STUDIORUM PROGRESSUS

A Study on Amino Acids in Gastric Mucosa During Differentiation and Their Significance. I

FUJIE et al.[1] using the 33% alcohol method, carried out experiments in which they isolated the surface epithelial cells from the mucous membrane of rat gland stomach and reached the conclusion that the gastric hormone 'productin' (FUJIE) which is contained in these cells was extracted into the alcohol. That is to say, a promotive effect on the production of secretory granules in the gastric chief cells can be observed in starved rats after a s.c. injection of the NaCl-solution obtained from the evaporated alcohol.

Vacuoles are seen in the subnuclear portion of the gastric surface epithelial cells and these show an increase or a decrease under respective experimental conditions. FUJIE surmises that the content of these vacuoles will be his 'productin'. The existence of an imidazol group and a phenol group in the subnuclear portion of the surface epithelial cells where the 'productin' vacuoles were seen was proved histochemically[2], and this would suggest further research of 'procudtin'.

To find the effective substance in the 33% alcohol mentioned above, FUJIE et al.[3] analyzed the alcohol by the amino acids analyzer and proved the presence of histidine (imidazol group), tyrosine (phenol group), dozens of other amino acids and several related compounds. MABUCHI[4] examined the effect of each of these amino acids on the gastric chief cells of fasting rats by the s.c. injection of amino acid solution and obtained results that lysine, histidine, arginine and tyrosine all promoted the production of secretory granules in the chief cells. His results seemed to suggest a substance similar to 'productin' component.

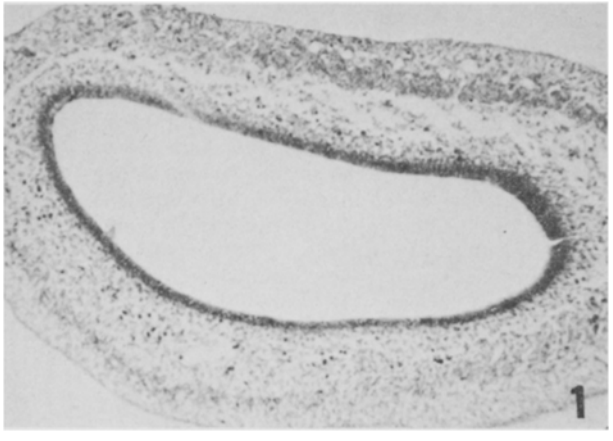

Fig. 1. The stomach of a 15-day-old embryo. H + E staining. ×100.

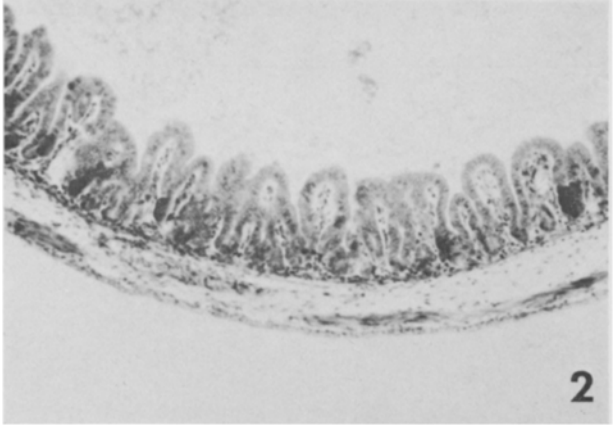

Fig. 2. The stomach of a 21-day-old embryo. Epithelium and the almina propria mucosae produce many small plicae. ×100.

Continuing from the above results, the author, using fetal and neonatal rats, investigated amino acids in the gastric mucosa during the gastric development and the relationship between the initiation of the secretory granule production in the chief cells and the amino acids in the mucosa.

Materials and methods. Adult Wistar rats were used. The estrus cycle of the female rat was examined by vaginal smear stained with Giemsa solution. The estrous female rat was kept in a wire cage with a male for 1 night. The next day was calculated as the 1st day of pregnancy.

Materials were taken from fetal rats and neonatal rats during a period of from 15 days after fertilization to 23 days after birth. To take materials for the histological observation, the thorax was opened under deep ether narcosis and Luna's liquid was injected into the artery from the left heart ventricle for the vital fixation. Then the stomach was taken out, small pieces were taken from the glandular portion of the stomach (gland stomach) and they were put into Zenker's fixative or Kolster's fixative for post-fixation.

