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STUDIES ON INSULIN ACTION.
IV. COOPERATIVE TRANSITION IN ADSORPTION: A THEORETICAL INTERPRETATION OF THE PRIMING ACTION OF GLUCOSE TREATMENT AT 25°C ON THE SUBSEQUENT ACCUMULATION OF LABELED GLUCOSE BY INSULINIZED FROG MUSCLE AT 0°C WITH A DISCUSSION OF BACTERIAL PERMEASE INDUCTION.

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SUMMARY

heincubation with D-glucose does not change the level of labeled D-glucose subsequently taken up at **0°C;** preincubation with insulin alone causes a relatively small increase; preincubation with both insulin and D-glucose simultaneously or separately (insulin treatment first followed by D-glucose treatment) further increases the steady level of labeled glucose uptake at **0°C.** This "priming" action of preincubation with D-glucose is duplicated by a nonmetabolizable sugar 3-0-methyl glucose and is not altered by the presence of protein synthesis inhibitors and m-RNA synthesis inhibitor D-actinomyosin. A molecular mechanism for this phenomenon is offered in terms of the association-induction hypothesis.

Most living cells derive their energy from the sugars in their environment. The first step in the consumption of these sugars is their transport from the external medium into the cells. The control of this process has been intensively investigated in two systems: (1) In microbes, which adapt to new sugars in their environment, control is exercised through the release of certain genes from a "repressed" state.[1-4] (2) In vertebrate muscle cells, control of sugar transport is achieved through the action of hormones like insulin.[5-9] Thus far, studies of these two systems have largely remained separate.

The key observation in the control of sugar transport by microbes is the ***induction phenomenon.***[1-3] Wild type ***E. coli*** possess neither the ability to accumulate lactose nor the enzymes to metabolize it. After growth in a medium containing lactose, however, there is dramatic change. The cells become "induced"; that is, they acquire a greatly enhanced ability to accumulate the sugar (in this case, lactose) to which they have been

exposed.

In studying the effect of insulin on the accumulation of glucose by frog muscles at 0°C, we have recently observed a phenomenon bearing a certain resemblance to microbial induction. At this temperature the phosphorylation of glucose is reduced to an undetectable level,[7] allowing the steady level distribution of glucose to be studied in isolation from its metabolic utilization. In order to demonstrate the effect of insulin it is necessary to preincubate the muscles at 25°C in the presence of this hormone before incubating with glucose at 0°C. Further, in order for insulin to exert its full effect on subsequent glucose accumulation at 0°C, *glucose itself (or one of its close analogues) must be in the preincubation medium.* Thus, just as in microbial induction, previous exposure to sugar enhances the ability of the tissue to accumulate subsequently the sugar to which it has been exposed. To prevent confusion, we shall refer to this phenomenon as *priming.*

In this paper, we shall describe the experimental results pertaining to the phenomenon of priming. We shall then examine the mechanisms thus far proposed for the insulin control of glucose transport, as well as the mechanism proposed for the control of sugar transport in bacteria, to see whether these mechanisms can provide an explanation for the observed results. Finally we shall examine the results in the light of the association-induction hypothesis.[10–12]

METHODS

The methods used here have been described in detail elsewhere.[7] Briefly, intact frog muscles are preincubated for up to 8 hours at 25°C in Ringer solution, which may or may not contain insulin or glucose. The muscles are then transferred to vials containing Ringer solution with varying concentrations of radioactively labeled glucose. After overnight incubation at 0°C, the muscles are analyzed for their labeled glucose uptake. This period of time is long enough for *steady level* of tissue glucose to be reached at all external glucose concentrations.[7] The incubation procedures, which are basically similar to those used by Narahara and his co-workers,[13] but more prolonged, produce no damage to the muscles as judged by the K^+-ion content and resting potential of the cells.[7]

