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Professzor József BALÓ
(1897—1979)

On October 9, 1979, József BALÓ, member of the Hungarian Academy of Sciences, emeritus professor, Kossuth-Prize winner, owner of the golden degree of the Order of Labour had died at age 84. He started as an instructor at the Institute of Pathology, University of Szeged in 1928 and later became director of the 1st Institute of Pathology and Experimental Cancer Research, Semmelweis University Medical School (1945—1967). Generations of medical students have widened their pathological knowledge based on his excellent text-book. Besides educating countless would be physicians he kept on some internationally acknowledged scientific research work.

His activities concerning the diseases of the nervous system led to the discovery of the concentric demyelinisation in the brain, which was named after him (Baló's disease). He worked out a genuine theory regarding the nervous system origin of certain skin diseases. His projects included the etiology of arteriosclerosis. With his wife, Dr. Ilona BANGA, he discovered the "elastase enzyme" that has been claimed to play an important role in the development of arteriosclerosis as well as in the pathological courses of the connective tissue. Professor BALÓ was one of the experimental tumour researchers in Hungary. He was the first in Hungary who pointed out the role of viruses in the development

of tumours carrying out significant activities in the field of the pathology of lung cancers. He was the first to recognize the tumour inducing effect of certain compounds, e. g. hydrazine derivatives. He had also played a prominent role in the antineoplastic drug research. Several new drugs have been tested at the Institute under his leadership; many of which have proven useful in the treatment of human tumours. His activities as professor and director of a research institute resulted in the establishment of a pathological and experimental school. Many of his students have since then become professors or occupy other leading posts in Hungarian or foreign institutes. His death is a great loss to the Hungarian medical society and the whole Hungarian Scientific life.

DR. K. LAPIS

Acta Morphologica Acad. Sci. Hung. 28 (1—2) 3—9, 1980

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CHANGES IN NUCLEAR VOLUME OF PURKINJE CELLS IN THE CEREBELLUM OF THE WATER FROG (*RANA ESCULENTA* L.) IN THE ANNUAL CYCLE

K. Dziubek, H. Lach and S. Krawczyk

(Received January 22, 1979)

In sexually mature female and male *Rana esculenta* L. frogs directly from natural habitat, in six characteristic periods of their life cycle, nuclear volume in Purkinje cells of the cerebellum was determined. Nuclear volume in Purkinje cells changed distinctly in the course of the year.

Nuclear volume was greatest in females in the breeding period (3rd decade of May), and in males in the middle of the period of active life (2nd decade of July). Nuclear volume was the smallest at the beginning of hibernation (3rd decade of October).

Introduction

Purkinje cells are one of the most important structural elements in the cerebellum. They form a characteristic layer surrounded by basket cells [19], which coordinate their action [32]. Owing to their interesting morphology and physiology, the Purkinje cells have been studied extensively, particularly in higher vertebrates with regards to the number and arrangement in space [26], to electrophysiologic phenomena [10, 21, 35], the influence of various chemical compounds and drugs on their activity [11, 12, 16], and enzymatic localization [7, 14, 34, 36]. The histologic and ultrastructural details of the cerebellum are well known [37] and so are the electrophysiologic reactions of Purkinje cells to electrical, chemical and natural stimulation [32, 33, 41]. There is however, a lack of information regarding the changes of activity in these cells in amphibians under normal environmental conditions throughout the year. The present study is concerned with the changes in volume of nuclei of Purkinje cells during the annual cycle in the water frog (*Rana esculenta* L.), in which the cycle is clearly divided into a period of active life and a period of hibernation.

Materials and methods

Experiments were carried out with 30 male and 30 female, sexually mature, water frogs (*Rana esculenta* L.), in six characteristic stages of their life cycle, viz., 3rd decade of January (middle of hibernation), 1st decade of April (end of hibernation), 3rd decade of May (breeding period), 2nd decade of July (middle period of active life), and 3rd decade of October (beginning of hibernation). The division into periods was adopted from the work of Juszczyk [15].

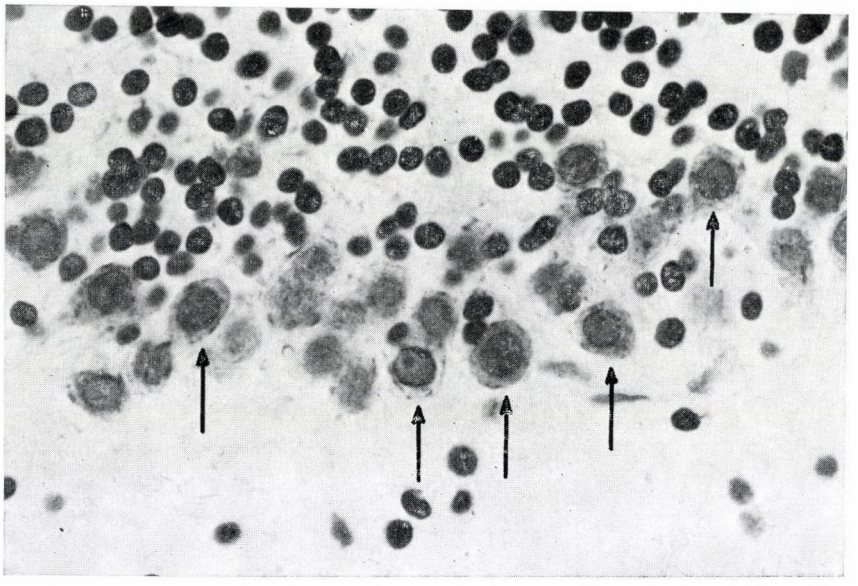

Fig. 1. Purkinje cells (arrows) in the cerebellum of *Rana esculenta* L. Luxol Fast Blue and cresyl violet staining, ×620

Five female and five male frogs from each period under investigation were captured directly from their natural habitat in the environs of Cracow (50° 04′ N, 200—220 m above sea level). The frogs were killed by decapitation, always at the same time of the day. The dissected brains were fixed in Bouin's fluid, 10% formalin neutralized with lithium carbonate, and in Carnoy's fluid, and embedded in parafin. Sections 7 μm thick were stained with Gomori's chrome haematoxylin and floxin by a modification of Bergmann's method [30], Luxol Fast Blue and cresyl violet [17], and with toluidine blue.

The sections were used to evaluate the activity of Purkinje cells in the cerebellum (Fig. 1) on the basis of changes in nuclear volume, regarded as a morphometric index of functional changes in cell activity [1, 2, 5, 18, 22, 25, 38, 39].

Nuclear volume was calculated by measuring the long axis (L) and short axis (B) of each cell nucleus and substituting these values in the equation ($V = \pi/6 \times LB^2$) [24]. One hundred nuclei of Purkinje cells were counted in the midle part of cerebellum of each frog, giving a total of 6000 measurements. From these data, arithmetical means, standard deviation and mean error were calculated. Significance of the differences in nuclear volume between females and males in each period of the cycle and between neighbouring periods was statistically tested using the Student—Gosset t test. Differences were considered significant if probability of their chance occurrence was equal to, or less than 0.01.

Results

Mean values of nuclear volume in Purkinje cells are assembled in Table I and illustrated in Fig. 2.

The mean volume of nuclei of Purkinje cells was greatest in females (438.367 μm^3) in the 3rd decade of May (breeding period), and in males (424.854 μm^3) in the 2nd decade of July (middle period of active life). In subsequent periods, mean nuclear volume in the cells continued to drop until

Table I

Volume of cerebellar Purkinje cell nuclei in female and male Rana esculenta L. during the annual cycle

Group	Period of investigation	Number of frogs	Sex	Volume of the nuclei in μm^3			Standard deviation	Mean error	t
				min.	mean	max.			
1	3rd decade of January	4	♀	365.767	398.072	455.892	40.03	17.95	5.35*
		4	♂	285.247	333.801	362.708	35.56	15.95	
2	1st decade of April	5	♀	381.118	415.201	431.771	20.94	9.39	1.66
		5	♂	358.818	402.234	447.761	32.89	14.75	
3	3rd decade of May	5	♀	426.402	438.367	450.862	11.03	4.95	2.03
		5	♂	367.276	420.022	487.100	43.73	19.61	
4	2nd decade of July	5	♀	375.178	403.468	483.661	46.15	20.69	1.86
		5	♂	389.916	424.854	455.573	30.05	13.47	
5	1st decade of September	5	♀	327.561	358.221	402.130	27.99	12.55	0.70
		5	♂	341.337	362.997	385.874	19.12	8.57	
6	3rd decade of October	5	♀	305.258	344.029	380.005	30.67	13.75	9.57*
		5	♂	249.165	276.143	293.801	17.58	7.88	

* Statistically significant at $p < 0.01$

the 3rd decade of October (beginning of hibernation). At the same time, in females (344.029 μm^3) and males (276.143 μm^3) mean nuclear volume in the cells was minimal. Beginning in the 3rd decade of October (beginning of hibernation), mean nuclear volumes increased again until they had reached maximum values in the 3rd decade of May (breeding period) in females, and in males in the 2nd decade of July (middle period of active life).

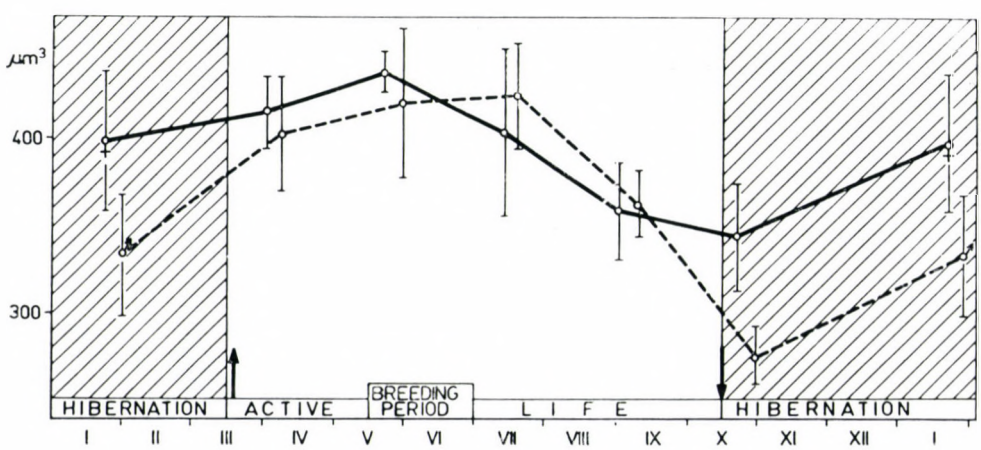

Fig. 2

Table II

t Values for differences between means in different periods

Periods compared	Student's t test	
	Purkinje cells	
	Sex	
	♀	♂
3rd decade January—1st dec. April	1.89	7.08*
3rd decade January—3rd dec. May	4.84*	7.63*
3rd decade January—2nd dec. July	0.44	9.75*
3rd decade January—1st dec. September	4.11**	3.60**
3rd decade January—3rd dec. October	5.35*	7.24*
1st decade April—3rd dec. May	4.88*	1.62
1st decade April—2nd dec. July	1.16	2.53
1st decade April—1st dec. September	8.13*	5.14*
1st decade April—3rd dec. October	9.55*	16.86*
3rd decade May—2nd dec. July	3.67**	0.45
3rd decade May—1st dec. September	13.29*	5.96*
3rd decade May—3rd dec. October	14.43*	15.23*
2nd decade July—1st dec. September	4.18**	8.66*
2nd decade July—3rd dec. October	5.35*	21.31*
1st decade September—3rd dec. October	1.70	16.67*

* — statistically significant at $p < 0.01$
** — statistically significant at $p < 0.05$

Mean nuclear volume in cells from different periods of the cycle showed statistically significant differences in the majority of females as well as males. On the whole, mean nuclear volume was higher in females than in males. Amplitude of the changes in nuclear volume was higher in males than in females.

Discussion

Analysis of the findings showed that the mean nuclear volume in Purkinje cells of the cerebellum in *Rana esculenta* L. undergoes cyclic changes in the course of the year. A particularly high increase takes place in females in the breeding period, and in males in the period of active life, followed by a sharp decrease reaching a minimum at the beginning of hibernation.

Alleva et al. [3] and Barraclough [4] showed that the reproductive cycle in female vertebrates reflects the marked differentiation between sexes, manifested among others, in behaviour [6]. In the water frog, in the nuptial

period activity is comparatively high. It is interesting that this motor activity coincides with the maximum activity of the Purkinje cells in the middle of the period of active life, i.e. in the period of intense feeding and motor activity. During this period, activity of the Purkinje cells decreases only slightly in females in comparison with that in the breeding period.

In homeothermic vertebrates, ambient temperature has a marked influence on hormonal activity, especially of the hypophysis [23], thyroid [20], and adrenals [13]. Increased hormonal activity accelerates metabolism and enhances protein synthesis [27, 28]. TABUCHI et al. [40] reported that s-100 protein is synthetized in Purkinje cells, mainly in the synaptic networks, i.e. in the area of intense metabolic and electrical activity. Among others, enhanced synthesis may be responsible for the marked increase in nuclear volume in Purkinje cells during the period of intense active life.

In both sexes, activity of the cells was minimal in the initial period of hibernation, when the water frogs after wandering to their winter lair begin to hibernate. These radically different periods in the life cycle of this species (active life and beginning hibernation) are probably one of the factors causing the decrease of these cells. Another factor seems to consist of lowered protein synthesis in amphibians in autumn [29].

The differences in nuclear volume in Purkinje cells between females and males are probably due to the sexual dimorphism of the brain. A similar phenomenon has been observed in mammalian brain [8, 9, 31].

In summary, activity of Purkinje cells is correlated with motor activity and metabolism in the annual cycle of the water frog.

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VERÄNDERUNGEN DES KERNVOLUMENS DER PURKINJE-ZELLEN IM KLEINHIRN DER RANA ESCULENTA L.

K. DZIUBEK, H. LACH und S. KRAWCZYK

Das Volumen der Nuklei von Purkinje-Zellen im Kleinhirn von geschlechtsreifen, aus ihrem natürlichen Milieu stammenden männlichen und weiblichen Fröschen (Rana esculenta) wurde in sechs charakteristischen Perioden ihres Lebenszyklus studiert. Innerhalb eines Jahres wurden im Kernvolumen der Purkinje-Zellen wesentliche Abweichungen verzeichnet.

Bei den weiblichen Exemplaren war das Kernvolumen in der Fortpflanzungsperiode (Mai, drittes Viertel), bei den Männchen in der Mitte ihrer aktiven Lebensperiode (Juli, zweites Viertel) am größten. Im Gegensatz dazu wurde das kleinste Volumen am Anfang der Hibernation (Oktober, drittes Viertel) gemessen.

ИЗМЕНЕНИЯ ЯДЕРНОГО ОБЬЕМА КЛЕТОК ПУРКИНЬЕ В МОЗЖЕЧКЕ ЛЯГУШЕК (RANA ESCULENTA)

К. ДЗЮБЕК, Х. ЛЯХ и С. КРАВЦЫК

У половозрелых лягушек (Rana esculenta) мужского и женского пола, взятых из естественной среды, авторы изучали изменения ядерного объема клеток Пуркинье мозжечка в шести типичных периодах их жизненного цикла. В течение года наблюдались значительные изменения ядерного объема клеток Пуркинье.

У самок ядерный объем наибольшим в период размножения (май, третья четверть), а у самцов в середине активной жизни (июль, вторая четверть). Наименьший ядерный объем был выявлен в начале периода гибернации (октябрь, тьетья черверть).

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HISTOLOGICAL ANALYSIS OF THE OVERLAPPING EFFECT OF HYPOPHYSEAL HORMONES ON THE COCKEREL'S TESTES

O. Dobozy, G. Csaba, M. A. Shahin and G. Lazáry

Histological examination of cockerel testes revealed the overlapping effects of FSH and TSH. Within the dose-range studied (20—160 μg FSH and 27.5—220 μg TSH), both hormones increased the cell number in the seminiferous cords including the number of spermatogonia, primary spermatocytes and pre-Sertoli cells. They also enhanced the mitotic activity of spermatogonia and accelerated spermatogenesis. Peak effects were observed after treatment with 160 μg FSH and 55 μg TSH dose. Liquefaction of cords due to hormone treatment was indicative of an acceleration of testicular ontogeny. Cross-effects of the two hormones were explained by receptor immaturity i.e. in the early stage of ontogenesis the receptors can bind both hormones due to the similarities in their structure. The maximum effects of the hormones were different, that of FSH being more marked.

