Studies of Liver Glucose 6-Phosphatase

III. SOLUBILIZATION AND PROPERTIES OF THE ENZYME FROM NORMAL AND DIABETIC RATS*

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In the last several years, a number of reports have appeared regarding alterations in the activity of liver glucose 6-phosphatase under various conditions. Thus, Cori and Cori (3) have been able to relate the accumulation of liver glycogen characteristic of von Gierke's disease to a deficiency of this enzyme. On the other hand, marked elevations in activity were found in the livers of diabetic animals (4, 5). Effects on glucose 6-phosphatase activity of modification of other endocrine states were modest or entirely lacking (5-7). Some interesting "adaptation"-like responses in glucose 6-phosphatase activity have been reported by Freedland and Harper (8) in animals fed diets low in glucose residues or high in fructose residues.

Our interest in this enzyme has been from the point of view of the mechanism of the hormonal influence thereon. As has already been observed (1), the elevation in activity of glucose 6-phosphatase in diabetic animals might be visualized as a reflection of an increased quantity of enzyme, or of an alteration in the properties of the enzyme in the direction of increased catalytic potency, or a combination of both. As an approach to this problem, we have undertaken an investigation of the properties of the enzyme and their alteration consequent with insulin deprivation and administration, first in whole homogenates of liver, and then in subfractions, as long as differences in properties persisted. The results have led us to the view that modifications in the structure or composition of the microsome itself accompany and perhaps underlie the altered kinetics and activities of glucose 6-phosphatase in the diabetic state. A brief report of some of these results has already appeared (1).

EXPERIMENTAL

Enzyme Preparations—Animals were killed by decapitation and the blood allowed to drain. Livers were chilled in cold 0.25 M sucrose, blotted and weighed, and homogenized in 9 volumes of 0.25 M sucrose in a Servall Omni-Mixer for 30 seconds at 0°. The whole homogenate is referred to as Fraction H.

For separation of microsomes, the homogenate was first centrifuged at 5000 × g for 10 minutes at 0° in the high speed head of an International centrifuge, model PR-2, to remove mitochondria and heavier particles. The supernatant fluid (Fraction S1) was then recentrifuged at 30,000 r.p.m. for 40 minutes at 0° in the No. 30 rotor of the Spinco ultracentrifuge, model I. The supernatant fluid thus obtained was decanted, and the microsomes, after a preliminary washing in the sucrose solution, were resuspended in a volume of 0.25 M sucrose equal to one-half the volume of S1 centrifuged (Fraction M). Further treatment of the microsomes was accomplished by the addition of the required reagent to Fraction M or, on occasion, by suspending the centrifuged microsomes directly in the reagent.

Assay Procedure—The reaction was carried out at 30° for 10 minutes unless otherwise stated, in a volume of 2.0 ml. containing 150 μmoles of sodium cacodylate buffer of the desired pH, sodium or potassium glucose 6-phosphate, and a suitable dilution of the enzyme, which was added last. The reaction was terminated by the addition of 0.4 ml. of 30 percent trichloroacetic acid, precipitated protein was removed by centrifugation, and phosphate was determined by the method of Fiske and Subba-Rao (9). When glucose liberation was to be measured, the reaction was stopped and the solution deproteinized by the addition of 10 percent ZnSO4 and 1 N NaOH, and glucose was determined by the method of Nelson (10) and Somogyi (11). There was no difference in the results obtained with the whole homogenate when the reaction was carried out in a Dubnoff Metabolic Shaking Incubator or in stationary tubes, nor when the Na or K salt of the substrate was used. For the determination of the kinetic parameters, the substrate concentration was varied from 1 to 20 mm, or from 0.5 to 10 mm, and the calculations performed as previously described (1).

Protein was determined by the method of Lowry et al. (12). A bovine albumin preparation was employed as a standard.

Materials—Sodium cacodylate, N. F., and digitonin were purchased from Fisher Scientific Company, glucose 6-phosphate from Sigma Chemical Company, Triton X-100 from Rohm and Haas Company, and deoxycholic acid from Matheson, Coleman, and Bell, Inc. The insulin preparations were obtained from Eli Lilly and Company, cortisone was obtained from Merek Sharp and Dohme (cortone acetate), and hydrocortisone, as the Na monohydrate, was obtained from The Upjohn Company (Solu-Cortef).