RESULTS

Figure 1 shows the steady level of glucose taken up (0°C) by muscles at varying external glucose concentrations following 8 hours preincubation at 25°C. When neither glucose nor insulin is present in the preincubation medium, the steady level of glucose reached is linearly related to the final concentration of labeled glucose (Curve C), as was demonstrated in the preceding paper of this series.[9] The inclusion of insulin (0.1 u/ml) but not glucose in the preincubation medium increased the subsequent accumulation of glucose at, 0°C (Curve B); further, the steady level attained is no longer linearly related to the external glucose concentration. If glucose is now added to the insulin-containing prein-

cubation medium, the steady level of subsequent glucose accumulation at 0°C is further increased (Curve A). That this is not a specific glucose effect added to the insulin effect is shown by the data in Table 1. Without insulin, preincubation with concentrations of glucose ranging from 0 to 150 mM produces no change in the distribution of glucose at 0°C. Thus, glucose in the preincubation medium functions to enhance the action of insulin on the subsequent uptake of glucose at 0°C, i.e., it acts as a *primer* for the insulin action.

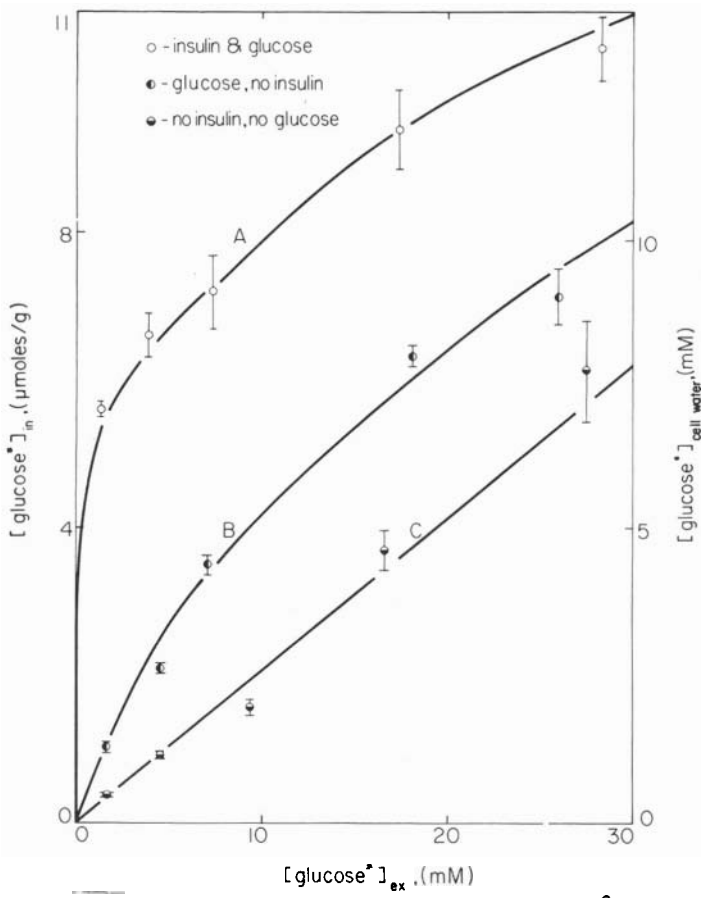

Figure 1. Effect of insulin and glucose in the preincubation medium at 25°C upon the steady levels of labeled glucose subsequently accumulated in muscle cells at 0°C.

To insure complete removal of glucose, muscles were washed in Ringer phosphate solution without glucose for 7 hours at 25°C, and then divided into 3 groups for preincubation (5 hours) at 25°C. For Curve C, the preincubation solution contained neither glucose nor insulin; for Curve B it contained 0.1 u/ml insulin; for Curve A, it contained 0.1 u/ml insulin and 24 mM glucose. The muscles were then incubated in different concentrations of labeled glucose for 18 hours at 0°C and assayed for their radioactivity. Each point represents the average of 7 determinations ± standard error. The left-hand ordinate gives labeled glucose concentration in micromoles per gram of fresh muscle tissue; the right-hand ordinate, in micromoles per millimeters of tissue water. The water content of muscle tissues in this and following experiments remains within 80% ± 1%.