Abbreviations:

ICCT: Intercordal connective tissue
Sem C: Seminiferous cord
SG: Spermatogonium
SC: Primary spermatocyte
PC: Peritoneal cell
PS: Pre-Sertoli cell
Bar scale = 10 um

Introduction

The role of the endocrine system in the regulation of organ functions is well known in the adult. However, much less information is available concerning the structure and the role of the endocrine system in the early stages of ontogenesis (intrauterine and neonatal age) and about the physiological and non-physiological role of hormones as well as the effect of exogenous hormones on the ontogeny of the endocrine system. The role of steroids, in particular of sexual steroids, in the determination of sex has been studied extensively [10, 14, 19]. These hormones are known to act through gene activation but if such hormones were present in non-physiological ratio, in certain critical periods of development they could modify the genetically determined sex.

More recently the problem of ontogenetic significance of polypeptide and amino acid type hormones binding to membrane receptors in adult has been investigated. During ontogenesis the cell membrane has specific developmental changes [11] and the maturation of receptors till reaching their specificity is attained during this period. Maturation of membrane receptors starts in the third trimester of intrauterine life and is completed only after birth.

The immaturity or lack of membrane receptors might be responsible for the phenomenon that in the hepatic cells of 15-day-old rat fetuses only 11% of the insulin is bound compared to the adult and glucagon is not bound at all. Shortly after birth, the hormone binding capacity is still low [3], in good agreement with the finding that in the rabbit, insulin enhances glycogen formation only after birth [16]. To this maturation process the developing of membrane receptors seems to require the presence of specific or nonspecific hormones [5, 6, 15]. Maturation is a critical period for membrane receptors since at this time they are not yet specific but still structurally versatile allowing substances similar to the future specific hormone to bind to them. This in turn may alter further maturation of the receptors, resulting in a reduced [5, 7] or enhanced [6] action of the specific hormone in adulthood.

The chemical resemblance of TSH and FSH has repeatedly been stressed [4, 17, 18]. The alpha subunits of both hormones are identical whereas specificity is ensured by their beta subunits. Similar sequences, however, are present even in the beta subunits of the two hormones. This might be the reason why these hormones act mutually upon the target cells of each other. Along this line in adult a weak TSH activity of human chonriogonadotropin [2, 13] and a specific binding of bovine TSH to receptors of the rat testis [1] has been demonstrated.

Earlier we showed an overlapping effect between FSH and TSH [8] using an *in vivo* bioassay [12]; the effect was more pronounced in case of TSH.

In the present work we investigate the effects of FSH and TSH on the histology of the testes and on the spermatogenesis of newly hatched cockerels.

Materials and methods

Newly hatched Hubbard-broyler cockerels were injected subcutaneously at 12 h intervals for 3 days with 20, 40, 80 and 160 μg porcine FSH (Nutritional Biochemical Co., Cleveland, Ohio) or with 27.5, 55, 110 and 220 μg TSH (Ambinon, Organon; porcine and bovine TSH with only vasopressin contamination), both dissolved in 0.2 ml physiological saline solution. The controls received injections of 0.2 ml saline solution. Twelve hours after the last injection, the left testis was removed, fixed in Bouin's solution and emeddded in paraffin. From the middle part of the testis 5 μ sections were cut and stained with haematoxylin–eosin. The observations were evaluated qualitatively and quantitatively.

The number of differentiated cells counted in 10 seminiferous cords per animal. Absolute numbers of cell types were related to the number of cells found in the 10 cords, in other words the percentage ratio of the cell types was determined. From this value the mean cell number per cord cross section and the mean number of constituent cell types in every group were determined. The relative frequency of different cell types was also calculated. Differences between the experimental groups were evaluated using the two-sample Student "t" test.

Results

a) *Controls*

The histological observation of the testes shows the normal structure of this age (Fig. 1). The testes were surrounded by a well developed tunica albuginea and composed of seminiferous cords and intercordal connective tissue. In some cords liquefaction is seen, but we can not say that it is true cavity. The germinal epithelium of the seminiferous cords consists mostly of undifferentiated cells (peritoneal cell) with a few differentiated ones, in which about 80% spermatogonia were located on the basement membrane bordering the cord. Only 14% primary spermatocytes were found. In this age no differentiated Sertoli cells, only their precursors were seen (pre-Sertoli cell) amounting to 3.4% of the total cell population.

b) *FSH-treated groups*

There was no basic difference in structure between FSH-treated and control testes but the size of the testes and the quantity and size of cells were markedly increased (Fig. 2). In groups treated with 80 and 160 μg doses, marked liquefaction started (Fig. 2a). No additional cell types were found as compared to the control.

The cell number per cord was found to increase with increasing FSH doses, reaching a maximum in the 80 μg group and declining slightly in the 160 μg group. The ratio of spermatogonia showed a dose-dependent significant ($p < 0.001$) decrease, with the highest FSH dose it fell below 20%. The number of spermatogonia per cord increased in response to 20 and 40 μg FSH ($p < 0.01$, relative to the control), a further increase of the dose caused a rapid decrease: in the 160 μg group it was the half of that in the control ($p < 0.001$). At the

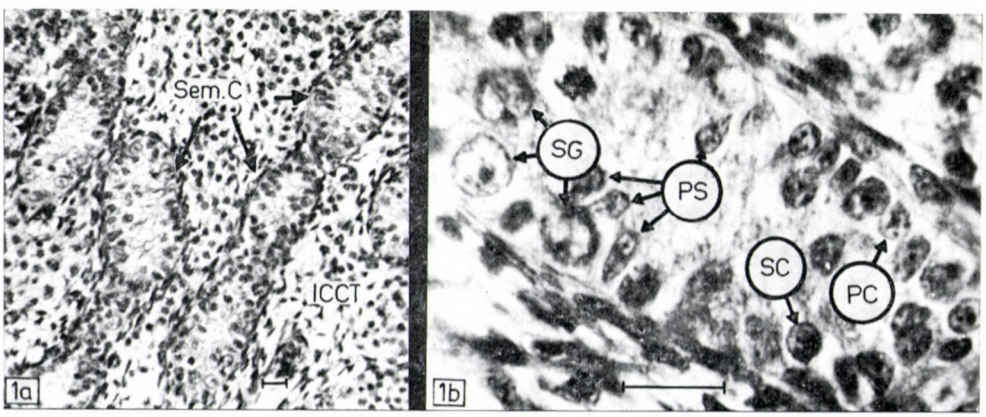

Fig. 1. Seminiferous cords of the control group contain various cell types of the germinal epithelium. Note the intercordal connective tissue between the cords

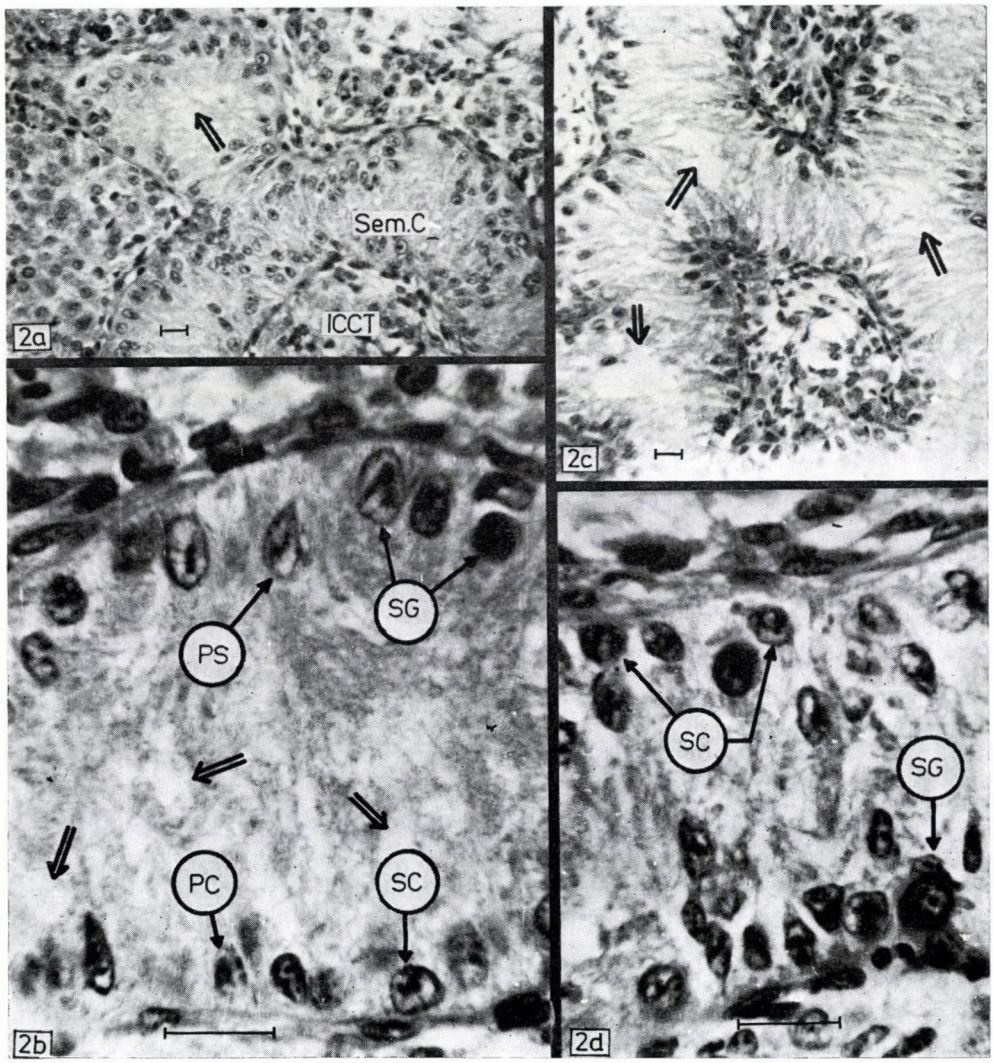

Fig. 2. Effect of FSH on testicular histology. A and B = 80 μg treatment; C and D = 160 μg treatment. Arrows point at sites of liquifaction

same time, the proportion and number per cord of primary spermatocytes increased markedly. In the 40 and 80 μg groups an increase occurred; it was significant ($p < 0.001$) as compared to the control group and to each other. The highest dose was less effective than the 80 μg dose but the difference was not significant statistically. Treatment with doses up to 40 μg caused the number and proportion of pre-Sertoli cells to increase. In the 160 μg group these cells constituted nearly 30% fraction of the total population but mature Sertoli cells were not encountered.

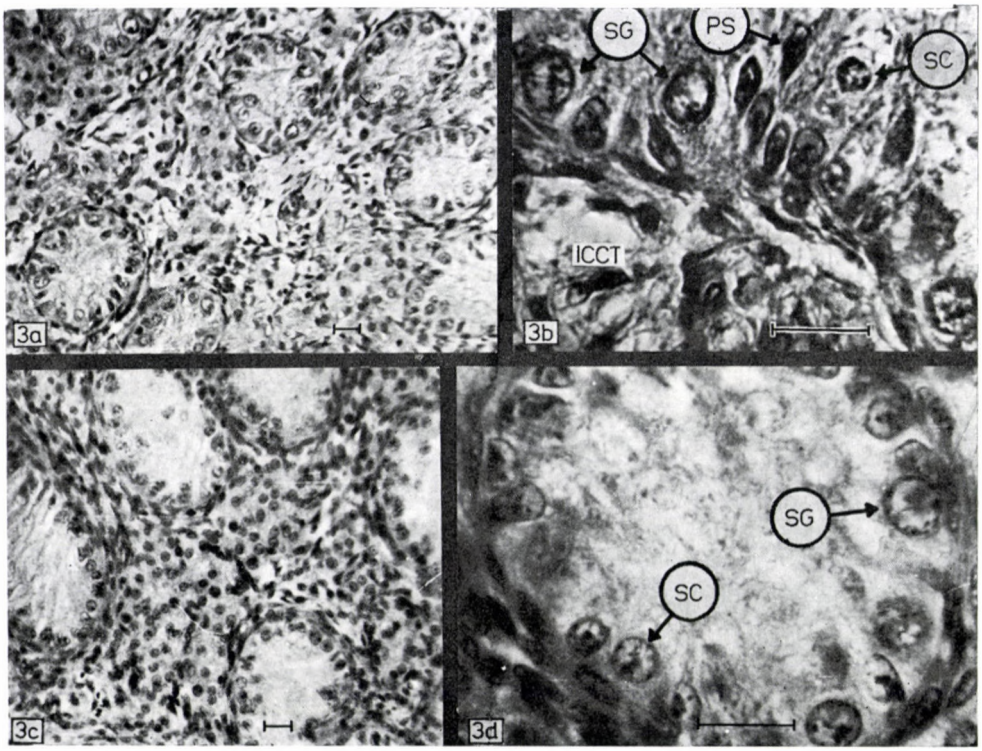

Fig. 3. Effect of TSH on testicular histology. A and B = 55 μg treatment; C and D = 220 μg treatment. Effect on cords and connective tissue are evident.

c) *TSH-treated groups*

The overall pattern of TSH-treated testes was similar as in the controls. Similarly to FSH, there was an increase in the size and cell number of the testes (Fig. 3). In contrast to FSH, TSH had a substantial effect on the connective tissue between the cords; the tissue spaces became wider and of more compact structure (Fig. 3a, c). The 55 μg dose had the highest effect on connective tissue.

The lowest TSH dose brought about a 50% increase in cell number whereas 110 μg caused a further 100% rise. Doses higher than 110 μg had a reducing effect. In animals treated with the lowest dose the proportion of the cell types corresponded to that in the control. Accordingly, the mean number of spermatogonia per cord increased significantly ($p < 0.01$). The 55 μg dose decreased significantly the ratio of spermatogonia ($p < 0.001$) and of primary spermatocytes ($p < 0.001$) but increased that of the pre-Sertoli cells ($p < 0.1$). The 110 μg dose had a similar but more pronounced effect. The lowest number of spermatogonia per cord was found in this group. After

220 μg doses the number and ratio of spermatogonia increased again ($p < 0.001$ and $p < 0.02$, as compared to the 110 μg group). While the number of primary spermatocytes and pre-Sertoli cells decreased (at the limit of statistical significance).

In neither of these young animals were Leydig cells distinguishable from other cells of the intercordal connective tissue.

Discussion

Among the parameters studied, the mean spermatogonium number is a function of the speed of two processes: the mitosis of spermatogonia and the transformation of spermatogonia to primary spermatocytes. The rate of spermatogenesis can be characterized by the number of spermatocytes per cord and the ratio of spermatogonia to primary spermatocytes. These parameters may thus be indicative of hormonal effects. On this basis it may be concluded that TSH and FSH enhance the mitotic activity of spermatogonia and accelerate spermatogenesis. There are, however, differences between the two hormones regarding the effect on the two processes, in dose-dependence and relative intensity.

The first step of spermatogenesis, the transformation of spermatogonia to primary spermatocytes, has started though on a minor scale, also in the controls.

The lowest dose of FSH caused a slight enhancement of both processes. Increasing doses gradually increase of the number per cord of primary spermatocytes (i.e. enhance of spermatogenesis). Only the 160 μg dose had a weaker (but not significantly weaker) effect than that of the 80 μg dose. The 40 μg dose enhances the mitotic activity of spermatogonia to a degree when the decrease in the number of spermatogonia was fully compensated by massive mitoses. Thus, the number of spermatogonia per cord was higher than in the former group. Elevating the dose to 80 μg the rate of mitosis was in balance with the enhanced spermatogenesis: the number of spermatogonia corresponded to that in the control. With the highest dose of FSH a roughly equal number of primary spermatocytes and half of the spermatogonia were present than in the 80 μg group. This suggests that the division of spermatogonia was less enhanced by the 160 μg dose than by the 80 μg dose. This may explain the decrease in the number of primary spermatocytes in spite of enhanced spermatogenesis.

