ENZYME HISTOCHEMISTRY IN EXPERIMENTAL DIABETES MELLITUS

II. FINDINGS ON SEVERAL ENZYME ACTIVITIES IN THE PARENCHYMAL ORGANS, MUSCULAR SYSTEM, INTESTINE, AND ADRENAL GLAND IN DIABETIC RABBITS

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SYNOPSIS

Reported here, are the observations on various histochemical enzyme reactions in several organs (the liver; the kidney; the cardiac, diaphragm, and femoral muscles; the duodenum; the adrenal gland) of rabbits which had been rendered diabetic for from 3 days up to 1 year or more. Independent of the diabetes-inducing reagents (Alloxan or Dithizone) used, the following similar results were obtained: With SD, LD and DPND (which are related to the Krebs cycle activity) and with such phosphatases as ACPase and ALPase, a distinct activity increase could be seen in the liver, the kidney, and the muscular system; except with SD, however, this increase tended to become indistinct or even slightly reduced when 1, 2, or 4 months had elapsed after the induction of diabetes. With G-6-PD (which is related to HMP cycle activity), a distinct decrease in the liver and the kidney could be seen throughout all the diabetic stages. The changes in Ph-rylase activity ran roughly parallel to those in glycogen content, but contradictory changes were found between activity and content in the lipemic heart. Regarding ATPase activity in the lipemic animals, a heavily stained membrane activity could be noted in the liver, the kidney and the cardiac muscle. The lipemic animals also revealed a remarkable increase in non-specific esterase. On the findings in the adrenals, the activities of G-6-PD, SD, ACPase, and ALPase had all been reduced, particularly in the fasciculata when diabetes had persisted for more than 2 or 3 weeks, and ran fairly parallel with the urinary 17-KS and 17-OHCS contents.

In addition to these findings, such interesting phenomena were noted as the tendency for the specific zonation of hepatic enzyme distribution to become obscure, the presence of an intimate relation between the activity changes in the liver and the kidney, and the presence of relatively specific activity distribution patterns in the various organs of rabbits having sustained experimental congenital diabetes for more than 1 year.

The specific cytotoxic effects of alloxan on pancreatic islets were first studied by Dunn, Sheehan and McLetchie (1943). Thereafter, Brunschwig et al. (1943) and Bailey and Bailey (1943) reported the inducement of a persistent diabetic state in animals. This milestone was soon followed by a wealth of studies and dis-

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coveries concerning experimental pancreatic diabetes, i.e., absolute insulin deficiency.

The investigation of this subject has been conducted on two different themes: The one theme concerns the mechanism of how alloxan and other zinc-combining reagents selectively destroy islet cells, and is represented by the work of Okamoto (1949, 1951 and 1965) and his associates who produced experimental diabetes by using dithizone, oxine and other reagents, and who established the unique theory that these reagents, when administered to animals by injection, reach the islet cells and react with the available zinc to produce their cytotoxic effects. Lazarow (1963) established a differing theory that alloxan, by reacting with the dithiol site of the cell membrane, alters the membrane structure to increase permeability, and thereby produce serious metabolic alterations and B-cell death. The other theme concerns metabolic abnormalities in diabetic animals, and is represented by biochemical, pathophysiological and experimental pathological studies. Especially numerous are the biochemical studies on enzyme activities in the diabetic liver, but studies on other organs (kidney, skeletal muscles, endocrine organs, etc.) are scanty because of the great biochemical difficulties encountered due to the lack of structural uniformity in these organs.

However, the advent of the histochemistry of enzyme activity, originated by Takamatsu (1938 and 1939) and Gomori (1939) separately, has led to many improvements in methodology. The present author has here attempted to elucidate morphologically the metabolic abnormalities of experimental diabetes by making use of these newly available histochemical methods and by stressing:

1. Changes in each phase of enzyme activity in the various organs of animals which have been rendered diabetic for from 3 days to 1 year or more.

2. Findings in the parenchymal organs (liver and kidney), in the cardiac and the skeletal muscles (diaphragm and quadriceps femoris), in the endocrine organ (adrenal gland).*

MATERIALS AND METHODS

The present study was conducted on New Zealand white rabbits of either sex, 6 to 18 months old, weighing 2 to 3.5 kg (rabbits in which diabetes had persisted for more than 1 year, however, were 18 months to 3 years old). They had either been bred in our laboratory or kept there for more than 1 week after purchase from a dealer. Their diets consisted of bean-curd refuse, carrots, oats, and weeds.

Methods of inducing alloxan and dithizone diabetes

The experimental animals were fed only bean-curd refuse for 3 or 4 days and starved for at least 12 hrs. prior to the administration of either of the following reagents.

1. Alloxan monohydrate (Eastman Kodak) 100 mg/kg as a 5% aqueous solution.
2. Diphenyl thiocarbazone (dithizone) (E. Merck) 100 mg/kg as an aqueous ammoniac solution (Okamoto, 1951).

Hypoglycemic convulsion was prevented by previous intravenous injections of glucose every 2 hrs. Urinary sugar was measured every morning by Benedict’s reagent (Kanai, 1958) or sometimes by “Test-tape” (Lilly) (Ando et al., 1959; Baba et al., 1958). All animals selected for the present experiment presented at least a very strong (++) positive urinary sugar reaction,

* Findings in the pancreatic islet cells will be reported in another paper (Ihara, 1965).
i.e., sustained diabetic condition. When the animals began to show a decrease in appetite during the diabetic course, it was assumed that lipemia or pneumonia might be present, and such animals were repeatedly given 2 units of protamine zinc insulin and mycillin.*

Diabetic animals showing an apparent weight loss were given more bean-curd and weeds, while animals sustaining a relatively slight diabetic state were given more oats. Then, animals which had sustained the established diabetic condition for various prescribed periods of time were sacrificed by air embolism early in the morning. The number, sex, periods of established diabetes, and blood sugar levels after Somogyi's method (Saito, 1953) just before sacrifice are shown in Table 1. Twenty-eight untreated rabbits were used as controls and were sacrificed concurrently with those treated with the diabetogenic substances.

Table 1. Animals sacrificed for the experiment

<table>
<thead>
<tr>
<th>No. of the diabetic animals</th>
<th>Methods for production of experimental diabetes</th>
<th>Durations of diabetes [d: days, w: weeks, m: month(s), y: year(s)]</th>
<th>Blood sugar levels (mg/dl) just before sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 ♀</td>
<td>Alloxan</td>
<td>3 d.</td>
<td>420</td>
</tr>
<tr>
<td>A3 ♀</td>
<td>Alloxan</td>
<td>3 d.</td>
<td>292</td>
</tr>
<tr>
<td>A32♀</td>
<td>Alloxan</td>
<td>3 d.</td>
<td>313</td>
</tr>
<tr>
<td>A5 ♂</td>
<td>Alloxan</td>
<td>7 d.</td>
<td>326</td>
</tr>
<tr>
<td>A6 ♀</td>
<td>Alloxan</td>
<td>7 d.</td>
<td>500</td>
</tr>
<tr>
<td>A7 ♀</td>
<td>Alloxan</td>
<td>10 d.</td>
<td>350</td>
</tr>
<tr>
<td>A9 ♂</td>
<td>Alloxan</td>
<td>2 w.</td>
<td>294</td>
</tr>
<tr>
<td>A11♂</td>
<td>Alloxan</td>
<td>2 w.</td>
<td>512</td>
</tr>
<tr>
<td>A30♂</td>
<td>Alloxan</td>
<td>2 w.</td>
<td>216</td>
</tr>
<tr>
<td>A12♀</td>
<td>Alloxan</td>
<td>3 w.</td>
<td>264</td>
</tr>
<tr>
<td>A14♀</td>
<td>Alloxan</td>
<td>3 w.</td>
<td>310</td>
</tr>
<tr>
<td>A15♂</td>
<td>Alloxan</td>
<td>1 m.</td>
<td>323</td>
</tr>
<tr>
<td>A16♀</td>
<td>Alloxan</td>
<td>1 m.</td>
<td>244</td>
</tr>
<tr>
<td>A18♂</td>
<td>Alloxan</td>
<td>2 m.</td>
<td>348</td>
</tr>
<tr>
<td>A20♀</td>
<td>Alloxan</td>
<td>2 m.</td>
<td>236</td>
</tr>
<tr>
<td>A23♂</td>
<td>Alloxan</td>
<td>4 m.</td>
<td>402</td>
</tr>
<tr>
<td>A25♀</td>
<td>Alloxan</td>
<td>4 m.</td>
<td>268</td>
</tr>
<tr>
<td>F1 ♂</td>
<td>Alloxan*</td>
<td>1 y.</td>
<td>440</td>
</tr>
<tr>
<td>F2 ♂</td>
<td>Alloxan*</td>
<td>1 y.</td>
<td>256</td>
</tr>
<tr>
<td>F3 ♂</td>
<td>Alloxan*</td>
<td>2 y.</td>
<td>219</td>
</tr>
<tr>
<td>F4 ♀</td>
<td>Alloxan*</td>
<td>2 y.</td>
<td>282</td>
</tr>
<tr>
<td>F5 ♀</td>
<td>Alloxan*</td>
<td>2 y.</td>
<td>296</td>
</tr>
<tr>
<td>D1 ♂</td>
<td>Dithizone</td>
<td>1 w.</td>
<td>410</td>
</tr>
<tr>
<td>D2 ♂</td>
<td>Dithizone</td>
<td>2 w.</td>
<td>306</td>
</tr>
<tr>
<td>D12♂</td>
<td>Dithizone</td>
<td>2 m.</td>
<td>380</td>
</tr>
<tr>
<td>D20♀</td>
<td>Dithizone</td>
<td>2 m.</td>
<td>236</td>
</tr>
<tr>
<td>A24♂</td>
<td>Alloxan**</td>
<td>1 w.</td>
<td>788</td>
</tr>
<tr>
<td>A31♂</td>
<td>Alloxan**</td>
<td>2 w.</td>
<td>806</td>
</tr>
</tbody>
</table>

* Treated with alloxan (60 mg/kg) after recovery from congenital diabetes mellitus induced experimentally (Okamoto, 1960).
** Affected with lipemia after the induction of diabetes.

* penicillin and streptomycin.
**Enzyme histochemical methods**

The organs or tissues (the liver and the kidney; the cardiac, diaphragm, and femoral muscles; the intestine, and the adrenal glands) were removed immediately after sacrifice, and from them small pieces were taken and frozen in petroleum ether kept at $-70^\circ$C in a bath of carbon dioxide (dry ice) and acetone. Cryostat sections at 10 $\mu$ were affixed to slides or coverslips and stained by the following enzymatic histochemical techniques:


For the staining of the liver, 25% reagent alcohol was added to the substrate solution as indicated by Takeuchi and Kuriaki (1956).

1b. Glycogen staining: in addition to the histochemical detection of Ph-rylase, glycogen staining was carried out on the liver, and kidney, and on the cardiac, diaphragm, and femoral muscles of several animals. Immediately after removal, the tissue blocks were fixed in the Carnoy solution that Lison (1960) recommended. The PAS technique together with salivary digestion test was applied. Thus, the findings of Ph-rylase staining and of glycogen staining were compared.

2. Succinic dehydrogenase (SD): by the method of Wachstein and Meisel (1954) or Nachlas et al. (1957).

3. Diphosphopyridine nucleotide diaphorase (DPND) and Lactic dehydrogenase (LD): by the method of Nachlas et al. (1958a). In place of sodium lactic dehydrogenase and DPN, an equivalent mol of DPNH was used.

4. Glucose-6-phosphate dehydrogenase (G-6-PD): by the method of Nachlas et al. (1958b).

5. Acid phosphatase (ACPase): by the method of Gomori (1950a) (lead nitrate method) and the standard coupling azo-dye technique of Grogg and Pearse (Pearse, 1961a).

For the coupling azo-dye technique, naphthanyl diazo blue B was used as a coupling reagent. In either case, frozen sections were first fixed in 6% CaCO$_3$-neutral formol for 30 mins. and then incubated for from 1 to 2 hrs.


7. Glucose-6-phosphatase (G-6-Pase): by the method of Chiquoine as modified by Wachstein and Meisel (1956).

8. Adenosine triphosphatase (ATPase): by the method of Padicula and Herman (1955a and b). After the sections were fixed in 3% neutral formol including 0.005 mol-MgCl$_2$ for 15 mins. (Lazarus and Barden, 1962), they were incubated in the substrate solution.

9. Non-specific esterase: by the method of Gomori (1950b) (Pearse, 1961b), and using $\alpha$-naphthyl acetate as a substrate. Unfixed frozen sections were incubated in the medium.

**RESULTS**

In order to represent the enzyme activity levels the following signs have been used in this paper:

- : Very slight reaction
+ : Slight but distinct reaction
++ : Somewhat strong reaction
### : Moderately strong reaction
#### : Very strong reaction
##### : Extremely strong reaction
1a Phosphorylase (Ph-rylase)

A. Alloxan diabetes
(1) Diabetes established for 3 days
   General and remarkable changes could already be seen.
   The diabetic liver: The activity was so remarkably reduced that reactive
cells could hardly be seen throughout all the lobules (Figs. 1a and b).
   The diabetic kidney: A significantly intensified reaction was observed in the
   collecting tubules or Henle’s thick limbs of the medullary rays. There was also
   an apparent increase in the collecting tubules of the marginal zone,* but no distinct
   activity changes in the intermediate and the papillary zones because these portions
   presented a very strong reaction even in the normal kidney.
   The diabetic muscles: The cardiac muscle showed a markedly increased
   activity throughout all the areas; the changes in the outer layer of the cardiac
   wall were more prominent since normally the activity in this layer is somewhat
   weaker than that in other portions. These findings were obtained in all the
diabetic animals through all the stages, except in the lipemic animals whose findings
will be described below. In contrast to the cardiac muscle, the diaphragm dis-
played a tendency for the reactivity to decrease although the percentage of muscle
fibers showing a distinct staining reaction was somewhat similar to that of the
control animals. Amongst these distinctly reactive fibers some rather weakly
reactive ones appeared in various numbers so that clear difference between the
two groups of the fibers observable in normalcy (that is, remarkably reactive ones
and very weakly reactive ones) could not be found. However, these changes in
the diaphragm were not so remarkable and constant as those seen in the cardiac
muscle.