Table 1. The Effect of Glucose Concentration in the Preincubation Medium at 25°C on the Steady Level of Labeled Glucose Accumulated Subsequently at 0°C in Noninsulin-Treated Muscles.
Isolated frog muscles were washed for 1 hour at 25°C to remove pre-existing glucose and insulin and then for 16 hours at 0°C and then again for 6 hours at 25°C in a total of 4 changes of solution. The muscles were transferred to 100 ml of Ringer phosphate solution containing various glucose concentrations for 3 hours and 40 minutes. The muscles were then incubated in labeled glucose (24 mM) for 16 hours at 0°C before assay. The final results were expressed as the ratio of tissue labeled glucose in micromoles per gram of fresh muscle to final external labeled glucose concentration. Each experiment consists of 5 individual determinations.

Glucose Concentration in Preincubation solution (mM)	$\frac{[\text{glucose}^*]_{tis}}{[\text{glucose}^*]_{ex}}$ (liter/kg) (mean ± S.E.)
0	0.32 ± 0.04
15	0.31 ± 0.01
30	0.23 ± 0.01
60	0.34 ± 0.01
90	0.31 ± 0.03
120	0.29 ± 0.01
150	0.28 ± 0.03

In Figure 2 the steady level of glucose uptake (0°C) is shown as a function of the concentration of glucose in the insulin-containing preincubation medium. In all cases, the incubation medium contained an initial concentration of labeled glucose of 10 mM. The steady level of glucose accumulated rises sharply with the concentration of glucose in the preincubation medium to about 40 mM before leveling off.

The glucose in the preincubation medium does not produce its effect by providing a source of energy. Thus, a nonmetabolizable glucose analogue,[14] 3-0-methyl glucose, also shows priming action (Table 2).

The possibility that glucose acts by promoting insulin entry into cells was investigated. Muscles were preincubated with insulin but no glucose at 25°C for 4 hours. After a brief rinse in Ringer solution free of both glucose and insulin, the muscles were again "preincubated" at 25°C with 24 mM glucose but no insulin for varying lengths of time, followed by overnight incubation with labeled glucose (24 mM) at 0°C. The final labeled glucose content of the muscles is shown in Figure 3 as a function of the duration of preincubation with glucose. The priming effect of glucose appears 10 minutes after introduction of the muscles into the second preincubation medium containing glucose and reaches a maximum in about 4 hours. In these experiments, the muscle tissues were exposed to the priming action of glucose after the exposure to insulin had already terminated. Thus,

insulin entry into the cells does not depend on the presence of glucose.