TSH also increased spermatogenesis but its maximum effect was weaker than that of FSH. Moreover, there was no parallelism between the doses of TSH and spermatogenesis. While with 55 μg doses a marked rise in the number of primary spermatocytes, a decrease occurred on a further elevation

Table I

Effect of FSH and TSH treatment on the cell types of seminiferous cords of newly hatched cocks
S = mean ±SE

Treatment		No. of animals	Total cell No.	Mean cell values and ratios per animal								SG/SC	Cell density No./$\mu^2 \times 10^{-3}$
				Total cell No.		Spermatogonia		Primary spermatocytes		pre-Sertoli cells			
				No.	rel. per cent	No.	rel. per cent	No.	rel. per cent	No.	rel. per cent		
FSH (μg)	160	8	1089	136.1 ±9.64	100	25.3 ±2.33	19.0 ±2.18	72.4 ±4.53	53.6 ±1.96	38.5 ±6.18	26.6 ±3.21	0.36 ±0.04	4.45
	80	8	1170	146.25 ±13.16	100	44.4 ±4.54	32.9 ±5.53	79.8 ±11.33	52.5 ±4.90	22.1 ±4.38	14.6 ±2.06	0.81 ±0.29	4.80
	40	12	1291	107.6 ±7.64	100	53.4 ±2.39	51.2 ±2.33	39.4 ±5.33	34.9 ±2.57	14.8 ±1.84	13.9 ±1.61	1.60 ±0.17	4.53
	20	8	464	58.0 ±3.78	100	45.5 ±3.84	77.8 ±2.68	9.8 ±1.34	17.3 ±2.56	2.8 ±0.62	2.7 ±1.17	5.51 ±1.01	2.69
	Control	9	473	52.6 ±5.07	100	41.2 ±3.42	80.1 ±3.40	7.9 ±1.82	14.1 ±2.63	3.4 ±1.35	3.4 ±1.95	8.07 ±1.76	3.97
TSH (μg)	27.5	12	849	70.6 ±5.18	100	58.4 ±4.21	82.9 ±1.38	8.9 ±1.14	12.6 ±1.40	3.4 ±0.76	4.5 ±0.88	8.33 ±2.40	2.38
	55	12	1457	121.4 ±11.76	100	55.9 ±4.54	47.9 ±3.08	51.4 ±7.57	40.9 ±2.94	14.1 ±2.51	11.1 ±1.92	1.30 ±0.18	3.80
	110	10	950	95.0 ±7.38	100	33.2 ±3.27	36.5 ±3.58	43.2 ±5.52	47.1 ±4.51	17.1 ±3.31	17.3 ±2.59	0.83 ±0.09	2.92
	220	11	937	85.2 ±11.45	100	42.9 ±1.95	55.6 ±5.96	31.6 ±7.52	32.8 ±4.33	10.6 ±3.67	10.1 ±1.92	2.69 ±0.71	2.38

* SG/SC = spermatogonia/spermatocytes

of the dose. In contrast to FSH, even the smallest dose of TSH was able to increase the number of spermatogonia. Considering that the primary spermatocyte number was comparable to the control, low doses of TSH seem to increase exclusively the mitosis of spermatogonia. Elevation of the TSH dose from 27.5 to 55 μg increased the spermatogenesis markedly but there was no reduction in the number of spermatogonia, due to a proportionate increase of mitoses. Under the effect of treatment with 40 μg, the number of both spermatogonia and primary spermatocytes was reduced, as compared to the former group. The fact that the number of spermatogonia decreased even more, indicated that although spermatogenesis was further accelerated, the reduced mitotic activity led to a decrease in number of both spermatogonia and primary spermatocytes. After the highest dose of TSH, the number of spermatogonia reached again the control value but the number of primary spermatocytes decreased further. (This was still an elevated figure as compared to the control.) Hence this high TSH dose had a minor effect in accelerating spermatogenesis.

Both hormones increased Sertoli cell formation. Within the seminiferous cords, the absolute number and ratio of pre-Sertoli cells were increased. Except for the highest dose, the effect of the two hormones was similar. Elevation of the FSH dose from 80 to 160 μg resulted in a doubling of the absolute ($p < 0.05$) and relative ($p < 0.01$) number of pre-Sertoli cells, whereas the effect of the highest TSH dose was 30% weaker than that of 110 μg treatment ($p < 0.05$).

In a previous study [8] a dose-dependent increase in the seminiferous cord diameter was observed to follow FSH and TSH treatment. Considering the cord cross section as roughly circular and the various cell components as uniform in size, mean cell number per unit, cord area can be calculated from the mean diameter and the data provided by the present data (Table I). From this it appears that the lowest doses of both hormones cause a substantial increase in diameter not accompanied by a proportionate increase in cell number. Accordingly, the mean cell size increases. This ratio is slightly higher than that in the control after other FSH doses. This would imply that although the cell are smaller than in the controls, their growth and division is in balance. This is not the case with TSH, as after the 55 μg treatment the cell size did not differ from the control. This increased significantly only upon increasing the doses.

Observations concerning the effect of TSH on connective tissue were in good agreement with the data of DAVIES et al. [9] who have shown TSH to bind to interstitial cells.

From the present work it appears that the perinatal effect of TSH on an alien target organ (on the testis) is similar to that of FSH. This implies that at this age the receptors of the interstitial and germinal cells of the testis are

not yet specific to react only with their own hormones. Differences in intensity of action between the two hormones may be due either to differences in beta-subunits or to differences in the amount of active receptors on various cell types (germ cells or interstitial cells).

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HISTOLOGISCHE ANALYSE DER ÜBERLAGERUNGSWIRKUNG DER HYPOPHYSENHORMONE AUF DEN HAHNENHODEN

O. DOBOZY, G. CSABA, M. A. SHAHIN und G. LÁZÁRY

Bei der histologischen Untersuchung des Hahnenhodens wurde die Überlagerungswirkung des FSH und des TSH nachgewiesen und festgestellt, daß im untersuchten Dosisbereich (20—160 μg FSH bzw. 27,5—220 μg TSH) beide Hormone die Zellzahl in den Hodenkanälchen erhöhen und in diesem Rahmen auch die Vermehrung der Spermatogonien, der primären Spermatozyten, der Urzellen der Sertoli-Zellen, der Prä-Sertolizellen bewirken, ferner die mitotische Teilung der Spermatogonien und die Spermatogenese steigern. Die größte Wirkung entfaltete die Behandlung mit 160 μg FSH bzw. 55 μg TSH. Die im Zusammenhang mit der hochdosierten Hormonbehandlung beobachtete Höhlenbildung in den Strängen deutet gleichfalls auf die beschleunigte Ontogenese des Hodens hin. Die einander überlagernde Wirkung der beiden Hormone läßt sich damit erklären, daß in der Frühphase der Ontogenese die noch in Entwicklung begriffenen, unreifen Rezeptoren infolge ihrer ähnlichen Struktur beide Hormone binden. Die maximale Wirkung der beiden Hormone ist nicht gleich, die des FSH ist ausgeprägter.

ГИСТОЛОГИЧЕСКИЙ АНАЛИЗ ПЕРЕКРЫВАЮЩИХСЯ ДЕЙСТВИЙ ГИПОФИЗАРНЫХ ГОРМОНОВ НА ЯИЧКИ ПЕТУХА

О. ДОБОЗИ, Д. ЧАБА, М. А. ШХАХИН и Д. ЛАЗАРИ

При гистологическом изучении яичков петуха авторы выявили, что действия гормонов PSH и TSH перекрывают друг друга. Было установлено, что при применяемых для анализа количествах (20—160 μг FSH и 27,5—220 μг TSH) оба гормона вызывают повышение числа клеток в семенных канальцах, в том числе повышение также числа сперматогоний, первичных клеток первичных сперматоцитов и клеток Сертоли, предклеток Сертоли, усиленное митотическое деление сперматогоний и ускорение сперматогенеза. Наибольший эффект был получен при даче FSH в количестве 160 μг или TSH в количестве 55 μг. Образование полостей в тяжах, наблюдаемое при введении высоких доз гормонов, также указывает на ускоренный онтогенез яичнов. Перекрывающие друг друга действия двух гормонов предположительно объясняется тем, что в ранней фазе онтогенеза еще развивающиеся незрелые рецепторы, вследствие их подобной структуры сязывают оба гормона. Максимальное действие гормонов неодинаковое, более выраженный эффект вызывает FSH.

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COMPLEX MORPHOLOGICAL STUDY OF THE EFFECT OF GLUTAURINE IN MAST CELLS

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Glutaurine produces rapid and intensive degranulation of mast cells without affecting the composition of their granules. The mast cells of the individual organs differ in the type and extent of degranulation. There is a wide scale between excessive degranulation (mast cells of subcutaneous connective tissue and of the peritoneum), and unresponsiveness (mast cells of thyroid and lymph nodes), and there are also differences in time. The peritoneal mast cells stain positively with safranin, swell excessively and assume bizarre forms in response to 5 μg/rat glutaurine intraperitoneally. Frequent occurrence of lymphocyte–mast cell contacts seems typical.

Glutaurine increases ^{3}H-histidine uptake of the peritoneal fluid cells in general and ^{35}S sulphate incorporation in the peritoneal mast cells in particular. Accumulation of these substances is more marked at 1 hour than at 3 hours after application.

Introduction

Glutaurine (gamma-L-glutamyl-taurine) is a recently discovered hormone of the parathyroid [5, 6]. It is similar in its effects to vitamin A and acts as an antagonist to cortisone [8] and thyroxine [9]. Since both formation and degranulation of the mast cells are affected by vitamin A as well as by the aforementioned hormones [1, 11, 13, 15]. it seemed of interest to examine whether *a)* glutaurine had any effect on the mast cells; *b)* if so, whether this effect was a direct or indirect one.

Materials and methods

A. *Histochemical studies*

Eight inbred male Wistar CB rats weighing between 150 and 180 g were administered 1 μg of glutaurine intraperitoneally. One and 6 hours after administration of the hormone the following materials were secured from two animals for light, fluorescence and dark-field microscopy: peritoneal fluid, mesentery, subcutaneous connective tissue, lymph node, thyroid.

The materials were fixed and processed according to the individual study. The histochemical methods applied were: alcian-blue–safranin method of Spicer [14, 15]; Azur-A (0.1% alcohol) according to Pearse [10]; colloid-iron method according to Pearse [10]; Reinecke salt (dark-field method) according to Schauer and Werle [12]; formaldehyde induced fluorescence (FIF) according to Falck [4].

B. *Isotope studies*

B/1. Four male inbred Wistar CB rats weighing between 150 and 180 g were administered 500 μCi $Na_2{}^{35}SO_4$ in 1 ml saline intraperitoneally. One hour later the peritoneal fluid and tissues of two animals were processed, smears were prepared from the peritoneal fluid, and the sediment obtained by centrifugation was embedded in Araldite (Durcupan ACM,

Fluka, AG., Buchs, Switzerland); specimens excised from mesentery, subcutaneous connective tissue and thyroid were embedded in paraffin. The two other animals received 1 μg glutaurine in 1 ml physiological saline intraperitoneally 1 hour after administration of the isotope. One hour later the peritoneal fluid and the tissues were processed as above. The preparations were covered with K5 Ilford emulsion (Kodak Ltd., Kirkby, Liverpool, England) detected after 3 weeks' exposure and stained with toluidine blue.

B/2a. Four inbred Wistar CB rats weighing between 150 and 180 g were administered 200 μCi ^{3}H-histidine (Amersham Co., Amersham, England) in 1 ml saline intraperitoneally. One hour later 10 ml heparinized Parker's 199 solution was injected intraperitoneally and 5 min later the animals were exterminated by an overdose of ether and the peritoneal fluid was withdrawn with a pipette. The Parker's solution containing the peritoneal cells obtained from two animals on each occasion was mixed and this cell-suspension was sedimented on plates placed in Bellco tubes left to stand for 2 hours, then the fluid was replaced according to the following scheme:

6 tubes: control nutrient (Parker 199)
6 tubes: 0.1 μg glutaurine/ml Parker 199
6 tubes: 1.0 μg glutaurine/ml Parker 199

The plates were removed from the tubes 1 and 3 hours later in the corresponding proportion fixed in Carnoy's solution, washed and, after rinsing with distilled water, covered with K5 emulsion for radio-autography. Time of exposure: 1 month. Staining with toluidine blue.

B/2b. Ten male Wistar CB rats weighing between 150 and 180 g were administered 500 μCi/ml/animal of $Na^{35}SO_4$ (Amersham Co., Amersham, England). Harvesting of the peritoneal cells was done as above. The cells were sedimented on the plates, incubated for 2 hours, then the fluid was replaced according to the following scheme:

12 tubes: control nutrient (Parker 199)
12 tubes: 1.0 μg glutaurine/ml Parker 199.

Processing was done as indicated under B/2a.

C. *Cell cultures*

C/1. The rats were anaesthetized with ether and injected intraperitoneally with 10 ml of the culture medium of the following composition:

90% Parker (TC 199) medium
10% calf serum (Phylaxia, Budapest, Hungary)
200 I.U./ml penicillin (Biogal, Debrecen, Hungary)
4 μg/ml heparin-Na (Richter Budapest, Hungary).

Anaesthesia was then continued until death of the animals (approximately 5 min), then, after laparatomy the intraperitoneal fluid was withdrawn with a Pasteur pipette under sterile conditions and transferred into Bellco tubes (1.5 ml per tube) with a coverglass at the bottom of each. After sedimentation for 2 hours in a thermostat the fluid was replaced by the control and by glutaurine 0.1 and 0.5 μg per ml containing nutrient, respectively. At 1, 3 and 24 hours after fluid exchange the cells on the coverglass were fixed in Carnoy's solution and stained with iron-alum–alcian-blue–safranin.

C/2. The control rats received 1 ml Tyrode's solution, the test animals 1 μg or 5 μg glutaurine, in 1 ml Tyrode's solution intraperitoneally. One hour later 10 ml of the nutrient described for group A was injected intraperitoneally. The subsequent steps were the same as for group A, with the difference that exchange of the nutrient in the Bellco tubes was unnecessary, which allowed to fix the cells at 1, 3 and 24 hours after explantation.

Results

A) *Histochemical studies*

Alcian-blue–safranin method

This method allows the differentiation of mast cells at different stages of maturation and is also suited for the demonstration of degranulation. Immature young mast cells are alcian-blue positive. During maturation they

show mixed staining and mature cells are safranin-positive. After degranulation the safranin-positive granules become alcian-blue positive. As mast cell groups are placed irregularly, their counting was baseless; their quantity was estimated by general impressions.

Peritoneal fluid

One hour after glutaurine administration there were few mast cells present and alcian-blue positive ones did not occur. The majority of safranin-positive mast cells underwent degranulation, the alcian-blue granules clustered at the marginal zones of the cells. By the end of the 6th hour the exclusively safranin-positive cells increased in size to several fold of the size of the controls or of the cells found at 1 hour. The majority of the mast cells were in apparently close contact with one lymphocyte (Fig. 1), occasionally with several lymphocytes (Fig. 2), or with a single one which joined other lymphocytes by its processes (Fig. 3).

Mesentery

One hour after glutaurine administration exclusively safranin-positive mast cells are visible, the majority of which shows no degranulation. At 6 hours the majority of the cells, which are likewise exclusively safranin-positive, showed degranulation in contrast to the control.

Subcutaneous connective tissue

One hour after glutaurine administration the appearance of safranin-positive mast cells was observed. They were intact and showed no sign of degranulation.

At 6 hours after glutaurine treatment the exclusively safranin-positive mast cells showed degranulation, manifesting in some cases with the appearance of marginal alcian-blue granules. In other cases degranulation involved the entire cell surface.

Thyroid

At 1 and 6 hours after glutaurine administration only safranin-positive mast cells were present; none of them showed degranulation. There was no difference between test animals and controls.

