transported D-glucose would thus gain access to an additional volume into which the low-affinity L-sorbose could move only slowly, so that the latter sugar would for the most part pass through the columns somewhat more quickly than the glucose, as observed. However, the portion of the sorbose which had entered the vesicular spaces would emerge only quite slowly, in accord with the observed protracted retention of the terminal portions of this sugar.

Fig. 2 illustrates the wide variability in both the initial glucose retardation and the late sorbose retardation (and the parallelism between the two in this variability) among a number of columns. The presentation here is comparable to that in the lower panel of Fig. 1, except that, in order to allow ready comparison of the curves, the abscissae have been normalized by use of the cumulative glucose elutions in lieu of the (trivially variable) numbers of cuts or eluate volumes. (The late sorbose deficit is, of course, greatly compressed along the horizontal axis by this conversion in the abscissae.) The upper three of the four curves shown in Fig. 2 for the pink (Levine-Stein) ghost preparations refer to columns prepared with the usual

Celite (L-665-A), whereas the remaining (lowest) curve was obtained with the specific type of DEAE-cellulose (Eastman Organic Cellulose N,N-diethylaminoethyl ether) recommended to us by Dr. W. D. Stein for use with the protein extracts. Each of these, as indicated, carried a similar load of the ghost material, but rather marked differences in the differential sugar elution are seen. Even more notable is the fact that an approximately *six-fold-heavier* load of the more thoroughly lyzed and washed ghosts (as prepared by Bonsall & Hunt, 1966) was required (on the usual Celite material) to show an activity comparable to that of the poorest of the cruder ghost preparations. Most discouraging of all were the results with the protein extracts on either of the varieties of DEAE-cellulose (alone or in combination with Hyflo-Supercel or various Celite mixtures): neither the dialyzed NaI extracts nor the Triton X-100 extracts ever elicited any selective sugar retention demonstrably exceeding that which was at times observed on plain columns lacking any ghost material. The range indicated by the note near the x-axis in Fig. 2 covers the extremes ever observed in the course of numerous tests on such extracts, although these were prepared, so far as we were able, precisely according to the procedures specified by the Manchester group (Bobinski & Stein, 1966; Bonsall & Hunt, 1966; Levine & Stein, 1967). The only occasion on which the protein-laden columns appeared in our hands to show selective glucose retention was during a brief period when an aging stock solution of ^{3}H-D-glucose became contaminated with a non-glucose tritiated component. The apparent glucose retention on these particular columns corresponded approximately to the proportion of the impurity in the labeled sugar as revealed by paper chromatography. Conceivably such a complication might have contributed to the dissonance between our results with extracts and those reported from Manchester, since neither Bobinski and Stein nor Bonsall and Hunt specifically cite verification of the radiochemical purity of their sugar preparations.

Several experimental variables were manipulated to test the vesiculation interpretation of the retardation behavior seen with the crude ghosts. Fig. 3 shows the effects of varying the elution rate in a given column in immediately serial runs (plotted as in Fig. 2). An increased elution rate apparently augments somewhat the differential treatment of the two sugars; we suggest that this is because of the reduction in the time for approach toward equilibrium across the vesicular surfaces at each position in the column, with a consequent enhancement of the differences in distribution occasioned by the two sugars' differing facilities for traversing these barriers. In any case, the observed effect is quite inconsistent with the thesis of a simple binding, since any limitation in the time for adsorption-desorp-

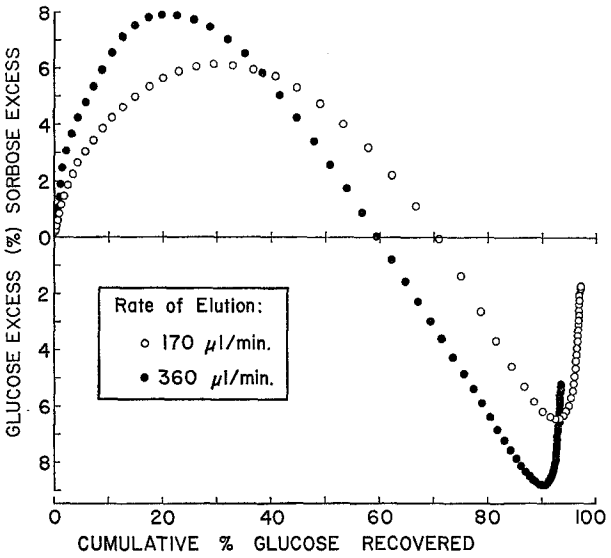

Fig. 3. Effects of varying elution rate on differential glucose-sorbose elution pattern. The same ghost-Celite column was run successively, first at the lower rate, then immediately after rinsing at the higher rate of elution (by increase of applied N_2 pressure). Plotted as in Fig. 2, but showing individual-cut points

