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# THE ACTIVITY OF GLUCOSE-6-PHOSPHATASE, ADENOSINE TRIPHOSPHATASE, SUCCINIC DEHYDROGENASE, AND ACID PHOSPHATASE AFTER DEXTRAN OR POLYVINYLPYRROLIDONE UPTAKE BY LIVER *IN VIVO*

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**Abstract**—Solutions of the macromolecular substances dextran and polyvinylpyrrolidone were injected intraperitoneally in mice of the $O_{20}$ strain. The activities of glucose-6-phosphatase, adenosine triphosphatase, succinic dehydrogenase, and acid phosphatase in the liver were investigated biochemically and histochemically.

The localization of the macromolecular materials stored in the liver was investigated histochemically.

As a result of the storage of dextran or polyvinylpyrrolidone, the total activity of acid phosphatase was significantly increased compared to that in control animals. The total activity of glucose-6-phosphatase, adenosine triphosphatase and succinic dehydrogenase however, was not significantly changed by such storage. The storage of the injected substances caused enlargement of the liver.

In the liver of control animals acid phosphatase activity, was found predominantly in the cytoplasm of the Kupffer cells. After storage, a rise in acid phosphatase activity was found in the cytoplasm of parenchymal cells. The activity of glucose-6-phosphatase and succinic dehydrogenase was found almost exclusively in the cytoplasm of the parenchymal cells. The activity of adenosine triphosphatase is localized mainly in the cytoplasm of the endothelial cells of the hepatic sinusoids and the small and large vessels of Kierman's portal lobules. After storage of dextran or polyvinylpyrrolidone, no alteration in the activity and in the localization of the three enzymes was found. The macromolecular substances were stored in the cytoplasm or the Kupffer cells and dextran storage was also found in the cytoplasm of the parenchymal cells.

Correlation was found between the biochemically and histochemically demonstrated activities of the four enzymes studied.

AFTER intraperitoneal injection of solutions of macromolecular substances into mice of the $O_{20}$ (Amsterdam) strain, biochemical and histochemical investigations showed that part of the injected substances is stored in the liver and spleen.[1, 2] As a result of this storage, the activity of acid phosphatase and $\beta$-glucuronidase in the liver and spleen was significantly increased.[1, 3, 4]

The storage of macromolecular substances has been related to small cytoplasmic granules, called dense bodies, peribiliary bodies etc.[5–9] This type of granule, as far as it has been studied cyto- and biochemically, has been found to be rich in acid phosphatase and $\beta$-glucuronidase.[10, 11] In our opinion, these granules are possibly identical with the lysosomes studied by de Duve.[11, 12]

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Our investigations are intended to determine whether it is particularly the enzymes present in the lysosomes that are increased by the storage. This suggested a further study to determine whether the activity of enzymes not found in lysosomes shows differences, with regard to storage, from that of the enzymes acid phosphatase (APase) and β-glucuronidase present in the lysosomes. Therefore, in the investigation reported below, the activity of glucose-6-phosphatase (G-6-Pase), adenosine triphosphatase, (ATPase) and succinic dehydrogenase (SDHase) was studied in the liver of control and injected mice of the $O_{20}$ strain (Amsterdam). The presence of these three enzymes in lysosomes has not yet been demonstrated.[12, 13] The activity of acid phosphatase was also determined. The activities of the enzymes were determined biochemically and histochemically. Determinations with the biochemical method yielded quantitative data concerning the possible changes in the activity of the enzymes in tissue homogenates as a result of the storage of the macromolecular substances. These results, however, represent mean values for a variety of cellular elements and extracellular fluids. To translate these results in terms of the structural units of tissue, histochemical methods are used to determine in which of these units the activity is located.

## MATERIALS AND METHODS

Mice of the $O_{20}$ (Amsterdam) strain, from 2 to 3 months old, were used. After intraperitoneal administration of the macromolecular substances, the activities of glucose-6-phosphatase, adenosine triphosphatase, succinic dehydrogenase, and acid phosphatase in the liver were biochemically and histochemically studied. In addition, the cellular storage sites of the macromolecular substances were studied histochemically.

*Macromolecular substances*

1. *Dextran*, (average molecular weight 200,000) from Poviet & Co., Amsterdam.
2. *Polyvinylpyrrolidone*, a polymer of vinylpyrrolidone (average molecular weight 12,600) from Bayer, Leverkusen.

Both substances were used as a sterile 6% solution in 0·9% NaCl.

*Injection scheme*

Four groups of animals were used, each consisting of seven animals. The animals were killed by cervical dislocation on the tenth day after the first injection.

