A Histochemical Study of Peptidase in Liver, Kidney and Pancreas of Starved Mice

by

Toshio Yasoda

Department of Anatomy, Tokyo Women's Medical College, Shinjuku, Tokyo.

(Director: Prof. Morio Ihnoma)

Gomori (1954) has succeeded to demonstrate chromogenic aminopeptidase using glycyl α- and β-naphtylamine as substrates histochemically. Also Burstone and Folk (1956) have demonstrated aminopeptidase using L-leucyl-β-naphtylamide and DL alanyl-β-naphtylamide as substrates. Bergmann et Furton (1941) and Bergmann (1942) classified exopeptidases by the properties, i.e. specificity to substrates, activation and inhibition. They confirmed, that all exopeptidases acted at alkaline range. Since Johnson (1936) and his collaborators have discovered the activation of peptidases by metallic ions, it has been made clear, that many peptidases need metallic ions when they act. For the histochemical demonstration of exopeptidases suitable peptides to their enzymatic action must be used as substrates, but there are many difficulties to utilize these substrates histochemically. Thereafter the histochemical studies of exopeptidases are not sufficient. According to the investigations of Smith (1948) and James et al. (1950) that some peptidases are strongly activated by cobalt and this activation may be owing to the bridging of cobalt between enzyme and substrate, Hanabusa and Mochizuki (1955) have succeeded to demonstrate some peptidase using polypeptide as a substrate histochemically. Later Hanabusa (1956) ascertained, that his method was the reaction being ascribed to the enzymatic action of peptidase which decomposed polypeptides in peptone studying the several properties, i.e. the specificity, activation, inhibition and so on, of this method. Further he clarified the coincidence between the histochemical and the biochemical observations in the enzymatic distribution in normal organs of various animals. The author carried out this study to examine
the specificity of Hanabusa and Mochizuki's method for peptidase and to observe the changes of the activity of some organs of starved mice.

**Material and method**

Adult mice, which were fed with wheat and fish meal and about 25 g in body weight, were starved being given water only, and then killed by decapitation after 1, 3, 6, 12, 24, 48 and 72 hours. Immediately pieces of organs were placed in cold acetone. As the control of the examination, the organs, which were taken from the non-starved animals, were fixed similarly.

The peptidase activity was demonstrated by the following method of Hanabusa and Mochizuki (1955).

1. Small pieces of fresh materials are fixed with ice cold acetone for 24 hours, then dehydrated with absolute acetone for 24 hours at room temperature.
2. Clear in benzene for 15 minutes.
3. Immersed in paraffin at 52°C for 30 minutes. Cut sections at 5 micra.
4. Remove paraffin from the slides by immersion in petroleum ether, 2 times. Immerse in absolute acetone for one minute, then in 95% and 85% acetone. Rinse in distilled water.
5. Immerse in the substrate solution for 4 hours at 37°C.
6. Wash in distilled water for 20 minutes.
7. Immerse in 0.01% acetic acid for a few seconds, then wash in distilled water for 5 minutes.
8. Immerse in diluted yellow ammonium sulfate solution for a few minutes.
9. Wash in water for 10 minutes.

Sites of peptidae activity are indicated by a brown black deposit.

Composition of the incubating mixture:

The solution which contains M/10 cobalt chloride is made. 2 g of peptone is dissolved in 50 ml of this mixture. The peptone solution is dialyse in 150 ml of the mixed solution of ammonium chloride and cobalt chloride for 3 to 4 days in an ice box. When it is used,
ammonia water is added to adjust the hydrogen ion concentration. And then this solution is filtered. According to the original method, the reaction is the strongest at pH 7.5, but the author has obtained the best result at pH 7.8 comparing the reacting manners of the substrate solutions of various hydrogen ion concentrations to the above mentioned organs. The hydrogen ion concentration 7.8 coincides with the adequate hydrogen ion concentration of various peptidases biochemically. As almost all cobalt of the preparations is dissolved by immersion for only a few seconds in 0.1% acetic acid, which is used to avoid the unspecific staining of cobalt in the original method, in this experiment, 0.01% acetic acid, which shows good result, is used. As a control of the staining the inactivated sections were stained simultaneously. Namely by the previous treatments with heating, 10% formalin, with cyanide no staining is observed. Without the peptone the reaction for the enzyme were all negative. The sections were incubated in the substrate solution of pH 7.8 for 4 hours. The results were compared with that obtained by the substrate solution of pH 7.5, but no difference about the positive sites was observed.

Observations

Liver:
The peptidase activity in the control mouse (fig. 1) is moderate at the cytoplasm. In general the nuclei of the liver cells shows weaker reaction than the cytoplasm, but sometimes stronger. Most of the stellate cells have strong activity. Scarcely any difference in the activity is observed between periphery and center of the lobule. The activity of the cytoplasm of the epithelium of the interlobular bile duct is weak but the activity of its nuclei is strong.