Production of Diabetes—Diabetes was produced by the subcutaneous injection of a freshly prepared solution of alloxan monohydrate in 0.1 m citrate buffer, pH 5.5, at a dosage of 18 mg. per 100 gm. of body weight. The rats were of the Wistar strain, purchased from Carworth Farms, Inc., and were fasted overnight before injection. All the animals were maintained on...
Purina laboratory chow and water ad libitum. Blood glucose determinations (Nelson-Somogyi (10, 11)) were performed on tail blood before injection, at random intervals after injection, and immediately before the animals were killed. Approximately half the animals succumbed to the injection in 2 to 5 days and half become diabetic. Only a few animals survive the injection without exhibiting diabetic symptoms. The criteria of diabetes were blood sugar levels of 400 mg. per 100 ml. or greater, failure to gain weight, and polydipsia.

RESULTS

Proportionality to Time and Enzyme Concentration and Stoichiometry of Reaction—Surprisingly, many of the reports dealing with glucose 6-phosphatase activities fail to indicate whether the experiments were carried out under conditions where the amount of product formed was a valid quantitative measure of enzyme activity. The proportionality of phosphate and glucose formation to time and enzyme concentration in these experiments is illustrated in Fig. 1, with a homogenate of normal liver as the source of the enzyme. The stoichiometry of the reaction is also apparent from the figure. Similar results were obtained with all fractions tested, from both normal and diabetic animals. When the reaction was carried out under strict zero order conditions, a

broad pH optimum was obtained over the range of 4.9 to 7.0, with a slight peak at pH 5.8.

<table>
<thead>
<tr>
<th>Status</th>
<th>$K'_t$</th>
<th>Units of glucose 6-phosphatase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Per 2.5 mg. liver</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (6)</td>
<td>1.72</td>
<td>0.306</td>
</tr>
<tr>
<td></td>
<td>(1.20–2.25)</td>
<td>(0.200–0.400)</td>
</tr>
<tr>
<td>Diabetic (12)</td>
<td>3.50</td>
<td>0.644</td>
</tr>
<tr>
<td></td>
<td>(1.82–5.10)</td>
<td>(0.442–1.025)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (2)</td>
<td>1.56</td>
<td>0.274</td>
</tr>
<tr>
<td></td>
<td>(1.52–1.60)</td>
<td>(0.273–0.275)</td>
</tr>
<tr>
<td>Diabetic (4)</td>
<td>3.83</td>
<td>1.013</td>
</tr>
<tr>
<td></td>
<td>(2.89–4.80)</td>
<td>(0.220–1.170)</td>
</tr>
<tr>
<td>Diabetic + insulin (4)</td>
<td>2.18</td>
<td>0.311</td>
</tr>
<tr>
<td>Cortisone-treated (4)</td>
<td>1.43</td>
<td>0.412</td>
</tr>
<tr>
<td></td>
<td>(1.30–1.50)</td>
<td>(0.348–0.435)</td>
</tr>
<tr>
<td>Hydrocortisone-treated (4)</td>
<td>1.34</td>
<td>0.390</td>
</tr>
<tr>
<td></td>
<td>(0.84–2.00)</td>
<td>(0.199–0.415)</td>
</tr>
</tbody>
</table>

* A unit of activity is defined as the amount liberating 1 μmole of orthophosphate per 10 minutes at infinite substrate concentration (extrapolated).

Effect of Diabetes and Hormone Administration on Kinetic Parameters—In Table I are presented the values of the glucose 6-phosphatase activities and the apparent Michaelis constants ($K'_t$) of the system for normal, diabetic, and hormone-treated animals. A large number of additional determinations have been performed subsequently with normal and diabetic animals, and all fell within the ranges indicated. The course of insulin injection, given subcutaneously, was as follows: 1st day, 10 units of protamine zinc insulin + 10 units of crystalline insulin; 2nd day, 5 units of protamine zinc insulin; subsequent days, 2 units of protamine zinc insulin. The animals were killed 48 to 72 hours after initiation of insulin treatment; 3 to 4 hours before this an additional 5 units of crystalline insulin were injected. Cortisone and hydrocortisone were injected intramuscularly at a dosage of 25 mg per day for 4 days and 5 mg per day for 7 to 21 days, respectively. It can be seen from Table I that insulin treatment promptly restored the altered kinetics of glucose 6-phosphatase in the diabetic animals toward normal and that hydrocortisone had little or no effect on the system. Cortisone produced the modest increases in activity previously observed (8, 9), but this increase, unlike that in the diabetic animals, was not accompanied by an increase in the apparent Michaelis constant.
TABLE II