One control group (I) received no treatment. A second control group (II) was given daily intraperitoneal injection of 1 ml 0·9% NaCl solution for 9 days and fasted for 18 hr before being killed on the tenth day. Group III was given daily intraperitoneal injections of 1 ml dextran solution for 9 days and fasted for 18 hr before being killed on the tenth day. Group IV was given daily intraperitoneal injections of 1 ml polyvinylpyrrolidone (PVP) solution for 9 days and fasted for 18 hr before being killed on the tenth day.

*Biochemistry*

*Glucose-6-phosphatase.* Liver samples weighing 100 mg were homogenized for 2½ min in a ground glass Potter–Elvehjem homogenizer with 8 ml citrate buffer solution at ca. 0°. Into an incubation tube 0·1 ml homogenate and 0·1 ml substrate solution

were added, both of which had been preincubated at 37° for 3 min. After incubation at 37° for 10 min, the reaction was stopped by adding 2 ml of 12·5% silicotungstic acid. In another tube a control was identically prepared without incubation. In each tube duplicate determinations of the inorganic phosphate content were made according to Lindberg and Ernster.[14] The activity was estimated by measuring the amount of inorganic phosphate released during the incubation period.

The citrate buffer solution consisted of a $5 \times 10^{-2}$ molar solution containing $1 \times 10^{-3}$ molar versenate at a pH of 6·5. The substrate solution consisted of a $6 \times 10^{-2}$ molar solution of $Na_2$ glucose-6-phosphate in distilled water at a pH of 6·5. Since the activity of G-6-Pase can be strongly inhibited in homogenates by glucose or inorganic phosphate, we used a purified preparation.

The dried sodium salt assays at about 99 per cent, and inorganic phosphate does not exceed 0·5 per cent of the total phosphate.

*Adenosine triphosphatase.* Samples of liver weighing 100 mg were homogenized, as described for the G-6-Pase estimations, with 10 ml Tris–HCl buffer solution. After homogenizing, more Tris–HCl buffer was added to bring the tissue concentration to 100 mg tissue per 50 ml buffer solution. Into an incubation tube 0·4 ml homogenate and 0·4 ml substrate solution were added, both of which had been preincubated at 37° for 2 min. After incubation at 37° for 10 min, the reaction was stopped by adding 2 ml 12·5% silicotungstic acid. In another tube a control was prepared identically without incubation. Duplicate determinations of the phosphate content were made for each tube.

The Tris–HCl buffer solution consisted of a $5 \times 10^{-2}$ molar solution also containing $6 \times 10^{-3}$ molar $MgCl_2$, at a pH of 7·4. The substrate solution was a $1 \times 10^{-2}$ molar solution of ATP in distilled water at a pH of 7·4.

Non-specific phosphatases (acid and alkaline phosphatase) can also split inorganic phosphate from G-6-P and ATP. For this reason it was desirable to determine which part of the free phosphate formed during the incubation period derived from the activity of the non-specific phosphatases. The activity of these non-specific phosphatases was determined under exactly the same incubation conditions as those described for the G-6-Pase and ATPase determinations. The G-6-P substrate solution, however, was replaced by a $6 \times 10^{-2}$ molar $Na_2$ $\beta$-glycerophosphate solution and the ATP substrate solution by a $1 \times 10^{-2}$ molar $Na_2$ $\beta$-glycerophosphate solution. Data in the literature indicate that the rate of hydrolysis caused by the non-specific phosphatases is not constant for the various substrates.[15–17] With purified acid phosphatase and alkaline phosphatase (Sigma, St. Louis, USA), the rates of hydrolysis were investigated for the three substrates under the conditions prevailing during the determinations. The data obtained from these investigations demonstrated that for the G-6-Pase determinations about 1 to 3 per cent of the phosphate liberated during incubation derived from the effect of the non-specific phosphatases; for the ATPase determinations this amount was about 2 to 6 per cent.