One hour starvation: The activity of the liver cell cytoplasm becomes somewhat weaker. In no other portions is there any difference of the activity observed.

3 hours starvation (fig. 2): The activity of the cytoplasm of liver cells is very weak compared with the control and the one hour case. The activity of the cytoplasm is the weakest in all experimental cases. The activity of the nuclei of the liver cells is stronger than that of cytoplasm, but is weaker as compared with the control. In general the activity of the stellate cells is weak, but sometimes there is moderate activity. Not any difference in the activity between
the center and the periphery of the lobule can be observed. The epithelium of the interlobular bile duct shows no change in activity.

6 hours and 12 hours starvation: The activity of the stellate cells is very weak. The activity of the cytoplasm of the liver cells is stronger than of the 3 hours case and that of the nuclei of the liver cells is almost similar to the 3 hours case. No change is observed other than the above mentioned.

24 hours starvation (fig. 3): The activity of the liver cells is weaker than that of the control. The activity of nuclei is stronger than that of cytoplasm. The stellate cells have weak activity. Not any reagional difference is observed in the hepatic lobule. The activity of the epithelium of the interlobular bile duct is weak and is similar to the control group.

48 hours starvation: The findings in this group is almost similar to the 24 hours group.

72 hours starvation (fig. 4): The activity of the liver cells is strong, especially the activity of nuclei is very strong. The bile capillaries can be seen by their strong activity. The activity of the interlobular bile duct is stronger than the control.

Kidney:
The activity of peptidase of the kidney of control animals is strong in the convoluted urinary tubule, especially strong in the proximal convolution (fig. 5). The brush border of the proximal portion of the proximal convolution shows strong activity, but that of the distal portion shows hardly any activity. The activity of the cytoplasm of the proximal portion is stronger than that of the distal portion. The descending limb of Henle’s loop shows hardly any activity. The ascending limb of this loop has moderate activity. The distal convoluted urinary tubule shows a stronger activity than that of the ascending limb, but weaker than that of the proximal portion of the proximal tubule. Hardly any activity is observed in the collecting tubule. The glomeruli show moderate or weak activities. The glomerular capsule has moderate activity. The basement membrane of the proximal portion of the proximal convolution shows strong activity. The activity of the nuclei of the convoluted tubule is stronger than that of the straight tubule.

One hour starvation and 3 hours starvation (fig. 6): Hardly any difference is observed as compared with the control.

6 hours starvation: The activity of the convoluted urinary tubule becomes weak.
12 hours starvation (fig. 7): The activity of the convoluted tubule decreases except the strong activity of the proximal portion. The activity of the glomerulus is very weak.

24 hours starvation (fig. 8): Again the activity of the convoluted tubule and glomerulus increases.

48 hours starvation (fig. 9): The remarkable activity, which is observed in the 24 hours group, of the convoluted tubule decreases again, but the activity of the proximal portion of the proximal tubule is unchanged. The activity of the glomeruli becomes weak.

72 hours starvation (fig. 10): The activity of the convoluted tubule is weaker than in the previous group. The brush border of the proximal portion of the proximal convolution is strong, but the activity of the cytoplasm is weaker than in the other experimental group. The activity of the glomeruli decreases as compared with that of the previous group. The activity of the nuclei of the urinary tubule shows hardly any difference.

Pancreas:
The peptidase activity of the control animals (fig. 11) is observed moderately in the basal portion of the exocrine cell. No activity is observed in its nucleus. The cytoplasm of the endocrine cells does not show any activity, but their nuclei have moderate activity.

One hour starvation: The activity of the exocrine cell decreases a little.

3 hours starvation (fig. 12): No change is observed in the endocrine cells. The activity of the exocrine cells decreases remarkably.

6 hours starvation: The findings in this group is almost similar to the previous one.

12 hours starvation (fig. 13): Hardly any changes are observed in the endocrine cells. The cytoplasm of the exocrine cells increases its enzyme activity as compared with the previous group.

24 hours starvation (fig. 14): The increase in the activity of exocrine cells is remarkable especially in their basal portion but that activity is also observed in the luminal portion. The nucleus of the centro-acinous cell shows moderate activity.

48 hours starvation (fig. 15): The activity of the exocrine cell becomes stronger than in the previous group. The activity is very strong around the nuclei. The nuclei of the endocrine cells have moderate activity but the cytoplasm has hardly any activity.

72 hours starvation (fig. 16): The activity of the exocrine cells is limited around the nucleus. The nuclei show strong activity.
Hardly any difference is observed in the activity of the endocrine cells as compared with the control. The nuclei of the centroacinuous cells show strong activity.