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Normal</th>
<th>Diabetic</th>
<th>Normal</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_+^{'})</td>
<td>Recovery</td>
<td>(K_+^{'})</td>
<td>Recovery</td>
</tr>
<tr>
<td>H</td>
<td>mM</td>
<td>%</td>
<td>mM</td>
<td>%</td>
</tr>
<tr>
<td>S</td>
<td>1.65</td>
<td>0.44</td>
<td>100</td>
<td>1.15</td>
</tr>
<tr>
<td>M</td>
<td>1.80</td>
<td>0.37</td>
<td>74</td>
<td>3.67</td>
</tr>
<tr>
<td>Digitonin suspension of M.</td>
<td>—</td>
<td>1.54</td>
<td>70</td>
<td>0.80</td>
</tr>
<tr>
<td>Supernatant fraction of No. 4, above</td>
<td>0.76</td>
<td>1.82</td>
<td>39</td>
<td>0.75</td>
</tr>
<tr>
<td>AS,</td>
<td>2.02</td>
<td>31</td>
<td>0.82</td>
<td>3.27</td>
</tr>
</tbody>
</table>

* Specific activity is defined as the units per mg. of protein (see Table I). For this table the activities were calculated at 10 mM rather than infinite substrate concentration, since the data were not available for the latter calculation in all cases.
† = Not determined.
‡ The low recovery in this fraction was due to losses in dialysis against distilled water instead of buffer.

Properties of Subcellular and Solubilized Fractions—In order to determine whether the altered kinetics of the diabetic enzyme persisted in preparations isolated from the influence of the supernatant fraction of the homogenate, the microsomes were separated, as described above, and the kinetic parameters determined. The bulk of the glucose 6-phosphatase activity was recovered in the microsomes, as has been reported by others (5, 7, 13). In addition, as can be seen from Tables II, III, and IV, the apparent Michaelis constants of the enzyme in the isolated microsomes were the same as in the corresponding homogenates. In order to purify the enzyme further, methods of solubilizing the activity were sought. Resuspension of the microsomes in water, in salt solutions, or in high concentrations of ethylenediaminetetraacetic acid, repeated freezing and thawing, or extensive sonic treatment, all failed to produce a preparation in which the activity remained in the supernatant fraction after high speed centrifugation. Resuspension in 1 per cent digitonin solution, on the other hand, solubilized approximately half of the total activity. Moreover, the \(K_+^{'}\) values of the digitonin-treated preparations from both normal and diabetic animals were similar and were reduced to levels lower than those from even normal microsomes (Table II). In contrast to the elimination of the differences in \(K_+^{'}\) between the normal and diabetic system, the higher specific activities of the latter remained, in both digitonin suspensions and the soluble preparations therefrom. Suspension in digitonin occasioned no loss in total activity and, in fact, would appear to produce a marked increase, if assays were performed at lower substrate concentrations, because of the decrease in \(K_+^{'}\).

Pomicitination of the activity from the digitonin solution could be effected by the addition of solid ammonium sulfate to 90 per cent saturation, after a dialysis of 4 hours against tris(hydroxymethyl)aminomethane buffer, pH 7.5. The sediment obtained after centrifugation at 40,000 r.p.m. for 20 minutes in the No. 40 rotor of the Spinco ultra centrifuge was redissolved in this buffer (Fraction No. 4) and contained almost all of the activity initially present in the digitonin solution. In order to rule out a specific effect of digitonin on \(K_+^{'}\), other agents which disrupt microsomes were tested. The addition of an equal volume of 1 per cent Triton X-100 solution, buffered at pH 7.5, or of one-fourth of a volume of 1 per cent deoxycholate solution, buffered at pH 8.3, to a sucrose suspension of microsomes led to results closely comparable with those from the digitonin experiments. That is, there was a solubilization of a large fraction of the activity and a reduction in the \(K_+^{'}\) values in both the detergent suspensions and the solubilized enzyme fractions therefrom (Tables III and IV). After centrifugation

1 The balance of the activity could be recovered in the residue after high speed centrifugation of the digitonin-treated microsomes, and additional activity could be solubilized by a second extraction. Optimal conditions for solubilization with digitonin have not been determined. Langdon and Weakley (14) have also reported a solubilization of glucose 6-phosphatase with digitonin.