Routine dehydration by ethanol and embedding in paraffin followed. Serial sections of 7μm were cut from the stomach fixed by Zenker's fixative and were stained by hematoxylin-eosin for general histological observation; sections of 4μm were cut from the material fixed by Kolster's fixative and these were stained by Heidenhain's iron hematoxylin for cytological observation of the gastric gland.

Also, the thorax and abdomen of other rats were opened under deep ether narcosis, and 0.9% NaCl solution was perfused from the left heart ventricle under constant pressure. The liver was cut into many lobi and the NaCl solution perfusion was continued until only a colorless fluid flowed out of the incized liver. Then the gland stomach was taken out, opened from the lesser curvature and washed in physiological saline solution. Then the materials were placed in 33% alcohol. In the case of a small stomach 2.5 ml alcohol per a stomach, of a big stomach 5.0 ml alcohol per stomach was used. Several stomachs were placed in a bottle.

After a 12 h immersion at 4 °C, the stomachs were removed. Alcohol was centrifuged for 20 min at 4000 rpm, the supernate was evaporated and the residue dissolved in pH 2.2 sodium citrate buffer and filtered. The sample thus obtained was analyzed by the Yanagimoto Amino Acids Analyzer LC-5.

1. *Histological observations.* The stomach of a 15-day-old embryo is a single vesica consisting of inside epithelium and outside mesenchymal tissue (Figure 1). No distinction between the fore-stomach and the gland-stomach can be

[1] K. FUJIE, T. KOIKE and Y. MABUCHI, Archum. histol. jap. *27*, 247 (1966).
[2] K. FUJIE, T. YAMASHITA, K. TOJYO and I. YAMAGATA, Archum. histol. jap. *13*, 175 (1957).
[3] K. FUJIE, Y. MABUCHI, K. ISHIMURA and J. HIRAOKA, Wakayama med. Rep. *12*, 99 (1967).
[4] Y. MABUCHI, Archum. histol. jap. *31*, 255 (1970).

seen. The epithelium is an irregular stratified epithelium showing nuclei of various forms, round, elliptic or oval etc. A differentiation of the tunica muscularis in the mesenchymal tissue can be seen. The irregular stratified epithelium changes gradually into a single columnar epithelium, the tunica muscularis becomes plain and a connective tissue between the epithelium and the tunica muscularis develops during the 16th–20th embryonic day period.

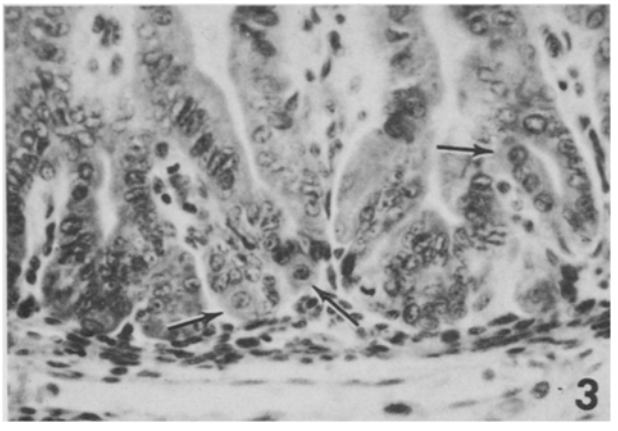

Fig. 3. Acidophil cells in the epithelium and in the cell proliferation. Three cells are shown by arrows. ×400.

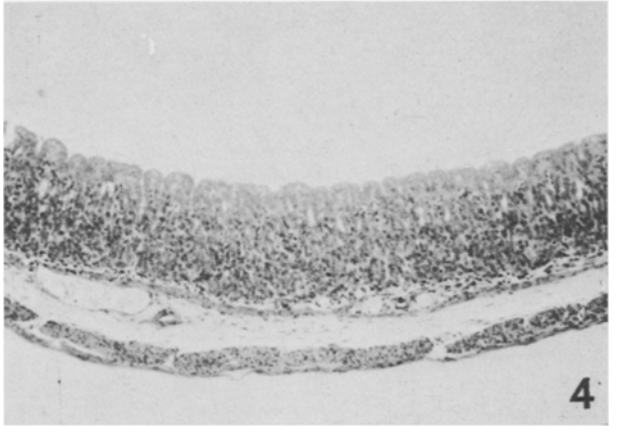

Fig. 4. The gastric mucosa of the 13th postnatal day rat. ×100.

