STUDIES ON INSULIN ACTION

V. STRUCTURAL REQUIREMENTS OF PRIMERS FOR SUBSEQUENT ACCUMULATION OF D-GLUCOSE AT O°C IN INSULINIZED FROG MUSCLES

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• *Systematic analysis was made of structural requirements for possible priming action of* D*-glucose and 40 related compounds in the presence of insulin to enhance subsequent labeled* D*-glucose uptake at* **O°C.** *It was concluded that a major portion if not all of the H-bonding groups on the* D*-glucose molecules are involved although their relative importance varies. Free OH and H on* C-1, *and upward orientation of OH and downward orientation of H on C-3, are the most important. Orientation of OH, H, and* CH_2OH *on C-2, C-4, and* **C-5** *are also important, but their perturbations are not nearly as detrimental to the priming action as those on* C-1 *and C-3.*

INTRODUCTION

In a preceding paper of this series,[1] the phenomenon of "priming" in frog muscles was described. Briefly, to achieve the full effect of insulin on subsequent uptake of labeled D-glucose in frog muscles at *O°C* (at which temperature D-glucose is not metabolized), the tissues must first be preincubated with insulin at a higher temperature (e.g., 25°C) in the presence of D-glucose or other structurally related "primers." The priming action of these sugars and derivatives was shown to involve no *de* novo synthesis of proteins or DNA. Nor does the priming depend on utilization of the primer as an energy source or a "counter-current" exchanger. On the other hand, the data agree with the model suggested in the association-induction hypothesis, in which the primer molecules play a major role in the cooperative transition controlled by the cardinal adsorbent, insulin.[2-5]

In this communication, we report our investigation of the structural requirements for a molecular species to function as an effective primer for subsequent labeled D-glucose accumulation in frog muscles.

MATERIALS

Sartorius, semitendinosus, tibialis anticus longus and iliofibularis muscles from North American leopard frogs *(Rana pipien pipiens,* Schreber) were used in all experiments.

Insulin containing 24 units/mg was obtained from Sigma Chemical Co., St. Louis, Mo. ^{14}C and ^{3}H labeled D-glucose were obtained from Calbiochem, Los Angeles, Calif.; Internal Chemical and Nuclear Corp., City of Industry, Calif.; and Schwartz, Syracuse, N.Y. Sugars and other related non-radioactively labeled compounds were obtained from Pfanstiehl, Waukegan, Ill.; Sigma, Calbiochem, and Mann Biochemical, New York; Nutritional Biochemicals, Cleveland, Ohio; and Fisher Scientific Co., Chicago, Ill.

METHODS

As a rule, a group of 4 muscles comprising one of the 4 types mentioned above, each muscle from a different frog, was preincubated and incubated in one flask but the muscles were analyzed separately.

Preincubation was carried out at 25°C for 6 hours with shaking in three changes of the preincubation Ringer solution. The volume of Ringer solution in each flask was 250 ml for insulin-free media,[6] but only 5 ml for muscles preincubated with insulin (0.1 unit/ml) and primer.

Incubation followed at O°C for 16 hours with shaking.[6] The incubation solution was a normal Ringer phosphate solution containing 24 mM D-glucose labeled with either ^{3}H or ^{14}C.

Methods for the extraction of labeled sugars from muscles with 5% TCA, and for the assay of radioactivity on a β-scintillation counter with Bray's scintillation fluid, were as described previously.[6]

RESULTS

Table I shows that when the preincubation medium contained neither insulin nor D-glucose, the concentration of labeled D-glucose accumulated in subsequent incubation at O°C in a Ringer solution containing 25 mM labeled D-glucose was 5.63 ± 0.25 μmoles per gram of fresh muscle. This value was moderately increased. by the in-

TABLE I. Effect of Insulin and D-glucose in Preincubation Media upon the Steady Levels of Labeled D-glucose Subsequently Accumulated at 0°C. (Initiated labeled D-glucose concentration in the 0°C incubation media was 24 mM in all cases.)