   On the other hand, the quadriceps femoris presented a more or less different
reaction from that in the diaphragm. Either a slight reduction in parallel to
that found in the diaphragm or almost no change was observed. The reduction
in activity was estimated by the reduced percentage in the number of distinct
positive fibers and a tendency for uniform reactivity to disappear.

(2) Diabetes established for 7 days, 10 days, 2 weeks, and 3 weeks
   The diabetic liver: Clearly showed a reduced enzyme activity throughout
these stages without exception. The higher the blood sugar level, the more re-
markable the changes.
   The diabetic kidney: Activity changes similar to those found in diabetes
established for 3 days were observed, chiefly in the medullary rays of cortex.
Presumably there was a progressive intensification in activity from the collecting
 tubules to the distal convolutions as well as to the Henle loops. The activity in

* To facilitate the description of the localization patterns of enzyme activities in the rabbit
kidneys, the following zonation of the medulla will be used: the marginal zone, the intermediate
zone and the papillary zone from cortical surface to renal pelvis.
the smooth muscle cells of the arciform arteries was found to parallel with that in the tubules in some cases.

The diabetic cardiac muscle: The activity changes were found similar to those seen in the previous stage.

The diaphragm: A reduction similar to that seen in diabetes established for 3 days was observed in almost all cases. There was a tendency for the activity changes to correlate with the blood sugar levels.

The quadriceps femoris: A tendency similar to that seen in the former group was shown, but one case (A11) presented an intensified reaction: It was found that the strongly reactive fibers had increased in proportion to the weakly reactive fibers, which fibers also showed an increase in enzyme activity.

(2a) Diabetes under the lipemic condition 1 or 2 weeks after the induction of diabetes mellitus (A24 and A31)

These 2 cases suffered from an extreme lack of appetite 1 and 2 weeks respectively after the occurrence of diabetes. The venous blood showed a milk-white colored lipemic turbidity visible even to the naked eye.

The activity changes in the liver and the diaphragm was of the same pattern as that seen in cases not complicated with lipemia, through each corresponding stage. In the quadriceps femoris on the contrary, there appeared no marked change (A31), or significant increase in activity (A24).

In spite of the above findings, the kidney and the cardiac muscle of this group presented specific findings: the former showed no distinct increase in activity, and the latter showed a rather slight decrease (Figs. 2a and b). No correlation could be found between the distribution of Ph-rylase activity and glycogen content in these organs, because an associated increase rather than a decrease was found in the histochemically demonstrable glycogen (Figs. 3a and b). The thick limbs of Henle in the kidney and the cardiac muscle fibers showed a remarkable deposition of fat droplets, and this finding correlated with the reduced enzyme activity. Furthermore, these droplets were also deposited in the arterial smooth muscle fibers of the cardiac wall, which also showed correlative changes in enzyme activity.

(3) Diabetes established for 1, 2 or 4 months

The diabetic liver: The activity showed an evident recovery towards a normal level during these stages, a few cases still presented a distinct reduction, and others, a reaction similar to that seen in the controls (relatively so many cases) i.e., an intensified reaction. Although a strict correlation could not be obtained between blood sugar levels and enzyme activity, cases (A20 and A25) showing a significant recovery in blood sugar level showed the same tendency in enzyme activity level.

The diabetic kidney: showed a reaction similar to that seen in diabetes established for from 1 to 3 weeks.
The diaphragm: About half of the cases ceased to show any distinct reduction in activity and one (A20♀) case even showed an increase.

The heart and the quadriceps femoris: A tendency similar to that seen in the previous stages was found.

(4) Diabetes established for more than 1 year.

The diabetic liver: The findings were somewhat indefinite; two cases (F1♂ and F5♀) presented an apparent reduction in activity, two cases (F2♂ and F3♂), presented an intensified reaction in the central or peripheral zones as well as in the intermediate zone, and one case showed a distribution pattern similar to that seen in the controls.

The diabetic kidney and the cardiac muscle: Almost the same reaction as that seen in all the preceding stages was found.

The diaphragm: A distinctly reduced activity was shown in all but one case (F3♂), which yielded an indistinct change in enzymatic reaction compared with that of the controls.

The quadriceps femoris: The changes were similar to those found in cases established for from 1 to 3 weeks.

B. Dithizone diabetes

The finding were similar to those found in alloxan diabetes in each corresponding stage.

1b. The finding on glycogen staining

The question dealt with here is: To what extent can a correlation between enzyme activity and histochemically demonstrable glycogen be found in the various organs or tissues?

(1-2) Diabetes established for 3 days (A1♂), 1 week (A6♀), and 3 weeks (A14♂)

Liver: A good correlation was found; there was a remarkable decrease both in enzyme activity and in glycogen content, and the lobular distribution patterns of the positive hepatic cells were similar. However, the correlation between each individual cell is unknown, because no investigation on serial sections was carried out.

Kidney: A distinct parallel between both findings could also be seen in this organ. But, there was relatively a large increase in glycogen content compared with the increase in enzyme activity during these and the later stages described below. Glycogen was found to have increased even in the collecting tubules of the intermediate and the papillary zones (A1♂), and also in the limbs of Henle of the medullary rays and the marginal zone (A6♀ and A14♂).

The cardiac muscle: Both activity and content showed a good parallel in respect to their histological distribution. However, some contrasting results were
obtained in so far as the distribution pattern in each fiber was concerned. The Ph-rylase staining showed a characteristic distribution of alternating stronger and weaker reactions within each individual segment or fiber. On the other hand, the glycogen staining showed a significant variation in the densities of the reaction granules amongst the respective segments. A similar relationship was also found in the diabetic cardiac muscle.

The diaphragm: There was a good correlation between both findings on the histological level, but some dissonance with regard to each individual fiber, that is, on the cytological level. An outline of the results obtained is as follows:

<table>
<thead>
<tr>
<th>Glycogen staining</th>
<th>Control sections</th>
<th>Diabetic sections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Almost no reactive glycogen: Abou 1/10 of the total fibers</td>
<td>(a) No changes</td>
</tr>
<tr>
<td></td>
<td>Strongly or somewhat weakly positive fibers: The remainders</td>
<td>(b) Uniform reduction in content</td>
</tr>
<tr>
<td>Ph-rylase staining</td>
<td>Almost no reaction on the whole length of the fibers: Less than 1/10 of the total fibers</td>
<td>(c) No changes</td>
</tr>
<tr>
<td></td>
<td>Strongly reactive fibers: About 1/2~1/3 of the total fiber</td>
<td>(d) Somewhat weakly reactive fibers</td>
</tr>
<tr>
<td></td>
<td>Weakly reactive fibers: The remainders</td>
<td>(d’) More weakly reactive fibers</td>
</tr>
</tbody>
</table>

The (a) fibers presumably correspond to the (c) ones, while the (b) fibers, to the (d) and the (d’) ones.

Regarding the individual fibers, the Ph-rylase staining presented a banded distribution pattern of strong and weak reactions, so that no sharp distinction could be drawn between the strongly reactive fibers and the weakly reactive ones. In the diabetic cases, most of these “strongly reactive bands” in a fiber had disappeared, having presumably been altered to weakly reactive ones.

Quadriceps femoris: The fibers of this muscle generally contained more dense glycogen granules than did the diaphragm muscle. The number of fibers showing a low glycogen content was less than 10% of the total number as was the case with the diaphragm. The remaining fibers were distinctly positive and the strongly reactive ones accounted for about 50%.

In the diabetic cases, if there was any change, it was a slight increase in glycogen content which was indicated by an increase in the numerical ratio of strongly reactive fibers. But, there also appeared a tendency toward a relatively irregular distribution of the glycogen granules.

(2a) Diabetes under the lipemic condition (A24Γ and A31Γ)
Liver and kidney: Intracellular distribution of glycogen granules were ser-
ously disarranged in the central and the intermediate zones of the diabetic liver and in the thick limbs of Henle of the kidney because small and large fat droplets were abundantly deposited in these portions.

Cardiac muscle: A striking deposition of numerous fat droplets and an even more striking glycogen reaction were noted in comparison with the controls. This was more remarkable in the outer layer of the cardiac wall. The glycogen granules became more coarse and, in addition, their intracellular distribution varied quite markedly even in each individual fiber by showing a more remarkably and irregularly banded distribution pattern on the longitudinal sections. Massive or diffuse distributions of such granules were also demonstrable on the cross section, excluding the cytoplasmic portions where lipid vacuoles had been formed (Figs. 3a and b).

On the other hand as described above, a significant reduction in Ph-rylase activity was demonstrated throughout all the layers of the diabetic heart. These facts indicate an unbalance between histochemically demonstrable glycogen and enzyme activity. (Figs. 2a and b).

Diaphragm: The findings were similar to those of diabetes not complicated with lipemia.

Quadriceps femoris: As could be seen in the diabetic (non-lipemic) cardiac muscle the glycogen granules became significantly coarse, and they became somewhat irregular in their distribution within the individual fibers owing to the deposition of many fat droplets. The glycogen content, being significantly increased, correlated roughly with the enzyme activity level.

(3) Diabetes established for from 2 to 4 months (A182 and A232)

Liver: There was a fairly remarkable reduction in content throughout all the lobules, especially in the peripheral (A182) or the central region (A232). However, it showed a distinct recovery as it was not so severe as in diabetes established for from 3 days to 3 weeks. On the other hand, a fairly strong Ph-rylase activity was already shown, which was only slightly reduced in the peripheral or the central region of the hepatic lobules. This fact indicates that the enzyme activity showed a more marked recovery towards the normal level than did the glycogen content. And besides, cells yielding an especially strong glycogen reaction were found to be scattered in the lobules. They seemed to correspond to the cells which yielded a bluish-violet staining reaction for Ph-rylase.

Kidney: The findings were similar to those seen in the previous stages.

2. Succinic dehydrogenase (SD)

A. Alloxan diabetes

(1) Diabetes established for 3 days

A complete diabetic distribution pattern was already found in the various organs examined.

Liver: The activity was found to be markedly increased throughout all the
Table 2. Enzymatic activity changes in the liver

<table>
<thead>
<tr>
<th>Enzyme activities</th>
<th>Diabetics or controls</th>
<th>Durations</th>
<th>Activity changes in the hepatic lobules</th>
<th>Activity changes in the Glisson's capsules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Peripheral zone</td>
<td>Intermediate zone</td>
</tr>
<tr>
<td>Contr.</td>
<td></td>
<td>3d.~3w.</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Diab.</td>
<td></td>
<td>1~4 m.</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1~4 y.</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Contr.</td>
<td></td>
<td>3d.~3w.</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Diab.</td>
<td></td>
<td>1~4 m.</td>
<td>↑ or→ or ↓</td>
<td>↑ or→ or ↓</td>
</tr>
<tr>
<td>Contr.</td>
<td></td>
<td>3d.</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Diab.</td>
<td></td>
<td>1~3 w.</td>
<td>↓↓</td>
<td>↓↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1~4 m.</td>
<td>↓↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>Contr.</td>
<td></td>
<td>3d.~3w.</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>ACPase</td>
<td></td>
<td>1~2 w.</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1~2 y.</td>
<td>↑</td>
<td>↑ or→</td>
</tr>
</tbody>
</table>
### ALPase

<table>
<thead>
<tr>
<th></th>
<th>Contr.</th>
<th>3d.~3w.</th>
<th>1~2w. (with lipemia)</th>
<th>1~4m.</th>
<th>1~2y.</th>
<th>Contr.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3d.~3w.</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>1~2w.</td>
<td>→</td>
<td>↓</td>
<td>↓</td>
<td>→</td>
<td>→ or ↓</td>
</tr>
<tr>
<td></td>
<td>1~4m.</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>→</td>
<td>→</td>
</tr>
<tr>
<td></td>
<td>1~2y.</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>→</td>
<td>→</td>
</tr>
</tbody>
</table>

### ATPase

<table>
<thead>
<tr>
<th></th>
<th>Contr.</th>
<th>3 d.</th>
<th>2 w. (with lipemia)</th>
<th>2 m.</th>
<th>3 d.</th>
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<tbody>
<tr>
<td></td>
<td>3 d.</td>
<td>↓ or→</td>
<td>↓ or→</td>
<td>↓ or→</td>
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<tr>
<td></td>
<td>2 w.</td>
<td>↓</td>
<td>↓</td>
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<td>↑ or→</td>
<td>↑ or→</td>
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### Esterase

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<th>1 m.</th>
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<tbody>
<tr>
<td></td>
<td>2 w.</td>
<td>↑</td>
<td>↑</td>
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</tbody>
</table>

**Symbols:**
- †††: Grades of enzyme activities as illustrated in p. 4.
- ↑, ↑: Apparent changes in activities.
- ↓↓, ↑↑: Remarkable changes in activities.
- ↓, ↑: Very slight changes.
- →: Indistinct or little changes.
- Contr.: Controls.
- Diab.: Diabetics.
- d.: days.
- w.: weeks.
- y.: years.
- B.C.: Bile ducts.
- S. L.: Sinusoidal linings.
### Table 3. Enzymatic activity changes in the kidney

<table>
<thead>
<tr>
<th>Enzyme activities</th>
<th>Diabetics or controls</th>
<th>Durations</th>
<th>R.C.</th>
<th>Segments of the renal tubule</th>
<th>Vessels</th>
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<tr>
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<td>+</td>
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<tr>
<td>3d ~ 3w.</td>
<td></td>
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<td>→</td>
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<tr>
<td>Diab.</td>
<td></td>
<td></td>
<td>→</td>
<td>→</td>
<td>↑↑</td>
</tr>
<tr>
<td>1 ~ 2w. (with lipemia)</td>
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<td></td>
<td>→</td>
<td>→</td>
<td>↑</td>
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<tr>
<td>1 ~ 4m.</td>
<td></td>
<td></td>
<td>→</td>
<td>→</td>
<td>↑</td>
</tr>
<tr>
<td>1 ~ 2y.</td>
<td></td>
<td></td>
<td>→</td>
<td>→</td>
<td>↑</td>
</tr>
<tr>
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<td>+~ +</td>
<td>+</td>
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<tr>
<td>3d ~ 3w.</td>
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<td>↑</td>
<td>↑</td>
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<tr>
<td>Diab.</td>
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<td></td>
<td>→</td>
<td>→</td>
<td>↑ or→</td>
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<tr>
<td>1 ~ 4m.</td>
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<td>Diab.</td>
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<td>1 ~ 3w.</td>
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<td>↑ or→</td>
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<tr>
<td>1 ~ 2w. (with lipemia)</td>
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<td>↑</td>
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<tr>
<td>1 ~ 3w.</td>
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<td></td>
<td>→</td>
<td>→</td>
<td>↑</td>
</tr>
<tr>
<td>1 ~ 4m.</td>
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<td>→</td>
<td>→</td>
<td>↑</td>
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<tr>
<td>1 ~ 2y.</td>
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<td>→</td>
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<tr>
<td></td>
<td>Contr.</td>
<td>3 d.</td>
<td>1 ∼ 2 w. (with lipemia)</td>
<td>Diab. 1 ∼ 3 w.</td>
<td>1 ∼ 4 m.</td>
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<tr>
<td>ALPase</td>
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<td>↑</td>
<td>↑ or → ↑</td>
<td>↓ or ↓</td>
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<tr>
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<td>+ ~ # (P.Z.) ↑ + ↑ + + + ~ #</td>
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</tr>
</tbody>
</table>

\(↑ ∼ ↪\) : Grades of enzyme activities as illustrated in p. 4.