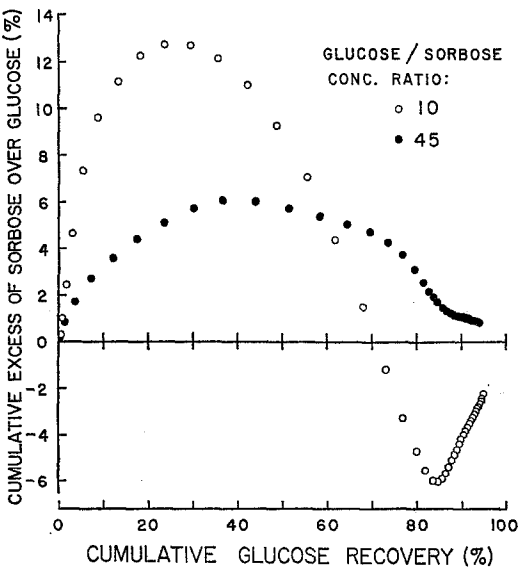

Fig. 4. Effects of varying glucose concentration on differential glucose-sorbose elution pattern. The same ghost-Celite column was run successively, first with bolus of 22 mM glucose/2.2 mM sorbose, then 102 mM glucose/2.2 mM sorbose. Plotted as in Fig. 3

tion reactions would tend to *diminish* the specific retardation rather than enhance it. Moreover, the increase in retention with the faster elution appears to apply equally to the initial glucose retardation and to the late sorbose retardation.

The effects of varying the glucose concentration in the bolus are shown in Fig. 4. The most significant aspect of this is that an increase in the glucose level suppressed the sorbose retention, completely abolishing it when the glucose/sorbose ratio was set at 45. This accords with the vesicle-penetration interpretation of the phenomena, since high levels of glucose would be expected to inhibit competitively the entrance of sorbose into the vesicles, leaving the latter sugar to be eluted rapidly in a single uncomplicated pulse. However, confident interpretation of these experiments is vitiated by the finding that, following treatment with any sugar level exceeding 20 mM, the capacity of the columns to separate glucose and sorbose in the usual 2 mM:2 mM mixture was greatly diminished. The basis for this apparently irreversible disturbance is not clear; as will be brought out

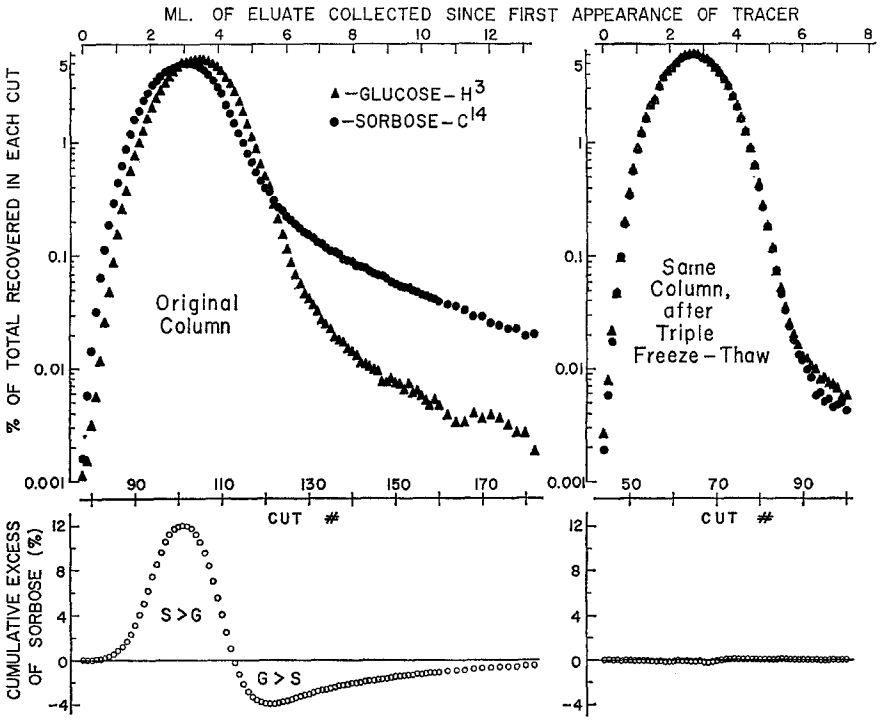

Fig. 5. Abolition of the differential elution behavior by freeze-thaw treatment. The same ghost-Celite column was run successively on the same day before and after rapid freezing and thawing three times (using liquid N_2). Experimental arrangement as for Fig. 1; plotting same as for Fig. 1, except that here logarithmic scale is used for ordinates in upper (individual cut-recovery) panels

below, repeated use of the same column for over two weeks at the 2 mM level did *not* substantially diminish the retardation of both sugars, as long as the column was left mechanically undisturbed.