Lymph nodes

At 1 and 6 hours after glutaurine administration alcian-blue positive as well as safranin-positive cells were found together with cells of mixed granulation. The results obtained with the alcian-blue–safranin method with

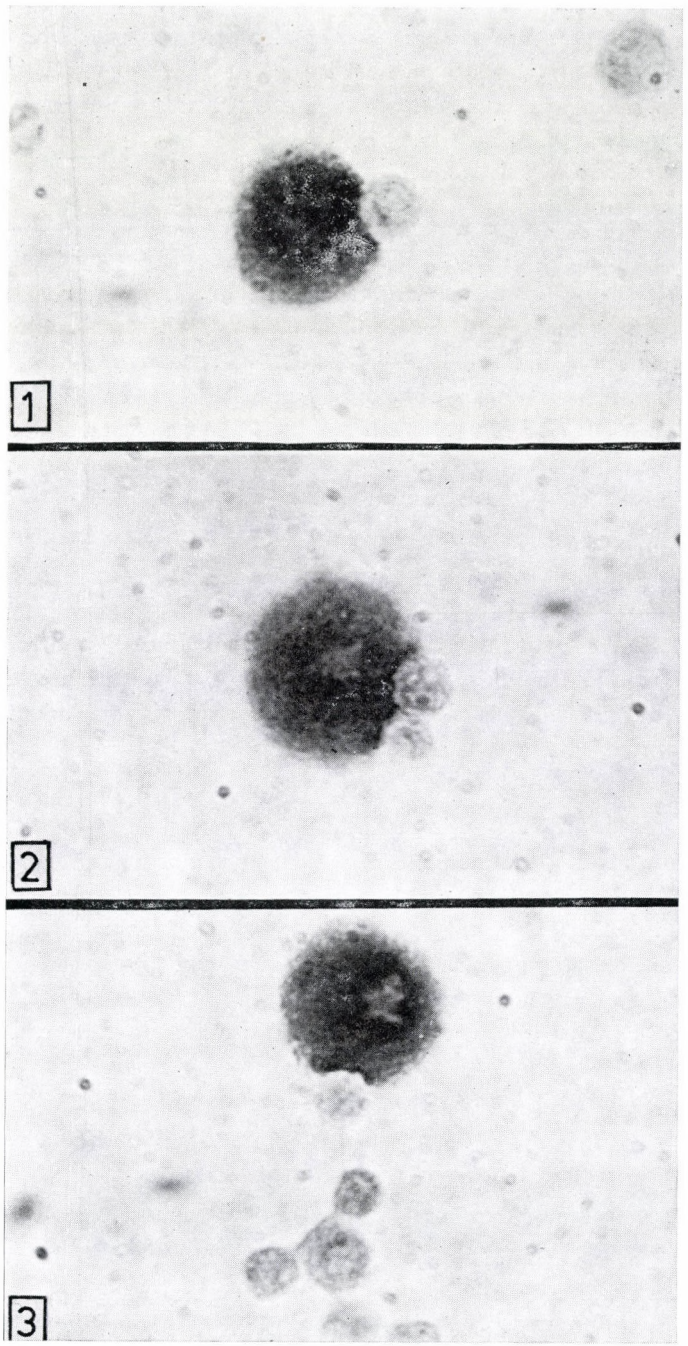

Fig. 1. Safranin-positive swollen mast cell 6 hours after glutaurine treatment. The lymphocyte appears to be wedged into the cytoplasm of the mast cell. AS, ×1200

Fig. 2. Safranin-positive mast cell, swollen to excessive size, 6 hours after glutaurine treatment. The mast cell is in close contact with two lymphocytes. AS, ×1200

Fig. 3. Mast cell swollen to excessive size, 6 hours after glutaurine treatment. Note the lymphocyte attached to the mast cell and forming contacts with other lymphocytes by its projections. AS, ×1200

regard to degranulation were confirmed by those of the other four procedures and revealed no difference between the granular components in the test animals and the controls.

B) *Isotope studies*

B/1. No difference was seen between the test group and the controls in the uptake of labbelled sulphate by the mast cells of the thyroid, mesentery and subcutaneous connective tissue. Though the peritoneal fluid is little suited for the demonstration of quantitative differences, degranulation, in other words elimination of sulphate-containing granules, seemed to be increased in the glutaurine-treated group (Figs 4, 5).

B/2a. Histidine is taken up not only by the mast cells but also by other cells of the peritoneal fluid. Granules on the surface of the cells of 1 and 3 hour cultures were sparse, and little sulphate was taken up (Fig. 6). The cultures treated with 0.1 μg glutaurine showed many grains on top of the cells at 1 hour (Figs 7, 8) in contrast to the control (Fig. 9).

By the end of the 3rd hour the granules decreased in quantity but were still in excess of those found in the controls. Though treatment with 1 μg glutaurine caused less striking differences, increased uptake of labelled sulphate in the glutaurine-treated cultures was none the less distinct as compared with the controls. The cultures treated for 1 and 3 hours showed no significant difference.

B/2b. At 1 and 3 hours massive accumulation of labelled sulphate was seen in the mast cells. The cells appeared intact, the uptake was elective, 0.1 μg glutaurine produced definite degranulation by the end of the first hour, while the amount of sulphate, was similar as in the controls. The 3 hour cultures yielded similar results. Treatment with 1 μg glutaurine resulted in an excessive uptake of sulphate by the end of the first hour. The mast cells appeared to be coated with the granules. Degranulation was also intense (Figs 10, 11, 12). At 3 hours the cells contained sulphate in moderate amounts and degranulation was also less marked. The grainules seemed to be similar in quantity as in the controls (Fig. 13).

C) *Cell cultures*

C/1. One hour after addition of 0.5 μg glutaurine in 1 ml saline the number of mast cells was significantly higher in the test group than in the control groups (Figs 14, 15). A significant proportion of the mast cells was in diverse stages of degranulation. The surface of some cells was rippled, which might be an early sign of degranulation, other cells were found to discharge granules, the sites of ejection were tapering off, the cell membranes uneven. In other cases degranulation was more marked and was clearly seen over the

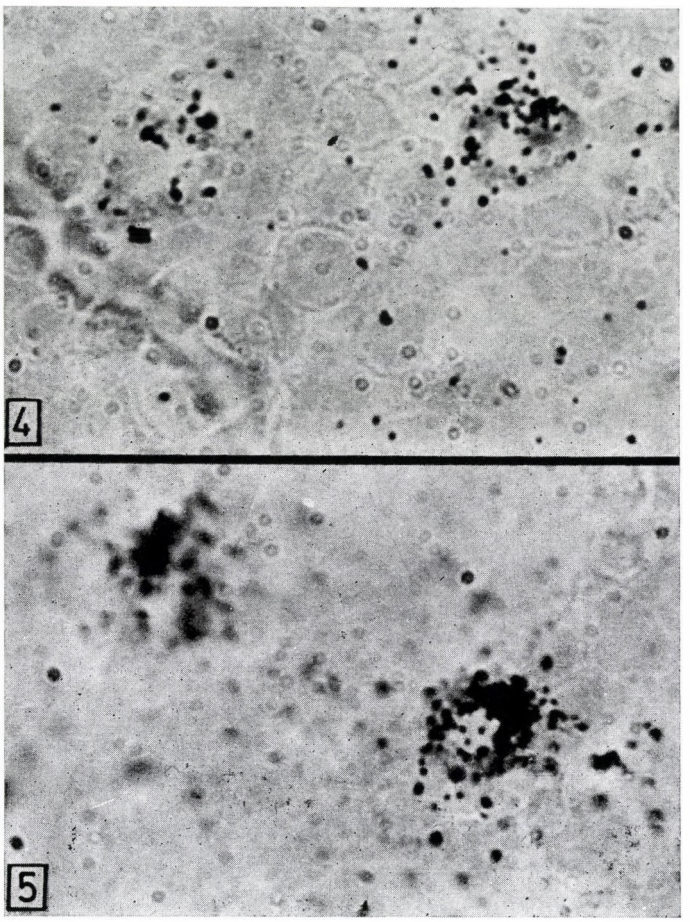

Fig. 4. Semi-thin autoradiogram of the peritoneal fluid of a glutaurine treated rat. The mast cells show marked degranulation but are covered by granules indicative of sulphate. Toluidine blue, ×1200

Fig. 5. Semi-thin autoradiogram of peritoneal fluid of control rat. Sulphate has been taken up electively by the cells. There are more granules on the cells than after glutaurine treatment. Toluidine blue, ×1200

entire cell surface (Figs 16, 17), and the cells even disrupted. Safranin-positive cells were predominant, alcian-blue positive cells were sporadic and those of mixed granulation, also sparse.

At 3 hours after treatment with 0.5 μg/ml glutaurine the quantiative ratio was similar as at 1 hour: the test cultures contained a far larger number of prevalently safranin-positive mast cells than did the control cultures (Figs 18, 19). While degranulation in the control cultures was confined to sporadic cells, in the test cultures degranulation was marked.

At 24 hours, degranulation in the control cultures was very marked and many cells were completely disrupted. On the other hand, degranulation

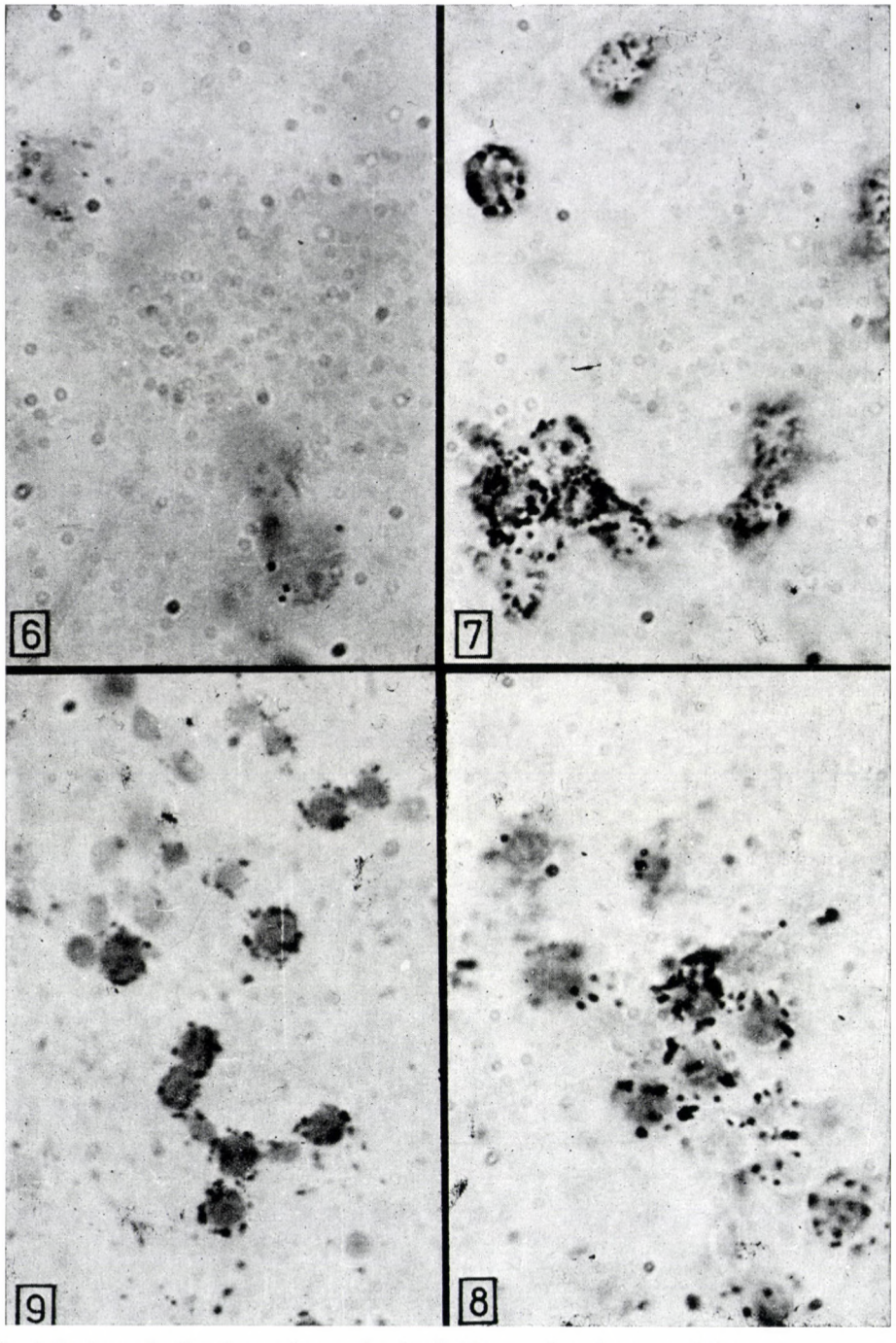

Fig. 6. Peritoneal cell culture of control animal, 3 hours after the start of glutaurine treatment. Sporadic grains are seen on the cells. Toluidine blue, ×1200

Fig. 7. Granular mass on top of a peritoneal cell culture, 1 hour after the start of glutaurine treatment (0.1 μg). Toluidine blue, ×1200

Fig. 8. After treatment with 1 μg glutaurine, granules are seen in massive amounts on the cells of the peritoneal cultures. Toluidine blue, ×1200

Fig. 9. One hour after harvesting the control cultures they contain less granules than do the glutaurine-treated cultures. Toluidine blue, ×1200

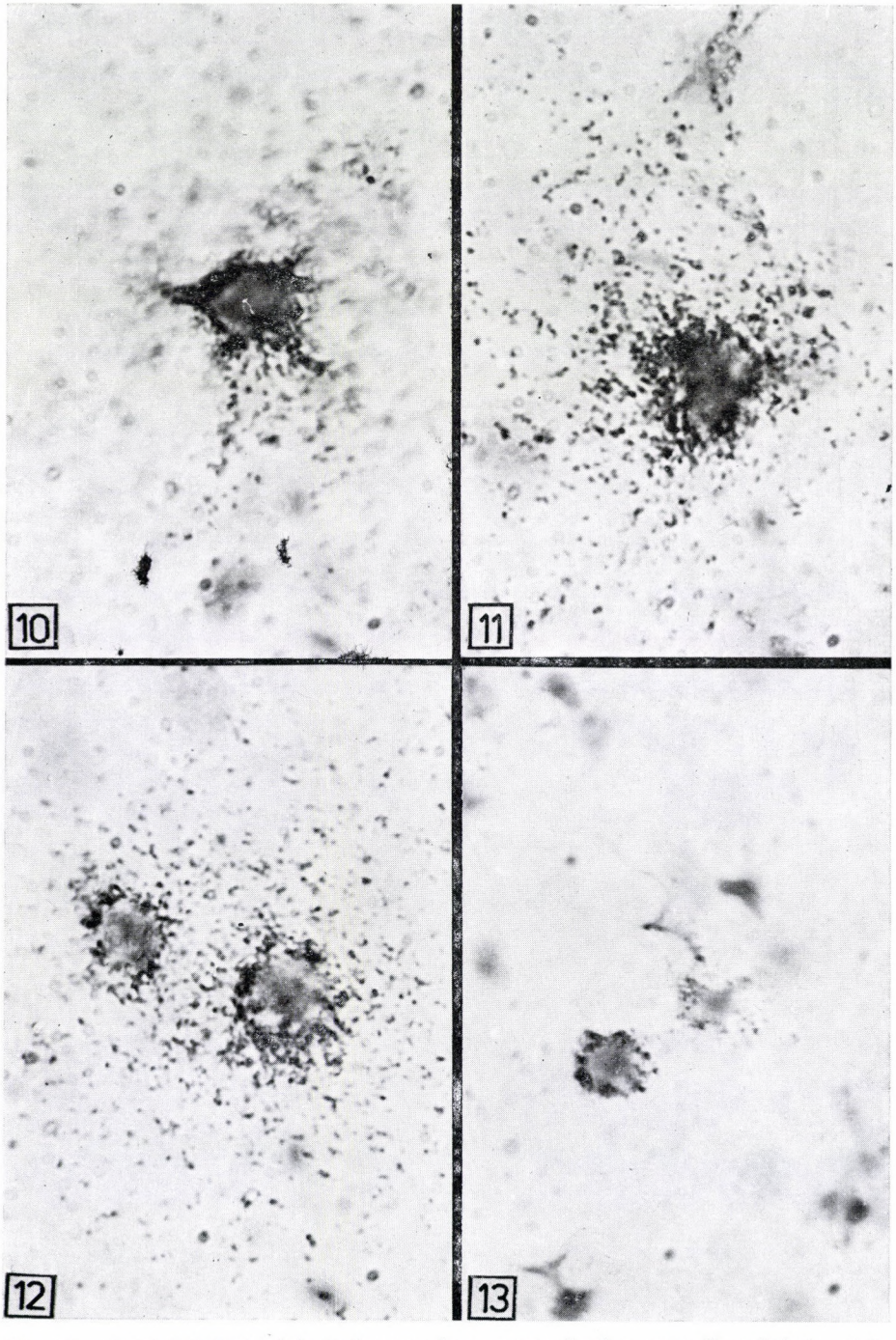

Fig. 10., 11., 12. Labelled sulphate in excessive amounts in the mast cells and their neighbourhood in peritoneal cell cultures treated with 1 μg glutaurine. Total degranulation of the cells. Toluidine blue, $\times 1200$

Fig. 13. Three hours after glutaurine treatment the granular mass on top of the cells has decreased and there is less degranulation. Toluidine blue, $\times 1200$

of the glutaurine-treated cultures lagged far behind that found at 3 hours when the number of cells was greater than is the control cultures (Figs 20, 21). Numerous macrophages were seen to contain phagocyted mast cell granules.

