added to the perfusate, the quantity of the perfusate used, and the flow rate through the liver are maintained constant in all the experiments reported upon, we may assume that the changes observed in perfusate radioactivity reflect changes in clearance ratios only.
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22. The marked deceleration of the clearance rate of fibrin and thrombin 15 to 30 minutes after the start of the liver perfusion initially puzzled us. However, we found that the radioactive material of the perfusate not bound to protein (soluble in trichloroacetic acid) increased linearly with time during perfusion with both thrombin and fibrin. This increase in free activity assumed a considerable magnitude. On the average, it amounted to 20 to 25 percent of the initial perfusate activity at the end of a 120-minute perfusion. If we consider that the total activity of the perfusate does not change after 12 to 30 minutes (which is not true, since the total activity does indeed decline slowly), the protein-bound radioactivity of the perfusate, which constitutes the difference between the total perfusate and the free radioactivity, declines with an average hourly rate of 10 to 12 percent of the initial radioactivity in the perfusate. In reality, this decline is somewhat larger since the curve of perfusate radioactivity does not really become perfectly horizontal.
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Trehalose Regulation of Glucose-6-Phosphate Hydrolysis in Blowfly Extracts

Abstract. *The presence of trehalose enhances the rate of glucose-6-phosphate hydrolysis in extracts of fat body and other tissues of adult* Phormia regina *Meigen. The activation appears to be specific, and increasing the concentration of trehalose changes both* V_{max} *and* K_m *for glucose-6-phosphate. There is, however, no easily recognizable phosphotransferase in the extract.*

α,α-Trehalose [(α-D glucosido)-α-D-glucoside] is the predominant hemolymph carbohydrate in a large number of insect species and is known to be synthesized in certain of them (*1*) through the same series of reactions that has been demonstrated in yeast (*2*):

glucose-6-phosphate + uridine diphosphate glucose → trehalose-6-phosphate + uridine diphosphate (1)

trehalose-6-phosphate → trehalose + P_i (2)

Reaction 1, catalyzed by UDP glucose: D-glucose-6-phosphate 1-glucosyltransferase (2.4.1.15), has never been completely separated from reaction 2, whereas the second enzyme, trehalose-6-phosphate phosphohydrolase (3.1.3.-12), catalyzing the hydrolytic cleavage of trehalose-6-phosphate, has been isolated and partially purified (*3*).

Since insect fat body appears to be an important site of production of α,α-trehalose, research concerned with the control of trehalose synthesis has necessarily centered around this organ. In the course of a recent investigation of this problem in the adult blowfly, *Phormia regina* Meigen, I observed that the in vitro release of trehalose by fat body was decreased in the presence of relatively small amounts of added trehalose (*4*). Murphy and Wyatt, having previously noted a similar phenomenon in Cecropia larvae, were able to demonstrate that trehalose in some way inhibited the trehalose synthetase complex (reactions 1 plus 2) prepared from fat body extracts (*5*).

There is now reason to speculate that in the blowfly, at least, one of the ways in which trehalose may exert a measure of control over trehalose synthesis is through its action on a system responsible for the hydrolysis of glucose-6-phosphate, since a preparation has been obtained from adult *Phormia* fat body and other tissues in which the rate of hydrolysis of this important substrate is specifically increased by trehalose (*6*).

This discovery provides an explanation for some heretofore poorly understood results described in another report (*3*). In those experiments crude adult blowfly tissue extracts were shown to catalyze reactions resulting in the liberation of inorganic phosphate (P_i) from adenosine triphosphate (ATP) at a rate which was greater in the presence of trehalose than glucose. Enzymes have now been found in these extracts which rationalize the trehalose effects. These include low levels of trehalose (catalyzing the reaction: trehalose → 2 glucose), hexokinase (glucose + adenosine triphosphate → glucose-6-phosphate + adenosine diphosphate), and a trehalose-activated glucose-6-phosphate hydrolyzing system (glucose-6-phosphate → glucose + P_i).

Table 1 contains the results of an experiment in which a partially purified extract was incubated with the substrates noted. The presence of a phosphatase activated by trehalose and inhibited by glucose is established in the last three lines of the table. In Table 2 it may be seen that trehalose does not change the balanced liberation of glucose and P_i, and in Table 3 the specificity of trehalose in this activation process is demonstrated. In this experiment the inhibition of P_i liberation by maltose is due to glucose contamination of the maltose preparation.