*Succinic dehydrogenase.* SDHase activity was determined according to a modification of the method described by Slater.[18] Samples of liver tissue weighing 100 mg were homogenized, as described for the G-6-Pase estimations, with 5 ml, of a succinic acid phosphate buffer solution. After homogenization, 0·2 ml of the homogenate was mixed in a 1-cm cuvette with 2·8 ml of a solution containing $K_3Fe(CN)_6$. The rate of reduction of $K_3Fe(CN)_6$ was colorimetrically measured at 400 m$\mu$ and 20° between

2 and 10 min after mixing the two solutions. The rate of the change in extinction is a measure of the activity of the enzyme. It is not known exactly what components of the succinic oxidase system are involved in the reduction of $K_3Fe(CN)_6$, in all probability the succinic dehydrogenase is the most important of them.[19, 20]

The succinic acid phosphate buffer solution contains $3 \times 10^{-2}$ molar phosphate and $1 \times 10^{-1}$ molar succinic acid at a pH of 7·4. In order to prevent denaturation of the enzyme during homogenization, the tissue was homogenized according to Cooperstein *et al.*,[21] in a buffered solution containing succinate. The concentration of the $K_3Fe(CN)_6$ solution was $1 \times 10^{-3}$ molar. This solution also contained KCN ($1 \times 10^{-2}$ molar) to inhibit cytochrome oxidase, succinic acid ($3 \times 10^{-2}$ molar), and phosphate ($13 \times 10^{-2}$ molar), at a pH of 7·4.

*Acid phosphatase.* Acid phosphatase activity at pH 4·8 was determined as reported previously.[22] Disodium β-glycerophosphate was used as substrate. The product contained 2·1 per cent of the α-isomer (determined according to Toal and Phillips[23]).

The validity of the four methods was verified in control experiments which showed that splitting of the substrates was proportional to both enzyme concentrations and incubation time.

*Histochemistry*

Pieces of liver tissue were quickly frozen on dry ice and series of sections with a thickness of 10 μ were made. The activity of G-6-Pase and ATPase was demonstrated according to Wachstein and Meisel.[24] Activity of SDHase was demonstrated according to Nachlas *et al.*[25] and the demonstration of ATPase according to Gomori.[26] The cryostat sections used for the determination of ATPase and APase were fixed prior to incubation in an aqueous solution of 4% formaldehyde, 2% $CaCl_2$ and 6% dextran (M.W. 18,000). The dextran was added for osmotic protection according to Scarpelli and Pearse.[27]

For the demonstration of dextran, sections were fixed in absolute ethanol and stained with the alcoholic periodic acid Schiff method.[28] We were unable to stain PVP histochemically, but in the haematoxylin-stained sections an accumulation of basophilic substance could be seen in the Kupffer cells. This may indicate the site of the stored PVP.

## RESULTS AND DISCUSSION

*Biochemistry*

The results of the G-6-Pase, ATPase, SDHase and APase determinations are given in Table 1. In order to compensate for fluctuations in hepatic weight and for the use of animals of varying body weight, the specific activity of the enzymes was multiplied by the weight of the intact liver and divided by the body weight of the animal. This calculation normalized the values to give the total enzyme activity in the liver per g body weight.[29] Table 1 gives the activities for the whole organ per g body weight, the activity of the non-specific phosphatases having been substracted.

The data in Table 1 (column 2) show a decrease in the liver weight under the influence of fasting; and, under the influence of the storage of the macromolecular substances, an increase of the decreased weight induced by fasting. The total activity of acid phosphatase in the liver (column 6) does not vary significantly as a result of fasting and injection with 0·9% NaCl solution. Under influence of storage, the

TABLE 1. THE AVERAGE QUOTIENTS Q AND THE AVERAGE ENZYME ACTIVITIES OF THE LIVERS OF THE VARIOUS GROUPS OF MICE (±S.D.)

$$\text{s.d.} = \sqrt{[\Sigma(x - \bar{x})^2/(n - 1)]}$$

| Group | Q* | Activity | | | |
|---|---|---|---|---|---|
| | | G-6-Pase† | ATPase† | SDHase§ | APase† |
| | | pH 6·5 | pH 7·4 | pH 7·4 | pH 4·8 |
| I Controls (without fasting) | 48·9 ± 2·6 | 20·1 ± 1·9 | 51·3 ± 10·2 | 0·195 ± 0·022 | 2·2 ± 0·2 |
| II Controls (0·9% NaCl) | 37·2 ± 2·7 | 22·2 ± 1·8 | 56·6 ± 11·4 | 0·170 ± 0·037 | 2·3 ± 0·3 |
| III Dextran | 47·8 ± 5·7 | 21·6 ± 2·4 | 62·3 ± 12·5 | 0·207 ± 0·035 | 4·9 ± 0·9 |
| IV PVP | 44·9 ± 3·8 | 21·4 ± 2·3 | 59·5 ± 9·8 | 0·198 ± 0·033 | 4·2 ± 0·6 |
| P‖ (groups I and II) | <0·01 | 0·10 > P > 0·05 | 0·40 > P > 0·30 | 0·20 > P > 0·10 | >0·50 |
| P‖ (groups II and III) | <0·01 | >0·50 | 0·20 > P > 0·10 | 0·10 > P > 0·05 | <0·01 |
| P‖ (groups II and IV) | <0·01 | 0·50 > P > 0·40 | 0·50 > P > 0·40 | 0·20 > P > 0·10 | <0·01 |