Concentrations in Preincubation Media		Number of experiments	Number of samples	Levels of labeled D-glucose accumulated in muscle cells (μmoles/g)
Insulin (unit/ml.)	D-glucose (mM)			
0	0	10	40	5.63 ± 0.25
0	24	7	29	8.29 ± 0.58
0.1	0	8	32	7.99 ± 0.34

clusion in the preincubation medium of either 0.1 unit/ml of insulin or 24 mM D-glucose as shown in Table I. Thus the level of labeled D-glucose accumulated in response to the inclusion of 0.1 unit/ml of insulin (alone) in the preincubation medium, according to a total of 8 sets of experiments comprising 23 samples, was 7.99 ± 0.34 μmoles/g. This value serves as the base line to measure primer effectiveness.

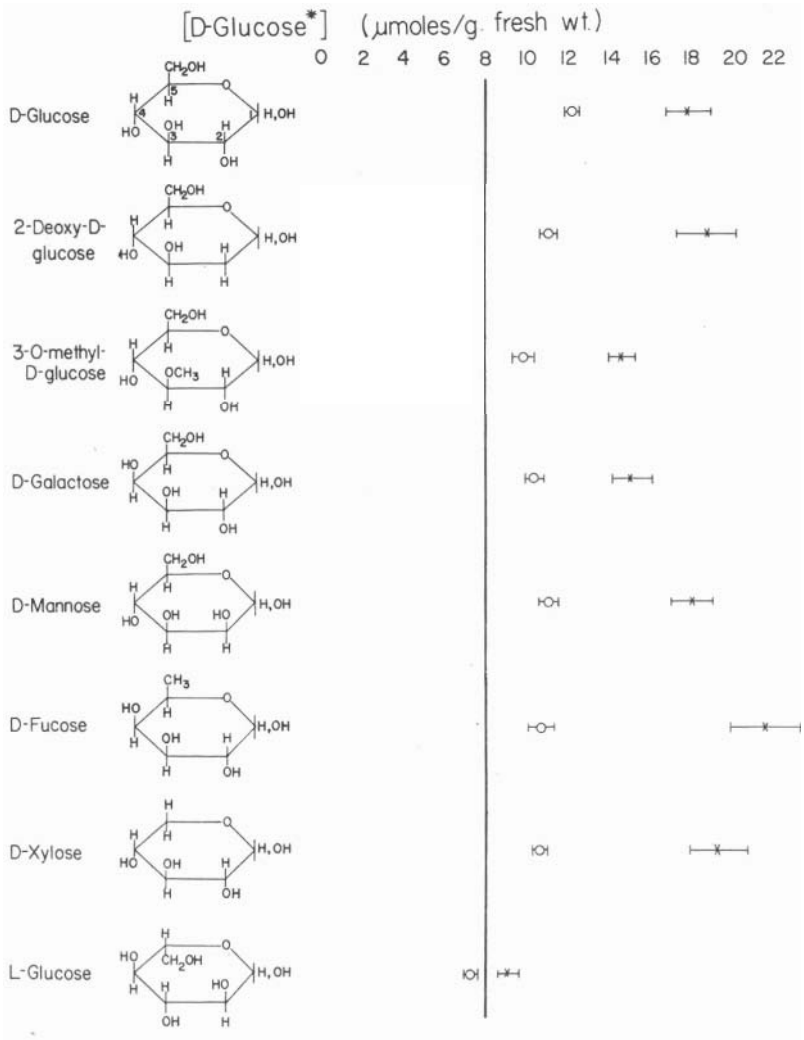

FIGURE 1A. Effects of various sugars and related compounds in the preincubation media upon the subsequent steady levels of labeled D-glucose accumulated in frog muscles at 0°C.

For results shown in this Figure, and in Figures 1B and 1C following, the preincubation media (kept at 25°C) contained 0.1 unit/ml of insulin and either 24 mM (I-O-I) or 100 mM (I-X-I) of the primer indicated on the left. The incubation medium, kept at 0°C, contained 24 mM labeled D-glucose but no insulin. The vertical line represents the level of labeled D-glucose accumulated in the tissue following preincubation in a medium containing only 0.1 unit/ml of insulin but no D-glucose or other primers (see Table I). More complete data are given in Table II.

In Table II are tabulated the results of our investigation of the priming effect (if any) of 14 hexoses, 6 pentoses, 7 acyclic polyols, 1 alicyclic polyol, 5 glycosides, 5 disaccharides, and 2 trisaccharides. For clarity, parts of the data given in Table II are reproduced graphically in Figs. 1A, 1B, and 1C, including those applying to all the hexoses and pentoses studied as well as representative members of each of the other categories.