2 Crane, on the other hand, has reported no alteration in the \(K_+^{'}\) for the enzyme with galactose 6-phosphate as the substrate, in the presence of a Triton X-100 concentration of 0.4 per cent in the

TABLE III

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<thead>
<tr>
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<tr>
<td></td>
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<td>%</td>
<td>mM</td>
<td>%</td>
</tr>
<tr>
<td>S</td>
<td>1.80</td>
<td>0.37</td>
<td>74</td>
<td>3.67</td>
</tr>
<tr>
<td>M</td>
<td>1.80</td>
<td>1.46</td>
<td>68</td>
<td>4.20</td>
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<td>Digitonin suspension of M.</td>
<td>—</td>
<td>1.54</td>
<td>70</td>
<td>0.80</td>
</tr>
<tr>
<td>Supernatant fraction of No. 4, above</td>
<td>0.76</td>
<td>1.82</td>
<td>39</td>
<td>0.75</td>
</tr>
<tr>
<td>6. AS,</td>
<td>2.02</td>
<td>31</td>
<td>0.82</td>
<td>3.27</td>
</tr>
</tbody>
</table>

* Specific activity calculated as in Table II.
† = Not determined.

TABLE IV

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
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<th>Diabetic</th>
<th>Normal</th>
<th>Diabetic</th>
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<tr>
<td></td>
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<td>Recovery</td>
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<td>mM</td>
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<td>%</td>
</tr>
<tr>
<td>S</td>
<td>1.65</td>
<td>0.44</td>
<td>100</td>
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</tr>
<tr>
<td>M</td>
<td>1.80</td>
<td>0.37</td>
<td>74</td>
<td>3.67</td>
</tr>
<tr>
<td>Digitonin suspension of M.</td>
<td>—</td>
<td>1.54</td>
<td>74</td>
<td>3.43</td>
</tr>
<tr>
<td>Supernatant fraction of No. 4, above</td>
<td>0.76</td>
<td>1.82</td>
<td>39</td>
<td>0.75</td>
</tr>
<tr>
<td>6. Loose sediment from No. 4 . .</td>
<td>0.61</td>
<td>3.53</td>
<td>52</td>
<td>0.88</td>
</tr>
</tbody>
</table>

* Specific activity calculated as in Table II.
† = Not determined.
of the deoxycholate-treated microsomes, an appreciable fraction
of the glucose 6-phosphatase activity was associated with a
fluffy sediment which overlay a tightly packed layer and could
be separated from the latter and most of the supernatant ma-
terial. This fraction exhibited a high specific activity and a $K'_v$
value characteristic of disrupted microsomes, even though the
activity was still associated with particulate matter as in the
dehydrate experiments.

DISCUSSION

It seems clear from the results presented that the altered
kinetics of glucose 6-phosphatase in diabetic livers are not a direct
result of an altered extramicrosomal milieu, since the high $K'_v$
values obtained with diabetic liver homogenates persisted in
isolated, washed microsomes therefrom. Nor, on the other
hand, do they seem to be an intrinsic property of the diabetic
drug per se, since, upon disruption of the microsomes, the
Michaelis constants of the diabetic and normal enzymes become
indistinguishable.

Several possible explanations for these results suggest them-
selves. A decrease in $K'_v$ such as obtained here in proceeding
from intact to disrupted microsomes, might be the result of the
destruction or dilution of a competitive inhibitor. If such an
inhibitor is indeed present in microsomes, it must be a substance
to which the microsomal membrane is not freely permeable,
since suspension of the microsomes for several hours in buffer or
sucrose solution at 0° produced no alteration in the kinetics,
whereas suspension in reagents which disrupt the membrane
brought about an immediate reduction in the apparent Michaelis
constant.

The accumulation of a competitive inhibitor in the microsomes
of diabetic livers might also be responsible for the increases in
activity of glucose 6-phosphatase observed with these prepara-
tions, as well as for the altered kinetics. Ample precedent exists
for the induction of enzyme activities as the result of the pres-
ence of a competitive inhibitor of that enzyme (for references see
(17)). The observed relationship between the total activity and
apparent Michaelis constants in individual animals (1) is in
harmony with this view. On the other hand, attempts to obtain
direct evidence for the presence of a glucose 6-phosphatase in-
hibitor in extracts of diabetic microsomes have thus far been
unsuccessful.