Discussion

Hanabusa and Mochizuki (1955) have succeeded to demonstrate the activity of peptidase histochemically by the affinity to metal, using a polypeptide as a substrate. Berger et Johnson (1939, 1940), Robinson et al. (1953), Birmingham et Grade (1954) and others have observed the aminopeptidase, which is activated by Co-ion. Neuman et Smith (1951) reported about the remarkable activating function of Co-ion to aminopeptidase. Johnson et al. (1942), Maschmann (1941) and Smith (1948) reported that the glycylglycin dipeptidase was strongly activated by Co-ion. Also the fact, that the diglycylglycine tripeptidase was activated by Co-ion, was reported by Maschmann (1941) and Fleisher (1955). The activity, which is demonstrated histochemically by Hanabusa and Mochizuki's method, is derived from several peptidases or one of them.

The activity of nuclei of some normal organs is recognized by Hanabusa (1956). Gomori (1951) pointed out that the alkaline phosphatase reaction of the cell nuclei was due to diffusion. Siebert et al. (1953) have found the existence of some peptidase in nuclei biochemically. Among the peptidases found in nuclei, the glycylglycine dipeptidase is strongly activated by Co-ion. If Co-ion becomes the bridge between the enzyme and the substrate, the positive reaction of the nuclei is approved. The author's experiment shows that the activity of the nuclei of liver cells is almost the same as that of the cytoplasm, and the activity decreases for several hours in starvation. At this stage the nuclei show stronger activity than the cytoplasm. At 72 hours starvation the activity of the nuclei is very strong. The cytoplasm of the endocrine pancreatic cells shows hardly any activity but their nucleus has moderate activity. The activity in the nuclei of the exocrine pancreatic cells is observed at least from 48 hours in starvation, and the activity increases remarkably, being limited to nucleus and around nucleus. In the kidney the activity of nuclei is moderate in the cortex and medulla. The kidney is a peptidase rich organ. Nagatani and others (1956) observed cathepsin- and trypsin-like enzymes, which acted at the alkaline
A Histochemical Study of Peptidase in Liver, Kidney etc.

range by Takamatsu and Wada’s method (1954) for endopeptidase in the organs of normal rabbits and found, that only in the kidney the nuclei showed strong activity. The distribution of proteinase in the kidney is interesting. Takamatsu (1950) pointed out the affinity of metallic ions, which are used in histochemical studies, to tissues and cells. In Hanabusa and Mochizuki’s method attention must be paid to the affinity of cobalt ion to cells and nuclei. The author could deny the affinity of cobalt-ion in this method, by using the substrate without pepton for over 24 hours. Also the inactivated sections showed negative reaction.

Burstone and Folk (1956) reported on the aminopeptidase activity of normal organs of man, monkey and rat histochemically. According to them the activity is observed in the cytoplasm of liver cells and not in the capsule of liver and bile duct in man, monkey and rat. In the kidney of the monkey the activity of aminopeptidase is localized at the cortex, and the site of activity is different, when L-Leucyl-naphthylamide and DL-alanyl-β-naphthylamide are used as substrates respectively. Weak activity is observed in glomerulus and Bowman’s capsule, but the reaction of the ascending limb of Henle’s loop is negative. The luminal cytoplasm of the pancreatic cells of man and monkey shows strong activity of aminopeptidase, also the interlobular duct demonstrates positive reaction. In some portion of Langerhans’ islet the activity distributes itself granular. In the pancreas of the rat the same distribution of the activity is observed but is far weaker than that of man and monkey. Weil and Jennings (1941) observed the activity of catheptic aminopeptidase in the kidney of the rabbit and said, that the activity of proximal and distal convolutions were two times stronger than that of ascending and descending limbs of Henle’s loop and four times stronger than that of the collecting tubule, and that the activity of dipeptidase distributed itself in the same manner as catherine aminopeptidase, except at the collecting tubule, where no positive reaction was observed. Maschmann (1941) described the existence of several peptidases, which were activated by Co-, Mn-, Mg- and Zn-ions, in the liver and kidney of the mouse. Abderhalden (1942) reported, that the increase or decrease of di- and polypeptidase in blood plasma, liver and muscle of rats, which were fed with the diets with sufficient protein and insufficient protein respectively, did not be observed. Bargoni (1950) fed male rats with such food as: sugar 87%, olive oil 8%, table salt 4%, yeast and vitamin.
B, K, A, D, E 1%, for 25 days, and compared these rats with the
rats fed with the food containing casein instead of sugar and re-
ported the 7.9% decrease of the dipeptidase activity of the liver in
the former rats.