The data show that the priming activities of the substances tested fall roughly into two general categories. Effective primers administered at 24 mM concentration in an

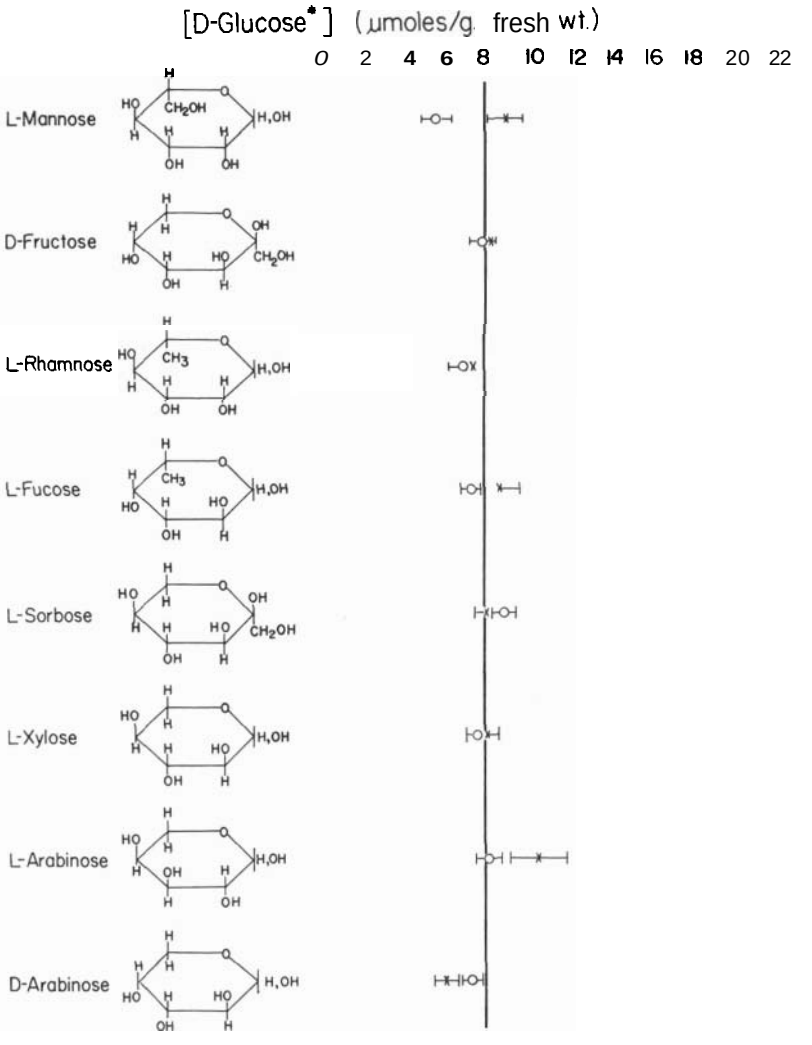

FIGURE 1B. Effects of various sugars and related compounds in the preincubation media upon the subsequent steady levels of labeled D-glucose accumulated in frog muscles at 0°C, *cont.*

insulin (0.1 unit/ml) containing preincubation medium elevate the subsequent level of labeled D-glucose accumulation from approximately 8 μmoles/g to 10 μmoles/g. Equally distinctive is the much higher level of the labeled D-glucose accumulation (14 μmoles/g or higher) to be observed if the concentration of the effective primer in the preincubation medium is raised to 100 mM.

On the other hand, ineffective primers when added at a concentration of 24 mM in the preincubation medium with 0.1 unit/ml of insulin do not increase subsequent labeled D-glucose uptake at 0°C beyond the 8 μmoles/g level produced by preincu-