\(↓, ↑\) : Apparent changes in activities.

\(↓↓, ↑↑\) : Remarkable changes in activities.

\(↓, ↑\) : Very slight changes.

\(→\) : Little changes.

Contr. : Controls.

Diab. : Diabetics.

d. : days.

w. : weeks.

y. : years.

R.C. : Renal corpuscle.
lobules of the diabetic liver, especially in the central zone as can be seen in Table 2. Accordingly, the zonal distribution pattern observable in the normal liver had become indistinct. Regarding intracellular distribution, somewhat coarse bluish granules, as well as densely deposited fine purplish ones, appeared and were deposited in the peripheral zone.

Kidney: There was a markedly increased activity in all portions of the nephrons which would show a distinct reactivity in normalcy. This activity was especially remarkable in the cortex and the marginal zone of the medulla. Concerning cellular and the segmental distribution, formazan granules were more densely deposited in the proximal convoluted and straight portions, bluish coarse granules being intermingled at the same time. Activity distribution in the individual segments or portions of the tubule is shown in Table 3.

Cardiac muscle: The activity changes could not be judged accurately, for the formazan granules deposited were somewhat coarser and sparsely arranged. However, there were some fibers in which blue-toned granules appeared in abundance.

Skeletal muscles: In the diaphragm, as could be seen in the cardiac muscle, the deposited granules became relatively coarse and were somewhat sparsely arranged, so that the muscular fibers appeared to be somewhat lighter, compared with the normal section, although the ratio of distinctly reactive fibers in which bluish coarse granules were deposited was increased and they accounted even for 1/4~1/3 of the total reactive fibers in some cases. A similar tendency was also observed in the quadriceps femoris.

Adrenal gland: An intensified reaction was shown throughout all the cortical zones (Table 4), especially in the zona glomerulosa and fasciculata (A12) or in the zona fasciculata and reticularis (A32).

(2) Diabetes established for 7 days, 10 days, 2 weeks and 3 weeks

Liver: The findings were similar to those seen in diabetes established for 3 days. The higher the blood sugar levels, the more marked the observed increase in activity.

Kidney: A remarkably increased reaction was observed in 6 out of the 8 cases examined (Figs. 4a and b). The other 2 cases showed no distinct variation in activity (A12) but for a slight increase in the proximal tubules (A30). The diabetic animals which presented a marked increase in the kidney presented more marked changes in the liver so that the findings in the one generally correlated with those in the other. The results are summarized in Table 3. In almost all portions where intense reaction was observed, abundantly deposited bluish coarse granules were found.

Cardiac muscle: It was impossible to judge the activity changes accurately. Many cases seemed to show very a slight decrease in activity, but in one case, a slight increase was noted.

Skeletal muscles: The formazan granules deposited became coarser and were more sparsely arranged, but in about 1/5 of the fibers abundant coarse bluish granules appeared together with the purplish, relatively fine granules, so that
further investigation is required to judge the activity level on the whole muscle section. Similar findings were also noted in the quadriceps femoris.

Adrenal gland: In diabetes established for from 7 to 10 days, almost no change (A6♀ and A7♂) or only a very slight increase in the zona fasciculata was observed. In cases established for 2 or 3 weeks, there appeared a more or less distinct reduction in the whole cortex, especially in the fasciculata (except in case A9♂) (Table 4).

(2a) Diabetes under the lipemic condition

Liver: The reaction was so remarkable that a deposition of abundant bluish coarse granules appeared throughout all the lobules. In the centrilocular cells, there was an equivocal appearance of fat droplets, in the border of which such formazan granules were abundantly deposited. In the bile ducts, especially in the ductular epithelia, a marked reactivity was also observed.

Kidney: As can be seen in the Table 3, there appeared a more marked increase in activity than that seen in the former group as mentioned above. Bluish granules were abundant in the tubules, especially in the thick limbs of Henle and the distal convolutions wherein many fat droplets had appeared.

Cardiac muscle: The deposited formazan granules were seriously disarranged owing to the deposition of fat droplets and had become coarser and blue-toned. Accordingly, it was very difficult to accurately judge the enzyme activity level (Figs. 5a and b).

Skeletal muscles: The findings were similar to those seen in diabetes not complicated with lipemia in these stages.

Adrenal gland: A distinct reduction could be found in the 1 case (A31♂) investigated.

(3) Diabetes established for 1, 2, and 4 months

Liver and kidney: As is shown in Tables 2 and 3, the findings were similar to those seen in diabetes established for from 7 days to 3 weeks.

Cardiac muscle: There also appeared deposits of bluish coarse formazan granules as seen in the previous stages. This reaction was regarded as an increase in activity for 7 cases. But, it was impossible to judge accurately the activity level for the other 2 cases (A18♀ and A23♂), because the granules were somewhat sparsely deposited.

Skeletal muscles: Changes were found to correlate with those in the cardiac muscle.

Adrenal gland: A slight decrease was observed in the cortex, especially in the fasciculata, secondarily in the reticularis with the exception of 1 case (A20♀) (Table 4).

(4) Diabetes established for more than 1 year (F-rabbits)

Liver and kidney: The findings were similar to those seen in the other groups
Table 4. Enzymatic activity changes in the adrenal gland

<table>
<thead>
<tr>
<th>Enzyme activities</th>
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<th>Medulla</th>
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<td>Z. fasc.</td>
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<td>+++</td>
<td>+++</td>
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<td>Diab.</td>
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<td>↑</td>
<td>↑↑</td>
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<td></td>
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<td>2 ~ 3 w.</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 ~ 4 m.</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>Contr.</td>
<td></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 d.</td>
<td>→</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 ~ 3 w.</td>
<td>↓</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>1 ~ 4 w.</td>
<td>↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>G-6-PD</td>
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<td></td>
<td>+++</td>
<td>+++</td>
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<td>1 ~ 2 w.</td>
<td>↑</td>
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<td>(with lipemia)</td>
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<td>3 d.</td>
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<td>1 ~ 2 w.</td>
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<td></td>
<td>(with lipemia)</td>
<td>1 ~ 3 w.</td>
<td>→</td>
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<td>1 ~ 4 m.</td>
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<tr>
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<td>+++</td>
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<td>1 ~ 3 w.</td>
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<td>1 ~ 4 m.</td>
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<td>↓↓</td>
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<td>1 ~ 2 y.</td>
<td>→</td>
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<td>3 d.</td>
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<td>(with lipemia)</td>
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<tr>
<td></td>
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<td>1 m.</td>
<td>→</td>
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</tbody>
</table>

++~+++: Grades of enzyme activities as illustrated in p. 4.  Contr.: Controls.
↓, ↑: Apparent changes in activities.  Diab.: Diabetics.
↓↓, ↑↑: Remarkable changes in activities.  d.: days.
↓, ↑: Very slight changes.  w.: weeks.
→: Indistinct or little changes.  y.: years.
S.L.: Sinusoidal linings.
(Tables 2 and 3). A good correlation was also found between these organs. However, 2 cases (F1 and F4) showed a slight reduction in the renal tubules owing to the occurrence of nephrosis.

Cardiac and skeletal muscles: A distinctly reduced activity was observed. The formazan granules deposited became coarser and were more sparsely arranged. One case (F3) showed no distinct changes.

B. Dithizone diabetes

The findings in dithizone diabetes established for from 1 week to 2 months were found similar to those seen in alloxan diabetes in each correspondent stage.

3. Lactic dehydrogenase (LD) and diphosphopyridine nucleotide diaphorase (DPND)

A. Alloxan diabetes

Similar results were generally obtained for both the LD and DPND activities. Therefore, the LD findings will be described first and only the points of DPND reaction differing from those of LD will be described in addition.

(1) Diabetes established for 3 days

A typical diabetic distribution pattern had already been formed.

Liver: As can be seen in Table 2, a markedly intensified reaction was observed in the parenchymal cells throughout the whole of the lobules. There was a deposit of somewhat coarse blue-toned granules together with fine deep purplish ones observable in the normal liver. In the bile duct, especially in the ductular epithelial cells contiguous to the peripheral zone, an clear increase was found but it was not so remarkable as that found in the parenchymal cells. Similar changes were also found in DPND activity.

Kidney: As shown in Table 3, in all the tubular segments, observable changes were more or less in parallel with those in the liver. The deposit of coarse blue-toned granules were also present in every portion in which enzyme activity had been remarkably intensified. An observable increase was also found in the interstitial cells in both the cortex and the medulla as well as in the arterial wall. Similar but more remarkable changes in DPND activity could be seen in all the portions.

Cardiac muscle: There appeared a remarkable increase in activity with the deposit of abundant coarse (blue-toned) granules in addition to fine purple ones present in the normal section. The arterial wall (the smooth muscle cells and the endothelial cells), as well as the interstitial cells showed changes more distinct than those in the other organs examined.

Skeletal muscles: In the diaphragm muscle the strongly reactive fibers accounted for about the same ratio as those in the normal muscle. However, many fibers showed a deposit of coarse bluish granules as well as fine purplish ones
which were more densely deposited (Figs. 7a and b). Granules of both types could also be found densely deposited in the interstitial cells, fat cells, and the arterial wall. Similar results were obtained in the quadriceps femoris as well.

(2) Diabetes established for 7 days, 10 days, 2 weeks and 3 weeks
Liver and kidney: A marked increase similar to those seen in the previous stages was found (Tables 2 and 3). In the kidney, an intensified reaction had also become very distinct in the collecting tubules. The changes in both parenchymal organs as well as in the muscular system described below were roughly proportional to those in the blood sugar level.
Cardiac and skeletal muscles: In both LD and DPND activities a marked increase was shown in the cardiac muscle and the diaphragm without exception. But the changes in the quadriceps femoris was somewhat indistinct, showing no detectable changes in the 3 cases, a slight increase in 2 cases and a presumably very slight decrease in 1 case.

(2a) Diabetes under the lipemic condition
Generally, changes more remarkable than those found in diabetes not complicated with lipemia were observed during the equivalent stages.
Liver: As observable in diabetes not complicated with lipemia, abundant coarse granular deposits were found, which were blue-toned on the borders of fat droplets in the parenchymal cells of the central and the intermediate zones. Of course, an activity higher than that seen in the other diabetic groups was also

Figs. 1a and b. Ph-rylase in the liver obtained from Al$^+$ and the control animal. There is shown such a marked reduction in enzyme activity throughout all the areas of the lobules that the distinct reaction can hardly be seen in the parenchymal cells. In the photographs below, the control sections are shown on the left ("a"), and the diabetic sections ("b"), on the right. $\times150$.

Figs. 2a and b. Ph-rylase in the cardiac muscle obtained from A24$^+$ affected with lipemia and the control. Numerous fat droplets are deposited within the diabetic muscle fibers, and the enzyme activity is also reduced. $\times150$.

Figs. 3a and b. Glycogen histochemically demonstrable in the cardiac muscle obtained from A24$^+$ and the control. An apparently segmental distribution of glycogen in the control muscle and addition, a markedly increased content in the diabetic muscle is observed. This finding is ostensibly contradictory to the enzyme activity seen in Figs. 2a and b. $\times150$.

Figs. 4a and b. SD in the renal cortex obtained from A9$\dagger$ and the control. There is a distinctly increased activity in all the proximal portions. $\times150$. 
observed in the peripheral zone. With regard to DPND, the formazan granules were more sparsely deposited in the central zone which contained abundant fat droplets, while a more remarkably increased activity was observable in the peripheral zone when compared with the LD activity. The reaction in the ductular epithelial cells in Glisson's capsules was similarly and markedly intensified for both the LD and DPND.

Kidney: A more marked increase was observed in all portions of the tubules, especially in the proximal straight portion of the medulla (†††), in the thick limbs of Henle (or the distal convolutions which contained many fat droplets), and in the collecting tubules. This was also the case for the findings on DPND staining (Figs. 6a and b).

Cardiac muscle: Here, the coarser (blue-toned) granules were even more densely deposited than those seen in diabetic groups not affected with lipemia, although they were markedly disarranged owing to the fat droplets.

