Zusammenfassung. Es wird gezeigt, dass die elektronenopaken Matrizen der adrenergen Vesikel nach Inkubation mit ATP und Magnesium gut erhalten sind, und zwar trotzdem der Gehalt an Katecholamin bis zu 75% verloren ging. Die Elektronendichte kann mit Uranylazetatfärbung gesteigert werden.

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A Study on Amino Acids in the Gastric Mucosa During Differentiation and their Significance. II

As a means of isolating the epithelial cells of the mucosa, the 33% alcohol immersion method is well known. FUJIE[1] has succeeded in extracting some contents in the surface epithelial cells of the rat gastric mucosa by this method, and has proved by the amino acids analyzer that dozens of amino acids and several kinds of related compounds exist in the alcohol. MABUCHI[2] proved experimentally that lysine, histidine, arginine and tyrosine have the effect of promoting production of secretory granules in the gastric chief cells of rats.

The author[3] reported in a previous paper that the amount of amino acids in the differentiating gastric mucosa is very small; in the developing mucosa it increases regularly day by day, but the increase of the restricted amino acids – lysine, histidine, arginine, tyrosine and isoleucine – is delayed more than that of others, and the beginning of this increase coincides with the first appearance of rich secretory granules in the gastric chief cells.

To investigate further the effect of lysine, histidine, arginine, tyrosine and isoleucine, the author examined their influence on the embryos when pregnant rats were given with these amino acids.

Table I. Dose and kind of amino acids and related compounds injected into pregnant rats

Substance	Group A	Group B
Tryptophan		0.2
Lysine	1.6	
Histidine	0.4	
Arginine	0.7	
Cysteic acid		1.5
Taurine		23.7
Aspartic acid		2.9
Threonine		2.8
Serine		4.9
Glutamic acid		16.9
Proline		2.0
Glycine		9.8
Alanine		7.8
Valine		1.4
Cystine		0.7
Methionine		0.6
Isoleucine	0.7	
Leucine		1.7
Tyrosine	0.8	
Phenylalanine		0.8

Dose injected is $\times 10$ μM of the value ascertained from 1 g-stomach of adult rats fasting for 24 h.

Materials and methods. Wistar rats weighting 200–250 g were used for the experiment. The estrus female rat was kept in a wire cage with a male for a night. The next day was calculated as the 1st day of pregnancy. Pregnant rats were classified into A, B and C groups. They were injected with amino acids, as shown in Table I, throughout pregnancy. C-group rats were kept as controls.

Neonates of A, B or C group were classified in the same way into A, B or C groups, and materials were taken from the neonates on the 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 and 23 postnatal day. To take materials, the neonates were separated from their mothers for more than 6 h, while the infant rats were not given food for more than 12 h. After weighing, the thorax and the abdomen were opened under deep ether narcosis, and Luna's liquid was injected into the artery from the left heart ventricle for the vital fixation. Small pieces taken from the stomach (glandular portion, if distinguishable) were put into Zenker's or Kolster's fixative for the post fixation. Paraffin sections of 7 μm were cut from the material fixed by the former fixative and stained by hematoxylin-eosin for histological observation; 4 μm sections, on the other hand, were cut from the stomach fixed by the latter fixative and stained by Heidenhain's iron hematoxylin for cytological observation of the chief cells.

Observations. The body weight of the neonates and the infant rats is shown in Table II. All the neonates, regardless of grouping, are covered with short white hair on the 7th–9th postnatal day, their eyes open on the 13th–15th postnatal day period.