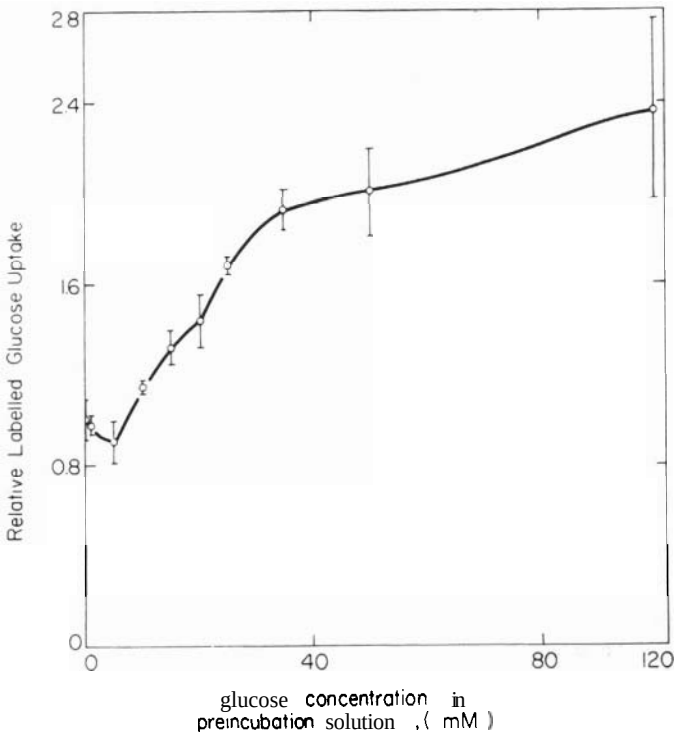

Figure 2. The effect of glucose concentration in the preincubation medium at 25°C on the steady level of labeled glucose subsequently accumulated in muscle cells at 0°C.
Each set of muscles was preincubated for 6 hours 45 min at 25°C in Ringer solution containing glucose (0 to 100 mM). Incubation at 0°C lasted 18 hours. The ordinate of the graph represents a ratio of the value of glucose uptake in these muscles to that in muscles preincubated in the absence of glucose. Since the "carry-over" from the preincubation solution changes the final glucose concentration in each vial to a different degree, the uptake for each set is normalized to correspond to the same final external glucose concentration. The abscissa represents the concentration of glucose in the preincubation solution. Each point is the average of 4 determinations ± standard error.

Finally, the possibility that the priming action of glucose is a result of the stimulation of ***de novo*** synthesis of protein was investigated by adding protein synthesis inhibitors to the preincubation and incubation medium (Fig. 4). Neither puromycin, cycloheximide, tetracycline, chloromycetin (all well-known protein synthesis inhibitors), nor β-thienylalanine (which prevents normal protein synthesis) had any effect on the induced uptake of glucose at 0°C. Nor did an inhibitor of m-RNA synthesis, actinomycin-D, have any effect. Thus the effect of preincubation with glucose and insulin cannot be due to ***de novo*** protein synthesis.

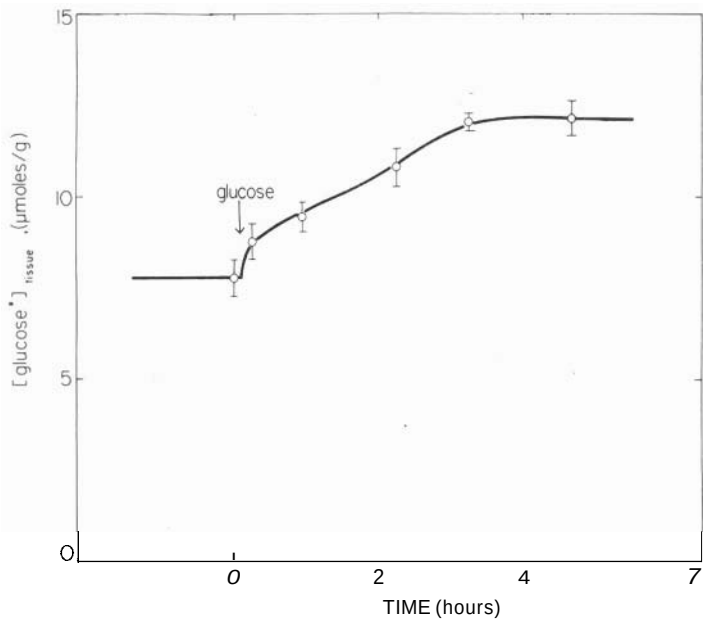

Figure 3. Separation of insulin and glucose treatment; the effect of the duration of preincubation with glucose on subsequent labeled glucose accumulated at 0°C.
Isolated muscles were preincubated in Ringer phosphate solution containing insulin (0.1 u/ml) but no glucose at 0°C for 24 hours and then at 25°C for another 4 hours. After washing in 3 changes of cold Ringer phosphate solution (0°C) containing neither insulin or glucose for 1 hour, one set of 10 muscles was taken out and incubated for 18 hours in 3 ml Ringer solution containing 24 mM labeled glucose. The remaining muscles were introduced into 100 ml of Ringer phosphate containing only 24 mM labeled glucose, but no insulin in a 250 ml flask (25°C). Samples of muscles were taken out at intervals and also incubated for 18 hours at 0°C in Ringer phosphate solution containing 24 mM labeled glucose. The final labeled glucose uptake in these muscles is plotted against the time of preincubation in the warm glucose-containing Ringer solution.