Skeletal muscles: As can be seen in Figures 7a and b, remarkable changes were demonstrable in the diaphragm as well as in the quadriceps femoris. An intense reaction was also observed in the interstitial and fat cells.

(3) Diabetes established for 1, 2 or 4 months

Liver: The changes were not so remarkable as seen in diabetes established for from 3 days to 3 weeks. Three cases (A15♂, A20♀ and A23♂) still showed an observably increased activity, but 1 case (A18♂) revealed almost no change, and the other 2 cases (A16♀ and A25♀), revealed only a slight or moderate decrease. Generally, the reaction in the bile duct or ductular epithelia presented no distinct changes (Table 2).

Kidney: The 3 cases which still showed an intense reaction in the liver, also yielded similar results, and the other cases showed more dense deposits of purple-bluish granules in the thick portion of Henle and in the distal convolutions.

Figs. 5a and b. SD in the cardiac muscle obtained from A31♂ affected with lipemia and the control. Moderately coarse and deeply violet-bluish formazan granules are deposited in the diabetic muscle fibers. They are somewhat irregularly arranged owing to the droplets. X150.

Figs. 6a and b. DPND in the renal cortex obtained from A31♂ and the control. A distinctly intensified reaction is shown in the proximal tubules, the thick limbs of Henle and the collecting tubules. X150.

Figs. 7a and b. LD in the diaphragm muscle obtained from A11♂ and the control. Bluish coarse granules are recognized in some fibers of the diabetic section. Also, purplish granules are more densely deposited. X150.

Figs. 8a and b. G-6-PD in the liver obtained from D12♂ and the control. The enzyme activity is significantly reduced throughout all the areas of the lobules. X150.
than in the normal cases, but few activity changes in other portions of the tubules (Table 3).

Cardiac and skeletal muscles: Very slight or obvious increases were still observable in the cardiac muscle and the diaphragm, but no distinct changes in the quadriceps femoris, in the 2 cases (A20♀ and A23♂). The DPND showed no distinct changes in any of the cases.

B. Dithizone diabetes

The findings obtained were similar to those seen in alloxan diabetes in each correspondent stage.

4. Glucose-6-phosphate dehydrogenase (G-6-PD)

A. Alloxan diabetes
(1) Diabetes established for 3 days

Liver: As can be seen in Table 2, a slightly intensified reaction was observed throughout all the lobules.

Kidney: As seen in Table 3, a very slight or an apparent increase was found in the tubular epithelia, especially in the thick limbs of Henle and distal convolutions where coarse blue-toned granules were also deposited. In 1 case (A3♀), an apparent increase in the arterial smooth muscle and endothelia was demonstrable.

Adrenal gland: An evident increase in the zona glomerulosa in 2 cases attracted much attention (A3♀ and A32♀) (Table 4).

(2) Diabetes established for 7 days, 10 days, 2 weeks, and 3 weeks

A specific distribution pattern of enzyme activity had just been completed in these stages.

Liver: As can be seen in Table 2, a significantly reduced activity was observed. This finding was somewhat remarkable in the peripheral zone. There was also a reduction in the bile duct epithelia which ran parallel to that of the parenchymal cells in some cases.

Kidney: A distinct reduction was observed in the proximal portions without exception (Table 3). The findings in the peripheral tubules were somewhat indefinite, showing a slight decrease (A5♂, A6♀, A7♂ and A30♂) or indistinct changes (A9♂, A11♂, A12♀ and A14♂).

Adrenal gland: A slight or moderate reduction appeared in the cortex, especially in the zona fasciculata and reticularis (Table 4) and well correlated with the findings in the liver and the kidney.

Pancreatic exocrine portions: According to the 2 cases investigated, (A30♂ and A9♂) the activity was found to be distinctly reduced in the glandular epithelia.
(3) Diabetes established for 1, 2 and 4 months

Liver: The activity was markedly reduced throughout all the lobules without exception (Figs. 8a and b) (Table 2). This was the more remarkable in the cases showing higher blood sugar levels. A similar tendency was noted in the bile duct epithelia.

Kidney: As can be seen in Table 3 and Figs. 9a and b, reduction in the proximal portions was observed in nearly every case. However, the changes were somewhat complex in the peripheral portions. Two cases (A16 and A20) showed a rather slight increase in the terminal straight portion where fine formazan granules were more densely deposited than in the controls. On the other hand, a slight reduction was generally present in the peripheral portions including the collecting tubules. Two cases (A15 and A18) showed a distinctly intensified reaction in the thick limbs of Henle in the marginal zone, but reduction in the other peripheral portions was obscure, and 2 cases (A23 and A25) showed an apparent increase in the thick limbs of Henle and the distal convolutions where the fine granules of formazan were densely deposited.

There were no noticeable changes in the collecting tubules, and no apparent changes in the arterial wall.

Adrenal gland: The activity changes were found to be similar to those in diabetes established for from 1 to 4 weeks and well correlated with those in the liver and the kidney (Table 4).

B. Dithizone diabetes

The changes were similar to those found in alloxan diabetes.

5. Acid phosphatase (ACPase)

A. Alloxan diabetes

This enzyme activity was detected through staining by both the metal salt method and the standard coupling azo-dye method, but in general, only the findings by the former method will be described except for points of difference.

(1) Diabetes established for 3 days

Liver: As can be seen in Table 2, a markedly increased activity was observed throughout all of the lobules, especially in the central zone, so much so that the zonal distribution demonstrable in the normal liver had become indistinct.

Kidney: A marked increase was shown throughout almost all portions of the tubules, especially in the proximal portions (Table 3). Both the nuclear and the cytoplasmic reactions were likewise intensified. In the glomerular tufts, the reaction in both the epithelial and the endothelial cells had also become intensified.

Cardiac muscle: Either a slight or significant increase was shown in all 3
cases investigated. Such a change was remarkable, especially in A12, which showed the highest blood sugar level (Figs. 10a and b). The arterial smooth muscle and the endothelial cells as well as the interstitial cells, correlating with the changes in the cardiac muscular fibers, gave either a moderately or remarkably increased reaction, which presented a tendency similar to that presented in LD and DPND. As a result, the changes in the arterial wall and the interstitial cells presumably depended on which organ was investigated.

Adrenal gland: As shown in Table 4, a distinct increase was found in the zona fasciculata and reticularis and also in the cells of the medulla.

(2) Diabetes established for 7 days, 10 days, 2 weeks, and 3 weeks

Liver: The reaction was similarly intensified in all cases, as it was in the cases established for 3 days (Table 2). Little alteration in activity was found in the endothelial cells of the central veins. An increase in parenchymal cells was generally recognized throughout all of the cytoplasmic portions, but the detailed changes depended on the individual case examined, i.e., the pericanalicular reaction in the periportal cells was especially intensified in 2 cases (A12 and A15), the cytoplasmic reaction was evenly increased in 3 cases (A6, A9 and A30), and, on the contrary, the reaction around the bile canaliculi became indistinct, due to a diffusely intensified reaction in 3 other cases (A7, A14 and A11).

Kidney: As can be seen in Table 3, the proximal convoluted portion of the tubules showed few changes in staining reaction but for a slight reduction in some cases (A9, A14 and A12). However, the straight portion in the marginal zone still demonstrated a distinctly intensified reaction (++) in correlation with the changes in the liver.

Cardiac muscle: Either slight or remarkable increases in activity were observed with the exception of 2 cases (A5 and A30). The structures of the myofibrils and the striations became very pronounced, although they were inconspicuously stained in the control section. The black-brownish fine granular deposits seemed to correspond to the A-bands.

Adrenal gland: Only indistinct changes, a slight decrease in some cases, (A9, A12 and A14), and even a slight increase in the cortex in some other cases were observed.

(2a) Diabetes under the lipemic condition

Liver: As shown in Table 2, the cytoplasmic reaction of the centrilobular cells, where fat droplets had been abundantly deposited, was either only slightly changed or slightly reduced. However, at and around the boundaries of those fat droplets, a black-brownish fine granular or linear staining reaction appeared, forming a striking contrast to the reaction in the other cytoplasmic portions. In the peripheral zone, the parenchymal cells showed a slight increase in activity, their cytoplasm diffusely stained and their bile canaliculi indistinct. Therefore, the activity increase was not so conspicuous as in cases not affected with lipemia. By using the coupling azo-dye method, a more marked reaction around the fat
droplets in the centrilobular cells, i.e., densely deposited fine granules, were observed.

Kidney: As can be seen in Table 3, a remarkably increased activity in the glomerular tufts; the epithelial and the endothelial cells, as well as in the endothelial cells of the vasa afferens attracted much attention. Here, also, abundant embolies of fat droplets could be seen. The proximal convoluted portion of the tubules showed either little change or only a partial and intermittent decrease in activity. However, the straight portion in the marginal zone showed a markedly intensified reactivity. There was a significant dilatation of the lumina in this portion. In the thick and the thin limbs of Henle, abundant deposits of relatively coarse reaction granules were observed, which were especially dense in the thick limbs which contained many fat droplets in the cytoplasm. These findings were remarkable in the medulla, but not in the cortex. After all, the marginal and intermediate zones were characterized by a distinctly intensified reaction in all the tubules (Figs. 12a and b). By using the coupling azo-dye method rather than the metal salt method, activity increase could be seen to be more pronounced in the terminal portion of the proximal straight tubules and the thick limbs of Henle, especially in the latter portion where fine granular reaction products were densely deposited around the fat droplets.

Cardiac muscle: An extremely intensified reactivity was found in the muscular fibers which showed an irregular or uneven staining due to the fat droplets. The activity in the arterial smooth muscle cells (+) and in the interstitial cells (++) was also significantly increased. In general, the lipemic cardiac muscle showed a much more intensified reaction than the non-lipemic cardiac muscle did.

Adrenal gland: An apparently increased reactivity was shown principally in the fasciculata.

Figs. 9a and b. G-6-PD in the kidney obtained from A16♀ and the control. The activity is also reduced in the proximal convoluted and straight portions of the diabetic kidney. ×150.

Figs. 10a and b. ACPase in the cardiac muscle obtained from F1♂ and the control. The muscular fibers in the normal section show only a weak reaction, while the fibers in the diabetic section, quite a remarkable reaction (standard coupling azo dye technique). ×150.

Figs. 11a and b. ACPase in the zona fasciculata of the adrenal cortex obtained from A23♂ and the control. The enzyme activity is markedly reduced in this zone (metal salt method) ×200.

Figs. 12a and b. ACPase in the kidney obtained from A31♂ and the control. These figures show the marginal zone of the renal medulla. A markedly intensified reaction is observed, especially in the proximal straight portion and the thick limbs of Henle (standard coupling azo dye technique). ×150.
(3) Diabetes established for 1, 2 and 4 months

Liver: The activity findings correlated well with those on LD and DPND (Table 2): a distinct increase was still demonstrable in 3 cases (A15♀, A20♂ and A23♂), an indistinct change in 1 case (A18♀), and even a slight decrease in the centrilobular and periportal cells in the other 2 cases (A16♀ and A25♂). These findings did not always strictly correlate with the blood sugar levels immediately before sacrifice.

Kidney: The staining reaction in the proximal convoluted portion varied somewhat according to each tubule or cell, but generally a slight reduction in activity was observed with the exception of 1 case (A25♂) which showed no detectable change. In the proximal straight portion of the cortex, no distinct changes were seen although in this portion of the marginal zone a distinct increase was observed in most cases, roughly correlating with the LD and DPND findings (Table 3).

Cardiac muscle: The activity was found to be increased in various degrees; especially intense were the changes in 2 cases (A18♀ and A23♂) which also showed higher blood sugar levels.

Adrenal gland: A distinctly reduced activity was shown in the cortex, especially in the fasciculata (Figs. 11a and b).

(4) Diabetes established for more than 1 year (F-rabbits)

Liver: Here too, there was a tendency towards activity increase, but the changes were not so great as seen in early severe diabetes; for example, a remarkable activity increase was found in F3♀ and F5♀, a slight decrease in the centrilobular cells and a slight increase in the periportal cells in F1♀ and F2♀, and

Figs. 13a and b. ALPase in the adrenal cortex obtained from A23♂ and the control. The staining reaction is distinctly weakened in the sinusoidal lining, the cell membrane, and the cytoplasmic portion of the fasciculata and the reticularis (which is shown on the left side of these figures). ×200.

Figs. 14a and b. G-6-Pase in the renal medulla obtained from A1♀ and the control. A markedly increased activity is also shown in the proximal straight portion. ×150.

Figs. 15a and b. G-6-Pase in the duodenal mucosa obtained from A1♀ and the control. Most epithelial cells in the diabetic section present the strongest reaction. ×150.

Figs. 16a and b. ATPase in the renal cortex obtained from A31♂ and the control. The activity in the brush border of the proximal convolution is markedly intensified. On the other hand, there are few changes in the basal membrane and in the glomerular epithelial cells. The cytoplasmic reaction (mitochondrial?) in tubular epithelial cells is also slightly increased. ×150.
no distinct change was found in F4Ω.

Kidney: As shown in Table 3, a slightly increased reactivity could be seen only in the proximal straight portion, while a partial or intermittent reduction in the proximal convoluted portion was observed.

Cardiac muscle: A slight or distinct increase was demonstrable in every case.

B. Dithizone diabetes

The findings corresponded with those in alloxan diabetes in each correspondent stage.

6. Alkaline phosphatase (ALPase)

A. Alloxan diabetes

(1) Diabetes established for 3 days

A characteristic distribution pattern could already be seen.

Liver: The activity in the bile canaliculi of the peripheral zone showed a most remarkable increase, and, in addition, this intensified reactivity extended to the intermediate and even to the central zones where little reaction had been shown in the normal cases. The activity in the endothelial cells of the central veins and the sinusoidal linings adjoining them was also increased, but the sinusoidal reaction was rather characterized by the finding that it extended to the intermediate and even to the peripheral zones. To sum up, the zonal distribution of the enzyme tended to become indistinct (Table 2).