1. *Histological observation.* The gastric mucosa on the 1st postnatal day is quite similar to that of the control rats, i.e. the mucosa forms very small plicae and acidophil cells can be distinguished in the epithelium. On the 3rd postnatal day, cell proliferation and pit formation at the basal part of the plicae can be observed. This may be interpreted as a tendency towards formation of the tubular glands. At this stage, this tendency seems more pronounced in rats of A group than in those of B and C groups. In the 5th postnatal day, the mucosal development is the same in the 3 groups. Thus, it may be presumed that the development from the 3rd to 5th postnatal day in B or C group rats may have already begun during the 1st–3rd postnatal day period in A group. The mucosa of A, B and C group rats on the 7, 9, 11, 13, 15, 17, 19, 21 and 23

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[2] Y. MABUCHI, Archum histol. jap. *31*, 255 (1970).
[3] T. NISHIOKA, Experientia *30*, 659 (1974).

postnatal days shows a similar development, regardless of the experimental conditions.

The differentiation of the gastric gland seems to be finished during 13 postnatal days, thenceforth the development seems to begin. The distance between the surface of the mucosa and the lamina muscularis mucosae, measured by the same method as described in the previous paper, will show these proceedings (Table III).

2. *Gastric chief cells.* As described in the histological observations, the epithelium of the mucosa consists of similar columnar cells regardless of the apex and the basis of the plicae and also of some acidophilic cells differentiated from these on the 1st postnatal day. Cell proliferation and pit formation can be seen adjacent to the base of the plice on the 3rd postnatal day. At this stage, the surface epithelial cells are columnar with oval nuclei and differ from the adjacent cells proliferated at the basis of the plica, which latter are cuboidal with big round nuclei. The author considered the latter cells may be the differentiating cells of the chief cells. The cells contain granular, short rod- and long rod-shaped mitochondria. In B and C group rats the cells show no great change until the 13th postnatal day, except that some enlargement of the cell can be seen and that big granular mitochondria are scattered in the cytoplasm of the 9th–13th postnatal day rats. In A group rats, however, most of the chief cells contain secretory granules on the 3rd postnatal day. Secretory granules increase in the cells on the 5th day. Thenceforth the secretory granules rich cells are present at all times in the gland. On the 11th postnatal day and thereafter, corpulent secretory granules can be also seen.

Table II. The body weight of the neonates and the infants (g)

Postnatal day	Group A	Group B	Group C
1	6.6 ± 0.4	7.6 ± 0.5	5.9 ± 0.4
3	4.4 ± 0.3	7.3 ± 0.7	8.3 ± 0.6
5	10.3 ± 0.7	10.1 ± 0.7	12.0 ± 0.7
7	11.5 ± 1.0	15.1 ± 1.2	14.2 ± 0.7
9	18.1 ± 0.8	16.2 ± 2.1	18.1 ± 1.5
11	17.8 ± 1.5	17.5 ± 0.6	17.8 ± 0.8
13	24.9 ± 2.8	25.9 ± 0.9	21.2 ± 0.7
15	23.9 ± 4.0	23.6 ± 1.2	22.1 ± 1.6
17	24.6 ± 4.4	24.6 ± 2.8	26.6 ± 0.9
19	33.8 ± 5.4	25.2 ± 3.9	36.9 ± 3.9
21	36.2 ± 1.8	23.8 ± 1.7	31.6 ± 3.1
23	41.5 ± 1.4	39.9 ± 3.0	35.7 ± 5.2

Table III. Distance between the surface and the *lamina muscularis mucosae* of the gastric mucosa (mm)

Postnatal day	Group A	Group B	Group C
1	4.1 ± 0.7	4.2 ± 0.5	4.4 ± 0.3
3	5.3 ± 0.8	4.6 ± 0.7	4.3 ± 0.5
5	5.5 ± 0.7	5.6 ± 0.5	6.0 ± 0.6
7	5.9 ± 1.3	5.7 ± 0.3	5.5 ± 0.5
9	5.9 ± 0.2	5.6 ± 0.2	5.7 ± 0.5
11	6.3 ± 1.0	6.4 ± 0.8	5.6 ± 0.5
13	6.7 ± 0.5	6.8 ± 0.4	6.4 ± 0.5
15	8.2 ± 1.8	8.1 ± 0.7	7.6 ± 1.0
17	9.7 ± 0.8	9.2 ± 0.6	9.1 ± 1.0
19	9.5 ± 1.3	8.8 ± 0.5	11.3 ± 0.5
21	10.8 ± 0.2	10.6 ± 0.5	11.1 ± 1.4
23	11.2 ± 0.3	11.4 ± 1.5	11.8 ± 0.5