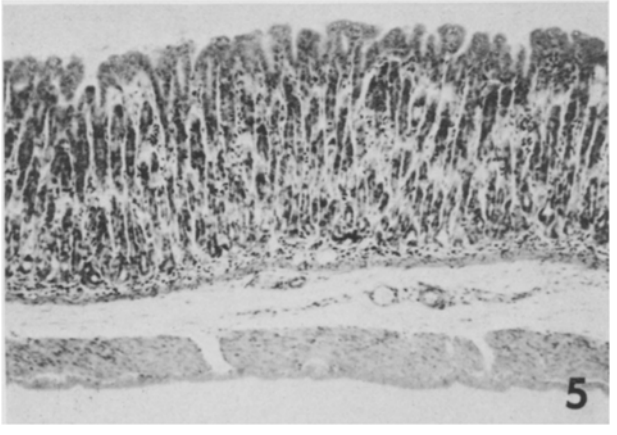

Fig. 5. The gastric mucosa of the 21st postnatal day rat. × 100.

In prenatal rats (21–22-day-old embryos), many plicae can be seen on the surface of the mucosa. Near the basis of these plicae the formation of the lamina muscularis mucosae is seen also. No differentiation of the epithelial cells can be seen (Figure 2).

From the 1st–3rd postnatal day, some proliferation of epithelial cells at the base of the plicae can be seen. In the epithelium, acidophil cells appear at the same time. It seems to be a histological differentiation of the epithelial cells into parietal cells (Figure 3). Other epithelial cells are columnar regardless of whether they are at the apex or the base of the plicae. In the proliferated part, a tubular pit begins to be formed for the lamina muscularis mucosae, consequently the distance between the surface of the epithelium and the lamina muscularis mucosae becomes greater. This process is connected with the formation of the tubular glands.

In the 3rd–5th postnatal day period, the form of the glands as yet seems not to be definite, but the epithelial cells of the tubular pit (except for the acidophil cells) become cuboidal, have big round nuclei and are different from the surface epithelial cells. This may show a morphological differentiation in the chief cells. It becomes more pronounced during the 7th–9th postnatal day period. On the 13th postnatal day, the constitution of the gastric mucosa (Figure 4) becomes similar to that of the adult rats. Figure 5 shows the gastric mucosa of the rat 3 weeks after birth. Comparing these, it seems that the differentiation of the gastric mucosa is complete within 13 days after birth, and thenceforth it proceeds to develop.

2. *Measuring of the distance between the surface of the mucosa and the lamina muscularis mucosae.* As the author observed an increase in the distance between the mucosa surface and the lamina muscularis mucosae because of the constitution of the tubular glands, he considered this may be connected with the process of differentiation or development of the mucosa. Colour films taken under the same magnification (×10 ocular, ×10 objective) were put on the illuminating box («der Schaukasten») and measured by the lucid measure. Readings obtained from several places in the same film were averaged. (Table I).

Readings will also be shown in the Figure 6. In the graph, some coincidence with the histological observations can be seen. That is to say, 1. the 1–3 day postnatal period is the non-differentiation stage, and in that period no increase of distance can be seen, 2. during the 5–13 day period which

Table I. The distance between the mucosa-surface and the *lamina muscularis mucosae*

Postnatal day	Value measured (mm)
	Mean ± S.D.
1	4.4 ± 0.3
3	4.3 ± 0.5
5	6.0 ± 0.6
7	5.5 ± 0.5
9	5.7 ± 0.5
11	5.6 ± 0.4
13	6.4 ± 0.5
15	7.6 ± 0.9
17	9.1 ± 1.0
19	11.3 ± 0.5
21	11.1 ± 1.4
23	11.8 ± 0.5

is a differentiation stage, the distance increases but not in a regular day-by-day fashion, 3. from the 13th day on, the distance increases in a marked day-by-day manner and a developmental stage is definitely present.