Kidney: The activity was increased more remarkably in the cytoplasm of the proximal tubular epithelia, which was diffusely stained, than in the brush border or the basement membrane (Table 3).

Adrenal gland: A markedly intensified reaction could be seen throughout all the cortical zones, but the detailed changes were found to differ among the various cellular components or zones; that is, the reaction in the fasciculata and the reticularis (A1Ω And A32Ω) was significantly increased, more remarkably in the cytoplasm than in the cell membrane or the sinusoidal lining. As for the one remaining case (A3Ω), relatively complex changes were observed in the outer region of the fasciculata, where a slight increase was seen in the cytoplasm; little change was found in the cell membrane, and a slight decrease was found in the sinusoidal lining and in the inner region of the zone where a distinct reduction was observed both in the cell membrane and in the sinusoidal lining (Table 4).

(2) Diabetes established for 7 days, 10 days, 2 weeks, and 3 weeks

Liver: Changes similar to those seen in cases established for 3 days were observed in the hepatic lobules. In Glisson's capsules, an increased reaction was shown in the interstitial cells surrounding the bile ducts (A6Ω) or in the capillary endothelial cells (A7Ω), but these results were not constantly obtained (Table 2).
Kidney: In about half of the cases examined (A6, A5, A9 and A30), a markedly increased reaction was seen in the brush border and in the cytoplasm of the proximal tubular epithelia, especially in the straight portion. However, little change was seen in the convoluted and the straight portions (A14), and even a reduced activity in the former portion (A7) was observed. In a word, there was a general tendency toward activity decrease in the convoluted portion.

Adrenal gland: In general, the activity tended to increase in the fasciculata, especially in the sinusoidal lining, with few detectable changes in the glomerulosa and the reticularis. This activity increase correlated roughly with that seen in the liver and the kidney, showing no specific change, although the adrenal cortex of 1 case (A14) revealed a reduction in the cytoplasm, the cell membrane, and the sinusoidal lining in the fasciculata, when compared with the control (Table 4).

(2a) Diabetes under the lipemic condition

Liver: On the whole, there was a slight reduction although the enzymatic reaction finding among the hepatic lobule specimens were not consistent. In the central zone, where fat droplets had been abundantly deposited, a distinctly increased reactivity was shown in the cytoplasm of the parenchymal cells, especially in the portion adjacent to the cell membrane, since it was there that the fine granular reaction products appeared in abundance. However, the reaction in the endothelial cells of the central veins and the sinusoidal linings was distinctly reduced, but that in the bile canaliculi in the intermediate and the peripheral zones showed little change (Table 2).

Kidney: As for the brush border of the tubular epithelia, a slight increase was observed in the proximal convoluted portion, while a slight decrease was observed in the straight portion, especially in its terminal region. The reaction in the cytoplasm of the proximal tubular epithelia showed little change.

Adrenal gland: A remarkable reduction in the fasciculata and the reticularis was shown. It was characterized by a reduced reaction in the cell membrane and cytoplasm, although the sinusoidal reaction was nearly unchanged, compared with the controls.

(3) Diabetes established for 1, 2 and 4 months

Liver: In these stages, changes somewhat different from those found in diabetes established for from 1 to 3 weeks were observed, that is, in diabetes established for 2 or 4 months (Table 2), a distinctly reduced activity was found in the bile canaliculi of the peripheral zone as well as in the sinusoidal linings and the cell membranes in the central zone, while in diabetes established for 1 month, little change (A16), or an intensified reaction, as before, was observed. This finding did not strictly correlate with that of ACPase.

Kidney: A tendency similar to that seen in the liver was found; the activity in the brush border of the proximal straight portion remained slightly increased in diabetes established for 1 month, while a distinct reduction in the proximal convolutions was shown in every case established for 2 or 4 months, although in
the proximal straight portion, few changes (A20 and A25), or a slight increase in the most peripheral region of the straight portion (A23 and A18), were observed (Table 3).

Adrenal gland: The activity was found to be distinctly reduced in every case. Such findings were significant not only in the glomerulosa, but also in the fasciculata, that is, in the cytoplasm, the cell membrane, and the sinusoidal linings, which all reacted to the ALPase staining (Table 4). In some cases (A23 and A18), similar findings were observed in the reticularis (Figs. 13a and b).

(4) Diabetes established for more than 1 year (F-rabbits)
The findings in these F-rabbits can not be considered as an extension of changes seen in the preceding stages.

Liver: An intensified reaction similar to that seen in diabetes established for from 2 to 3 weeks, was observed in every case; an increase in the cell membrane of the whole lobule (F3), a marked reaction in the endothelial cells of the central veins and the sinusoidal linings adjoining it (F1), and a marked increase in the bile canaliculi of the peripheral and the intermediate zones (F3 and F4) or in all portions (F2), were demonstrated. Therefore, the zonal distribution of the enzyme activity remained somewhat distinct, representing a difference from that observed in cases established for from 1 to 3 weeks (Table 2).

Kidney: Few changes were observed in the proximal convoluted portion, but for a distinct increase in the brush border and the cytoplasm of the epithelial cells in the proximal straight portion (Table 3).

Adrenal gland: The reaction was distinctly intensified in the sinusoidal linings of the fasciculata and the reticularis (Table 4).

B. Dithizone diabetes
The results obtained correlated roughly with those obtained in alloxan diabetes in each correspondent stage. In the pancreas investigated on D12, the staining reaction was found to be distinctly increased in the excretory tubules.

7. Glucose-6-phosphatase (G-6-Pase)

A. Alloxan diabetes

(1) Diabetes established for 3 days
A complete diabetic distribution pattern had already been well established.

Liver: A markedly increased activity was observed throughout all the lobules, especially in the centrilobular cells so that the zonal distribution pattern which could be seen in the control sections became indistinct.

Kidney: A distinct increase, corresponding to that in the liver, was also observed in the proximal convoluted and straight portions, being more remarkable
in the latter (Figs. 14a and b).

The duodenal mucous membrane: Here too, a distinct increase was recognized, for there was found an increase in the number of epithelial cells which showed a very strong reaction: markedly so in A1‰ (Figs. 15a and b) and less so in A3‰.

(2-3) Diabetes established for from 1 week to 4 months

Results similar to those shown in the cases established for 3 days were obtained in all diabetic animals. Furthermore, the higher the blood sugar levels (e.g., cases of A11‰, A18‰ and A24‰), the more remarkable the changes observed. In 3 cases (A9‰, A12‰ and A25‰) which showed relatively low blood sugar levels, the changes in the duodenum were very slight or indistinct.

(4) Diabetes established for more than 1 year (F-rabbits)

Similar tendencies were also found here, but the changes in the duodenum in 1 case (F3‰) was indistinct.

B. Findings in dithizone diabetes

Results similar to those shown in alloxan diabetes were obtained.

8. Adenosine triphosphatase (ATPase)

A. Alloxan diabetes

(1) Diabetes established for 3 days

Liver: A slightly reduced activity in the bile canaliculi, the cell membrane (A32‰) and the cytoplasm (A32‰ and A3‰) was observed (Table 2).

Kidney: The cytoplasmic reaction was slightly reduced in the proximal portions, while the activity in the brush border and the cell membrane was almost unchanged (Table 3). The reactivity in the arterial smooth muscle was also reduced.

Cardiac muscle: A slight increase was demonstrated; it was characterized by the appearance of a uniform reaction in both the central portion and the portion just beneath the sarcolemma of the muscle fiber, and also by the appearance of coarse granular reaction products somewhat concentrated on the A-band (a reaction to the Q-granules?). These findings was more remarkable in 1 case (A32‰). However, the activity in the arterial smooth muscle cells and in the endothelial cells was significantly increased in every case.

(2a) Diabetes under the lipemic condition

Liver: A quite remarkable increase was observed in the sinusoidal linings of
the central zone and the endothelial cells of the central veins, while a distinct decrease was observed in the bile canaliculi, especially in the centrilobular cells wherein many fat droplets had infiltrated.

Kidney: The reactivity was found to be distinctly intensified in the brush border and the basal membrane of the proximal convoluted and straight portions (Figs. 16a and b). In the former portion, the cytoplasmic reaction was also slightly intensified.

Cardiac muscle: In the cytoplasm of the muscle fibers, coarse granular reaction deposits were relatively sparse and, because of the presence of fat droplets, very irregular. On the other hand, the activity in the cell membrane was much pronounced, in comparison with the controls. An apparent staining reaction also appeared on the border of the fat droplets. Furthermore, an intensified reaction was found in the smooth muscle cells, in the endothelial cells of the vessels, and in the interstitial cells.

(3) Diabetes established for 2 months

Liver: Somewhat indefinite findings were obtained. A very slight increase was observed in the cytoplasm of the periportal cells (A18), or in that of the bile canaliculi (A20), and a slight decrease was observed in the sinusoidal reaction of the central zone (D12), although the cytoplasmic reaction was almost unchanged (Table 2).

Kidney: Few changes in activity was demonstrable, but a slight increase, in the membrane ATPase of the distal tubules was seen in 1 case (A18) (Table 3).

Cardiac muscle: A slight intensification was seen in the activity of the muscle fibers alone.

B. Dithizone diabetes

No experiments were conducted on dithizone diabetes.

9. Non-specific esterase

A. Alloxan diabetes
(1) Diabetes established for 3 days

Liver: In the parenchymal cells, the granular reaction products deposited were relatively sparse, but they were coarse and of a considerably deeper color-tone, compared with those in the controls. This finding shows that the higher activity was concentrated on the point where the granules were deposited.

Kidney: There were no detectable changes in the present investigation.

Adrenal gland: The activity in the fasciculata and reticularis was distinctly increased. Few changes were demonstrable in the medulla.
(2a) Diabetes under the lipemic condition

Liver: A distinctly increased activity was observed throughout all the lobules of the diabetic liver, especially in the centrilobular cells wherein abundant fat droplets had infiltrated (Figs. 17a and b) (Table 2).

Kidney: A significantly increased activity was shown in all portions of the tubules, especially in the proximal region of Henle's loops (Table 3).

Adrenal gland: A slight or distinct increase was also observed in this organ (Table 4).

(3) Diabetes established for 1 or 2 months

Liver: An apparently increased activity was still demonstrable throughout all areas of the lobules (Table 2).

Kidney: A slight increase was also recognized in the proximal portions (Figs.
18a and b), especially in the straight portion of medulla, showing a good correlation with that in the hepatic lobules. Furthermore, a slight increase in the thin limbs of Henle was demonstrated (Table 3).

Adrenal gland: A slightly intensified reactivity was shown in the fasciculata alone (Table 4).

B. Dithizone diabetes

No experiments were conducted on dithizone diabetes.


discussion

Included in the following discussion are: (1) a survey of the literature, (2) a comparison between our findings and those of others, and (3) the biological significance of the findings on each organ studied.

A. General findings

1. On the Ph-rylase findings

The Ph-rylase activity in the diabetic liver was found to be significantly reduced in early stages, but tended to recover in various degrees toward a normal activity level in the later stages. On the other hand, various findings on glycogen content have been reported by several investigators. According to Fitch et al. (1959), both glycogen content and phosphoglucomutase in the alloxan diabetic liver (rat) was apparently reduced. Midorikawa (1964) reported that there was almost no change but for a slight reduction in glycogen content in the alloxan diabetic liver (rat). According to Winehaus et al. (1963), the liver glycogen content of alloxan diabetic rats is high and does not readily “exchange” with blood glucose. The present author's preliminary data demonstrated that both enzyme activity and glycogen content were remarkably reduced in rat liver within several days after the induction of alloxan diabetes. Diabetes is severe in such early stages, showing some tendency to lipemia. However, rats which survived, began to show more or less recovery toward the normal activity level within 2 or 3 weeks and their livers went so far as to yield the reverse results, presenting more glycogen and more enzyme activity than the normal liver. Therefore, it is reasonable to suppose that variance among respective data referred to above may be attributed to differences in severities and durations of the disease.* Also, the diabetic rabbits did begin to show recovery from the disease but, only in the relatively later stages, that is, from 1 to 4 months after induction. Shimizu et al. (1951) reported on alloxan diabetic rabbits (7 days after the induction of diabetes, and 200~700 mg/dl of blood sugar level) to the effect that the higher the blood

* This variance may be also attributed to the nutritional state of the experimental animals as indicated by Fagundes et al. (1965).
sugar level, the larger the glycogen content in the diabetic liver, and that this increase was to be observed principally in the central and the intermediate zones of the lobules. Their results are, of course, in disagreement with the present data.

In respect of findings on the diabetic kidney, it has been reported that glycogen infiltrates or is deposited in the thick limbs of Henle and in the collecting tubules (Kashimura, 1958; Okamoto, 1951). The present study also demonstrated a similar increase in enzyme activity. However, the intensification in enzyme reaction was more limited in extent than the increase in glycogen content.

Takeuchi et al. (1955) described a significantly intensified reactivity in the cardiac muscle of the alloxan diabetic rabbit and, on the other hand, some reduction in skeletal muscle. In the present study, a markedly increased activity was found, especially in the outer layer of the cardiac wall so as to create a tendency toward uniform reactivity throughout all the layers, in contrast to the normal heart where the inner layer presents a relatively stronger reaction. The findings on glycogen staining correlated well with the activity changes on the histological (but not on the cytological) level, but this, as described above, was not the case with diabetic animals affected with lipemia. These facts seem to indicate that the enzyme activity is inhibited by the deposited fat droplets, or they practically present a cytological compensatory function against an absolute lack of sugar (not a passive behavior owing to general weakness), because several other enzymes (which will be discussed below) showed almost the same reaction as could be seen in diabetes not complicated with lipemia.

Regarding skeletal muscles, a distinctly reduced activity in the diaphragm muscle, especially in the severely diabetic animals, but no marked and definite changes in the thigh muscle could be observed. According to Krahl (1951), Krahl and Cori (1947), and Ville and Hastings (1949), an apparent decrease in glucose uptake as well as in synthetized glycogen content in the diabetic diaphragm was shown in in vitro experiments. This present study offers a histological ground for these findings.