The effect of 0.1 μg/ml glutaurine was similar in type as that of 0.5 μg, only less intensive.

C/2. After the injection of 1 μg glutaurine to rats their cultured mast cells showed marked degranulation as early as 1 hour after explantation. Subsequently, the number of mast cells did not exceed that of the controls and degranulation was also the same as in the controls.

The mast cells cultured after 5 μg glutaurine showed the same number as in the controls 1 hour after explantation. The cells were prevalently safranin-positive. No signs of degranulation were visible, the cytoplasm showed invaginations, thus appearing to form cellular orifices (Fig. 22). By the end of the 3rd hour the picture was the same, but by 24 hours the features demonstrable at 1 hour had become very marked. The most cells displayed invaginations at multiple sites, and some of the cells had a clover-leaf appearance with signs of intensive degranulation (Fig. 23).

Discussion

It appears from the histochemical studies that the quantity, or rather the histochemically demonstrable proportion, of granular mast cell components as visualized by alcian-blue–safranin staining, remains unaffected by glutaurine. On the other hand glutaurine produces rapid degranulation in the histochemical assays as well as in cell cultures. Later this fades and may disappear altogether, as indicated by the observation that degranulation in cells obtained at 1 and 3 hours is intensive whereas at 6 and 24 hours it is less marked. Degranulation appears to be physiological, since the cells, instead of being disrupted, seem to discharge the granules one by one.

It was striking to find different responses of the mast cells originating from different organs. While the mast cells of the peritoneal fluid showed degranulation after one hour but none at 6 hours, those from the mesentery and subcutaneous connective tissue differed in no way from the controls at 1 hour, but displayed degranulation at 6 hours. On the other hand, the mast cells of the thyroid and the lymph nodes were unaffected by glutaurine. This might be connected with the intraperitoneal route of administration which would account for the degranulation as early as at 1 hour. This is, however, at variance with the finding that in the mesenteric mast cells degranulation was absent until the end of the 6th hour, though here, too, the effect was a direct one.

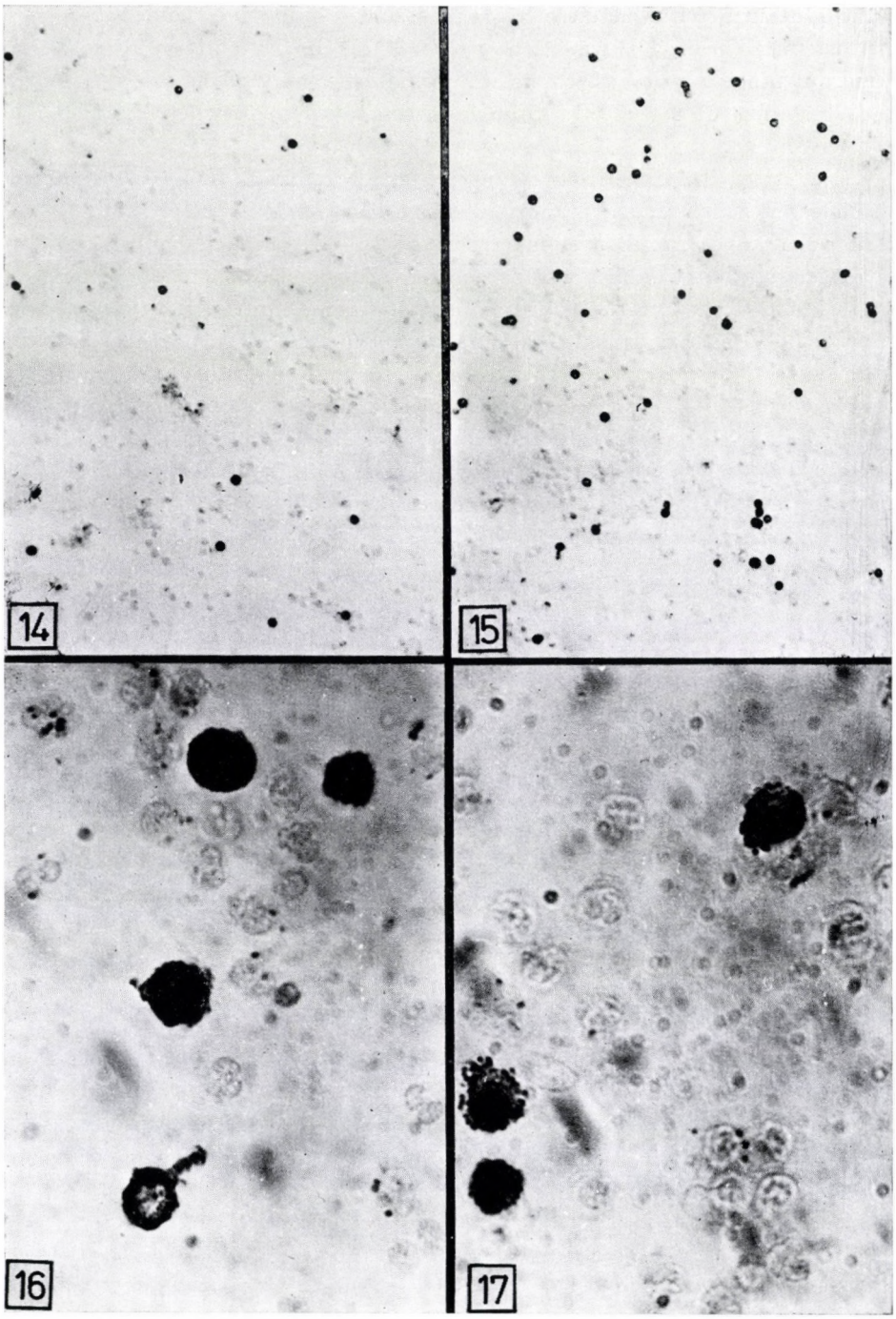

Fig. 14. One hour peritoneal cell culture from a control animal. Sporadic mast cells HS, ×100
Fig. 15. Peritoneal cell culture 1 hour after treatment with 0.5 μg glutaurine. Numerous mast cells are seen AS, ×100

There was a close contact between the mast cells and the lymphocytes, confirming our earlier studies [2] based on microkinematographic analysis of normal peritoneal mast cells where this connection resulted in degranulation in the majority of cases. In this case, however, the contact with the lymphocytes was formed by medium-sized, but never by large mast cells. The phenomenon is uncommon in peritoneal cell preparations. In view of the technique applied it may be assumed that the contact with the lymphocytes had been formed before the cells were removed and remained in this state during exsiccation. This is consistent with the observation that in experiments (see C/2) where the cells were removed as late as 1 hour after the addition of glutaurine, the phenomenon did not appear and in the histochemical studies too, it was demonstrable only in the 6 hour specimen. If in this, any degranulation was found, it was confined to the lymphocyte contacts. It may thus be assumed that lymphocyte contact, which is a physiological phenomenon, is enhanced by glutaurine. A lymphocyte being in contact with a mast cell may form contacts with other lymphocytes as well.

The safranin-positive mast cells obtained 6 hours after glutaurine treatment were all excessive in size in comparison with the lymphocytes. Such an enlargement of some the mast cells and the appearance of bizarre forms occurred in cell cultures too, but only sporadically.

Isotope studies are particularly suited for evaluation of the results of short-term cultures, from which it clearly emerged that the uptake of both labelled substances, histidine and sulphate, is enhanced by glutaurine. Histidine as an universal amino acid, is a component of numerous proteins, its uptake was, therefore, not confined to the mast cells. This, however, fails to account fully for the excessive histidine uptake by the peritoneal mast cells, particularly in the case of application of glutaurine in 0.1 μg doses, which would seem to indicate that glutaurine stimulates protein synthesis.

Since sulphate forms one of the groups of the other mast cell component, heparin, its uptake is an elective indicator of the synthesis of the latter. Glutaurine, particularly at high dose levels, significantly increased the uptake of sulphate, thus also the synthesis of heparin.

The labelled substances were introduced in all experiments *in vivo* and the cells were removed at 1 hour. Isotope was present in the washing fluid as well as in the cells by which it had been taken up. In those cases where

Fig. 16. Marked degranulation in a culture after one hour glutaurine treatment. Granules are discharged over the entire mast cell surfaces. Note discharge of a row of grains by the cell at the bottom AS, ×480

Fig. 17. Glutaurine-treated culture at one hour. Marked degranulation of mast cells. The one in the middle is in the phase of complete disruption, but its structure is preserved. Degranulation of the mast cell at the bottom has just started, whereas the mast cell on top has discharged numerous granules AS, ×480

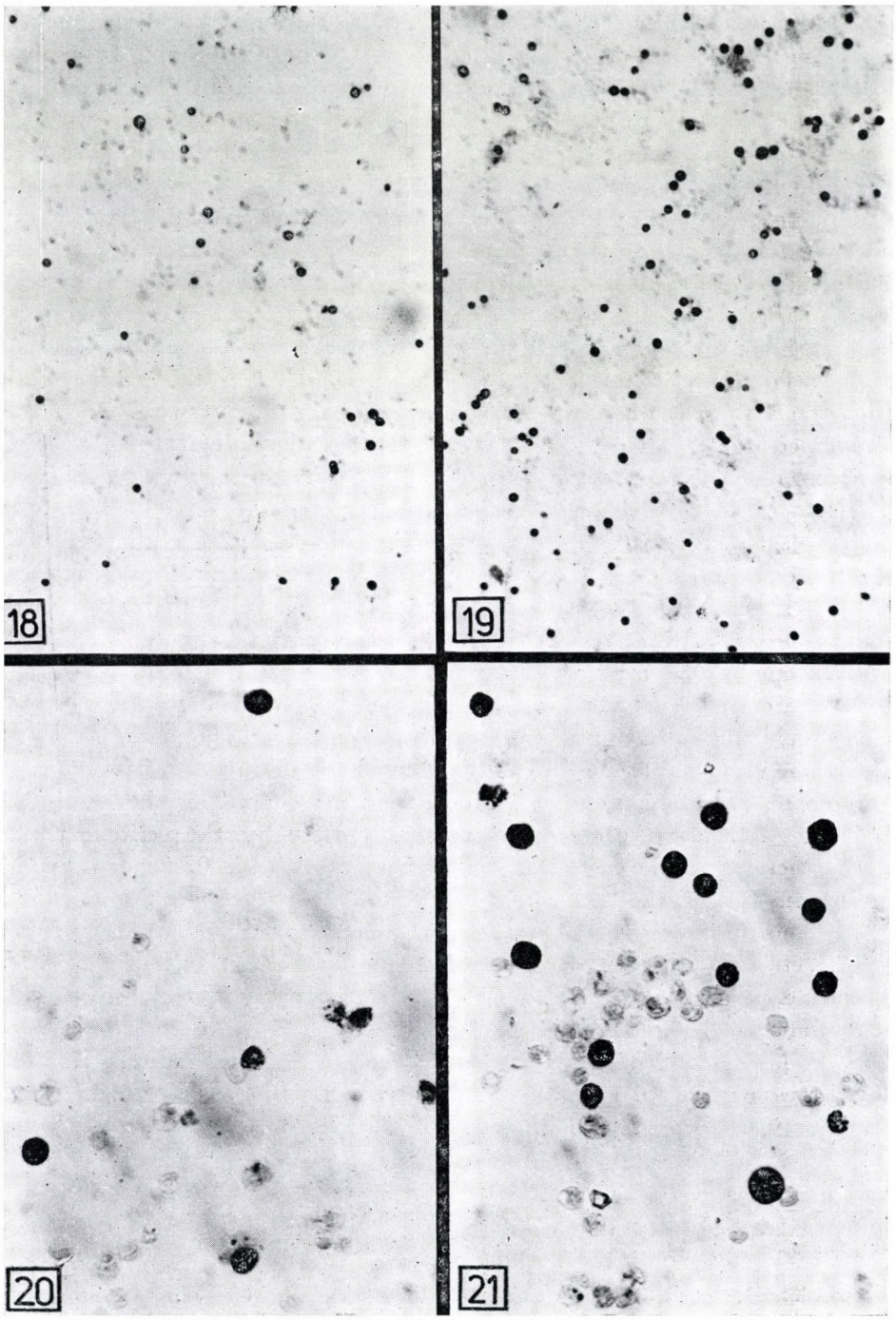

Fig. 18. Control peritoneal culture at 3 hours. Few mast cells AS, ×100
Fig. 19. Glutaurine-treated (0.5 μg) culture at 3 hours. There are more mast cells than in the controls AS, ×100
Fig. 20. Control cultures 24 hours after start of the experiment. Marked degranulation AS, ×240
Fig. 21. Glutaurine-treated (0.5 μg) culture 24 hours after start of the experiment. The number of mast cells has considerably increased, degranulation is less marked AS, ×240

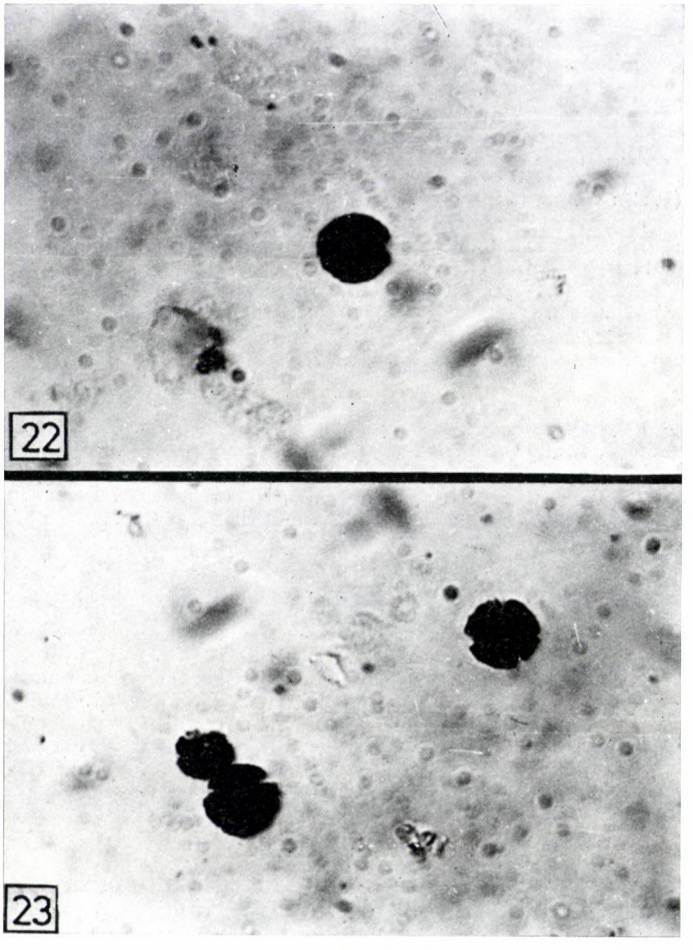

Fig. 22. Glutaurine-treated (0.5 μg) peritoneal cell culture at 24 hours. The mast cell shows an orifice-like invagination AS, ×480
Fig. 23. Glutaurine-treated culture at 24 hours. The mast cells have assumed a clover-leaf shape as a result of invaginations AS, ×480

the quantity of grains was increased, labelled sulphate had been taken up by the cells from the nutrient. Uptake reached its peak at 1 hour in both cases, which indicates that glutaurine takes effect rapidly (as confirmed also by all assays described in this report). The difference was, however, that for histidine, 0.1 μg/ml nutrient of glutaurine and for sulphate, 1.0 μg/ml nutrient proved more potent. We cannot explain this finding, but it is possible that the dose which still stimulates the synthesis of mucopolysaccharides, may be inhibitory to that of proteins.