Table 2. The Effect of 3-0-Methyl Glucose in the Preparation Medium at 25°C upon the Steady Level of Labeled Glucose Subsequently Accumulated in Muscle Cells at 0°C.
Each set of 4 muscles were preincubated for 7 hours in 3 changes of 100 ml of Ringer phosphate solution containing combinations of the several components (insulin, D-glucose, 3-0-methyl glucose) indicated below. The muscles were then incubated in various concentrations of labeled glucose at 0°C for 18 hours, after which the muscles were analyzed individually.

Insulin (u/ml)	D-Glucose (mM)	3-0-Methyl Glucose (mM)	No. of Expts.	No. of Detns.	$[Glucose^*]_{in}$ (μmoles/g) (Mean ± S.E.)
none	none	none	6	24	4.63 ± 0.45
0.1	none	none	6	24	6.78 ± 0.34
0.1	24	none	8	34	10.27 ± 0.44
0.1	none	24	3	12	9.83 ± 0.62

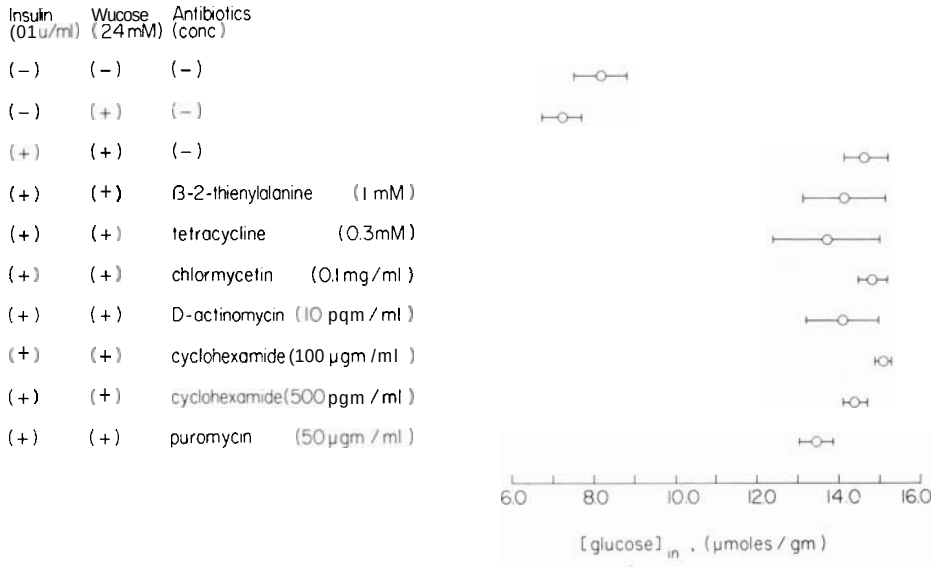

Figure 4. Effect of inhibitors of protein synthesis upon the primed accumulation of labeled glucose at 0°C.

Groups of eight isolated muscles were incubated in Ringer phosphate solutions containing various components as indicated, first at 0°C without shaking for 12.5 hours and then at 25°C with shaking for 7 hours with 3 changes of solution. After blotting, the 8 muscles were then incubated for 21 hours at 0°C in 4 ml of each of the above preincubation solutions plus 1 µc tritium-labeled glucose. Labeled glucose content is expressed as the mean ± standard error.

DISCUSSION

The predominance of the membrane theory has led most investigators in the field of glucose transport to postulate that the site of insulin action is located at the cell membrane (or possibly at intracellular barriers similar to the cell membrane). (For review of theories, see refs. 15 & 16.) There are two possible interpretations based on the general concepts of the membrane theory: (1) that the priming action presented here is the same as the induction phenomenon of microbes, and (2) that the priming action may be explained by Wilbrandt's "counter current flow" concept.[17]

I. THE PERMEASE INDUCTION PHENOMENA

We recall that Jacob and Monod[4] offered an explanation for an induced accumulation of sugar in bacteria on the basis of new protein ("permeases") synthesis (for evidence of de *novo* synthesis of protein in bacteria, see Kolber and Stein[18]). We have shown that there is no protein synthesis involved in the primed accumulation of sugar by muscles (Fig. 4). Thus, "priming" and "induction" are not identical phenomena. They may, however, be related phenomena (see below).