On the relationship between the enzyme activity of the diaphragm and of the thigh muscle, strongly reactive fibers in the former were found to account for about 1/2 of the total number and those in the latter, for more than 2/3. The oxidative enzymes examined showed quite the reverse results, that is, the existence of a reciprocal relationship between the Ph-rylase and the oxidative enzymes as Dubowitz and Pearse (1962) had already pointed out. Such a difference seems to be concerned with each functional specificity, which, in turn, seems to have a bearing on the different behavior of enzyme and of glycogen staining in the diabetic state. Another noteworthy fact is that the enzyme activity as well as the glycogen content in the lipemic thigh muscle presented an apparent increase similar to that found in the non-lipemic diabetic heart. The significance of this finding yet remains to be determined.

As mentioned above, the enzyme activity was found to correlate roughly with the histochemically demonstrable glycogen, but this is not the case in so far as the exact distribution patterns in the various organs are concerned. For example, no strict correlation could always be seen even in normal livers. This phenomenon may be due to (1) the sensitivity of the glycogen staining method, and (2) a
presumably unsettled balance between the product under the influence of the enzyme activity (the glycogen originally present in the living bodies) and the enzyme activity itself which appears on the frozen sections on the cellular order. Is there a prompt shift from Ph-rylase a to Ph-rylase b and vice versa (Grillo, 1961)? Further investigations on the serial section method, etc., are required to answer this question. It must also be taken into consideration that the grade of enzyme activity is judged by the reaction product amounts appearing on a frozen tissue section under entirely artificial conditions. Accordingly, there is a room for question when such a grade is discussed on the same level with a reaction product (glycogen) having originated in a living body.

Furthermore, next there are two pathways for polysaccharide synthesis: (1) the amylophosphorylase branching enzyme system, and (2) the UDPG-glycogen transferase system (Takeuchi and Glenner 1961).

Although the second system remains a problem for future investigation, the final products synthetized through the first histochemical system might also contain, more or less, the polysaccharide formed in the UDPG-glycogen transferase reaction, if UDPG or its precursor has naturally been present in the tissue sections.

2. On the SD findings

Copenhaver et al. (1951) demonstrated an apparent increase in SD activity in liver homogenates from alloxan diabetic rats. Rudolph (1959) investigated histochemically ALPase, A5'Pase (5-nucleotidase), ATPase and SD activities in the livers and kidneys of alloxan diabetic rats (6~72 hrs. and 6 days after injection). According to him, the SD activity in the liver already showed a significant increase 20 hrs. after injection. Rudolph pointed out that the activity was present principally in the peripheral zone of the normal liver, while a significantly intensified reaction was also found in the central zone. According to Hazama (1965) who investigated the activity changes in the livers of dd/s-obese hyperglycemic mice, an irregular but distinct increase was observed, which had a correlation with the findings in the kidney and the cardiac muscle. The present findings are quite consistent with the Rudolph's data on the cases established for more than 3 days, also with the biochemical findings obtained by Copenhaver et al., and with Hazama's data in part. With reference to the report (Pearson et al., 1959) that there was a markedly increased activity in liver SD during rapid growth following partial heptectomy, it is reasonable to think that such an increased activity represents an important biological function under the diabetic condition. Furthermore, the present study demonstrated a specific increase in the central zone of the hepatic lobules. As a result, the zonal distribution of enzyme activity clearly observed in the normal liver became indistinct. Novikoff and Essner (1960) who discussed the zonal distribution of several enzyme activities, pointed out that such distributions have an intimate relationship with blood flow and, for example, that SD and cytochrome oxidase, which show a significant reactivity in the peripheral zone, are present probably for the convenience of lobular oxidative respiration via the Krebs cycle. Furthermore, it is indicated that there are numerous long mitochondria in the cells of the peripheral zone, which fact, of course, well corresponds
with the findings on SD activity (Novikoff and Essner, 1960). Judging from this point of view, the activity changes in the central zone of the diabetic liver seem to represent a mobilization of the reserved action. The mitochondria in this zone are fewer and more rounded, and this condition leads to the interesting question: What are changes that have been brought about in such mitochondria?

Regarding the findings in the kidney, Rudolph (1959) demonstrated that clear decrease in activity, especially apparent in the cortex, was brought about with the lapse of time after alloxan injection. However, it is uncertain whether or not similar changes could be seen at 6 hrs., for such findings were not demonstrated at the time. He ascribed these activity changes to the lesion of the tubular epithelial cells brought about by the alloxan, because there had not only been a reduction in SD but also in ALPase, ATPase and A5'Pase, and in addition, more remarkable changes were to be observed when the injection dosis was increased. These observations are, of course, in clear disagreement with our own observations on rabbit kidney. Regarding the results obtained in this study, do they represent an actual increase in Krebs cycle activity? They are contrary to such findings as those of Joslin et al. (1959). If they do, how is the enzyme activity that appeared on the sections to be interpreted? This manifestation may be a fabricated activity produced by the entirely artificial circumstances under which that biochemical and also, perhaps the histochemical method of study is carried out. This phenomenon seems to signify an important compensatory function in the living body. But for such a mechanism, a much more serious handicap to Krebs cycle activity would be brought about.

The present study on the diabetic heart demonstrated that the dehydrogenase activity examined showed a tendency toward decrease in most seriously diabetic cases in the early stages or in most F-animals, while most cases in which diabetes had been established for from 1 to 4 months, by showing a stable or convalescent phase, presented a tendency toward increase. However, careful observations on intracellular distribution revealed the deposition of the blue-toned coarse granules in the muscle fibers of all diabetic cases. Such granules were found somewhat sparsely distributed in the former groups and more densely in the latter. Accordingly, it naturally followed that every site where the reaction products were deposited showed an increased reactivity in all cases. But no further explanation for this phenomenon is available as yet. It is also unknown as to what changes in their form or distribution take place.

In relation to the skeletal muscle examined, changes similar to those seen in the cardiac muscle were found. With reference to the report that there was a significant decrease in activity in the denervated muscle (rat soleus) (Nachmias and Padicula, 1958), it is naturally thought that the distinct increase in convalescence is of important biological significance.

In case of the adrenal gland, we found that a relatively high activity appeared throughout all the cortical zones 3 and 7 days after the occurrence of diabetes, which findings well correlated with the changes found in the liver and the kidney, while a rather apparent decrease in activity in the zona fasciculata and reticularis was shown in more than 2 or 3 weeks. Relating to these findings, Glick (1960), and Glick and Greenberg (1958) disclosed that the SD activity per unit protein
nitrogen in each zone showed no changes after the administration of cortisone acetate for 2 weeks, but a reduction in the zona reticularis appeared 7 days after hypophysectomy. However, according to Bourne (1955) who investigated by the histochemical method, the injection of cortisone in intact rats increases the SD reaction in all organs, but causes a reduction of that in the adrenals. These findings suggest that SD activity level has some connection with the functional level of the adrenal cortex. Furthermore, Akazawa, (1959) who investigated the urinary 17-ketosteroids, 17-ketosteroid fractions, 17-hydroxycorticosteroids, and uropepsin in alloxan diabetic rabbits, found that the values he obtained showed a temporary increase 7 to 14 days after the alloxan administration. As for fractions II, III, IV and V from alloxanized rabbits, II and III showed a similar tendency, but VI and VII showed a gradually and steady decrease during the illness, without any temporary increase. Accordingly, there is roughly a good correlation between our histochemical and Akazawa’s biochemical observations in so far as fractions II and III are concerned. Fractions VI and VII, however, show a good correlation with the G-6-PD activity.

3. On the LD and DPND findings

The staining reaction was generally found to be intensified in the parenchymal cells, the cardiac and the skeletal muscle cells, the fat cells and the interstitial cells, especially when the diabetic condition was severe. This fact suggests that diabetic abnormalities affect all the organs, tissues, and cells on the subcellular levels, and therefore leads us to raise the following questions: (1) What portions or components within the cells present an intensified reaction? (2) What is the significance of the fact that almost the same results were commonly obtained for LD and DPND reactions in all tissues and cells despite their different functional specificities? Is some relationship to the mechanism of insulin action involved here? Regarding the first question, noteworthy is the appearance of blue-toned, coarse formazan granules in addition to the fine purplish ones which had also increased remarkably in the diabetic condition. Might such granules be presumably formed in consequence of the condensation of the fine granules? If so, on what cellular component do they condense? No definite answer to this question could be obtained since the present study was carried out on the light microscopic level. On the latter subject, a few comments will be made from the standpoint of the present findings.

Schmidt (1963) who investigated the activities of LD and several other enzymes in diabetic human liver specimens obtained by biopsy, demonstrated a remarkable increase in LD and other enzyme activities related to the Embden-Meyerhof pathway in both adult and juvenile diabetes. According to the biochemical study by Spiro et al. (1958), the utilization of glucose by rabbit liver slices for conversion to glycogen, CO₂, and fatty acid are all impaired in the alloxan diabetes. The histochemical Ph-rylase findings described above are in accordance with these findings. On the other hand, it is difficult to interpret the LD and DPND as well as the SD findings. This fact is seemingly contradictory to the findings of Spiro et al. However, this is an apparent increase "on the frozen sections under
the histochemical circumstance.” Such activity changes presumably represent some
active compensatory mechanism in the living organism. Such a mechanism may
be unable to function under the diabetic circumstances, but may be able to do
so under the entirely artificial circumstances. This interpretation might, perhaps,
be extended to similar findings in other organs. Dolkart et al. (1964) have recently
reported that hepatic LD activity was significantly reduced in rat made diabetic
with alloxan and with anti-insulin serum (11–12 days after the injection, and
about 300–600 mg/dl of the blood sugar level), when calculated per total liver.
And besides, on the basis of liver protein, alloxan caused a more marked reduc-
tion in activity than did the anti-serum. These findings are, of course, quite in
disagreement with those of the present author. However, according to our pre-
liminary data, changes similar to those shown in rabbits were also observed in
severe diabetic rats in the early stages, while a decrease in activity was observ-
ed in the cases in which diabetes had persisted for more than 2 weeks. In the
diabetic rabbits, such a tendency was found only after 1 or 2 months.

In our own study regarding the diabetic kidney, a good correlation was found
between the hepatic parenchymal cells and the renal tubular epithelia, especially
the proximal straight portion.

Regarding the changes in the diaphragm, the various in vitro experiments
(Butterfield et al., 1958; Field, 1959; Riddick and Reisler, 1962; Soskin and Levine,
1937) and the present histochemical findings indicate an evident deficiency of sugar
level was remarkably increased. The noteworthy increase in LD and DPND is
thought to be intimately related to the Krebs’ cycle activity level.

The cardiac muscle also revealed the same tendency as the diaphragm did
although quite the reverse results were obtained on glycogen content and Ph-rylase.
In this respect, the findings in the normal condition are instructive; the cardiac
muscle gave a weaker LD and DPND reaction and a stronger Ph-rylase reaction
than did the diaphragm (as indicated by Blanchaer and Van Wijhe (1963), Van
Wijhe and Blanchaer (1963), the reciprocal relationship is found also here). The
quadriceps femorizes is a white muscle (Ogata, 1958) and shows findings somewhat
similar to those seen in the cardiac muscle. This fact seems to be connected with
the specificity of their functions and modes of energy acquirement.

Concerning the fat cells of the diaphragm, similar findings were obtained.

After all, such intensified LD and DPND activities were commonly found in
all the organs and tissues examined. Could this fact be interpreted as a primary
adaptation to the intracellular deficiency of sugar substances owing to the absolute
lack of insulin? If so, then insulin might exert a similar primary action upon the
cells of all organs and tissues despite the different findings obtained on Ph-rylase
or glycogen content etc. among the various organs.

4. On the G-6-PD finding

Pagriaro and Notarbartolo (1961) examined human hepatic tissues, obtained
by needle biopsy from normal and diabetic subjects, on their G-6-PD and G-6-
Pase contents, finding no difference in the former, but finding the latter to be
2 to 3 times as high in diabetes as in normalcy. On the other hand, according
to Schmidt (1953), the liver G-6-PD in yet untreated elderly and juvenile diabetes was found to be significantly reduced, especially in the latter and recovered to nearly normal after treatment. Similar findings were reported after the investigation on kk-lined congenital diabetic mice (Masuda et al., 1962). As for the alloxan diabetic rat (10 days after injection), the biochemical study of Glock and McLean (1955) also demonstrated a significant reduction in liver G-6-PD (either as concentrations or as total levels). The present histochemical study indicates that such G-6-PD findings were remarkable in the lobular parenchymal cells, but were obscure in the Kupffer cells and the endothelial cells of the central veins or in the arterial wall of Glisson's capsules.

Also in the renal tubules, similar changes were definitely observed, especially in the proximal portions which always corresponded with those in the hepatic lobules, while few changes were observed in the glomeruli and the vessels. In conclusion, such reductions were commonly obtained in the parenchymal cells (renal or hepatic). In this respect, Fitch et al. (1959) reported that G-6-PD as well as phosphoglucose isomerase were unaffected in the diabetic livers of rats which had been fed stock diets containing no free hexose. In such cases, does diet similarity influence the renal tubules?

As for the physiological significance of these facts (i.e., G-6-PD↓ in the parenchymal organs), Glock and McLean (1955) and Agranoff et al. (1955) suggested that there is an active competition between the glycolytic and the hexosemonophosphate oxidative pathways for available G-6-P. At the same time, much evidence indicates that there is, in fact, a metabolic block at the glucokinase stage in experimental diabetes as well as in the state of starvation (Renold et al. 1956). As a result, an absolute deficiency of intracellular G-6-P seems to be a decisive factor. Therefore, such a fact as G-6-PD↓ may be considered to have an intimate relation with the fact ; SD↑, LD↑ and DPND↑.