3. *Amino acids in the extract of the gastric mucosa.* Conditions and results of amino acids analysis are shown in Table II. Amino acids are proved to exist in small amounts and show a negligible fluctuation during the 1st–11th postnatal day period. Thereafter, they show a distinct tendency to increase. This tendency coincides with the histological development of the gastric mucosa. However, the beginning of increase of some amino acids differs from that of others; lysine, histidine, arginine, tyrosine and isoleucine show a later increase than others, whereas methionine and tryptophan show no visible tendency to increase at all during this period. These results are tabulated in Table III.

It is natural that the increasing tendency of amino acids goes hand in hand with the development of the gastric mucosa, but it will be noticed also that the initiation of the increase is late in the case of lysine, histidine, arginine, tyrosine or isoleucine.

4. *Observations on the gastric chief cells.* It can be surmised that the chief cells will differentiate from the epithelial cells following the development of the gastric mucosa. The differentiation of parietal cells, as mentioned above, can be observed on the 1st postnatal day, but differentiation of chief cells can only be seen in the 3rd–5th postnatal day period as different cells from the surface epithelial cells, being cuboidal and having big round nuclei. Using Heidenhain's iron hematoxylin staining (Figure 7), short and long rod shaped mitochondria in the perinuclear cytoplasm, granular mitochondria in the supranuclear cytoplasm and no secretory granules in the apical cytoplasm are seen. The cell figures show no change during the 5th–13th postnatal day period. In the latter half of this term, a few small granules, larger than the granular mitochondria, can be observed in some cells. Presumably they are corpulent mitochondria. On the 15th postnatal day, cells having distinct secretory granules can be observed. The number of the granules in each cell is large. Thenceforward, in all cases, the chief cells have abundant secretory granules, and some of the granules show a corpulency, suggesting accumulation in the cell. The appearance of secretory granules in the chief cells seems not to be a gradual day-by-day but a sudden increase.

Discussion. Studies of the differentiation of the gastric mucosa were made in Japan as early as 1931. HARA[5] observed Japanese fetuses and obtained results showing that the first differentiation of the gland cell can be seen in parietal cells in the embryo of a 7 cm crown-rump length, but the differentiation period of the chief cell cannot be observed because the cell has few morphological characteristics. Recently, NOMURA[6] reported that electron microscopic observation showed that the parietal cells began to develop from the undifferentiated pit epithelial cells at the bottom of the gastric pit of the fetus in 10 weeks of menstruation age.

The author observed rats' embryos and infant rats, and confirmed that the appearance of acidophil cells in the

[5] T. HARA, Acta anat. nippon. *4*, 28 (1931).
[6] Y. NOMURA, Z. Anat. Entw, Gesch. *125*, 316 (1966).

Table II. Amino acids and related compounds in the extract of the gastric mucosa of prenatal, neonatal and infant rats (μM/stomach)

Procedure of analysis	Neutral and acidic amino acids	Basic amino acids
Buffer solution	1st: pH 3.25 2nd: pH 4.25 (0.2 *N* citrate buffer)	pH 5.28 (0.35 *N* citrate buffer)
Column	0.9ϕ × 70 cm	0.9ϕ × 10 cm
Resin	Amberlite CG 120	
Flow rate	Buffer 80 ml/h, Ninhydrin 40 ml/h	
Column temperature	55 °C	