However, this ability of a given column to maintain its capacity to separate the sugars proved to be quite sensitive to various physical manipulations. Fig. 5 shows the effects of repeated freeze-thaw treatment of a column that had initially given a substantial retardation. Here (and in Fig. 6) the cut-contents data in the upper panels are given logarithmically to illustrate the contrasting *proportions* of the two sugars at the different stages of elution. The undisturbed column showed a 12% glucose retardation with a subsequent peak sorbose retention of about 4%. The freeze-thaw treatment completely abolished both phenomena, the elutions becoming virtually superimposed (right-hand panels). Phase-contrast microscopy showed that this treatment reduced the large ghost vesicles into much smaller fragments (lacking a definitely vesicular character). Fig. 6 shows

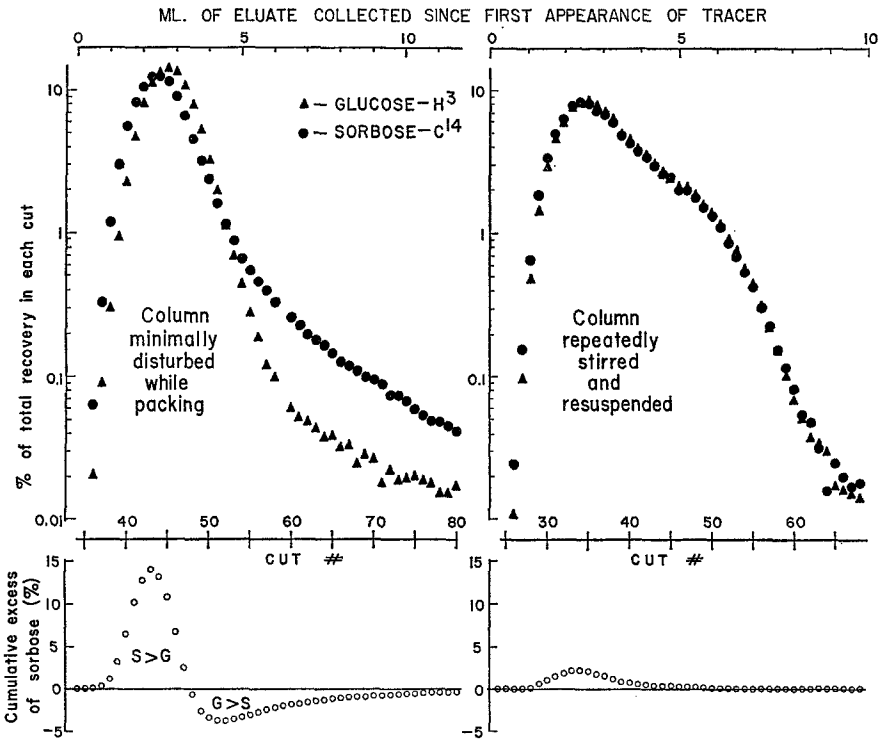

Fig. 6. Effects of mechanical agitation on differential elution behavior. A mixture of 20 ml packed Levine-Stein ghosts with Celite was divided evenly between two columns, one of which (left panels) was packed with minimal disturbance whereas the other (right panels) was vigorously stirred during packing. Immediate runs carried out as for Fig. 1; plotted as in Fig. 5

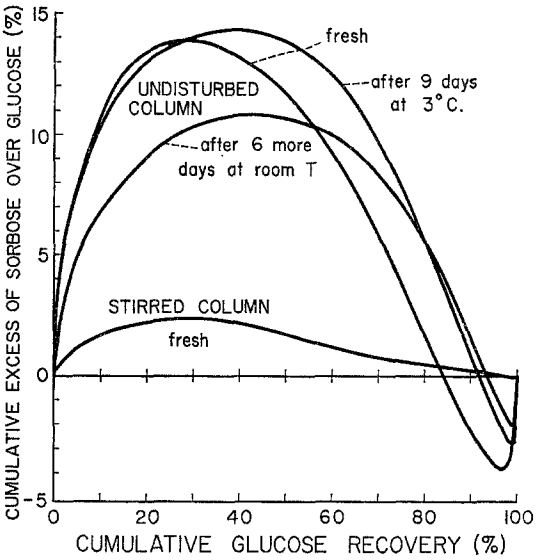

Fig. 7. Survival of differential elution behavior during column storage. Same pair of columns as in Fig. 6. Two curves for "fresh" columns are from same runs as in Fig. 6, plotted as in Fig. 2; other two curves show much later reruns (as labeled) on the column that had been subjected to minimal mechanical disturbance