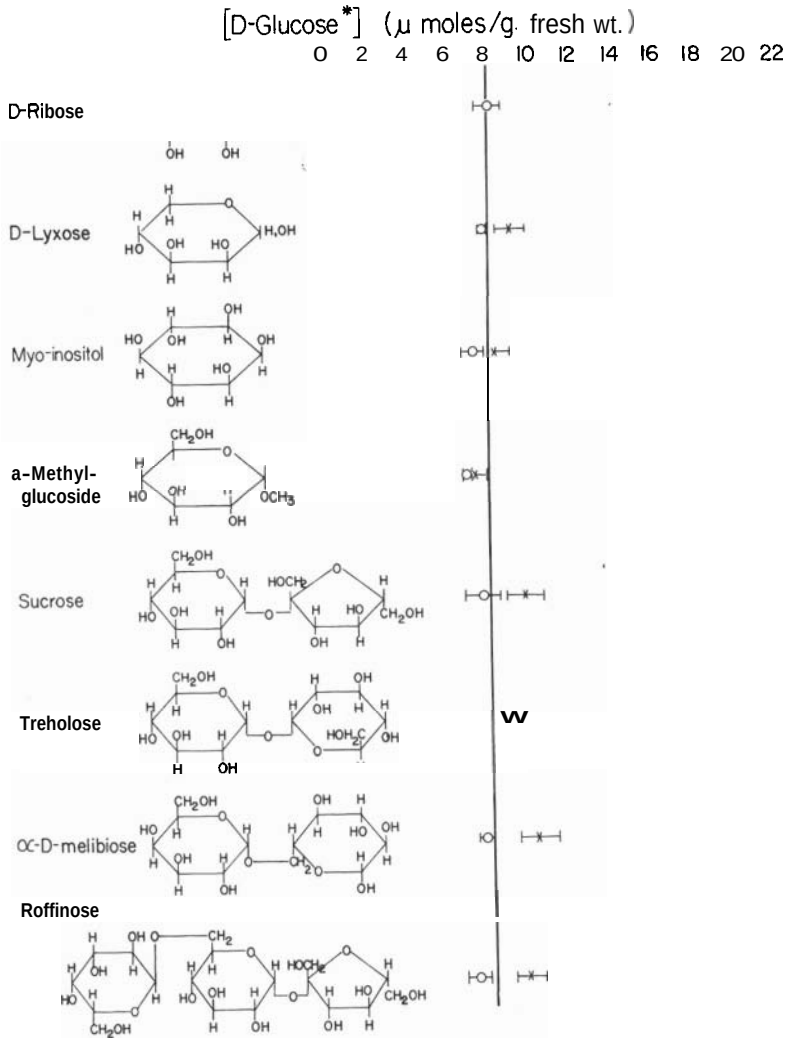

FIGURE 1C. Effects of various sugars and related compounds in the preincubation media upon the subsequent steady levels of labeled D-glucose accumulated in frog muscles at 0°C, *cont.*

bation with insulin alone. Furthermore, an increase of the concentration of an ineffective primer in the preincubation medium from 24 mM to 100 mM produces increases in subsequent labeled D-glucose uptake not beyond the level of 10 μmoles/g.

DISCUSSION

Of all the 41 compounds tested, only 7 proved effective primers: D-glucose (both α and β anomers) and its derivatives, 2-deoxyglucose, and 3-O-methylglucose, D-mannose, D-galactose, D-fucose, and D-xylose. The rest were either totally ineffective or merely marginally active. These data yield the following observations concerning the structural requirements of an effective primer.

1. *Ring Structure*

Seven acyclic polyols including one tetritol (erythritol), 3 pentitol (D-arabitol, xylitol and ribitol), and 2 hexitols (D-mannitol and sorbitol) were found inactive as primers, suggesting that the ring structure is essential for priming action.

TABLE II. Effect of the Inclusion of Various Compounds in the Preincubation Media (Containing 0.1 unit/ml Insulin) upon the Steady Levels of Labeled D-glucose Subsequently Accumulated at 0°C. (Under *A* the data are from experiments in which the concentration of the compound under study was 24 mM; under **B** it was 100 mM. The first numeral in parentheses refers to the number of separate experiments while the second numeral refers to the number of independent determinations. Initial labeled D-glucose concentration in the 0°C incubation media was 24 mM in all cases.)