II. THE "COUNTER CURRENT FLOW" INTERPRETATION

Wilbrandt's "counter current flow" experiment refers to the enhanced exit of labeled 3-0-methyl glucose from erythrocytes when nonlabeled glucose was added to the bathing medium. Thus, in this theory increased concentration of a sugar on one side of the membrane causes an increased flow of similar sugar in the opposite direction.

The question may be raised whether the (nonlabeled) glucose introduced into the muscle cell during preincubation might produce also a higher subsequent uptake of labeled glucose by a similar type of counter current flow. That this is not the explanation is shown in Table 1. Preincubation in solutions containing from 0 to 150 mM of glucose (but no insulin) did introduce a different amount of glucose into the cells (Curve C of Fig. 1). Yet the subsequent uptake of labeled glucose is materially the same. As pointed out above, the effect of priming is evident ***only*** if insulin is also present in the preincubation medium.

III. INTERPRETATION ACCORDING TO THE ASSOCIATION-INDUCTION HYPOTHESIS

In the preceding paper of this series we showed that, in the absence of insulin, the intracellular glucose level is a constant fraction of the external glucose (i.e., about 25%, see Fig. 1, Curve C). This fact was explained on the basis that the polarized multilayers of water found within the cell act as a poorer solvent than the water of the external medium (refs. 10, Chap. 2, & 19; for full discussion on this subject, see refs. 9, 12, 20). Treatment of muscle cells withinsulin and glucose adds another fraction of intracellular glucose. In the preceding paper of this series we have shown that if one subtracts Curve C from Curve A in Figure 1, one obtains a Langmuir adsorption isotherm (hyperbola).[9] Such an isotherm is characteristic of the adsorption of substances on a limited number of adsorption sites. This suggests that the function of insulin is to create specific adsorption sites for glucose within the protoplasm.

We must now consider the question: Is there a component in the protoplasm capable of providing enough specific adsorption sites to account for the glucose uptake of insulin-treated muscles? The only macromolecular component of protoplasm known to absorb sugars with a high degree of stereospecificity is protein.[21-24]

There is evidence to suggest that the stereospecific adsorption of sugars and other hydrogen bonding substances is a general property reflecting some fundamental attribute of all protein molecules. The enzymes of carbohydrate metabolism, for example, adsorb sugars in a highly specific manner. In general, however, only one or two molecules of sugar are bound to each molecule of enzyme protein, a fact which led Cohen and Monod[2] to argue that the proteins of bacterial cells could not provide enough sites to account for the large accumulation of galactosides seen in E. *coli* under certain conditions.

,However, there is no real reason why the number of *nonenzymatic* binding sites on protein should be so limited. Thus, H_2O is another H-binding substance like D-glucose.

Kuntz et al.[20] from NMR studies reported that among 9 proteins, a minimum hydration of 0.28 g of H_2O per g of protein was observed for bovine serum albumen at pH 2-3. Assuming an average amino acid residue weight of 110, this data corresponds to no less than $\frac{110 \times 0.28}{18} = 1.7$ water molecules adsorbed for each amino acid residue.

In the present experiments about 9 millimoles of sites per kg of muscle cells are required to produce the observed results (see legend, Fig. 1). In other experiments, as many as 16 mmoles of sites are required.[9] The maximum required binding site is only one glucose adsorption site for every $\frac{200/110}{16 \times 10^{-3}} = 110$ amino acid residues. When compared to the number of water binding sites the great majority of proteins possess, this modest demand on the number of sugar binding sites can hardly be considered so excessive as to make it altogether impossible.

The question is now, how do insulin and glucose produce new adsorption sites? The experiment shown in Figure 4 rules out the most obvious answer – de *novo* synthesis of protein. This leaves only the alternative of a change in the pre-existing proteins produced by insulin and glucose to provide new sites for glucose adsorption.