the effects of a much gentler form of mechanical disruption; here, a single ghost-Celite mixture was used to prepare two columns, one of which was initially stirred vigorously prior to pouring and again repeatedly (with a long steel wire) during the course of the column-packing process. The loss of both aspects of the retardation behavior in the stirred column is quite obvious. The unstirred column from this experiment was then rerun after 9-day storage at 3 °C, and again 6 days later after standing at room temperature in the interim; as illustrated in Fig. 7 (plotted in the summary fashion of Fig. 2), the long periods of storage without deliberate mechanical disturbance did not profoundly modify the column's retardation behavior.

Discussion

The protein extracts from erythrocyte ghost preparations gave no evidence whatsoever of preferential binding of D-glucose in comparison to the relatively non-transportable analogue L-sorbose. In the case of the Triton X-100 extracts, this is perhaps not suprising in view of Hunter's (1964) observation that this substance inhibits the transport system in intact cells; but it conflicts directly with the positive results of Bonsall and Hunt (1966). NaI extraction, which has been useful for the solubilization of enzyme

systems of various tissues (Nakao, Yashima, Nagamo & Nakao, 1965; Schwartz, 1965; Tashima, Nakao, Nagano, Mizuno & Nakao, 1966), similarly gave no indication here of having solubilized the sugar-stereo-selective component, even though on occasion we incorporated into a single column some five to seven times the amount of such extracts as was used for the comparable preparations reported by Bobinski and Stein (1966) and by Levine and Stein (1967) as exhibiting selective sugar retardation. The bases for these direct experimental conflicts have not come to light. The question of possible radiochemical contamination, already mentioned, may be considered. Also of some possible concern are the exceedingly low counting rates shown in the only illustration from the Manchester group which provides such numbers (Fig. 1 from Bobinski & Stein, 1966); these activities are given as only about 48 counts/min at the peak. Such rates would hardly suffice to resolve the differences dealt with in experiments of this type (in the present studies, typical counting rates for single cuts near the peak were in the range of 25,000 to 100,000 counts/min).

No similar difficulty was encountered in demonstrating selective glucose retardation on Celite-supported columns of unextracted ghosts; in fact, with the crude ghosts of the Levine-Stein type, such columns in our hands generally evinced this phenomenon rather *more* dramatically than had been suggested by the Manchester reports. However, our findings suggest strongly that this occurred only because the ghosts were still sufficiently structured that a functional transport system could still operate to convey the sugars into and out of closed compartments on the columns – that in fact the column retardation system simply provides an elaborate way of again examining the sugar-transport function that has long been known to survive undiminished in such ghosts (LeFevre, 1961*a*). The possibility of such an interpretation was raised originally by several discussants in unpublished response to the first oral reports from the Manchester group, but evidence bearing on the issue was then lacking. The chief experimental arguments provided in the present study may be restated in summary.

(1) The initial relative delay in elution of the high-affinity transport substrate, D-glucose, was followed by a prolonged delay (apparent "binding") of the poorly transported analogue L-sorbose. Typically, about 5% of the sorbose was eluted only after virtually all the glucose has passed through the columns.

(2) When the columns were mechanically disrupted, by either freeze-thaw treatment or stirring, there was a loss (in some cases complete) of the preferential "binding".

(3) Increasing the speed of elution enhanced the apparent preferentia "binding".

(4) Increasing the glucose level in the applied sugar mixture reduced the terminal sorbose retention, completely abolishing it when the glucose/sorbose ratio was about 50.

The loss of "binding" characteristics upon mechanical or freeze-thaw disturbance of the columns might conceivably be attributed to detachment of the stereoselective component and its subsequent washout from the columns. However, removal by such relatively gentle measures seems unlikely in the face of the prolonged stability of the retardation characteristics of undisturbed columns. Moreover, the usual extent of the peak glucose retardation observed here corresponds to about *10 times* the experimentally estimated *upper limit* for the number of sugar-fixing transport sites in intact ghosts (LeFevre, 1961*b*). The latter figure would appear to fall below the limits of reliable resolution of the retardation method except with extremely heavily loaded columns; thus, failure to detect any retardation after any given treatment cannot be taken as indicative of removal or inactivation of the true binding entity.

An anomalous feature of passing high levels of sugar through these columns was a partial irreversible loss of the specific retardations. This may have an osmotic basis, especially in view of the fact that, in spite of the disappearance of the red color from the eluate in the course of the initial rinsing of the columns with the plain phosphate buffer, there was at these high glucose levels a noticeable new loss of hemoglobin during the peak sugar elution.

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