Schematically this can be shown as follows:

Days after diabetic induction:

<table>
<thead>
<tr>
<th>3d.</th>
<th>10d.</th>
<th>60d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-6-PD↑</td>
<td>G-6-PD↑</td>
<td>G-6-PD↑</td>
</tr>
<tr>
<td>SD↑</td>
<td>SD↑</td>
<td>SD↑</td>
</tr>
<tr>
<td>LD and DPND↑</td>
<td>LD and DPND↑</td>
<td>LD and DPND, →* or ↓</td>
</tr>
</tbody>
</table>

The findings in the adrenal gland also showed a good correlation with those in the liver and the kidney. However, is this fact to be interpreted as coincidental or as representative of a cooperative function in the whole body? According to the opinion of Haynes and Berthet (1957), and Haynes (1958) on the mechanism of ACTH action, ACTH increases adrenal Ph-rylase activity, and thus increases G-6-P which, in turn, on oxidation by G-6-PD, increases TPNH and its dependent reaction leading to corticosteroidogenesis. Therefore, the fact that the G-6-PD activity is reduced in the adrenal gland in effect becomes a reduction in TPNH

* No remarkable changes.
content, leading also to a reduction in corticosteroidogenesis (although glycogen content and Ph-rylase are not yet determined). In this respect, Greenberg (1960), investigating the effect of ACTH treatment on G-6-PD as well as 6-phosphogluconic dehydrogenase activities in the rat adrenal gland by the quantitative histochemical technique, indicated that these activities were apparently increased, although the findings in the former are somewhat indistinct; besides the maxima of both enzyme activities shifted from the fasciculata to the fasciculata and reticularis border zones. Furthermore, Akazawa's finding (Akazawa, 1959) that there is a remarkable reduction in urinary VI and VII fractions of 17-ketosteroid in alloxan diabetic rabbits is well consistent with that of the present author. On the other hand, it has been found that adrenalectomy, in case of the diabetic animals, results in a reduced hepatic glucose production on the one hand (Krahl and Cori, 1947) and in a return to the normal level of the depressed glucose uptake in muscles on the other (Renold et al., 1956).

Finally, when the above findings are considered synthetically, it naturally follows that a reduction in the cortex of the adrenal gland as well as in the liver and the kidney represents a cooperative mechanism of a living system.

5. On the ACPase findings

In relation to the serum and liver phosphatases, it was first pointed out by Cantor et al. (1947), and by Drabkin and Marsh (1947) that both phosphatases were markedly increased in the alloxan diabetic rat. According to the former, such increase in the serum phosphatases, especially the alkaline enzyme, was correspondent well with those in the blood sugar level. According to the latter, significant increase in both enzyme activities was found to occur in rats with alloxan diabetes well established (i.e., more than 4 days after injection), and effective insulin therapy applied to the rats was found to restore both phosphatase activities to their normal levels. Vranic and Allegretti (1959) also demonstrated that the changes in the serum phosphatase were parallel to those in the blood sugar level, although the liver ALPase showed only a slight change. These experiments, as mentioned above, indicate parallel changes (a) in the ACPase and the ALPase activities and (b) in the serum and the liver phosphatases. On the (a) findings, the histochemical method also proved the existence of a similar interrelationship as will be described below.

According to the report of Shimizu et al. (1951), who investigated the histochemical findings on the liver ACPase and ALPase of alloxan diabetic rabbits (1 week after induction and 200-700 mg/dl levels of blood sugar), both enzyme activities were generally significantly increased in the hepatic lobules, including the Kupffer cells. This finding concurs with that of the present author. Rudolph (1959) also demonstrated that ACPase and ALPase activities were clearly increased in the liver of alloxan diabetic rat, while these activities were decreased in the kidney. This kidney finding is contradictory to that obtained by the present author. According to Hazama (1965) who investigated the activity changes in the livers of dd/s obese hyperglycemic mice, the distribution of the activity was somewhat patchy with no detectable changes in its level and, in addition, there were
no apparent changes in either the kidney or the cardiac muscle.

On the other hand, from the standpoint of the present findings, it naturally followed: (a) That, as with the other enzyme activities examined, a good correlation was preserved between the activity changes in the hepatic lobules and the renal tubules. (b) That the increased activity in the kidney and the liver was most significant in from 1 to 4 weeks, but became obscure in from 1 to 4 months, or rather, led to a decreased activity similar to the changes seen with LD. However, in case of the F-rabbits, there was a tendency to maintain such changes as could be seen in diabetes established for from 1 to 3 weeks. (c) That the changes in the adrenal gland correlated well with those in the liver and the kidney in the early stages, but that an apparent decrease in the fasciculata was shown after 2 or 3 weeks. This finding corresponded with that of 17-ketosteroid and its fractions obtained by Akazawa (1959). (d) That in the diabetic cardiac muscle, the activity was found to be increased in various degrees throughout all stages.

These complex findings in the various organs, in reference to the course of experimental diabetes, may be interpreted as follows: (a) A severe weight loss and a tendency to lipemia, i.e., a metabolic incompensation, are observable within 1 week after diabetic induction when a distinct increase can be seen in the activities in every organ examined, i.e., liver, kidney, heart and adrenal gland. (b) The stable diabetic state has been established in from 2 to 3 weeks when the adrenal gland begins to show a decrease in activity. (c) The animals recover from the severe diabetic state, their body weight being restored to the normal level (or rather to an increase over the normal) in from 1 to 4 months when the activity in the parenchymal organs is found apt to decrease.

Then, how may the enzymatic findings be interpreted in respect to their biological significance?

According to the recent investigation of Ogawa (1963), the main points on the functions of ACPase in cellular metabolism can be schematized as follows:

1. Digestion
   a) Circumscribed: phagocytosis.
   b) Cellular autolysis.

2. Secretion or excretion: Reversed pinocytic activity.


Vorbrodt (1958) enumerated several findings in illustration of his opinion that ACPase plays some role in the protein synthetizing process. He also demonstrated that the regenerative hepatic lobules after partial resection showed a distinct increase in activity.

Furthermore, the following facts are exemplified: In the starvation experiment on mice (Arizono, 1952), the ACPase activity was found to remain increased for 72 hrs. in the duodenum, the liver and the kidney (although the increased ALPase was rather reduced in the later stages). By various stimulations of the reticuloendothelial system (Thorbecke et al., 1961), [for example, frequent injections of endoxan, an injection of zymosan (yeast cell polysaccharide) and B.C.G., a subcutaneous transplantation of sarcoma 180, and the infection of Friend leukemia virus], ACPase activity in mouse liver (especially in the Kupfer cells), as well as that of ALPase was remarkably intensified.

In conclusion, by inference from the facts described above, it is suggested
that the changes in ACPase activity represent either compensatory or antagonistic actions on metabolic abnormalities brought about under diabetic and other conditions.

6. On the ALPase findings

As a result of the investigation of Shimizu et al. (1951) it has been well known that ALPase activity is remarkably increased throughout the hepatic lobules of alloxan diabetic rabbits 1 week after induction. The present observations are well consistent with theirs. As has been described before, Rudolph (1959) reported similar findings. In case of the kidney, however, his results are quite reverse to ours. According to Masuda et al. (1962) who investigated the activity in the hepatic lobules of kk-congenital diabetic mice, few distinct changes were found.

This time sequence study indicated that the activity changes in the organs examined are diphasic exactly as was shown in the LD and DPND, that is, first, a marked increase in activity can be seen, followed by a contrary decrease, although the activity in the adrenals alone began to turn into a lower level somewhat earlier than that in the other 2 organs. It is difficult to interpret this phenomenon. All that may be said at the present time is that this is undoubtedly related to the process of recovery from severe diabetes.

In relation to the physiological significance of ALPase, it is suggested, from the viewpoint of its localization in various tissues of the body, that in the regions of absorption or secretion there is a phosphate transfer mechanism which provides energy for the passage of substances across the cell membrane, being intimately connected with the activity involved (Bourne, 1955; Danielli, 1953; Denuce, 1953; VAN Robertson et al., 1950; Vorbrodt, 1958; Wachstein, 1959). Regarding the renal proximal tubules particularly, it is presumably involved in glucose transport (Pederson and Dalgard, 1960).

Therefore, as for an increased activity, for example, in the brush border of the proximal straight portion of the diabetic kidney, it is conceivable that this fact represents an increased glucose uptake across the cell membrane. However, no clearly observable activity revealed itself in the thick portion of Henle, the distal convolution, and the collecting tubules, where, in diabetes, increased glycogen deposits were to be found. Regarding this point, it is suggested that an enzyme activity is not always demonstrated histochemically on the same level even if the same physiological process can presumably be operative in each tissue or cell. As for the activity changes in the liver, it is conceivable that the glucose output was increase in this organ.

Regarding the activity changes in the adrenals, they may probably be related to changes in urinary 17-KS and its fractions as demonstrated by Akazawa (1959). On the other hand, Vorbrodt (1958) demonstrated, that on the surface of silk gland cells, ALPase activity is strongest in the sixth larval stage. This finding is highly instructive.

The findings in the condition of lipemia will be discussed below.
7. On the G-6-Pase findings

Ashmore et al. (1954), Ashmore and Hastings et al. (1956), Weber and Cantero (1954), and Segal and Washko (1959) found, by using the biochemical method, that G-6-Pase activity increased remarkably in the livers of alloxan diabetic or fasting rats. On the other hand, Chiquoine (1955) reported that no changes in the amount and distribution of the enzyme could be demonstrated by the histochemical method in rats sacrificed 24 hrs. after the alloxan injection. These differing findings seem to be dependent not so much upon the technique used but upon the interval between injection and sacrifice. In the preliminary data of the present author the histochemical method revealed a markedly increased activity in diabetic rat liver as early as 3 days after injection.

According to Fitch et al. (1959) who investigated the influence of diet, relative slight changes were observed in the livers of rats fed on diets containing much free hexose. This fact suggests that the relative deficiency of sugar substances in diabetes has an effect on the liver G-6-Pase level. The biochemical data reported by Masuda et al. (1962) demonstrated that somewhat indefinite changes appeared in kk-hyperglycemic mice livers, that is, significant increases in some cases and indistinct changes in others. Hazama (1965) showed that the activity in dd/s-hyperglycemic mice livers were apparently increased in many cases. According to Patrick and Tulloch (1957), Egeli and Alp (1958), Wallenfells et al. (1959), and Pagriaro and Natarbartolo (1961), who investigated enzymes in the diabetic human liver, the data obtained are all consistent in the point that there is a distinct increase in activity. Langdon and Weakley (1955) reported that an activity increase in the diabetic liver is observable both in whole liver homogenates and in microsomal fractions. In the present investigation, these subcellular changes could not be detected by light microscopy. The recent investigation of Dolkart et al. (1964) demonstrated that in the livers of rats made diabetic with alloxan (11 to 12 days after injection) and with guinea pig anti-insulin serum (24 to 60 hrs. after the first injection), the enzymic activity become distinctly intensified, being much higher in serum diabetes than in alloxan diabetes, which is based upon liver protein.

The present histochemical observations yielded diabetic liver changes similar to those mentioned above and, moreover, similar increases in the renal tubules. According to Drury et al. (1950), who experimented on eviscerated animals, it was concluded that the kidney also contributed toward maintenance of the blood sugar level. Moreover, the present investigation revealed that the small intestine played a similar role.

8. On the ATPase findings

According to the histochemical study of Rudolph (1959), ATPase activity was distinctly increased in the hepatic lobules of the alloxan diabetic rat within 6 days after the injection, while it was progressively depressed in the renal tubules with the passage of time.

The present investigation on rabbit livers and kidney yielded the following results: The cytoplasmic reaction in the hepatic parenchymal cells and the renal...
tubular epithelia was slightly depressed in 3 days, while such membrane ATPase activity as that shown in the cell membrane, the sinusoidal lining, the brush border, and the basal membrane etc., were relatively indifferent. However, in the state of lipemia, 1 and 2 weeks after induction, such membrane ATPase activity, including that in the cardiac muscle, was markedly intensified, while the cytoplasmic reaction was almost unchanged, or only slightly reduced, and the reaction in the bile canaliculi was distinctly reduced in all the areas of the hepatic lobules, especially in the central zone.

It is supposed that the cytoplasmic reaction is brought about by the ATPase activity in the mitochondrial, in the endoplasmic reticulum, or in both. On the other hand, membrane ATPase, especially that in the sinusoidal lining, according to Novikoff et al. (1961), is presented by A5'Pase as well as by the specific ATPase, and the ATPase in the bile canaliculi is presented by the non-specific ALPase as well as by the specific ATPase, furthermore, the activity in the brush border is presented by ALPase as well as by the specific enzyme.

Therefore, an increased reaction in the ATPase staining may presumably represent an increase not only in the specific ATPase but also in the non-specific ALPase and A5'Pase activities.

This enzyme is responsible for the break down of ATP to ADP (adenosine diphosphate) with the release of free energy. It has been suggested, moreover, that mitochondrial ATPase is in effect a reversal and diversion of the reaction responsible for the synthesis of ATP from ADP during oxidative phosphorylation (Lardy and Wellman, 1953; Ishikawa and Kurata, 1963). Thus, it is not accidental that the findings of the activity (↓) in early severe diabetes coincide with those of the oxidative phosphorylation level (↓).

9. On the nonspecific esterase findings

According to Hazama (1965), the lipase activity (using Tween 20 and 40 as the substrates) was slightly increased in the livers of dd/s-obese hyperglycemic mice. On the other hand, it has been reported (Midorikawa, 1964) that liver esterase activity in the steroid-diabetic rat showed some tendency towards reduction.

The present findings on the alloxan diabetic rabbits demonstrate (a) that the activity tends to concentrate in every cytoplasmic site where coarse granular, heavily stained reaction products appear, although the staining reaction seems to be relatively faint throughout the whole section; (b) that the remarkable changes appear in the parenchymal organs in lipemia, 1 to 2 weeks after induction, that is, a markedly increased activity in the central zone of the hepatic lobules and in the thick as well as in the thin limbs of Henle where many fat droplets are deposited; and (c) that an apparently intensified reactivity is also shown in 1 or 2 months.