The extract, as it is assayed, has little adenosine triphosphatase activity (see Table 1), but it does hydrolyze inorganic pyrophosphate (PP_i). Recent studies on rat microsomes have uncovered what appears to be a single enzyme which is responsible for both the synthesis and hydrolysis of glucose-6-phosphate through the coordination of three activities: glucose-6-phosphatase, inorganic pyrophosphatase, and glucose-PP_i phosphotransferase (*7*). The enzyme is nonspecific, mannose acting as a phosphate acceptor from both PP_i and glucose-6-phosphate. In view of this finding, the blowfly preparation was examined for similar activity, the examination including, however, an investigation of the influence of trehalose on the rate of hydrolysis of PP_i

Table 1. Inorganic phosphate released from various substrates by an extract of adult *Phormia regina* Meigen. The reaction mixture consisted of substrate in the final concentration shown below, $MgCl_2$ ($4.5 \times 10^{-3}M$), tris HCl (*p*H 7.2, $4.5 \times 10^{-2}M$), enzyme (0.2 ml) (40 to 60 percent ethanol fraction of centrifuged homogenate), and H_2O up to 0.45 ml. The mixture was incubated for 30 minutes at 32°C and P_i was determined by an unimportant modification of the method of Fiske and Subba Row (see *8*).

Substrate	P_i released (μg)
ATP ($3.3 \times 10^{-3}M$)	0.8
Glucose ($1.8 \times 10^{-2}M$) + ATP	4.3
Trehalose ($9 \times 10^{-3}M$) + ATP	12.1
Glucose-6-phosphate ($1.3 \times 10^{-2}M$)	26.4
Trehalose + G-6-P	55.4
Glucose + G-6-P	13.0

in the presence of glucose-6-phosphate. Table 4 contains the results of an experiment in which glucose-6-phosphate in the presence and absence of trehalose was incubated with an enzyme extract and saturating amounts of sodium pyrophosphate. In both cases the hydrolysis of each substrate proceeded semi-independently of the other, that is, P_i released in the presence of both substrates was greater than that released from either substrate alone. We were also unable to find any glucose-6-phosphate produced in a reaction mixture containing glucose (400 μmole), sodium pyrophosphate (10 μmole), a concentrated enzyme extract, buffer (pH 6.5), Mg^{++}, and enough glucose-6-phosphate dehydrogenase and triphosphopyridine nucleotide to trap and assay for small amounts of product.

In the absence of a relatively nonspecific glucose(trehalose)-PP_i phosphotransferase, there remains the possibility that the activation of glucose-6-phosphate hydrolysis by trehalose could come about through the presence in the extract of a trehalose-glucose-6-phosphate phosphotransferase coupled to a phosphatase which limits the rate of the overall reaction and with which trehalose-6-phosphate has a greater V_{max} than does glucose-6-phosphate. Results of an examination for phosphatase activity in this preparation indicate a high degree of specificity, phenyl phosphate, adenosine-5′-monophosphate, β-glycerophosphate, and ribose-5-phosphate remaining untouched. Trehalose-6-phosphate is, however, split at eight times the rate of glucose-6-phosphate, although the K_m is between five and ten times higher than that with glucose-6-phosphate as substrate.

Since one of the enzymes of the hypothetical activation system is present, a search for the phosphotransferase has been initiated by looking for effects of high glucose concentrations on trehalose-6-phosphate hydrolysis rates. Glucose, in concentrations as high as 100 times that of trehalose-6-phosphate does not produce a decrease in the rate of hydrolysis, nor is there any evidence of net synthesis of glucose-6-phosphate from trehalose-6-phosphate plus excess glucose (1000 times). However, more sensitive methods of analysis might yield such information, since the high trehalose-6-phosphatase activity and equilibrium constants of intermediate reactions could conceivably mediate against a measurement of net synthesis.