	A		*B*	
D-glucose	(9,38)	12.20 ± 0.28	(6,24)	17.82 ± 1.16
α-D-glucose	(5,21)	10.72 ± 0.34		
β-D-glucose	(5,22)	10.83 ± 0.34		
2-deoxy-D-glucose	(4,16)	11.08 ± 0.49	(1,4)	18.83 ± 1.56
3-O-methyl-D-glucose	(4,16)	9.82 ± 0.50	(1,3)	14.63 ± 0.68
L-glucose	(4,16)	7.24 ± 0.26	(2,8)	9.03 ± 0.49
D-galactose	(5,24)	10.35 ± 0.37	(1,5)	15.10 ± 1.03
D-mannose	(4,16)	11.07 ± 0.45	(1,4)	18.10 ± 1.13
L-mannose	(1,5)	5.72 ± 0.68	(1,4)	9.08 ± 0.93
D-fuctose	(5,20)	7.96 ± 0.52	(2,9)	8.27 ± 0.27
L-rhamnose	(2,9)	6.98 ± 0.67	(1,5)	7.48 ± 0.05
L-fucose	(2,9)	7.38 ± 0.48	(2,8)	8.79 ± 0.87
L-sorbose	(2,8)	8.98 ± 0.61	(1,4)	8.10 ± 0.56
D-fucose	(1,4)	10.72 ± 0.02	(1,4)	21.7 ± 1.86
L-fucose	(2,9)	7.38 ± 0.48		
D-xylose	(8,33)	10.60 ± 0.35	(1,4)	19.38 ± 1.42
L-xylose	(5,20)	7.62 ± 0.54	(1,5)	8.14 ± 0.45
D-arabinose	(6,24)	7.36 ± 0.43	(3,13)	6.16 ± 0.51

2. *O in Ring*

The ineffectiveness of myo-inositol may suggest that the oxygen atom in the ring is essential. However, since only one alicyclic polyol was studied, the suggestion at this stage is only tentative.

3. OH *on C-1*

Five glycosides, all derivatives of sugars that are themselves effective primers (see Table II and Fig. 1) were found inactive: a- and β-methyl-D-glucosides, a-methyl-D-mannoside, β-phenyl-D-galactoside, and α-methyl-D-xyloside. Their inactivity suggests that a free hydroxyl group on C-1 is essential. In general agreement with this conclusion is the fact that the following D-glucose and D-galactose derivatives also proved ineffective or merely marginally active: sucrose (a-D-glucopyranosyl β-D-fructofuranoside), melezitose (O-α-D-glucopyranosyl-(1→3)-O-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside), raffinose (O-α-D-galactopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside), melibose (6-O-α-D-galactopyranosyl-D-glucose) (see Fig. 1C).

TABLE II, *cont.*

	A		*B*	
L-arabinose	(7,29)	8.20 ± 0.62	(3,13)	10.69 ± 1.41
D-ribose	(4,16)	8.02 ± 0.61		
D-lyxose	(7,29)	7.74 ± 0.25	(2,9)	9.02 ± 0.66
myo-inositol	(2,9)	7.21 ± 0.48	(1,5)	8.27 ± 0.73
erythritol	(1,4)	5.92 ± 0.34	(2,9)	8.91 ± 0.53
D-xylitol	(1,4)	6.01 ± 0.92	(1,4)	8.31 ± 0.74
D-arabitol	(2,8)	6.90 ± 0.65	(1,5)	8.11 ± 0.93
D-ribitol	(2,8)	8.22 ± 0.52	(1,5)	7.98 ± 0.51
D-galactitol	(2,8)	7.56 ± 1.04	(1,5)	8.33 ± 0.52
D-mannitol	(1,4)	7.93 ± 0.80	(1,5)	8.29 ± 0.78
D-sorbitol	(2,8)	6.91 ± 0.55	(1,5)	7.99 ± 0.99
a-methyl-D-glucoside	(1,4)	8.89 ± 0.75	(2,9)	8.35 ± 0.46
β-methyl-D-glucose	(1,4)	6.91 ± 0.07	(1,5)	7.29 ± 0.38
α-methyl-D-mannoside	(1,4)	7.85 ± 0.43	(2,8)	9.68 ± 1.00
β-phenyl-D-galactoside	(1,4)	4.95 ± 0.92		
a-methyl-D-xyloside	(3,12)	7.74 ± 0.41	(2,9)	9.79 ± 0.55
sucrose	(3,12)	7.69 ± 0.91	(3,12)	9.70 ± 0.99
maltose	(3,12)	6.76 ± 0.70	(3,12)	8.29 ± 0.75
lactose	(2,11)	7.62 ± 0.36		
trehalose	(1,4)	8.72 ± 0.23	(2,8)	9.17 ± 0.46
melibose	(1,4)	7.66 ± 0.41	(2,8)	10.24 ± 0.84
turanose			(1,4)	8.66 ± 0.49
rafinose	(2,8)	7.21 ± 0.51	(2,8)	9.71 ± 0.69
melezitose	(1,4)	8.48 ± 0.34	(2,7)	10.07 ± 0.61

While the above data strongly suggest that a free OH group on C-1 is essential, its orientation does not seem important, as shown by the fact that α and β anomers of D-glucose have similar priming activities. However, it is possible that mutarotase in the muscle cells might convert one anomer completely to the other if one of the anomers were being continually removed from solution by adsorption.