Discussion. In a previous paper[3], it was observed that the morphological differentiation of the gastric chief cells could be noted from the 3rd–5th postnatal day, but the initiation of the function (appearance of the rich secretory granules) was from the 15th–17th postnatal day, and lysine, histidine, arginine, tyrosine and isoleucine in the gastric mucosa increased from the 15th–17th postnatal day, coinciding with the appearance of rich secretory granules in the chief cells. This latter fact seems to suggest that the presence of these amino acids may have some connection with the function of the chief cells. Thus the author attempted to study the effect of lysine, histidine, arginine, tyrosine and isoleucine given to the rat embryos on the secretory activity of the embryonic chief cells. In the amino acids and the related compounds shown in the gastric mucosa, the author puts lysine, histidine, arginine, tyrosine and isoleucine into A group and the others into B group. Pregnant rats were injected with the amino acids of A or B group from the 1st day of the pregnancy. Thus A or B group rats were made.

The neonates of A or B group rats do not show any remarkable difference from the control rats (C group) as regards body weight, date of eye-opening and histological differentiation and development of the gastric mucosa. However, the differentiation of the mucosa on the 3rd postnatal day in A group neonates is identical with that of the 5th postnatal day in other groups. This would seem to be indicative of an early initiation of the differentiation stage in A group. In the gastric chief cells stained by the iron hematoxylin, rich secretory granules can be observed on the 3rd postnatal day in A group neonates, then the granules increase in the cells from the 5th–7th postnatal day period. However, in B group neonates, the secretory granules in the chief cells appear, for the first time, on the 15th postnatal day. This is quite similar to what has been observed in the case of control neonates (previously reported). Thus, it may be worth noting that the differentiation of the gastric mucosa in A group seemed to be more pronounced at the 3rd postnatal day than in other groups.

Lysine, histidine, arginine, tyrosine and isoleucine injected into pregnant rats seem to promote the differentiation of the gastric glands of the embryos and to accelerate the initiation of the function in the embryonic chief cells. When the appearance of cells differing from the surface epithelial cells (cuboidal cells with big round nuclei) is considered as the morphological differentiation of the chief cells, the appearance of rich secretory granules in these cells is the initiation of the secretory activity, i.e. the functional differentiation; and the latter is about 10 days later in the normal neonates. Thus, it is presumed that in A group neonates the morphological and the functional differentiation of the chief cells occurs almost at the same time, because lysine, histidine, arginine, tyrosine and isoleucine injected into pregnant rats act effectively on the acceleration of the functional differentiation of the chief cells in the embryos.

Compared with the results obtained by MABUCHI[2], i.e. that lysine, histidine, arginine or tyrosine injected into adult fasting rats have the effect of promoting the production of secretory granules in the chief cells, there seems to be no discrepancy.

According to HELANDER[4], peptic activity in the homogenated rat embryonal stomach can be determined from the 19th day of gestation and increases thenceforth. In fact, he proves by means of the electron microscope that

[4] H. F. HELANDER, Gastroenterology *56*, 53 (1969).

the secretory granules in the zymogen cells differentiated at the same stage. Using the light microscope, no cells such as the chief cells (pepsine secreting cell) can be seen in 19–20-day-old rat embryos, and of course no secretory granules of the chief cells. Before the morphological differentiation of the chief cells, sometimes many secretory granule-like granules can be observed in the apical part or in the supranuclear portion of the epithelial cells (where it coincides with the mucous part of the surface epithelial cells of adult rats). However, the author has no evidence to prove that these granules may be the peptic granules. With the light microscope, it is difficult to relate the peptic activity with the morphological source.

