References and Notes

1. N. L. R. Bucher, *Intern. Rev. Cytol.* **15**, 245 (1963).
2. G. F. Leong, J. W. Grisham, B. V. Hole, M. L. Albright, *Cancer Res.* **24**, 1496 (1964); B. Sigel, F. J. Acevedo, M. R. Dunn, *Surg. Gynecol. Obstet.* **117**, 29 (1963); M. Virolainen, *Exp. Cell Res.* **33**, 588 (1964).
3. N. L. R. Bucher and M. N. Swaffield, *Cancer Res.* **24**, 1611 (1964).
4. W. C. Schneider, *J. Biol. Chem.* **161**, 293 (1945).
5. F. B. Siebert, *ibid.* **133**, 593 (1940).
6. K. Weinbren and E. Woodward, *Brit. J. Exp. Pathol.* **45**, 442 (1964).
7. W. C. Alston and R. Y. Thomson, *Cancer Res.* **23**, 901 (1963).
8. J. W. Grisham, G. F. Leong, M. L. Albright, J. D. Emerson, *ibid.* **26**, 1476 (1966).
9. B. Sigel, in *Gastroenterology* (abstract), **52**, 1142 (1967).

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Imine-Bonding in Membrane Transport of Monosaccharides: Invalidity of Kinetic Evidence

Abstract. *The proposition that carrier mediation of sugar transport may involve formation of imine complexes with specific cell membrane proteins has recently been advanced. However, the primary data presented accord more quantitatively with the presumption of a nonspecific reaction and furnish no evidence for the existence of the high-affinity glucose-binding sites which are essential to the proposed interpretation.*

Considerable study is being directed toward identification of the physicochemical reactions underlying the carrier-mediated transport of monosaccharides through cell membranes. An exciting and original lead in this connection has come from Langdon and Sloan's findings (*1*) that one or more proteins in the membranes of human erythrocytes or rat adipose cells can become associated with sugars by formation of (Schiff's base) imines, demonstrable by the production of stable secondary amines upon reduction with sodium borohydride. A possible role of this process in the transport events was suggested, with the corollary implication of one of the protein participants as the sugar carrier which has long been postulated from various kinetic characteristics of sugar permeation in these and other cells. The essential basis for this suggestion lies in the purported contrast between the kinetics of the imine formation in systems capable of such transport and that observed with nonspecific proteins. Thus, when mixtures of glucose-C^{14} at varying levels with indifferent proteins (such as serum albumin or hemoglobin) were incubated for a brief fixed period with an excess of the borohydride, and when the extent of the reaction was estimated by determination of the label incorporated into the subsequently isolated protein, the pattern of such incorporation as a function of the sugar concentration suggested, for such proteins, only an apparently homogeneous, low affinity for the sugar. But when similar experiments were conducted with either intact erythrocytes or their ghosts, the patterns observed led Langdon and Sloan to conclude that there was an additional, relatively small component of much higher apparent affinity (of the same order as estimated for the transport function in the same cells). However, I now point out that the figures offered by Langdon and Sloan (as representative of their data in support of this contention) do not corroborate the latter conclusion.

The points in each panel of Fig. 1 have been taken from Fig. 1 of Langdon and Sloan's report (*1*, p. 403). It shows the incorporation of uniformly labeled D-glucose-C^{14} into the membrane protein of intact human erythrocytes, during incubation at 37°C for varying periods up to 2 minutes, in the presence of 45.5 m*M* borohydride (*1*). In accord with their presumption that the glucose binds with two pools of sites—one (P_T) of low affinity (expressed in terms of a sugar-protein complex dissociation constant, K_p), and the second (C_T) consisting of specific (carrier?) sites of higher affinity (dissociation constant, K_C)—Langdon and Sloan applied to these data the equation:

$$PC^{14} = C_T\left\{1 - \exp\left(-\frac{k_r\,[BH]\,[G]t}{K_C + [G]}\right)\right\} + P_T\left\{1 - \exp\left(-\frac{k_r\,[BH]\,[G]t}{K_p + [G]}\right)\right\} \quad (1)$$