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KOMPLEXE MORPHOLOGISCHE ANALYSE DER WIRKUNG DES LITORALONS AUF DIE MASTZELLEN

L. FEUER, P. KOVÁCS, ZSUZSANNA NAGY, OTTILIA TÖRÖK und G. CSABA

Das Präparat Litoralon verändert nicht die Zusammensetzung der Mastzellenkörnchen, hingegen bewirkt es eine rasche und energische Degranulation der Mastzellen. Im Ausmaß und im Typ der Degranulation bestehen Unterschiede zwischen den in den verschiedenen Organen befindlichen Mastzellen, angefangen von einer außerordentlich hochgradigen Degranulation (subkutanes Bindegewebe und peritoneale Mastzellen), hin bis zur Reaktionslosigkeit (Mastzellen der Schilddrüse und der Lymphdrüsen), und es lassen sich auch zeitliche Verschiebungen beobachten. Die peritonealen Mastzellen sind Saffranin-positiv und schwellen stark an; unter der Wirkung von 5 μg Litoralon nehmen sie bizarre Formen an. Der häufige Kontakt zwischen Lymphozyten und Mastzellen scheint typisch zu sein.

Litoralon bewirkt einen allgemeinen Anstieg der Histidinaufnahme in die Zellen der Peritonealflüssigkeit und einen speziellen Anstieg der Sulfataufnahme in die peritonealen Mastzellen. Die Anreicherung dieser Substanzen ist nach einer Stunde stärker als nach drei Stunden.

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THE AMPHICRINE (ENDO-EXOCRINE) CELLS IN THE HUMAN GUT, WITH A SHORT REFERENCE TO AMPHICRINE NEOPLASIAS

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(Received January 6, 1979)

In the human gastrointestinal tract the amphicrine cells are described as a special form of endocrine cells. Depending on their behaviour under silver impregnation, they are divided into three subgroups: the mucoargentaffine, the mucoargyrophilic and the mucoargyrophobic cells. They were detected electron microscopically in 1969, but they were histologically verified and identified as mucus-excreting endocrine elements only in 1977. Since 1969 such cells have also been observed in normal and regenerating rat and mouse stomachs. Our own human material includes stomach (3 cases), appendix (12 cases), colon (1) and a series of amphicrine proliferations and tumours. Two cases of chronic gastritis and one chronic peptic ulcer with metaplastic and regenerating epithelium contained mucoargyrophilic cells with mucus below the nucleus in the atypical glands. The possibility of endocrine granules being sluiced out in the mucous grains is discussed. Of the appendices only two were normal (ages 6 and 7 years), 10 showed pathological changes: there were seven neurogenic appendicopathies (14—58 years), one lymphatic hyperplasia, and one hyperplasia of mucoargyrophobic cells with mucostasis. Mucoargentaffine cells far outnumbered the mucoargyrophilic and mucoargyrophobic cells. The mucus may have either an apical or basal location; in the latter case, paracrine secretion into the subepithelial lamina propria was seen. As neoplastic cells, the amphicrine cells form the rare amphicrine tumours (goblet-cell and muco-adenoid carcinoids) of the appendix and colon. They are also found in mucinous cystadenomas of the ovary [26,] in the enteral type of a nasal carcinoma [27, 28], and in a 5-HT-carcinoid of the ovary [15]. They are therefore to be regarded as a differentiation disorder of the endocrine cells under the pathological conditions of appendicopathy, hyperplasia, metaplasia and true neoplasias.

Introduction

Amphicrine cells are rare differentiation forms of FEYRTER's "Helle Zellen" ("clear cells"), which in their totality form the "diffuse endocrine epithelial organs" [5] or, as he later called them, the "peripheral endocrine (paracrine) glands" [6, 7].* FEYRTER's cell system is now best known in the gastrointestinal tract and is divided into three subgroups,

1. *The argentaffine enterochromaffin (EC) or 5-HT cells* which have been known for long and which may be demonstrated with MASSON's silver method.

* Other terms for this cell systems include "FEYRTER's disseminated endocrine (paracrine) cells" [10, 16, 20, 21, 22]. The latest WHO suggestion is "FEYRTER's diffuse endocrine system". Today in the Anglo-American and German literature the term "APUD-cells" has become customary. It is intended to summarize the characteristic histochemical properties of the cell granules: *A*mine *P*recursor *U*ptake and *D*ecarboxylation.

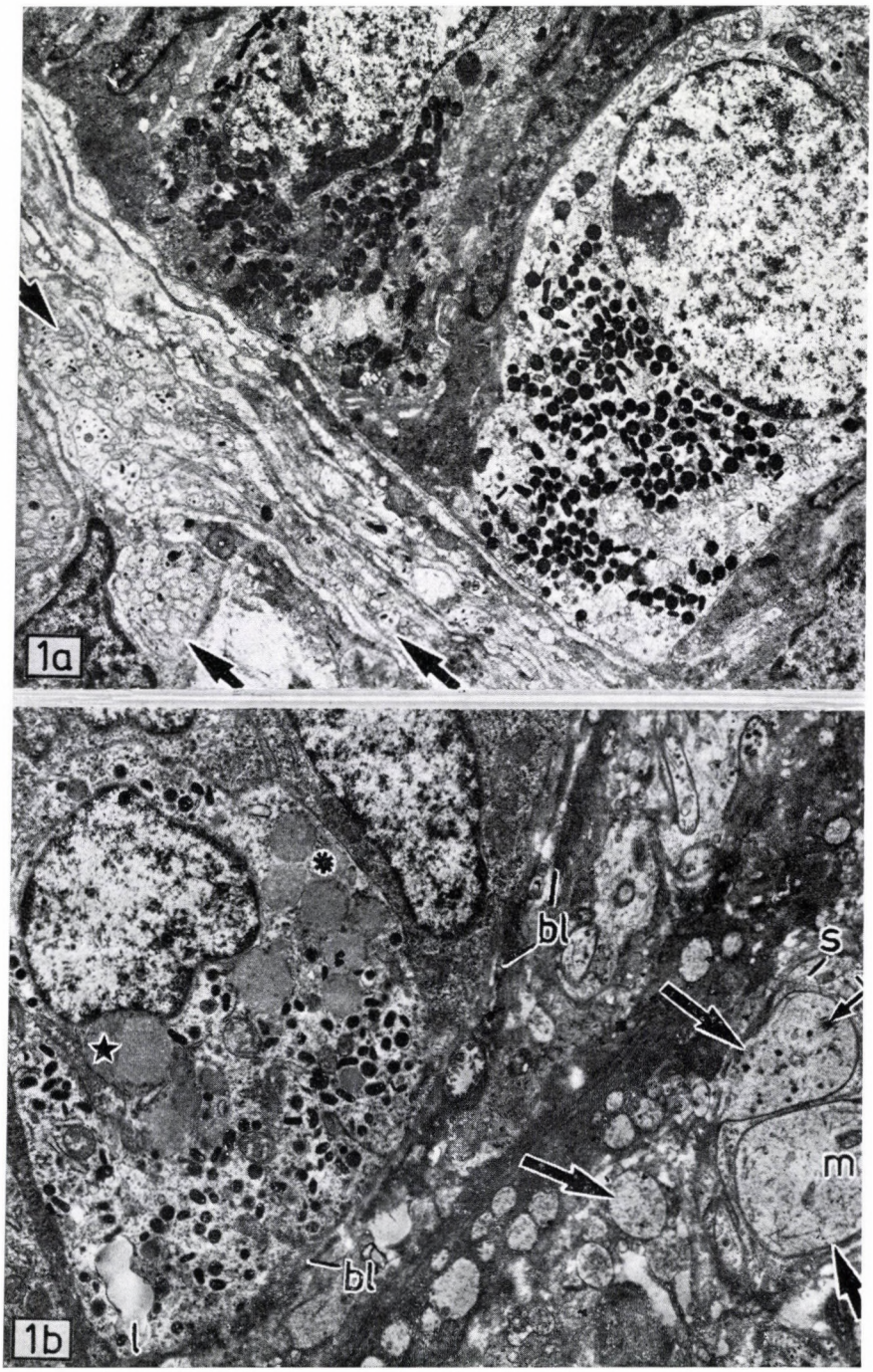

Fig. 1 a Appendix A3/A_5: for comparison: 2 ordinary basal granulated argentaffine cells (EC-cells) without mucus droplets. Note the abundance of normal subepithelial nerve fibres (arrows). ×9200

1 b Appendix A3/A_1: mucoargentaffine cell. Some large typical mucus droplets below the high-lying nucleus. This is indented by the largest droplet. Stars = lipid vacuoles; bl = basal lamina. Thick arrows point to the swollen axons of neurogenic appendicopathy with few mitochondria (m) and few neurosecretory granules (crossed arrows). Schwann-cell cytoplasm (S). ×5200

2. The large group of *argyrophilic cells*, demonstrable with silver impregnation according to BODIAN, GRIMELIUS and SEVIER-MUNGER; they form about a dozen different peptides (peptide cells) or histamine in ECL cells.

3. A rare third subgroup is the "leere helle Zelle" or "empty clear cell" [6, 29]. In these argyrophobic cells endocrine granules may not be demonstrated neither histochemically nor with silver impregnation. Membrane-surrounded inclusions in EC cells of appendix crypts were also found [24] (Figs 1—3). These inclusions lay between the EC granules on the cells base, and were identified as mucous grains. In neurogenic appendicopathy a similar cytoplasmic inclusion suggestive of mucus was found in an EC cell originated *via* "endophytia" FEYRTER's from crypt epithelium [2]. Until there, no such mucus-containing endocrine cells have been reported in man by electron microscope but at the same time peculiar cells have been observed in the digestive tract of mice and rats [13] and rarely in the normal stomach (fundus and pylorus), in the large sintestine and the regenerating gastric mucosa; these cells contained both endocrine granules and inclusions which were held to be mucus globules or zymogen granules. Later mucus-containing endocrine cells were observed in the human stomach, appendix and colon [22]; they have been introduced into literature as *amphicrine cells*. Meanwhile the pathologists [9, 32] detected the endo-exocrine carcinoids in the appendix.

In this paper the amphicrine cells of the human gastrointestinal tract are described and evaluated with particular regard to their formation of mucus. The cells were first studied electron microscopically, whereby they may easily be identified, then in histological preparations, where they may be recognized only after careful study using mucus staining and silver impregnation.

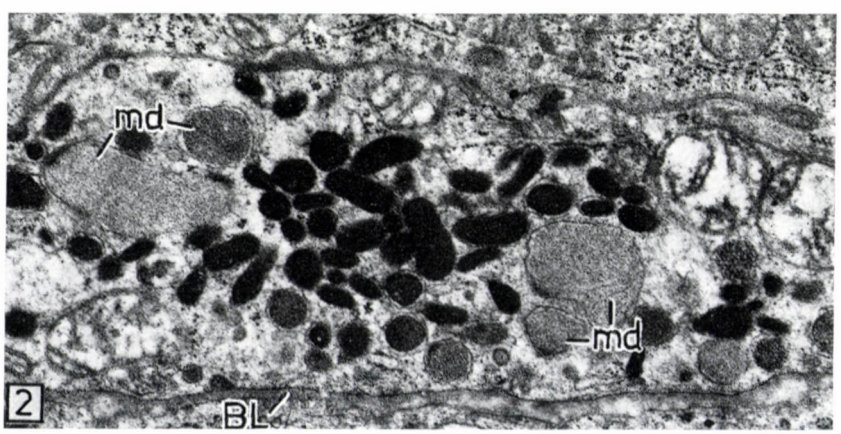

Fig. 2. Appendix A3/A_3: process of a mucoargentaffine cell from the basis of the crypt. Black pleomorphic 5-HT granules and mucus droplets (md). ×21,700

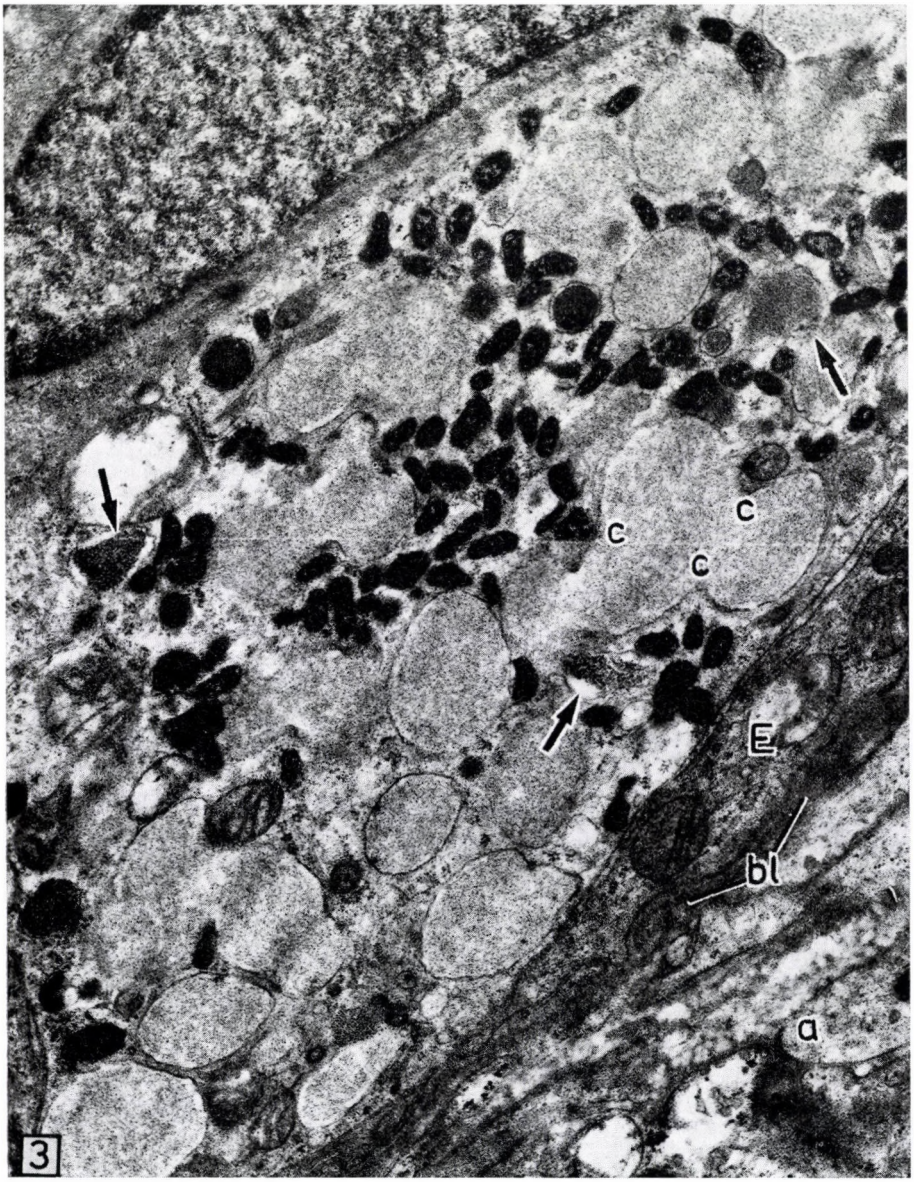

Fig. 3. Appendix A3/A_4: Basal granulated mucoargentaffine cell. Dense mixture of 5-HT granules and mucus droplets of various sizes; the latter are concentrated near the basis of the cell. The contents of the small droplets (arrows) are mostly denser than those of the large ones. C = cloverleaf-shaped beginning confluence of three mucus droplets; n = nucleus of a neighbouring cell; E = enterocyte; bl = basal lamina; a = axon. ×20,300

КОМПЛЕКСНЫЙ АНАЛИЗ ДЕЙСТВИЯ ЛИТОРАЛОНА НА ТУЧНЫЕ КЛЕТКИ

Л. ФЕУЕР, П. КОВАЧ, ЖУЖАННА НАДЬ, ОТТИЛИЯ ТЁРЁК и Г. ЧАБА

Литералон не изменяет зернистого состава тучных клеток, но вызывает их интенсивную и быструю дегрануляцию. В размере и типе дегрануляции наблюдаются отклонения между тучными клетками, находящимися в различных органах, начиная с чрезвычайно сильной дегрануляции (подможная соединительная такнь и перитонеальные тучные клетки) вплоть до отсутствия реакции (тучные клетки в щитовидной железе и в лимфатических узлах), и имеются также смещения во времени. Перитонеальные тучные клетки сафранин-положительны и они набухают в значительной мере. Под влиянием 15 μг Литоралона они принимают причудливые формы. Характерными кажутся частые контакты между лимфоцитами и тучными клетками.