Zusammenfassung. Lysin, Histidin, Arginin, Tyrosin und Isoleucin wurde schwangeren Ratten täglich injiziert. In den von den injizierten Tieren geborenen Ratten tritt die Sekretgranula in den Hauptzellen ca. 2 Wochen früher auf als in den Kontrollen.

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The Effect of Diphtheria Toxin on Primary Cell Cultures of the Adult Human Liver

The human liver is seriously damaged during diphtheria and, under this pathological condition, cloudy swelling, cytoplasmic vacuolation, fatty degeneration and, finally, necrosis of the hepatocytes occur. Moreover, diphtheria toxin induces considerable mitochondrial swelling in guinea-pig hepatocytes, followed by lesions which are very similar to those observed in the human liver[1,2].

Nevertheless, it is very difficult to ascribe some of these effects to the toxin per se rather than to post-mortal degeneration and to remarkable circulatory modifications causing anoxia.

The effect of diphtheria toxin was also studied on the human liver cell cultures of the Chang's strain, which constitute a biological substrate free from hormonic, nervous, circulatory and immunological influences. At the concentration of 0.005 MLD/ml diphtheria toxin causes, in these cell cultures, cytoplasmic vacuolation, fatty degeneration, disappearance of mitosis and death of the cells[3]. Unfortunately, the Chang's strain is a very dedifferentiated cell line, which has lost most of liver cells morphological and biochemical characteristics[4]. The cytopathic effect obtained in these cells by treatment with diphtheria toxin is, therefore, quite similar to that observed in other cell strains derived from human and animal tissues different from the liver[5,6].

Primary adult human liver cell cultures were successfully obtained not long ago[7] but actually they can be considered as a suitable experimental substrate which demonstrate the morphological features that are characteristic of liver parenchimal cells. In this paper we have studied the effect of different concentrations of purified diphtheria toxin on human hepatocytes cultured in vitro in order to gather further information on the relationships between diphtheria and hepatic lesions in the man.

Materials and methods. Cell cultures. Adult human liver cell cultures were prepared by the method of ZUCKERMAN et al.[8]. Liver tissue was obtained by surgical biopsies from patients hospitalized for peptic ulcer. Liver cells were grown in Eagle's medium (Difco) containing 50 µg/ml of cephaloridine and supplemented with 20% of fetal calf serum (Microbiological Associates) on polythene discs placed into glass cups containing 1.0 ml of nutrient medium and incubated at 35 °C in air atmosphere enriched with 5% CO_2. After about 2 weeks, a suitable layer of cells was obtained and the cultures were then utilized for the experiments.

Reagents. Highly purified diphtheria toxin (kindly supplied by the Eli Lilly, Indianapolis, U.S.A.) lot. 00098, containing 600 Lf/ml was used. This toxin was diluted in the nutrient medium to obtain a final concentration of 0.1 and 1.0 Lf/ml respectively. The final pH was 7.3. Anatoxin was obtained in our laboratory from this same toxin by routine procedure. Antitoxin (horse serum, supplied by the Sclavo Institute, Siena, Italy) containing 500 AU/ml was used.

Experiments. The nutrient medium was eliminated from the glass cups and substituted with 1.0 ml of toxin dilutions. The cell cultures were then placed in a 35 °C incubator. After 3, 6, 12, 24 and 48 h the cultures were extracted from each glass cup, twice washed with Eagle's medium at +4 °C, fixed and stained as reported elsewhere[9]. These specimens were examined under a Leitz Orthoplan phase-contrast microscope. Fluorescence microscopy was carried out by fixing some specimens in ethanol for 10 min and staining with acridine orange (Merck) 1:2000 in Michaelis buffer, pH 3.5. A number of preparations were also stained by Herxheimer's method to demonstrate

Fig. 1. Two normal liver cells in culture. Note the large nuclei and the numerous round mitochondria scattered in the cytoplasm.

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