where PC^{14} is the amount of protein-bound labeled sugar, [G] and [BH] are the glucose and borohydride concentrations during the incubation for time t, and k_r is a rate constant for the reduction of the complex by the borohydride (which is taken to be rate-limiting, the imine-complex formation being essentially at equilibrium). By successive approximation in application of this equation to the points in Fig. 1, they calculated the following values as most suitable for the five constants: C_T and P_T, respectively, 5 and 95 nmole per milligram of protein; K_C and K_p, respectively, 20 and 1000 m*M*; and $k_r = 6.5 \cdot 10^{-5}$ liters per millimole-second. But the very poor fit of these values with the data is illustrated in Fig. 1a. Conceivably, this incongruity might have arisen from a trivial miscalculation for [BH] (in that this figure was given in the report only indirectly, by way of the recipe for the incubation mixture); and it is true that if the constant exponential factor, k_r[BH], is arbitrarily increased by about 16 percent so as to force the agreement shown in the original figure with the highest data point (for 100 m*M* glucose at 120 seconds), the reasonably satisfactory fit illustrated in Fig. 1b is achieved. In fact, this revised pattern does not differ radically from the picture originally given by Langdon and Sloan.

However, in spite of the fair accord with the observations that is thus possible with the suggested equation, there is no basis here for serious adoption of this particular combination of values for the five constants. For example, the systematic overshooting of the points at the lower glucose levels in the upper two curves of Fig. 1b suggests an underestimation of K_C; and if, accordingly, the specific-site dissociation constant is taken as 30 m*M* instead of 20 m*M* (and k_r is again adjusted slightly to provide coincidence with the highest point), the fit is substantially improved (Fig. 1c). But the satisfactoriness of this fit is not at all unique: with suitable compensatory juggling of the values assigned to almost any pair of the constants, various solutions of approximately equivalent congruity with the observations can be proposed. Thus, it is clear that with these five independent parameters, simple fitting of the data by successive approximation leaves a very large uncertainty in the assignment of an optimum combination of values; and no experimental effect on any one parameter can be genuinely distinguished unless the situations are decidedly divergent.

Far more disturbing to the suggested interpretation, however, is the fact that the distribution of the points in Fig. 1 can be still more adequately accounted for if the alleged high-affinity sites are altogether dismissed from consideration. Thus, if C_T is set at zero (as in Langdon and Sloan's treatment of

their data for nonspecific proteins), successive approximation by test of various combinations of the three remaining constants in the single-term exponential-decay equation leads to the pattern in Fig. 1d, with the values for P_T, K_p, and k_r as given in the figure legend. Since with these figures the curves conform with surprising precision to every point given (including the few which had remained visibly deviant when the two pools of sites were presumed), these data cannot possibly be taken as providing an argument for the existence of any specific sites of unusual affinity.

The only other individual analyses in Langdon and Sloan's report are those in their Figs. 2 and 3 (*1*, p. 405) concerning the binding of glucose to erythrocyte ghosts and to bovine serum albumin. For both of these, recalculation from the assigned values yields curves matching the data points even somewhat more satisfactorily than is suggested by the original figures. Thus, the serum albumin performance is very adequately described by the presumption of the single, nonspecific pool of low-affinity sites, while that of the ghosts is shown to conform reasonably well with a population of two pools in approximately the same ratio as had been suggested for the intact-cell system in Fig. 1. However, again in this case, a nearly superimposable curve can be derived by suitable combinations of constants for the nonspecific low-affinity site only (for example, $P_T = 28$ nmole per milligram of protein; $K_p = 500$ mM; and $k_r = 3.6 \cdot 10^{-5}$ liters per millimole-second).

One additional means of demonstrating the reality of a small pool of high-affinity sites is emphasized by Langdon and Sloan. This involves preparation of the ghost protein in the usual manner, after incubation with labeled glucose at two widely differing levels bracketing the apparent dissociation constant of the C-component, and comparison of the distribution of the label upon electrophoretic fractionation of the solubilized preparations. However, the results of this are somewhat incompletely reported: although it is specified that over 50 percent of the isotope incorporated into ghosts incubated with 16 mM glucose was localized into a single electrophoretic band, no account is given of the distribution at the higher concentration (250 mM) at which the nonspecific component would presumably greatly predominate. Thus, the meaning of the findings at the lower sugar concentration is left uncertain.