Литоралом повышает поглощение гистидина клетками перитонеальной жидкости в общем, и специально поглощение сульфата перитонеальными тучными клетками. Накопление этих веществ по истечение часа сильнее, чем по истечению 3 часов.

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Materials and methods

Fresh surgical material with amphicrine cells

A. *Under non-neoplastic conditions*

1. *Fundus ventriculi*
   M_1: E 9052/69, E 9252/69, EM No. 220, 41 yrs., m.
   Chronic gastritis, previous vagectomy.
   M_2: E 637/74, EM No. 673, 63 yrs., fem.
   Chronic atrophic gastritis of the corpus with enteral metaplasia, hyperplastic argyrophilic cells and microcarcinoidosis [22]. Pernicious anaemia.
2. *Pylorus.* E 30240/69, 52 yrs., fem. Regenerating mucosa in the vicinity of a chronic peptic ulcer. Main disease: gastrin-producing carcinoid of the liver with metastases.
3. *12 appendices* A_1—A_{12}.
   A_1: E 5636/67 EM No. 110, 58 yrs., fem. Vermiform process with neurogenic appendicopathy (n. a.), without inflammation.
   A_2: E 5635/67, EM No. 111, 18 yrs., fem., n. a. with endophyty, oxyuriasis.
   A_3: E 9263/63, EM No. 138, 15 yrs., fem., n. a.
   A_4: E 9262/68, EM No. 139, 14 yrs., fem., n. a. with endophytia/FEYRTER.
   A_5: E 26777/68, EM No. 185, 7 yrs., m., appendicitis peracta (control case without amphicrine cells, only histological).
   A_6: E 20909/71, 31 yrs., fem., appendectomy in the course of sterilization. Hyperplasia of mucoargyrophobic cells.
   A_7: E 38982/76, 6 yrs., fem., appendicitis peracta.
   A_8: E 2709/77, 70 yrs., fem. Mucosa in the vicinity of a malignant argyrophob peptide carcinoid of the appendix.
   A_9: E 3412/77, 60 yrs., fem., n. a.
   A_{10}: E 13422/69, EM No. 241, 55 yrs., fem., n. a.
   A_{11}: E 4355/69, EM No. 205, 10.5 yrs., m., slight lymphatic hyperplasia.
   A_{12}: E 8000/69, EM No. 217, 48 yrs., fem., n. a.
   C: *Colon sigmoideum* E 3709/77, 47 yrs., fem.
   Normal mucosa 15 cm proximal to an adenocarcinoma.

B. *Neoplastic amphicrine cells*

1. *Amphicrine carcinoids of the appendix*
   A_{13}: E 2520/77, 48 yrs., fem. Mixed mucoargentaffine and mucoargyrophilic amphicrine carcinoid of the appendix. Mucoadenoid type.
   A_{14}: E 16607/76, 70 yrs., m., Goblet-cell carcinoid.
2. *Argyrophobic carcinoids of the colon with single cell mucin production*
   C_1: E 17642/75, 53 yrs., m. Relapse in colostomy.*
   C_2: E 771—776/79, 81 yrs., fem. Metastizing coecal tumour.*
3. *Mucinous cystadenomas of ovary as endodermal derivatives*, 25 cases [26]
4. *Nasal carcinoma of enteral type*
   E 42113/76, EM No. 915, 39 yrs., m. [27, 28].

Electron microscopy. The material was cut into 1 mm^3 pieces in the operating room and prefixed in 3% glutaraldehyde (cacodylate buffer pH 7.3) for 3 hours. After rinsing with cacodylate buffer the material was postfixed and precontrasted in a 1% osmium tetroxide solution for 2 hours, then after dehydration embedded in Epon 812. The sections prepared with an Om U2 Reichert ultramicrotome were occasionally postcontrasted with lead hydroxide and uranyl acetate solution. The electron microscopic pictures of the sections were made with a Philips EM 200 apparatus.

Light microscopy. Paraffin sections were stained with HE, van Gieson, PAS, and Alcian blue; silver methods were according to MASSON, BODIAN, GRIMELIUS, SEVIER-MUNGER, and combination of Alcian blue with the silver methods.

* Non-published observations.

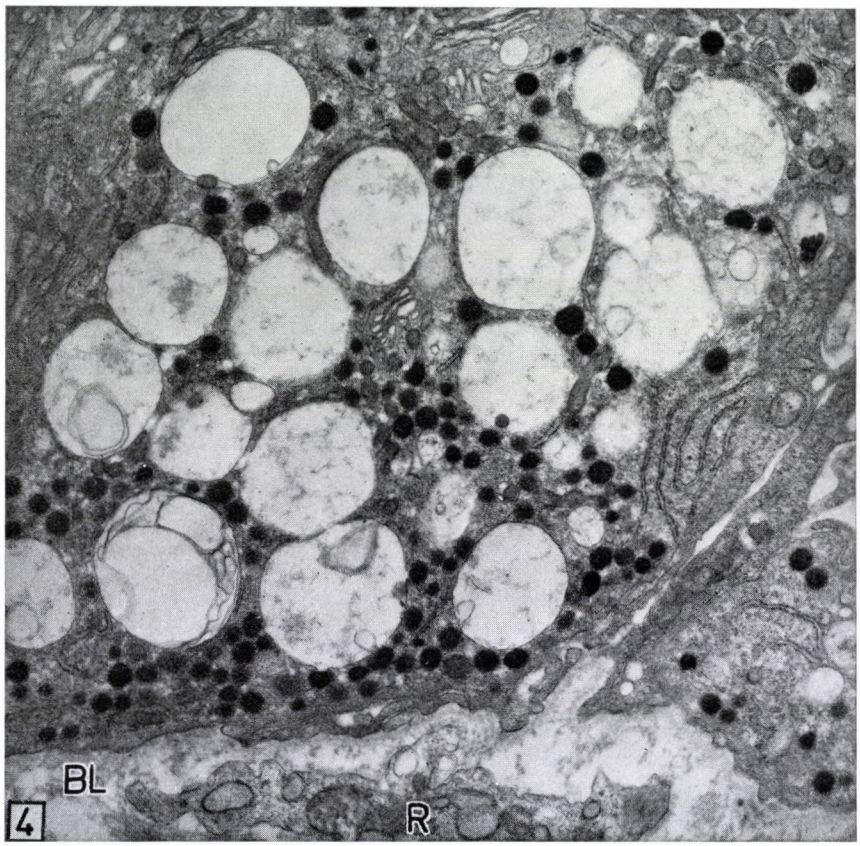


Fig. 4. Stomach A1/M_1: Chronic gastritis. Mucoargyrophilic cell, The round black peptide granules are intermingled with light mucus droplets. Lower corner right: basal part of an endocrine cell; bl = basal lamina; R = reticulum cell. ×15,200

Results

As the amphicrine cells were discovered with the electron microscope, these findings will first be discussed in correlation to the histological behaviour under silver impregnation. Depending on the nature of the specific granulation, the cells are subdivided into *mucoargentaffine, mucoargyrophilic and mucoargyrophobic amphicrine cells* (see Figs 10 and 11, Tables I and II).

A. *Ultrastructural results*

1. *Amphicrine mucoargentaffine cells.* We saw these repeatedly in the 12 appendices (Figs 1b, 2, 3). The mucus drops were found mixed with 5-HT (Fig. 2) or ECL granules, and were always in the basal parts of the cell, i.e.

Table I

Mucus localization in amphicrine cells and in amphicrine tumour cells. Location of findings in the gastrointestinal tracts

Mucus lies	Mucoargentaffine		Mucoargyrophilic		Mucoargyrophobic	
	apical	basal	apical	basal	apical	basal
Electronmicroscopical	— III/2*	+ II/2!	+ II/*1* III/3a	+ II/2 III/3a	+ II/2	—
Histological	+ II/*3* III/*1*	+ II/*1, 3* III/*1, 2, 3*	+ II/*1, 3* III/*1, 1—4*	+ II/*1, 3* III/*1, 1—4*	+ II/*1, 2, 3* III/*2*	+ II/*1, 2* III/*2*

The nunbers refer to the locations of findings in the organs as shown in Table II under normal and non-neoplastic conditions, and in Table III under neplastic conditions

* Reference [11]

Table II

A. *The subtypes of the amphicrine cells in the organs and their location and pathologic findings under non-neoplastic conditions*

Stomach fundus cases M_1, M_2	Predominant (A-like?) mucoargyrophilic cells	Chronic "Umbaugastritis" with intestinal metaplasia
Pylorus, 1 case	predominant mucoargyrophobic cells	regenerating mucosa near peptic ulcer
Vermiform process, 10 cases	mucoargentaffine cells predominantly of basal type A_1—A_4, A_6, A_9, A_{10}	neurogenic appendicopathy in 7 of 10 pathological cases with endophytia in A_2, A_4, A_{10}
	mucoargyrophilic cell A_8	near to a malignant argyrophobic carcinoid in this appendix
	mucoargyrophobic cells A_3, A_8	mucoargyrophobic cell hyperplasia with mucus hyperproduction (A_6)
Colon, 1 case	mucoargyrophobic cells in the crypts	near to an adenocarcinoma

below or next to the nucleus (for comparison see Fig. 1a: a common argentaffine cell). Large mucus drops may even make an indentation in the nucleus. Smaller, apparently younger mucus drops (Fig. 2) are somewhat coarser and more pronouncedly osmiophilic in their granulation; larger ones are finely granulated and less opaque. All mucus drops are surrounded by a 60 Å thick, continuous enveloping membrane, without 5-HT granules in the immediate vicinity. Larger mucus drops show a beginning confluence (Fig. 3) and then correspond to the histologically demonstrable fine mucus drops. The non-

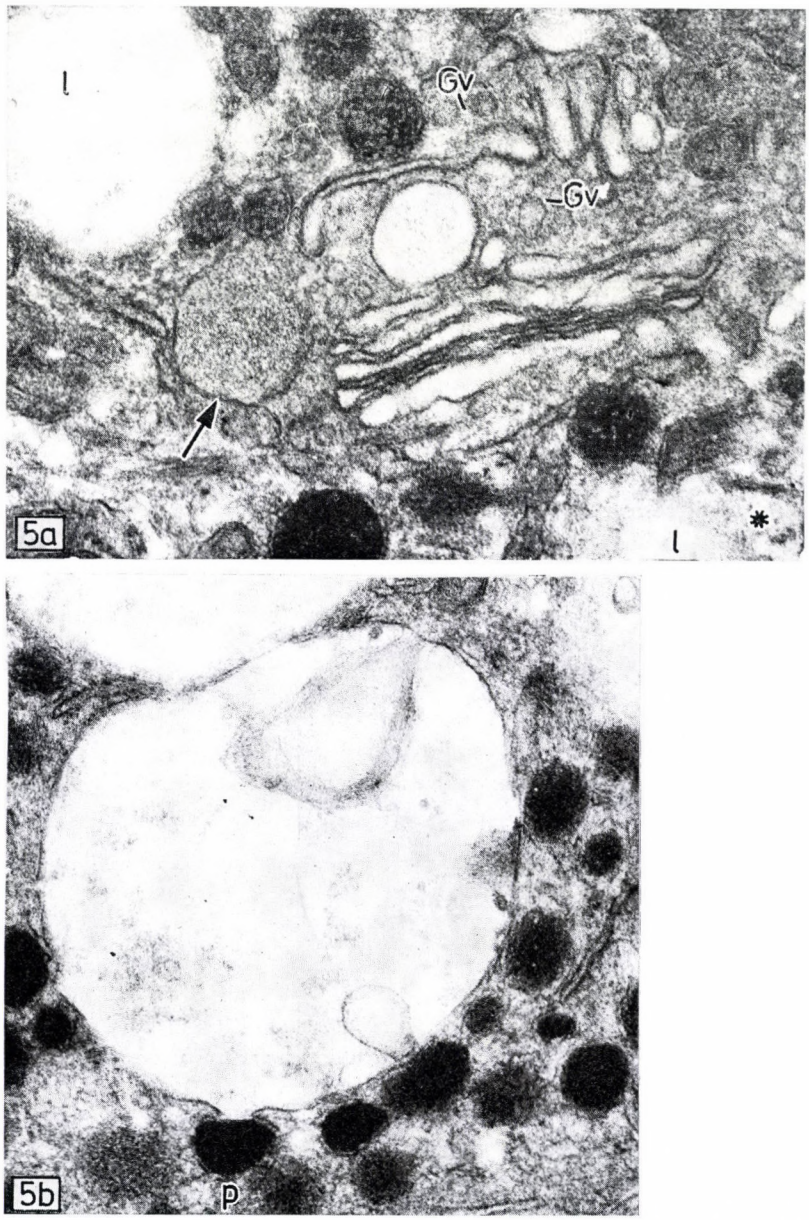


Fig. 5 Same cell as in Fig. 4. *A* — Origin of the mucus globules from the Golgi zone (centre). Left above and right, many small Golgi vesicles with contents (Gv). The thick arrow points to a middle-sized mucigenic granule with dense, fine granular contents. Very little and contrastpoor material inside the largest mucus droplets (l). Below right (*) a flat cut through the boundary membrane, ×62,300

Fig. 5 B. Same mucoargyrophilic cell as in Fig. 4. A large mucus droplet with ovoid, delicately cloudy profiles which adhere to the droplet membrane (above and below). The lower, pearshaped profile is above an endocrine granule. Above the peptide granule (p) seems to part the droplet membrane like lips (beginning expulsion of the granule into the mucus?), ×57,000

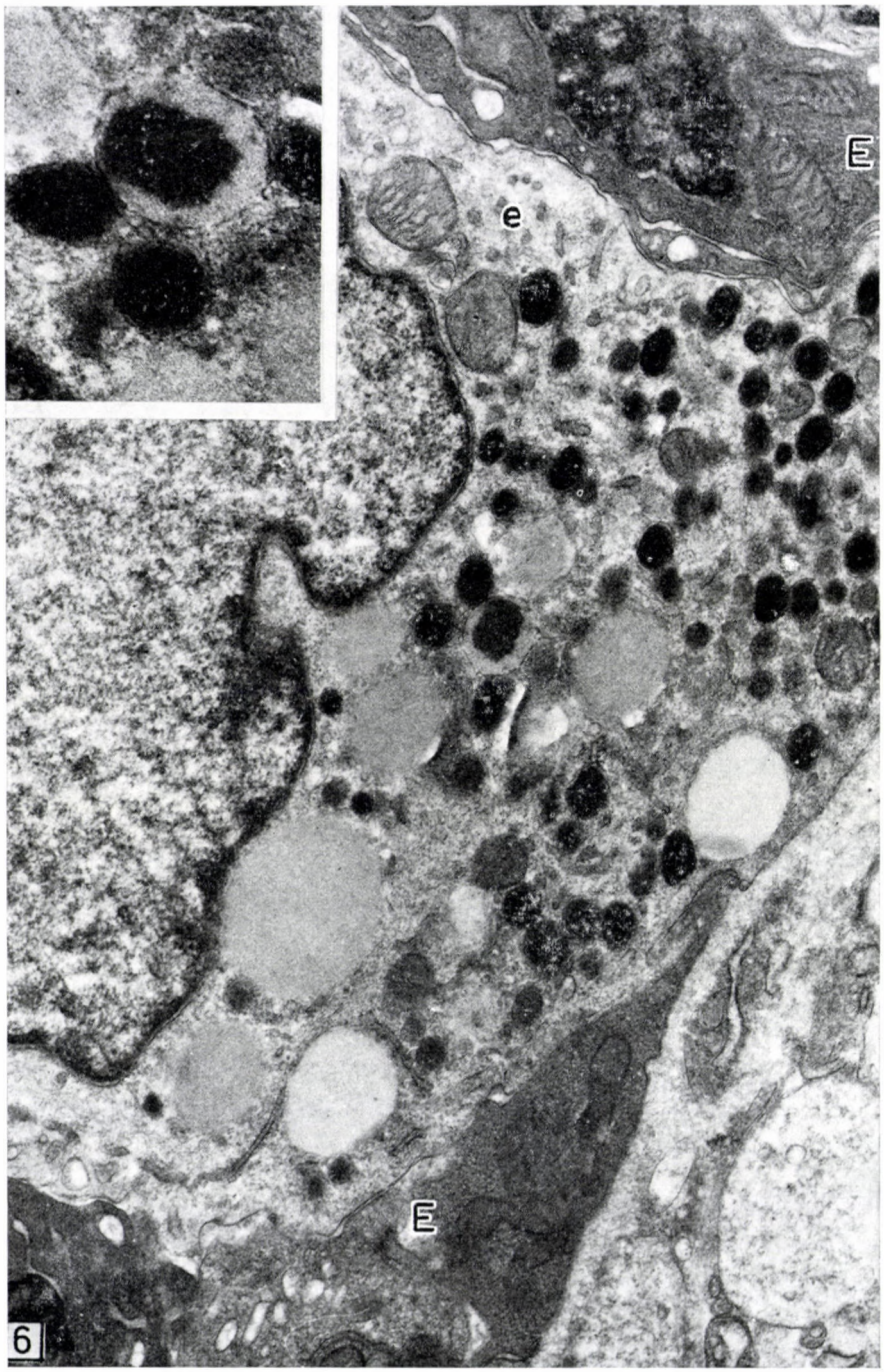


Fig. 6. Stomach $A1/M_2$: Atrophic fundus mucosa with intestinal metaplasia. Above and below enterocytes with dark ground plasm (E). Basal granulated mucoargyrophilic cell, clear ground cytoplasm. Most peptide granules are at the base of the cell. The mucus droplets are large and homogeneously grey; e = ergastoplasm. ×20,300. *Inset:* three black endocrine granules and mucus globules. In the central droplet a black patch without membrane ×43,000