* $\frac{\text{g(liver)}}{\text{g(mouse)}} \times 1000$ † $\frac{\mu\text{g P(liver)}}{\text{g(mouse) min}}$ § $\frac{\Delta E\text{(liver)}}{\text{g(mouse) min}}$

‖ has the usual connotation of a probability value referring to differences between the several groups of mice.

total activity rises significantly ($P < 0{\cdot}01$). These observations are thus in agreement with those made previously.[1, 3] The total activities of G-6-Pase, ATPase and SDHase also show no significant change ($P > 0{\cdot}05$) under the influence of fasting and injection with 0·9% NaCl solution. The small non-significant rise in G-6-Pase activity is of the same order of magnitude as that found by Freedland and Harper[30] in rat livers after fasting. The storage of the macromolecular substances also failed to effect a significant change ($P > 0{\cdot}05$) in the activity of G-6-Pase, ATPase and SDHase. Thus the activity of the three enzymes absent in lysosomes responds differently to the influence of storage than the activity of the two lysosomal enzymes acid phosphatase and β-glucuronidase.[1] The results of the present study thus support the hypothesis that particularly those enzymes present in the lysosomes, in which in our opinion the storage of the macromolecular substances in all probability takes place, increase in activity as a result of storage.

The homogenates of the livers of the mice injected with dextran or PVP contain small quantities of these macromolecular substances. It was investigated whether the presence of the latter in the various incubation media affects the activity determinations. In a previous study[2] an investigation was made into what quantities of the macromolecular substances dextran and PVP could be stored in the liver with the injection scheme in use. It is thus possible to calculate what concentration of these substances is present in the several incubation media for the groups of mice injected with solutions of the macromolecular substances. By the addition of known quantities of dextran or PVP to homogenates of the livers of untreated mice, it was demonstrated that the quantities of the stored substances were too small to be capable of altering the activities of the enzymes under investigation.

*Histochemistry*

Dextran was found to be stored in the cytoplasm of parenchymal and Kupffer cells in the form of small granules. The parenchymal cells located around the central vein stored more dextran than those lying peripherally. In the sections stained with haematoxylin, a weakly basophilic substance was observed in the cytoplasm of the Kupffer cells of animals which had been injected with PVP. This is probably the location of PVP storage, since the livers of animals which were not injected with this substance did not show this phenomenon.

In the livers of mice which were not injected with dextran or PVP, activity of APase was found predominantly in the cytoplasm of the Kupffer cells; in the cytoplasm of the parenchymal cells only small quantities of APase activity could be demonstrated. In the livers of mice injected with the macromolecular substances, APase activity was high in the cytoplasm of both the Kupffer cells and the parenchymal cells. Thus the activity of APase in these groups of animals differed from the control group in the considerably higher APase activity in the parenchymal cells. Because of the already high activity of APase in the Kupffer cells of control animals, it was impossible to determine whether this activity had risen as a result of the storage.

The storage of dextran or PVP produced no change in the localization and activity of G-6-Pase, ATPase and SDHase. G-6-Pase activity was found exclusively in the cytoplasm of the parenchymal cells, ATPase activity predominantly in the cytoplasm of the endothelial cells of the liver sinusoids and of the small and large vessels of Kierman's portal lobules. In several mice a slight activity of ATPase was observed in

the bile capillaries. The activity of SDHase was localized primarily in the cytoplasm of the parenchymal cells; limited activity was found in the cytoplasm of the epithelial cells of the billiary ducts and the cytoplasm of the Kupffer cells.

Concerning the variations in enzyme activity as a result of storage, it follows from the above observations that the visual determinations of the activity of the several enzymes obtained with histochemical methods correlate with those obtained by biochemical determinations of activity. Both methods showed that under the influence of storage of the macromolecular substances only acid phosphatase increased in activity. This correlation is particularly evident for the activity of the enzymes G-6-Pase and SDHase. Small variations (deviations of about 10 per cent from the average of a given group) in the activities of these two enzymes determined biochemically, can be observed visually with the histochemical methods. For the enzyme SDHase this correlation is noteworthy because the two methods used different substrates as electron acceptors.

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