This criticism does not constitute a rejection of the hypothesis that the binding of sugar to membrane protein by imine linkages is perhaps the essential mechanism of sugar-carrier complex formation. The challenge is simply to the validity of the asserted demonstration of the high-affinity sites from the binding data presented, and especially to the derivation therefrom of constants characterizing the sites under different experimental circumstances involving application of agents (phloretin, insulin, and others) known to modify the behavior of the monosaccharide mediated-transport systems. Moreover, an observation made in this laboratory several years ago (but hitherto reported only by personal communication) raises an additional experimental objection to the imine-formation hypothesis for the carrier-sugar interaction in the human red cell: a reasonably high affinity for the carrier sites (by the usual kinetic criteria) was displayed by 1,5-anhydro-D-glucitol (*2*), lacking the carbonyl function presumed essential to the interaction of the type proposed by Langdon and Sloan. Moreover, Crane and Krane (*3*) have shown that this "1-deoxy" sugar is absorbed from the hamster intestine even against a concentration gradient.

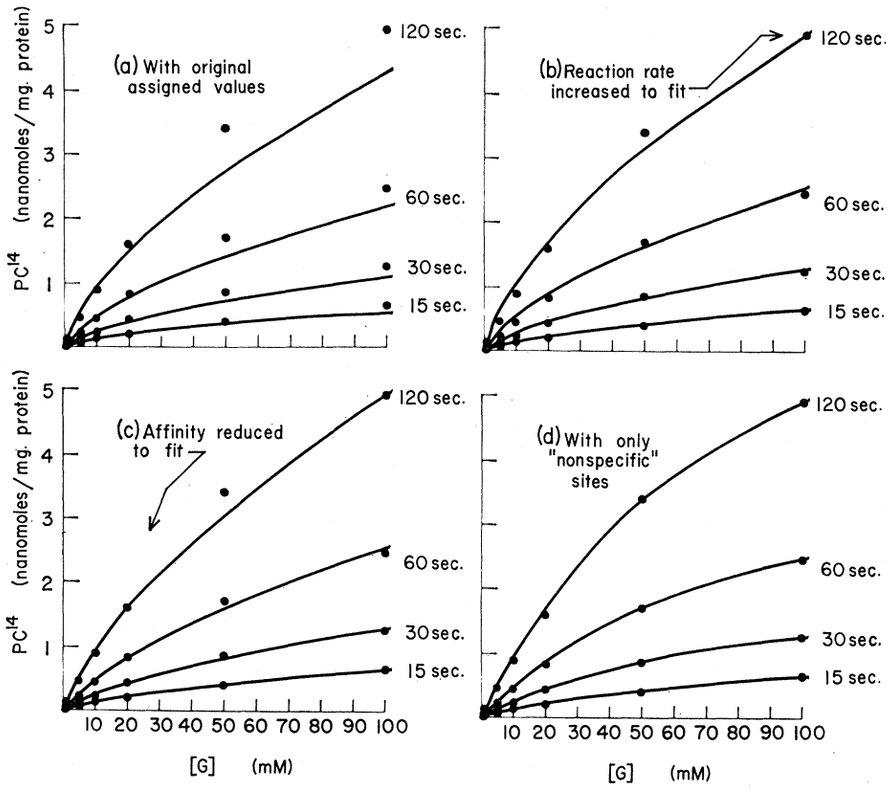

Fig. 1. Fitting of variously derived curves to the data of Langdon and Sloan's Fig. 1 (*1*), depicting the incorporation of uniformly labeled D-glucose-C^{14} into membrane protein of intact human erythrocytes, during incubation at 37°C for the period indicated with [BH] at 45.5 mM. Experimental points (taken directly from the original figure) are identical in each panel. Curves were calculated from Eq. 1, with the following respective presumptions regarding the five constants: (a) Langdon and Sloan's assigned values as given in the text; (b) the same values for C_T, P_T, K_c, and K_p, but with k_r raised to $7.55 \cdot 10^{-5}$ liters per millimole-second, so as to force the coincidence with the highest point shown in Langdon and Sloan's original presentation; (c) the same values for C_T, P_T, and K_p, but with K_c raised to 30 mM so as to accord better with points at lower [G]'s, and with k_r again adjusted up to $7.72 \cdot 10^{-5}$ liters per millimole-second; (d) complete elimination of the specific-site term and assignment of the following values: $P_T = 85$ nmole per milligram of protein; $K_p = 100$ mM; and $k_r = 2.2 \cdot 10^{-5}$ liters per millimole-second.

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References and Notes

1. R. G. Langdon and H. R. Sloan, *Proc. Nat. Acad. Sci. U.S.* **57**, 401 (1967).
2. This was synthesized and donated by Dr. Robert K. Crane.
3. R. K. Crane and S. M. Krane, *Biochim. Biophys. Acta* **31**, 397 (1959).

4. Supported by PHS research career development award 5K3AM-15,469 and by grants from NSF and NIAMD.

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