Substance	Prenatal day		Birth	Postnatal day											
	−2	−1	0	+1	+3	5	7	9	11	13	15	17	19	21	23
Tryptophan	0.003	0.004	0.003	0.004	0.006	0.005	0.010	0.006	0.008	0.019	0.008	0.011	0.015	0.007	0.038
Lysine	0.010	0.024	0.010	0.012	0.016	0.017	0.025	0.020	0.025	0.019	0.031	0.066	0.060	0.075	0.090
Histidine	0.002	0.007	0.004	0.006	0.009	0.011	0.013	0.010	0.009	0.012	0.017	0.020	0.025	0.029	0.035
Arginine	0.001	0.006	0.005	0.004	0.006	0.007	0.009	0.011	0.015	0.013	0.017	0.025	0.028	0.026	0.046
Cysteic acid	0.005	0.006	0.007	0.014	0.020	0.015	0.018	0.014	0.022	0.036	0.052	0.042	0.050	0.046	0.063
Taurine	0.066	0.138	0.122	0.135	0.190	0.280	0.343	0.282	0.262	0.430	0.689	0.856	0.979	0.932	0.903
Aspartic acid	0.011	0.019	0.013	0.017	0.027	0.030	0.029	0.024	0.032	0.050	0.064	0.105	0.129	0.113	0.225
Threonine + Serine	0.025	0.072	0.077	0.111	0.131	0.118	0.091	0.063	0.114	0.199	0.229	0.258	0.310	0.349	0.396
Glutamic acid	0.072	0.135	0.079	0.091	0.115	0.087	0.130	0.103	0.185	0.242	0.304	0.323	0.409	0.498	0.912
Proline	0.006	0.007	0.007	0.021	0.031	0.028	0.028	0.025	0.035	0.041	0.058	0.053	0.099	0.085	0.119
Glycine	0.025	0.049	0.053	0.070	0.079	0.073	0.087	0.077	0.077	0.113	0.154	0.185	0.239	0.360	0.454
Alanine	0.040	0.068	0.058	0.051	0.073	0.053	0.062	0.038	0.086	0.108	0.138	0.159	0.229	0.222	0.427
Valine	0.007	0.013	0.005	0.014	0.014	0.017	0.015	0.012	0.018	0.024	0.041	0.039	0.043	0.050	0.076
Methionine	0.003	0.010	0.007	0.012	0.016	0.019	0.020	0.012	0.013	0.018	0.030	0.018	0.017	0.019	0.048
Isoleucine	0.003	0.009	0.003	0.006	0.005	0.005	0.010	0.005	0.007	0.009	0.013	0.018	0.020	0.024	0.039
Leucine	0.006	0.014	0.010	0.019	0.019	0.025	0.026	0.022	0.033	0.044	0.065	0.060	0.062	0.088	0.104
Tyrosine	0.002	0.007	0.007	0.008	0.006	0.011	0.012	0.010	0.015	0.014	0.025	0.034	0.035	0.043	0.069
Phenylalnine	0.005	0.007	0.003	0.007	0.004	0.007	0.008	0.008	0.015	0.020	0.037	0.030	0.041	0.040	0.062

epithelium on the 1st postnatal day must be the differentiation of the parietal cells. HELANDER[7], however, reported in his electron microscopic observation that few primitive parietal cells can be clearly identified owing to the presence of intracellular canaliculi at an embryonal age o-19 days, and PENTTILA[8] recognized the first sign of parie. tal cells in 20.5-day-old rat embryo by electron microscopef

In the author's experiment with the light microscope, the epithelium at an embryonal age of 19–20 days consisted of columnar cells and with no sign of differentiating into morphologically different cells. The acidophil cells in the epithelium observed on the 1st postnatal day will be the parietal cells manifesting, for the first time, histological characteristics.

During the 3rd–5th postnatal day period as the formation of tubular glands proceeds, the cells (except the acidophil cells) become cuboidal, begin to have big round nuclei and differ from the columnar surface epithelial cells. The author considered these figures may be indicative of the morphological appearance of the primitive chief cells.

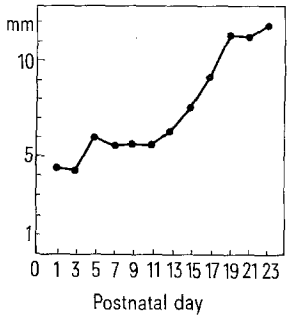

Fig. 6. Graph showing the distance between the mucosa surface and the lamina muscularis mucosae.

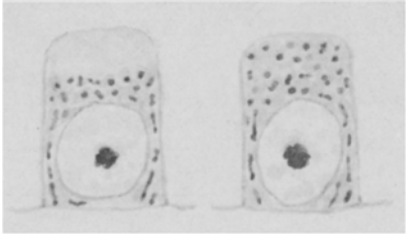

Fig. 7. The cells which seem to differentiate into the chief cells. At the 3–5 postnatal day period. Half-schematic representation of the cells obtained from the sections stained by iron hematoxylin.