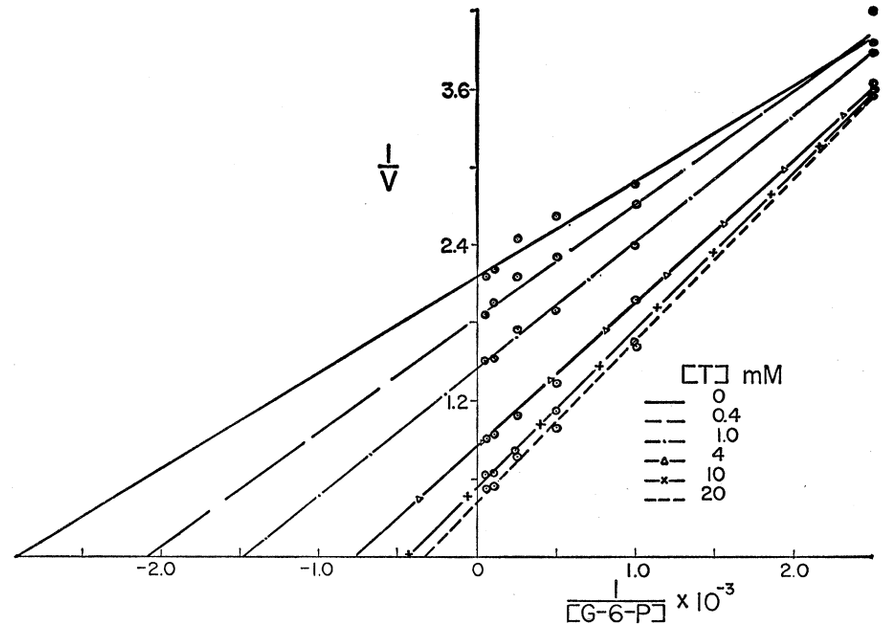

Fig. 1. Lineweaver-Burk plot of glucose-6-phosphate (G-6-P) hydrolysis in the presence of increasing amounts of trehalose (T). The reaction mixture consisted of glucose-6-phosphate and trehalose in concentrations noted on the figure, TES (N-tris[hydroxymethyl] methyl-2-aminoethane sulfonic acid) buffer ($4 \times 10^{-2}M$, pH 6.5), $MgCl_2$ ($8 \times 10^{-3}M$), enzyme as in Table 4 (0.15 ml), and H_2O to 0.5 ml.

Table 2. Balance study of glucose and P_i liberated in presence and absence of trehalose. The reaction mixture consisted of essentially the same constituents as in Table 1. Glucose was determined by a modification of the method of Keston (see *9*).

Substrate	Glucose liberated (μmole)	P_i liberated (μmole)
Glucose-6-phosphate	0.7	0.7
Glucose-6-phosphate + trehalose	1.35	1.37

Table 3. Activation of P_i release by various disaccharides. Reaction mixture similar to that described in Table 1.

Substrate	P_i released (μg)
Glucose-6-phosphate ($1.5 \times 10^{-2}M$)	19.5
G-6-P + trehalose ($1.5 \times 10^{-2}M$)	42.1
G-6-P + maltose ($7.5 \times 10^{-2}M$)	13.3
G-6-P + cellobiose ($7.5 \times 10^{-2}M$)	17.7
G-6-P + lactose ($7.5 \times 10^{-2}M$)	18.7
G-6-P + sucrose ($7.5 \times 10^{-2}M$)	17.0

Table 4. Interaction between hydrolysis of inorganic pyrophosphate and glucose-6-phosphate. Reaction mixture similar to that described in Table 1. Enzyme extract has been purified by diethylaminoethyl cellulose chromatography.

Substrate	P_i released (μg)
Na pyrophosphate ($1.2 \times 10^{-3}M$)	9.6
G-6-P ($4.8 \times 10^{-3}M$)	3.0
G-6-P + trehalose ($4.8 \times 10^{-3}M$)	7.5
PP_i + G-6-P	11.7
PP_i + G-6-P + trehalose	15.0

The kinetics of the activation process show certain complexities, a tenfold increase in K_m accompanying the increase in V_{max} as trehalose is added to the system (Fig. 1). Further purification of the extract should permit resolution of the question of whether these changes are due to the presence of a multicomponent system or a single enzyme whose substrate affinity and maximum velocity are oppositely affected by trehalose.

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