4. C-2

The presence of a downward oriented OH group on C-2 as in D-glucose is not absolutely essential for priming action. Thus both 2-deoxy-wglucose with OH group replaced by H on C-2 and D-mannose with an upward oriented OH are among the most active primers.

A comparison of the active D-xylose and the inactive D-lyxose shows, however, that the "right" orientation of OH group on C-2 is unessential only as long as the remaining structure and group orientations are exactly like those of D-glucose, as is the case for 2-deoxyglucose and D-mannose. The "wrong" orientation of the C-2-OH group when occurring in a molecule which has one (or more) additional departures from the D-glucose structure (e.g., replacement of CH_2OH on C-5 by H) produces an inactive molecule as is the case for D-lyxose, while D-xylose with only the replacement of CH_2OH by H remains active.

5. C-3

All active primers have an upward oriented OH group on C-3 as in D-glucose. All tested sugars with a downward oriented C-3-OH group were inactive. These observations indicate that the upward oriented OH and downward oriented H groups on C-3 are of marked significance.

The only partly tolerated modification of the C-3-OH groups is the substitution for the H of C-3-OH by the CH_3 group, as shown in the case of 3-O-methyl-D-glucose, which is active but less so than D-glucose itself.

6. C-4

There are two fully effective primers with an OH group oriented upward instead of downward as in D-glucose; they are D-galactose and D-fucose. The relative activity of D-galactose as a primer is below that of D-glucose, indicating that the downward orientation of the OH group and the upward orientation of the H group on C-4 are also important, though not as important as the OH and H groups on C-3.

Comparing the three pentoses, D-xylose, L-arabinose and D-lyxose, some idea can be arrived at concerning the relative importance of the orientation of OH and H on C-1 and on C-4. All three sugars lack the CH_2OH groups on C-5. D-Xylose is quite active, as the rest of the molecule is entirely like D-glucose. L-Arabinose is marginally active while D-lyxose is not. These differences show that the "wrong" orientation of OH and H on C-4 is not as detrimental as a similar wrong orientation on C-2.

7. C-5

Most of the effective primers have a CH_2OH group oriented upward and an H group oriented downward on C-5. However, CH_2OH is replaced by H in D-xylose

and by CH_3 in D-fucose. D-Xylose and D-fucose are both active.

Unlike D-xylose, which has only one departure from D-glucose (i.e., replacement of CH_2OH on C-5 by H), D-fucose has, in addition, a "wrongly" oriented OH and H on C-4. Both D-fucose and L-arabinose have wrongly oriented OH and H on C-4. The full effectiveness of D-fucose and marginal effectiveness of L-arabinose show that replacement of the upward oriented CH_2OH and H has an additional detrimental effect while replacement of CH_2OH by a CH_3 group has no detrimental effect.

CONCLUSIONS

From the above considerations, the following tentative conclusions can be drawn: To act as an effective primer for subsequent labeled D-glucose uptake at *0°C,* the molecule must be either D-glucose or its close analog. A free OH group on C-1 is quite essential and so is the correct upward orientation of the OH group and downward orientation of H on C-3. "Wrong" orientation of OH and H on C-2, C-4, and C-5 is tolerated if it occurs singly or, under certain circumstances, paired.

But the most important conclusion arising from the foregoing analysis is that a major part of the H-bonding groups on the D-glucose molecules reacts with the sites and is thus required for the priming action.

The full or nearly full involvement of all the H-bonding sites of the D-glucose molecules agrees with our interpretation of the priming action: to wit, that insulin adsorption on cardinal sites of certain proteins changes the electronic profiles of adjacent sites such that the preference of D-glucose (or other primer) adsorption is increased. This is the limited action insulin has if no primer is present in the preincubation solution. The presence of an effective primer in the medium makes possible the adsorption of the primer and an increased affinity for D-glucose or primer on another neighboring "downstream" site. This electronic and configurational transformation can then proceed over a number of sites in a step-by-step manner. As in other cooperative changes the priming action depends on "jumping" successive energy barriers and is thus temperature dependent as observed.[1–4]

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