Other possible explanations of the present observations have
been considered, based upon alterations in the intramicrosomal
environment other than the accumulation of a competitive in-
hibitor in the usual kinetic sense or upon alterations in the
microsomal membrane. In the latter connection, it might be
postulated that the $K'_v$ values obtained in the experiments with
intact microsomes reflect the substrate saturation characteristics,
not of the enzyme itself, but of a transport site on or in the
microsomal membrane, which has a lesser affinity for glucose
6-phosphate in the diabetic state. This would be the case if
transport across the microsomal membrane were the rate-limiting
step. Once the membrane has been disrupted, the $K'_v$
values would then reflect the intrinsic saturation characteristics
of the enzyme, which is the same whether from normal or dia-
abetic animals.

The results of the experiments on glucose inhibition (1, 2),
however, seem to us to detract from the likelihood of this pos-
sibility. Glucose inhibits glucose 6-phosphatase activity in both
normal and diabetic microsomes and in preparations from dis-
rupted microsomes, and with the same qualitative and quantita-
tive characteristics in all cases. Thus, if transport of the
substrate into the microsomes is the rate-limiting step, one is
apparently forced to conclude that glucose inhibits both the
transport and hydrolysis of glucose 6-phosphate in a closely
similar manner.

It has been noted above that the $K'_v$ values characteristic of
the enzyme in the solubilized preparations from either normal
or diabetic microsomes not only were essentially of the same
magnitude, but also represent a decrease from that of normal
intact microsomes, as well as from that of the diabetic counter-
part. This observation suggests that the feature of diabetic
microsomes which is responsible for the altered kinetics of glucose
6-phosphatase is merely a magnification of a normal property of
microsomes.

It is expected that further experimentation will allow a selec-
tion from among the several possibilities.

It is noteworthy that the decrease in $K'_v$ values obtained here
upon disruption of the microsomes did not seem to be associated
necessarily with a conversion of the enzyme from a particulate to
an apparently soluble state, as in the analogous case of the
inhibitor-binding constants in the brain hexokinase reaction (18),
since the effect was observed in particulate preparations obtained
from deoxycholate treatment of the microsomes, as well as in
solubilized preparations. Furthermore, there was no evidence
for the presence of a mixture of kinetic types in disrupted micro-
somes which still contained both soluble and particulate fractions.

The failure of corticoid administration in normal animals to
produce the effects of insulin deprivation on the activity and
kinetics of liver glucose 6-phosphatase, as reported here, is in
accord with the findings of others who measured enzyme ac-
tivity only (5-7). Similar results regarding picolinic carboxylase
activities of livers from diabetic and cortisone-treated animals
were obtained by Meehler et al. (19). (It would be of interest to
know whether there is also an alteration in the kinetics of this
system in which the enzyme is apparently in the soluble fraction
of the homogenate.) In both cases, however, adrenalectomy
reversed the effect of diabetes on enzyme activity. The
explanation for this apparent paradox is at present obscure. The
effect of cortisone administration in increasing glucose 6-phos-
atase activity appears to involve a different mechanism from
that of insulin deprivation, since in the former case there was no
alteration in the kinetic characteristics of the system, and the
degree of increase in activity was considerably less. It is of
interest in this regard that cortisone administration leads to
elevations of several-fold in the activity of a number of enzymes
which are also readily substrate-inducible (20, 21).
SUMMARY

1. The apparent Michaelis constant, and the total activity, of the glucose 6-phosphatase system, which are elevated in diabetic rat livers, were promptly restored to normal by administration of insulin.

2. Cortisone or hydrocortisone administration to normal animals failed to produce the alterations in the glucose 6-phosphatase system characteristic of the diabetic state.

3. The differences observed in the kinetic parameters of the glucose 6-phosphatase system between diabetic and normal liver homogenates persisted in the isolated, washed microsomes from these preparations.

4. A large part of the glucose 6-phosphatase activity of microsomes could be solubilized by treatment with digitonin, Triton X-100, or deoxycholate. Deoxycholate-treated microsomes also yielded a particulate fraction of high specific activity.

5. In disrupted microsomes from diabetic livers, as well as in solubilized enzyme therefrom, the apparent Michaelis constants were markedly reduced from those of the intact microsomes, and the difference in $K'$ values between the normal and diabetic systems was no longer present.

6. Possible interpretations of these results have been discussed.

REFERENCES

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