In 1961 the senior author of this paper presented a general theory, the association-induction hypothesis.[10,11,25] This theory hypothesized that the fundamental ability of living protoplasm to transmit information and energy over long distances resides in the ability of the protein-water system to undergo *cooperative* transitions between alternative metastable states (the indirect F-process). These transitions involve electronic polarization and depolarization (c-value change) of the protein molecule and are under the control of biological signals in the form of hormones, drugs, ATP, acetylcholine, etc. (For recent review, see ref. 12.)

The mechanisms involved in such cooperative transition have been explained elsewhere in detail.[10–12,26] Initially, the protein is conceived as existing in a "closed" configuration in which H-bonding sites provided by the carbonyl and amide groups of the backbone are saturated by forming H-bonds with complementary groups on another protein (or alternatively on the same protein or on other nonprotein molecules). Binding of insulin leads to a total change in the electronic polarization of the protein molecule that extends at least as far as a neighboring "closed" H-bonded site, causing this site to change its preference from its present partners to the glucose molecule. The adsorption of the glucose on the first site in turn brings about a change in the electronic polarization of a second site, leading to adsorption of another glucose molecule. This process proceeds until all the available sites on the protein molecule eventually finish in the glucose-adsorbing state.

It should be pointed out that the primary mechanism of cooperative change in this model is electronic, with stereospecificity playing a secondary modifying role. However, with the change of H-bonding partners (as well as other bonds), conformation change of the protein would generally occur as a consequence. Evidence for this type of hormone-

directed change of protein conformation is now well established through the work of Tomkins, Yielding, and their co-workers.[27]

For the sake of clarity, let us summarize the outstanding features of this model:

1. A specific cardinal adsorbent, insulin, tips the proteins toward a state favoring adsorption of glucose.

2. For the reaction to proceed "to completion," a sufficiently high concentration of glucose or its analogues must be available.

3. The cooperative transition from one state to another has a high activation energy and thus is highly sensitive to temperature.

All three of these features can be found in the evidence presented in this paper. Thus, by itself, insulin makes available a limited number of adsorbing sites for glucose. In the presence of glucose, a much larger number of sites become available. This cooperative transition is dependent on a high temperature of preincubation. It does not occur at 0°C within the time limits imposed by our experimental condition. Once the cooperative transition to a glucose-adsorbing state has occurred at 25°C, this state is preserved as long as glucose is present in the system. As in all equilibrium phenomena, there is a constant exchange between the glucose adsorbed and the glucose in the medium. This exchange takes place at O°C as well as at 25°C. Such exchanges involve a single glucose molecule at a time: they are not cooperative in nature and can therefore be completed at 0°C.

At present, the above model applies most clearly to glucose transport in vertebrate cells. Yet if "what is true of E. *coli* must also be true of elephants," then what is true of frog muscle must, in general, also be true of E. *coli*. Cohen and Monod[2] rejected an adsorption model for sugar accumulation in bacteria on the following grounds:

1. The extremely high level of galactoside accumulation in bacteria requires as much as one site per fractional "molecular" weight of 2,000. This corresponds roughly to a concentration of 30 mmoles/kg fresh cells, or one binding site for every $\frac{2000}{110}$ = 18 amino acid residues. This number is far smaller than the figure of 1.7 binding sites per amino acid residue for water, and therefore no ground for rejecting the adsorption model.

2. The initial rate of galactoside uptake is equal to the exchange rate in the steady state. Cohen and Monod argued that if an adsorption model is correct, the initial rate of uptake of labeled thiogalactoside should involve occupation of unoccupied sites and, thus, this rate should be faster than the rate of labeled thiogalactoside uptake during a maintained equilibrium when the sites are already occupied by labeled thiogalactoside. However, Cohen and Monod were dealing with induced cells; such cells have already been exposed to galactoside, and any adsorption sites present would already be occupied by the sugar. Thus, the initial rate of entry under the conditions of their experiment is really just a measure of the rate of exchange of sugar.