These facts suggest that the portions in which the enzyme activity is markedly increased may play some important roles in the metabolism of fats and proteins.
B. On some noteworthy findings obtained in diabetic animals with or without lipemia and in alloxan-diabetic F-rabbits

(1) The activity changes in the liver and the kidney

(a) On the zonal distribution of enzyme activities in the liver

In the normal liver, SD, ACPase and G-6-Pase showed relatively intense reactions in the periportal cells, while esterase showed a somewhat intense reaction in the centrilobular cells. As for ALPase, the reaction in the bile canaliculi was more remarkable in the peripheral zone than in the central zone, while, the reaction in the sinusoidal linings was vice versa.

Novikoff (1959) has tentatively suggested some functional interpretations of such quantitative differences, and pointed out the presence of an intimate relation with the direction of blood flow within the hepatic lobule. For example, the high SD (and cytochrome oxidase) of the peripheral cells (together with their numerous long mitochondria) suggests that in these cells, which are first cells exposed to the blood entering the lobule, oxidative respiration via the Krebs cycle may operate at a high level. In the peripheral cells of the lobule also, ACPase-rich lysosomes are larger and more numerous than in the more centrally located cells. This may reflect a higher rate of pinocytosis and other mechanisms by which proteins and other materials may enter the cells from the nutrient-laden blood in the periphery.

A higher level of G-6-Pase activity in the peripheral cells contrasts well with the relatively high Ph-rylase activity and high glycogen content in the centrilobular cells. Although the significance of ALPase remains unsettled at the present time, it may be supposed that the enzyme is also related to zonal functional specificity. On the other hand, a somewhat higher level of esterase is demonstrated in the centrilobular cells, in which lipid is frequently formed under the condition of lipemia.

In the diabetic condition, the zonal distribution of enzyme activities mentioned above are apt to become obscure, for the enzymes which are relatively strongly reactive in the peripheral cells, are proportionately increased in the centrilobular cells and vice versa. The enzymes which are almost similarly reactive throughout the whole lobule in the normal liver, for example, LD, DPND and G-6-PD, are increased or decreased almost evenly throughout the whole lobule of the diabetic liver. In other words, the zonal functional specificity in the hepatic lobule tends to disappear under the condition of diabetes.

From the standpoint of mitochondrial zonal differences in the liver, Nöel (1923) indicated that the central zone probably constitutes a region of reserve (permanent repose), while the peripheral zone, a region of permanent function. The zonal distribution of the several enzyme activities examined in the normal liver also substantiates this opinion.

In conclusion, the centrilobular cells, that is, the region of reserve, are specially mobilized and stimulated under the diabetic condition.

(b) Interdependence between the liver and the kidney

Although renal carbohydrate metabolism has not been studied as extensively
as hepatic carbohydrate metabolism, the available biochemical evidence indicate that these 2 tissues are quantitatively similar in this respect (Renold et al., 1956). For example, kidney slices from alloxan diabetic rats have been found to utilize less glucose from the medium and to produce more glucose when incubated in the presence of pyruvate than kidney slices from normal rats.

As shown in Table 5, the present histochemical investigation also demonstrated the presence of an intimate relation between the activity changes in the liver and the kidney.

(2) On the activity changes in the cardiac and skeletal muscles

As shown in Table 5, every muscle examined did not always present similar changes. For example, Ph-rylase activity was markedly increased in the cardiac muscle but distinctly reduced in the diaphragm, and the quadriiceps femoris showed indefinite or very slight changes in most cases or a tendency similar to that found in the cardiac muscle under the condition of lipemia. On the other hand, SD, LD and DPND activities showed similar changes in each muscle.

(3) On the activity changes in the adrenal glands

Of the several enzymes examined and already described, the changes in SD, ALPase and ACPase activities correlated roughly with those of urinary 17-KS and its fractions, although G-6-PD began to decrease at a relatively early stage, and esterase kept on increasing throughout all the stages.

In relation to these findings, the following facts require some explanation; (a) Except for the SD activity, the enzyme activities mentioned above showed no specific changes but rather changes similar to those found in the liver and the kidney. (b) According to several investigations, it has been demonstrated that as to G-6-PD, neither stress (cold exposure) nor hypophysectomy could affect its distribution in the adrenal cortex of the rat (Cohen, 1961), no matter how these treatments affected the distribution of glycogen and lipid, and that as for the SD, it could not be influenced by ACTH treatment (rat) (Dawson et al., 1961; Glick, 1960), and that as for ALPase, few changes could be demonstrated by the biochemical method after ACTH or DOCA administration (guinea pig) (Yoffey, 1955), although the distribution of an ascorbic acid-like substance was somewhat influenced by ACTH administration.

In short, (a) the activity changes in the adrenal cortex are not demonstrated as specific ones; (b) neither treatment with cortex-stimulating reagents nor hypophysectomy could bring about any changes in the enzyme activities themselves.

If this is the case, is it only fortuitous that a correlation could be found between the activity level and urinary 17-KS or its fraction? I think that there are two different pathways leading to the functional changes in the adrenals, that is, the direct one without influence on enzymes that are not directly related to specific adrenal gland function, and the indirect one leading to such changes by a side route, that is, by activity changes in those enzymes. It is conceivable that the functional level of an endocrine organ may even be influenced by such non-
Table 5. The enzyme activity changes in various organs from the rabbits which have been rendered diabetic for one to three weeks

<table>
<thead>
<tr>
<th>Enz.</th>
<th>Stg.</th>
<th>Liver</th>
<th>Kidney</th>
<th>Duodenum</th>
<th>Cardiac muscle</th>
<th>Diaphragm-muscle</th>
<th>Thigh-muscle</th>
<th>Adrenal gland</th>
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</thead>
<tbody>
<tr>
<td>Ph-rylase</td>
<td>↓↓</td>
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<td>↑</td>
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<td>↑</td>
<td>↑ or →</td>
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<td>SD</td>
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<td>LD(DPND)</td>
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<td>G-6-PD</td>
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<td>ALPase</td>
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<td>Esterase</td>
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Notes:—
- ↓↓, ↑↑: Marked changes
- ↓, ↑: Moderate changes
- ↑, ↓: Very slight changes
- →, ←: Indistinct changes
specific activities as those operative in the parenchymal cells.

(4) On the diabetic rabbits affected with lipemia

Some characteristic findings were obtained as described before, therefore these findings will be summarized here with some explanation.

Ph-rylase
In the cardiac muscle, the correlation between glycogen content and enzyme activity disappeared, that is, the former remained somewhat increased in spite of the irregular distribution of the reaction products, while the latter was rather somewhat reduced in most areas, compared with the controls. It may be supposed that lipid infiltration in the muscle fibers stands in causal relation to this phenomenon; however, it is unknown whether or not some other factors participate in it. In the kidney, no distinct increase in enzyme activity was shown although even more glycogen was deposited. In the quadriceps femoris, both the activity and the content were significantly increased, the former being more remarkable.

SD and LD (DPND)
These enzyme activities were markedly increased in the centrilobular cells of the liver, the distal portions of the renal tubule, and the cardiac muscle fibers in which lipid droplets had been deposited significantly. According to the review of Novikoff and Essner (1960) on SD activity, electronmicroscopic investigation made it possible to draw definite conclusions regarding the intimate relation between mitochondria and lipid droplets which is common in some circumstances. Palade (1959) speculated that the intimate contact reflects the oxidation of lipid, mobilized from droplets, by fatty acid oxidases of mitochondria. It may be naturally supposed that LD activity also participates in some similar mechanism.

ACPase
Similarly to the above dehydrogenases, this enzyme activity was also apparently increased in the cells in which lipid droplets were deposited. By the metal salt method, this finding was significant only around the droplets or on their boundaries, but by the azo-coupling method, the coarse granular reaction products could be seen densely deposited. Might these facts be related to the pinocytosis mechanism?

ALPase
In the central zone of the hepatic lobule, the reaction of the central vein and the sinusoidal lining was rather slightly reduced, while a fine granular reaction appeared in the cytoplasmic portion just beneath the cell membrane. At the present time, it is impossible to explain why membrane ALPase should be decreased here, but it may be supposed that it is transformed into the granular appearance moving towards the cytoplasmic portion.

Esterase
An especially intensified reaction was observed in the centrilobular cells of the
liver and in the peripheral tublar epithelia of the kidney. Therefore, it is reasonable to think that this finding may be related to the infiltration of the lipid droplets on which the esterase may be acting.

(5) Observations on F-rabbits

Some of the congenitally diabetic rabbits produced experimentally in our laboratory (Okamoto, 1960; Okamoto and Fukutome, 1955) spontaneously recovered from diabetes after a certain period (F-rabbits). Despite of their superficial recovery, however, the fact that they had a predisposition to diabetes is suggested by the following points:

1. 100 mg/kg of alloxan (Eastman Kodak) is required to produce diabetes in normal rabbits, while only 60 mg/kg is required in F-rabbits.

2. Most animals which have been rendered diabetic are on the road to recovery in from 2 to 4 months if they have not succumbed to lipemia, but F-animals show no such tendency for more than 1 year, that is, almost through life; sometimes they are affected with lipemia in even such late stages.

Therefore, it was estimated that the enzyme activity in various organs or tissues of these F-rabbits may, as a matter of course, show a characteristic distribution pattern. In fact, the results turned out as had been estimated; the findings obtained were roughly similar to those obtained in cases established for from 1 to 3 weeks. This was the case for the Ph-rylase activity in the heart and the diaphragm and also for the SD activity in the liver and the kidney. With regard to this enzyme activity in the cardiac muscle, the reaction products showed a relatively sparse distribution of the coarser granules, compared with the control section, as was shown in the diabetes of from 1 to 3 weeks’ duration.

On the other hand, the ACPase activity in the liver, the kidney, and the cardiac muscle was similar to that shown in diabetes established for from 1 to 4 months. However, the ALPase activity was similar to that seen in diabetes established for from 1 to 3 weeks. This enzyme in the adrenal cortex, especially in the zona fasciculata and reticularis, was significantly increased. This finding corresponds well with Ishizaki’s findings (Ishizaki, 1961) to the effect that the total 17-KS and its fractions (II, III, IV and VII) is significantly increased in alloxan diabetic F-rabbits when compared with simple alloxan diabetics.

SUMMARY

On the rabbits, which had been rendered diabetic for from 3 days up to 1 year or more, various enzymatic histochemical reactions in several organs have been observed. Independent of the diabetes-inducing reagents (alloxan or dithizone), similar results were obtained as follows:

Ph-rylase: A significant activity increase in the cardiac muscle of diabetic rabbits, regardless of the duration of the diabetic condition was found, but with some reduction in the diaphragm during the early and severely diabetic stages. Few changes were found in the quadriceps femoris in many cases. There was a
remarkable reduction in the whole lobule of the liver during the early stages, while recovery toward the normal level was more or less observable in the chronic stages. In the diabetic kidney, the activity in the thick limbs of Henle and in the distal convolutions, was significantly increased throughout all the stages. These changes were roughly parallel to those in glycogen content, but contradictory changes in the cardiac muscle under the lipemic condition were found between enzyme activity and glycogen content.

SD: The activity was distinctly increased in the diabetic liver (especially in the central zone of the lobules) and in the kidney (the tubular epithelia of the cortex and outer marginal zone of the medulla) throughout all the diabetic stages; also, a similar increase in the heart, the diaphragm and the quadriceps femoris of animals who had been diabetic for from 1 to 2 months, was detected.

LD and DPND: The histochemical findings on both enzyme activities showed approximately the same distribution. A remarkable increase was observed throughout the whole hepatic lobules, tubular epithelia of the renal cortex and medulla and muscular system in case of early severe diabetes. On the other hand, only a slight increase or even a decrease was shown in the cases established for more than 1 month's duration.

G-6-PD: The activity was found to be constantly and significantly reduced in the whole lobules of the diabetic liver and the tubular epithelia of the renal cortex except a part of the distal tubules in which the enzyme activity showed no decrease or even a distinct increase in the cases established for more than 1 week.

G-6-Pase: Through the whole course of the diabetic state, a remarkable increase was observed in the liver (especially in the central zone of the lobule), the kidney (the tubular epithelia of the cortex and the outer marginal zone of the medulla) and the mucous membrane of the small intestine.

ACPase: There was a distinct increase in the diabetic liver, particularly in the central zone of the lobules, and this increase was more remarkable in early severe diabetes. Almost no distinct changes in the renal cortex but for a significant increase in the proximal straight portion of medulla could be seen. This finding was also more remarkable in the early stages. However, they become indistinct when 1, 2, or 4 months had elapsed after the induction of diabetes. An activity increase in the heart was observed in most diabetic cases throughout all the stages.

ALPase: A marked increase was observed in the bile canaliculi throughout all the lobules of the diabetic liver, in the sinusoidal linings of the central zone, and in the proximal tubular epithelia of both the deeper zone of the cortex and the outer marginal zone of the medulla. Such increase was more remarkable in early severe diabetes.

ATPase: A relatively sparse distribution of the coarse granular reaction products was observed in the hepatic lobules and the renal proximal tubules in diabetes established for 3 days. In the cardiac muscle, however, a significantly intensified reactivity was found. A stronger activity of membrane ATPase could be seen in the liver, the kidney and the cardiac muscle of animals affected with lipemia. When diabetes had persisted for 2 months, the activity in the liver
showed a slight increase, although that in the kidney and in the cardiac muscle showed a few changes.

Non-specific esterase: Few changes were found in the liver and the kidney in diabetes established for 3 days. But a marked activity increase in the lipemic condition was observed. Furthermore, a relatively intense reaction was demonstrated in the cases established for 1 month as well.

Findings in the adrenal gland: The activities of G-6-PD, SD, ACPase and ALPase were all reduced, particularly in the fasciculata when diabetes had persisted for more than 2 or 3 weeks. Non-specific esterase in the fasciculata, however, remained increased.

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