Table III. Amino acids and related compounds classified as to the time of their increase in the gastric mucosa

Postnatal day	?	11–13	15–17
Amino acids and related compounds	Tryptophan Methionine	Cysteic acid Taurine Aspartic acid Threonine + Serine Glutamic acid Proline Glycine Alanine Valine Leucine Phenylalanine	Lysine Histidine Arginine Tyrosine Isoleucine

The gastric mucosa with its abundant tubular glands appears in the 11th–13th postnatal day period. At that time, it seems to be a miniature of the adult stomach. Thenceforth, the mucosa develops rapidly, increasing in thickness. The author thus considered that the differentiation of the mucosa consisting of the gastric glands begins from the 5th postnatal day and ends on the 13th postnatal day and then proceeds into the developmental stage. The distance between the surface of the mucosa and the lamina muscularis mucosae (Table I and Figure 6) proves that this process takes place. That is, in the 1st–3rd postnatal day period, though the epithelium makes small plicae, the gland is still not formed and only an appearance of the parietal cells can be noticed. Distance between the mucosa surface and the lamina muscularis mucosae is 4.3–4.4 mm (×100). During the 5th–11th postnatal day period, cell proliferation can be seen at the base of the plicae and then the pit is formed there. This is the first sign of the forming of the tubular gland. In the beginning, the epithelial cells of the surface and of the pit are similar but the parietal cells differ. Gradually the columnar cells of the pit turn into cuboidal cells having big round nuclei, suggesting a differentiation into chief cells. This cell proliferation, consequently, is responsible for the distance between mucosa surface and the lamina muscularis mucosae 5–6 mm (×100), but in this particular postnatal period there is no progressive day-by-day increase as shown in Figure 6. In other words, the gastric glands are formed in the mucosa during the 5th–11th postnatal day period but the mucosa with glands does not increase in thickness. The author considered this term may be the differentiation stage. Thenceforward, a rapid development can be supposed which keeps pace with the progressive increase in the distance between the surface of the mucosa and the lamina muscularis mucosae. This must be the development stage.

In the extract of the gastric mucosa immersed in 33% alcohol for 12 h, the same kinds of amino acids and the related compounds as those found in adult rats' gastric mucosa are found. The quantity and fluctuation of these substances are almost nil during the 1st–11th postnatal day period, i.e. in the non-differentiation and the differentiation stages. However, afterwards, most of these substances increase progressively and markedly day by day. This can be interpreted as an expansion of the stomach following the development, but it hardly explains why the obvious increase of the restricted amino acids – lysine, histidine, arginine, tyrosine and isoleucine – is postponed until the 15th–17th postnatal day period. It seems to suggest that these amino acids may have some different role from others.

On the other hand, the chief cells seem to differentiate from the epithelial cells a few days later than do the parietal cells. As shown in Figure 7, no secretory granules, big round nuclei and granular or rod shaped mitochondria are seen. It appears just a chief cell-like cell. The figure of the chief cells is maintained during the development stage of the mucosa. In the 15th–17th postnatal day period, suddenly, rich secretory granules can be observed in the cells. Thus we may presume that in the chief cells there may be a 10-day-interval between morphological differentiation

[7] H. F. HELANDER, Gastroenterology *56*, 35 (1969).
[8] A. PENTTILA, Z. Anat. Entw, Gesch. *132*, 34 (1970).

and actual functional initiation. It is very interesting to note that the time when the secretory granules appear in the chief cells (the 15th–17th postnatal day period) coincides with the time that lysine, histidine, arginine, tyrosine and isoleucine show an obvious increase in the mucosa (the 15th–17th postnatal day period).

As regards the differentiation period of the chief cells, HELANDER[9] described in his electron microscopic study that the zymogen cells in rat stomach can, with a little doubt, be identified in 20-day-old embryos, by reason of the fact the granules have a larger diameter than those of the mucous neck cells. He also reported that zymogen granules are present in large numbers in the cells at birth, but during the next 10 days they are sparse and that 20 days after birth the zymogen cells have attained a normal adult appearance.

In the author's observations with the light microscope, the rat gastric epithelium consists of columnar cells with long oval nuclei in 20–21-day-old embryos, regardless of the apex or base of the plicae. No cuboidal cells with round nuclei, like the primitive mucous neck cell or primitive chief cell, can be seen. However, sometimes, most of the epithelial cells contain many granules in the apical or supranuclear region of the cells. These granules are secretory granule-like and observable under the light microscope, but the author could not prove that they were zymogen granules.

The figure of the primitive chief cell as seen by the author showed no remarkable change during the differentiation stage, i.e. from the 5th to the 13th postnatal day period. This bears out what HELANDER says, i.e. the zymogen granules in the zymogen cells are few during 10 days after birth, and that peptic activity remains surprisingly constant during the same period.