They find, further, that the initial rate of entry of galactose into *E. coli* is saturable (see also refs. 10 & 28). They interpreted this finding as due to the presence in the cell

membrane of "permease" molecules endowed with a limited number of sugar binding sites. An implicit assumption in this theory is that the cell membrane is a universal rate-limiting barrier as postulated by Pfeffer some 90 years ago. Working with single muscle fibers, Ling and Shannon have recently shown that the kinetics of labeled D-glucose exchange does not support this assumption (see refs. 29 & 30). Indeed, in another system, Ling and Ochsenfeld[31] have shown that the initial rate of entry of another solute (Rb^+-ion) into a cytoplasmic protein-water system (actomyosin gel) also demonstrates saturation kinetics. Yet the actomyosin gel has no cell membrane and the rate of entry of Rb^+-ion is not surface limited. It possesses only polarized water and a limited number of binding sites distributed throughout the bulk of the gel system. Thus, saturation kinetics proves neither the existence of a rate-limiting cell membrane nor the presence in this membrane of a pumping permease.

3. Cohen and Monod argued that, on the basis of an adsorption model, the capacity of the bacterial cell for all galactosides should be the same. This argument assumes that all the sites are similar. However, if they are not of one type, a different number of maximum binding sites will be extrapolated for different galactosides (see Fig. 5).

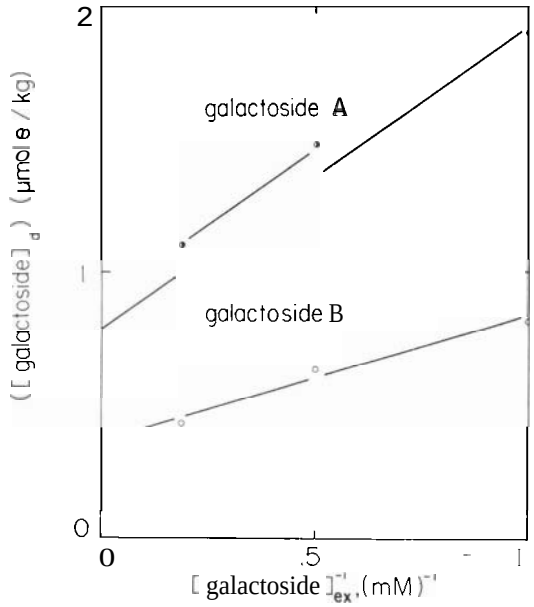

Figure 5. Theoretical demonstration that heterogeneity in adsorption sites leads to different apparent total numbers of adsorption sites from the study of two different galactosides.
In this theoretical model there are two types of galactoside-binding sites (I and II), each at the concentration of 10 mM. The adsorption constants for galactoside A and B on Type I sites are 10^4 and 10^5 (M^{-1}), those on Type II sites, 10^3 and 10^4, respectively. The unspecified competing ligands (e.g., H_2O, H-bonding sites on proteins) are such that the products of their adsorption constants and concentrations for Type I site is 10 and that for Type II site, 20. The data calculated from Langmuir adsorption isotherms are expressed as reciprocal plots of the calculated total adsorbed galactoside concentration. The extrapolated maximum number of galactoside binding sites are 27 and 13 mM respectively.

We might also point out that the general objections to the membrane model hold for microbes as well as for cells of higher organisms (refs. 10, 12, 19, 29 & 30). Thus, whether one postulates a Na^+ pump or a sugar permease, one must at least show that the cell possesses enough energy to deal with all the solutes that are in need of pumps.

We would like to suggest that in uninduced E. *coli* in a medium containing lactose, there is a maintained low concentration of the sugar in the polarized water of the microbial cells. Induced accumulation of sugars would then represent adsorbed sugar on certain cytoplasmic proteins.

One possibility is that the permease protein specified by the y-gene, in fact, functions in a manner like insulin on muscle cells as a cardinal adsorbent; its interaction with other proteins then tips them toward a physical state in which the adsorption of sugars becomes energetically more favorable. This idea is in agreement with the recent finding of Auraku et al., who isolated a protein from permease positive E. *coli*. When this protein is introduced into a permease negative strain of E. *coli* it causes this bacteria to accumulate galactose.[24]

ACKNOWLEDGEMENT

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