Maschmann (1942) observed the remarkable activity of
aminopeptidase in ascites in cases of ascites tumor. Gomori
(1954) reported the stronger chromogenic aminopeptidase activity
in the cancer of the kidney than in a normal kidney. Birnbaum
(1954) observed, that the activity of benign hepatome was similar
to that of the normal liver, but at malignant tumors the activity
was very strong. Aoki (1954) and his collaborators found the
increased activity of peptidase in various cancers. Until the
present time, no report has been published on peptidase during
starvation. The author has examined the changes of peptidase
activity of liver, pancreas and kidney during starvation. In the
author's experiment the distribution of the peptidase is wider spread
than that of the aminopeptidase of Burstone and Folk. This
result is due to the fact, that the peptidase reaction used by the
author is probably demonstrated by several enzymes which are ac-
tivated by Co-ion, and so the wide spread distribution seems to be
natural. The significance of the absence of the activity in Langer-
hans' islet is not clear. Hanabus did not observe the peptidase
activity in the glomeruli, but the author can demonstrate moderate
activity. When the hydrogen ion concentration of the substrate
solution moves to a neutral region, at PH 7.0 the activity can not
be observed, and at PH 7.5 the activity of the glomerulus is ob-
served but weak. The substrate solutions with various hydrogen ion
concentrations were used and it has become clear, that PH 7.8 is
the optimum hydrogen-ion-concentration for histochemical methods.

Among kidney, liver and pancreas, the activity of kidney is
the weakest. The activity of liver-cell-cytoplasm begins to decrease
just after fasting, it is weakest after 3 to 6 hours and it increases
again gradually after 12 hours. At 24 hours starvation, the activity
becomes somewhat strong but it is still weaker than that of the
control. After 24 hours the activity becomes stronger and at 72
hours it is stronger than that of the control. The activity of nuclei
-of liver cells are weaker than of the cytoplasm in the control, but
with the progress of starvation it becomes always stronger than
that of the cytoplasm. At 72 hours it is the strongest. The activity
-of the bile duct is weak till the 48 hours starvation but at 72 hours
it becomes somewhat stronger. The activity of the stellate cells is the strongest in the control and it decreases with passage of time. The enzyme activity of the convoluted urinary tubule shows the same degree as the control till the 6 hours starvation, but decreases remarkably at 12 hours. Hardly any change in the activity is observed in the proximal portion of the proximal convolution. The activity of the glomerulus decreases by 12 hours starvation. At 24 hours the strong activity as that of the control is observed in the cortex. Then its activity gradually decreases. At 72 hours starvation the proximal convolution and glomerulus show weak activity except strong activity at the brush border. The activity of the nucleus can be observed in the straight tubule but it is weaker than that of the convoluted tubule. The activity of nuclei increases with the progress of starvation and it is the strongest at 72 hours. But it is not so remarkable as in the liver and pancreas. The exocrine cells of the pancreas show remarkable decreasing activity till 12 hours. At 24 hours they show far stronger activity than that of the control, and the strongest at 48 hours. At 72 hours the cytoplasm around the nucleus shows strong activity. The activity of nuclei is not observable in the control and till 24 hours, but at 48 hours and 72 hours it is strong. The activity of the endocrine cells of the pancreas is almost negative, but their nuclei show moderate activity from the control, to 72 hours starvation. Many investigators reported, that the activity of peptidase in the tumor increased. The author has investigated the peptidase activity during starvation, and found the complex results above mentioned. The significance of these results must be clarified biochemically in future.

Summary

The peptidase activity of the liver, kidney and pancreas of starved mice has been investigated.

The activity of the liver, kidney and pancreas gradually decreases till 12 hours starvation. The activity at 24 hours increases in every organ. After 24 hours the activity decreases only in the kidney, and increases in the pancreas and liver with the progress of starvation. Especially the increase of activity in the pancreas is remarkable. In the pancreas the activity is noticeably stronger, than that of the control after 24 hours starvation. Only in the liver, the activity at 72 hours is somewhat stronger than that of the control.
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References


A histochemical Study of Peptidase in Liver, Kidney etc.


Explanation of Plate Figures

Fig. 1. The peptidase activity of the liver of the control.
Fig. 2. 3 hours starvation. liver.
Fig. 3. 24 hours starvation. liver.
Fig. 4. 72 hours starvation. liver.
Fig. 5. The peptidase activity of the kidney of the control.
Fig. 6. 3 hours starvation. kidney.
Fig. 7. 12 hours starvation. kidney.
Fig. 8. 24 hours starvation. kidney.
Fig. 9. 48 hours starvation. kidney.
Fig. 10. 72 hours starvation. kidney.
Fig. 11. The peptidase activity of the pancreas of the control.
Fig. 12. 3 hours starvation. pancreas.
Fig. 13. 12 hours starvation. pancreas.
Fig. 14. 24 hours starvation. pancreas.
Fig. 15. 48 hours starvation. pancreas.
Fig. 16. 72 hours starvation. pancreas.
Plate III

T. Yasoda