MABUCHI[4] examined the effect of amino acids on the secretory activity of the chief cells and obtained the result that lysine, histidine, arginine or tyrosine have a promotive effect on the production of secretory granules in the chief cells. The author's findings, i.e. that when these amino acids increase in the gastric mucosa, there is an abundance of secretory granules in the chief cells, may have some connection with MABUCHI's findings. The reason why lysine, histidine, arginine, tyrosine and isoleucine increase from this point onwards has not been ascertained in this study.

Zusammenfassung. Entwicklung und Aminosäuren der Magenschleimhaut an embryonalen und neugeborenen Ratten wurden untersucht und differenziert. In der Magenschleimhaut kann das nicht differenzierte Stadium, das Differenzierungsstadium und das Entwicklungsstadium beobachtet werden. Zahlreiche Aminosäuren nehmen während des Entwicklungsstadiums in der Schleimhaut zu, wohingegen Lysin, Histidin, Arginin, Tyrosin und Isoleucin erst zu einem späteren Zeitpunkt festgestellt werden konnten.

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

[10] Acknowledgments. I wish to thank Prof. K. FUJIE for consistent help and excellent suggestions.

PRO EXPERIMENTIS

Demonstration of Synchrony in the Cell Cycle of HeLa Cells by a Flow Microfluorometer

When as asynchronous HeLa cell population is inhibited before the S-phase by a double block of excess thymidine, release of the block would be expected to induce synchronous entry into the S-phase[1,2], This is normally detected by measuring changes in the rates of incorporation of radioactive precursors. However, such methods of monitoring DNA-synthesis are sometimes complicated by pool sizes and require a lot of time. Moreover, the measurement of DNA-synthesis in thymidine-inhibited cells is questionable because it is not quite certain whether the extent of radioactive thymidine incorporation in short pulse periods is a true measure of rates of DNA synthesis[3–5]. These studies were designed to demonstrate the usefulness of a high speed flow microfluorometric method, a technique which is independent of changes in the precursor pool, in synchronization experiments. (See also[6]).

Materials and methods. The investigations were carried out on HeLa S3 suspension cultures during the exponential phase of growth. The cells were grown at 37 °C in 250 ml Sovirell-glass-bottles in 150 ml modified Eagle's minimum essential medium (MEMS, Gibco) supplemented with 2% calf serum (Biocult). A nonheating magnetic stirrer (Bellco) was used to keep the cells in suspension. Cultures were inoculated with 1.7×10^5 cells/ml.

The cells were synchronized by double thymidine treatment, as already described[5,7]. Randomly growing cultures were exposed at 37 °C to 2 m*M* TdR (Serva) for two 12-h periods separated by a 10-h period in normal medium to allow the progression through one S-phase. Thymidine was removed after each 12-h period by centrifugation of the cells at $200 \times g$ for 8 min. The cell pellet was washed with control medium and resuspended in fresh medium. After reverse of the last blockade, 2 ml of cell suspension were pelleted at $200 \times g$ for 5 min and fixed with alcohol-sodium chloride (1:2) with vigorous shaking. The pellet was then washed in 0.1 *M Tris*-HCl buffer (pH 7.5) which was 0.1 *M* in NaCl, and exposed for 1 h to 2% (in the buffer mentioned above) RNase (Serva). After another wash in the same buffer, the cells were resuspended for 30 min in a solution of ethidium bromide (1 mg/100 ml; in *Tris*-HCl, pH 7.5). A 50 µm millipore filter was used to eliminate cell aggregates before measuring fluorescence intensity of DNA-bound dye, using the flow microfluorometer ICP 11 (Phywe AG,

[1] D. BOOTSMA, L. BUDKE and O. Vos, Expl Cell Res. *33*, 301 (1964).
[2] T. T. PUCK, Science *144*, 565 (1964).
[3] W. LANG, D. MÜLLER and W. MAURER, Expl Cell Res. *49*, 558 (1968).
[4] B. LEDERER, Beitr. Path. 142, 1 (1970).
[5] M. A. TOBIA, C. L. SCHILDKRAUT and J. J. MAIO, J. molec. Biol. *54*, 499 (1970).
[6] R. A. TOBEY and H. A. CRISSMAN, Cancer Res. *32*, 2726 (1972).
[7] T. PEDERSON and E. ROBBINS, J. Cell Biol. *49*, 942 (1971).