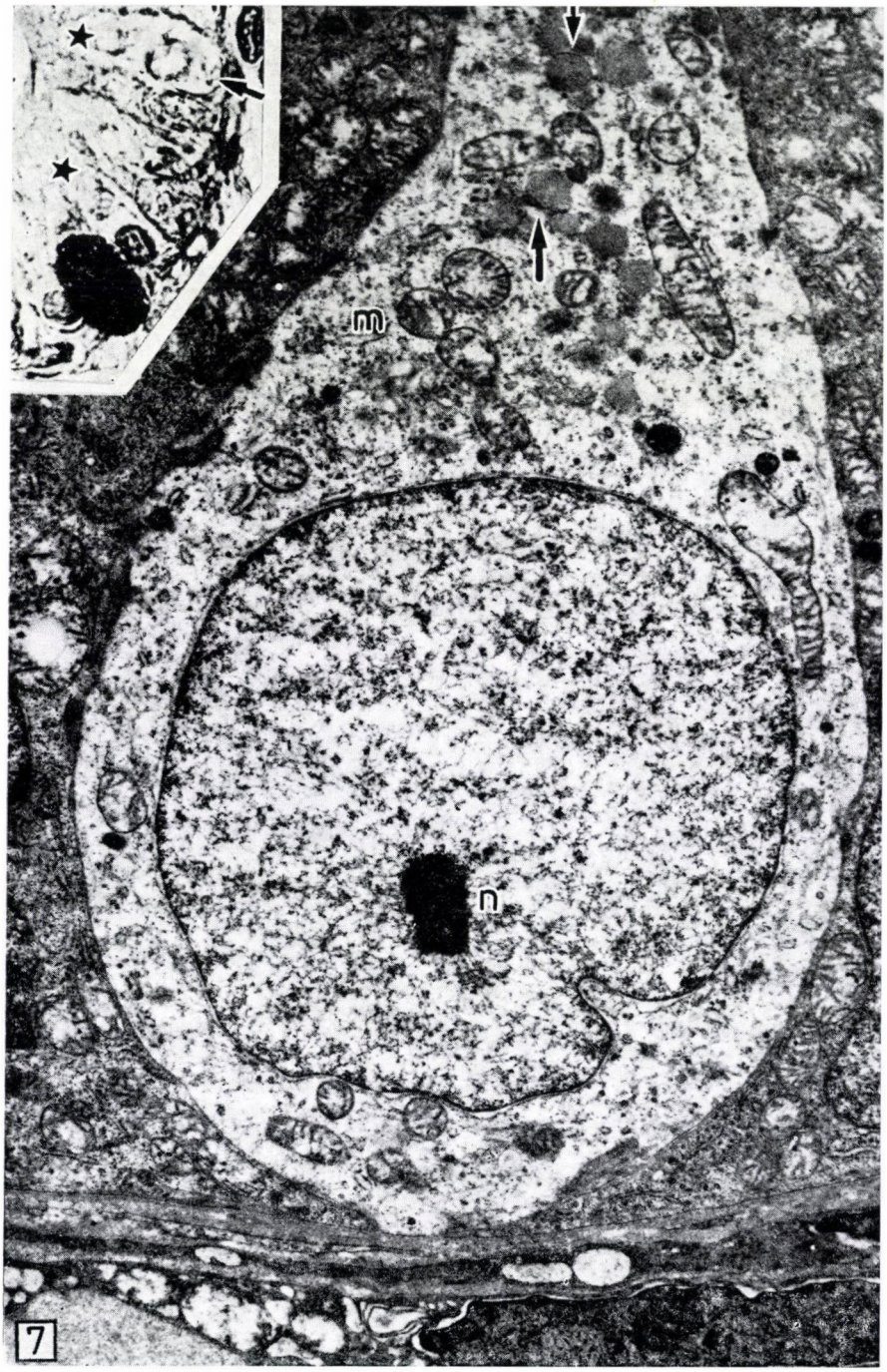


Fig. 7. Appendix A3/A_3: Argyrophobic empty (= "leere helle") cell of appendix crypt with questionable supranuclear mucous vesicles. No definite endocrine granules; m = mitochondria. ×13,100. *Inset:* taken from SPRAFKE [29]: Appendix crypt (partial view) with one argentaffine cell (black) and two empty clear cells (stars). Pushed-up, round nucleus. l = lumen. ca. ×400

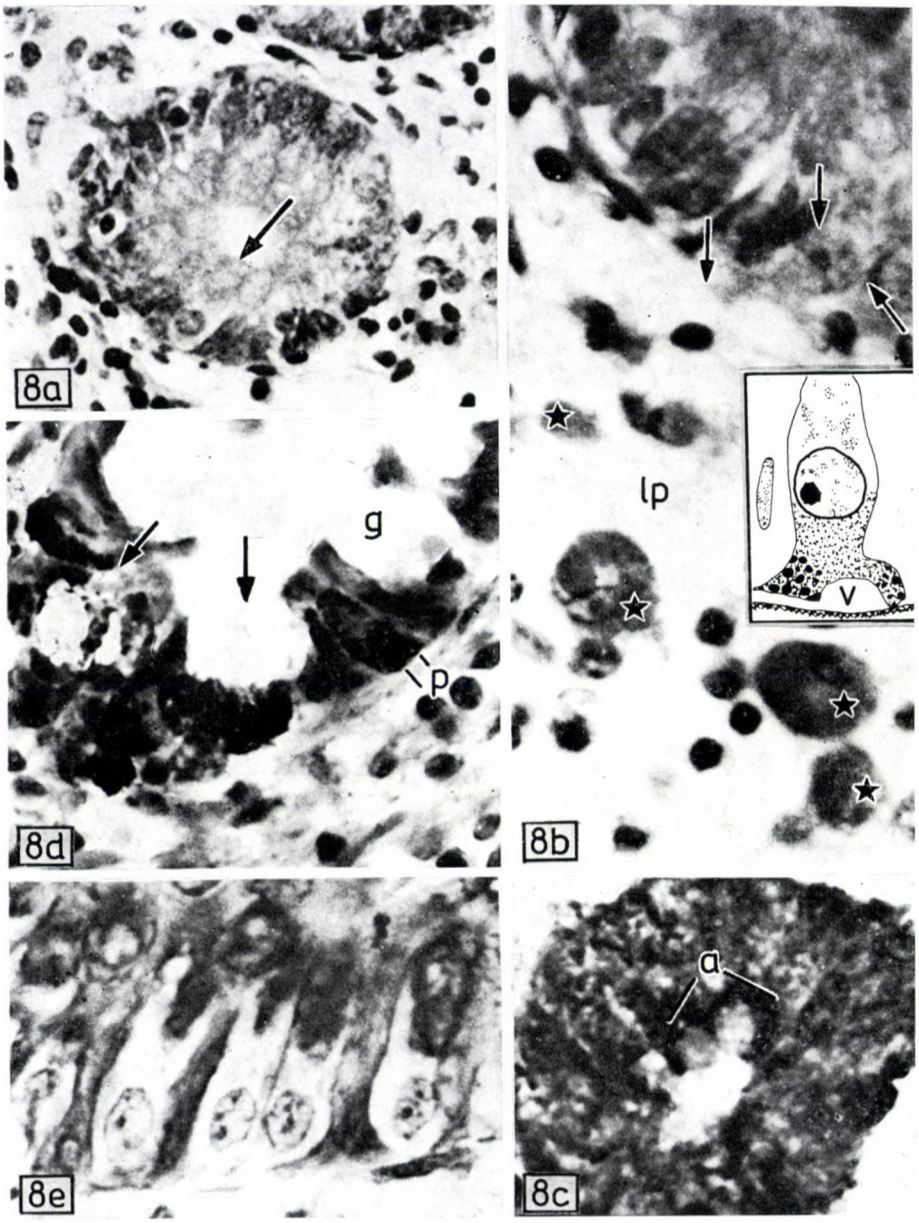
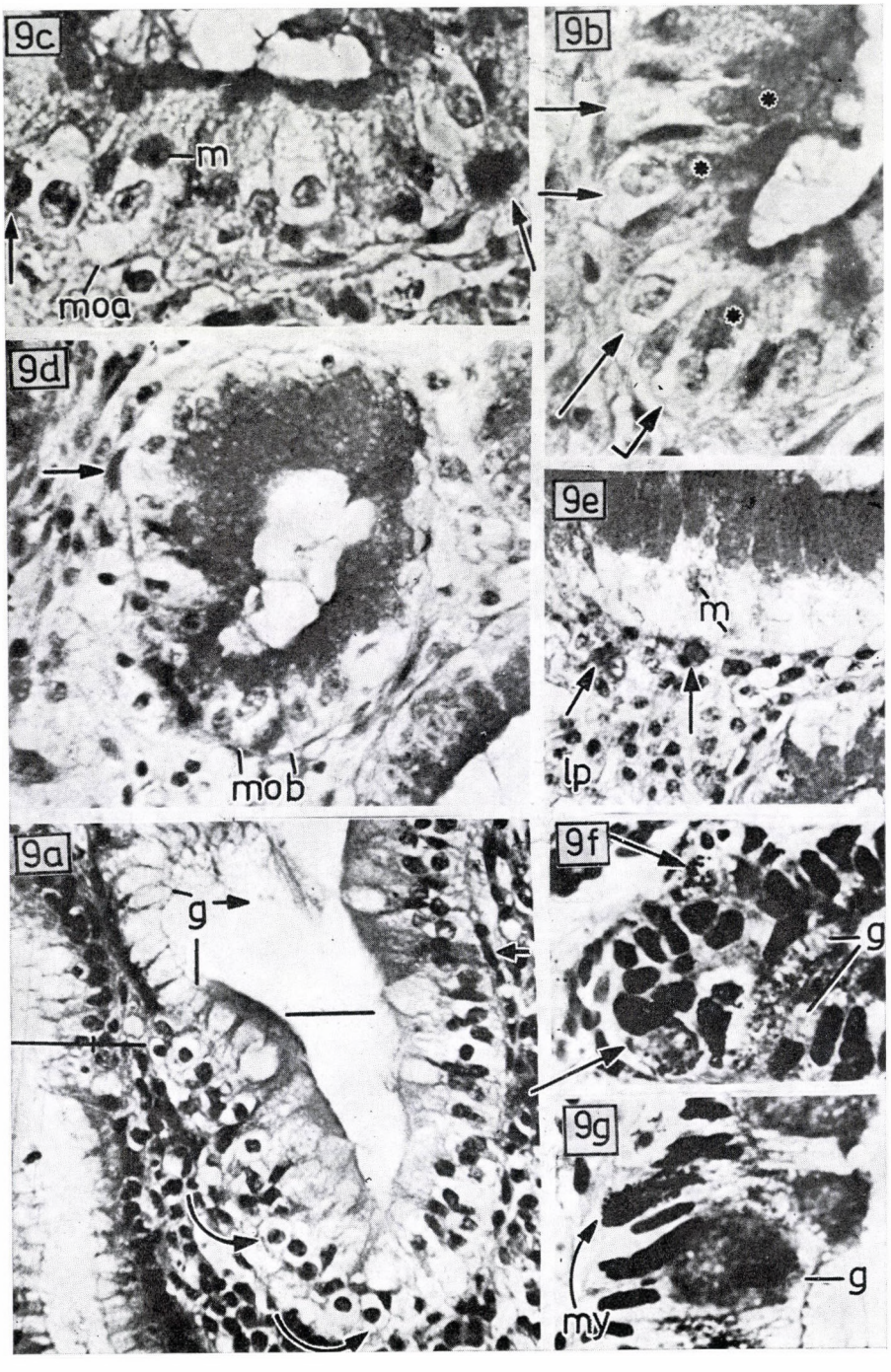


Fig. 8. Appendix A3/A_3: formol-PAS. *A:* apical subtype of mucoargentaffine cell with mucus plug as in goblet cell (arrow). 5-HT infranuclear granules grey (yellow from formol fixation). ×250. *B:* Basal subtype of mucoargentaffine cell (between the arrows and inset). Under the nucleus fine grey (yellow) 5-HT granules, to the left a few red, fine mucus granules (in the drawing as small dark spots), the fine 5-HT granules black, v = subepithelial vacuole. In the lamina propria (lp), four mucophages with fine and coarse mucus droplets (*). ×360 *C:* Stomach A1/M_1: Gastritis. Grimelius–Alcian blue. Argyrophilic granules on the base of the mucus plug (a) ×340. *D:* Stomach A1/M_2: Enteral metaplasia. Grimelius. Two mucoargyrophilic cells (arrows), the right one of the apical subtype; g = typical monocrine goblet cell, p = monocrine argyrophilic peptide cells, mostly with basal granulation. ×360 *E:* Appendix A3/A_6: Alcian blue, haematoxylin. Hyperplasia of mucoargyrophobic cells in a crypt. The Golgi zone is between the nucleus and the apical mucus plug. ×380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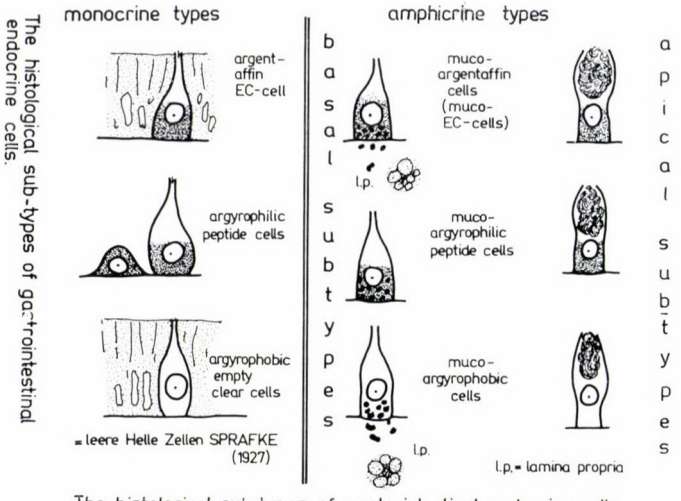

Fig. 10. Diagram of the histological subtypes of the gastrointestinal endocrine cells

homogeneous structure of the mucus bars confusion with lipid drops and vacuoles (Fig. 1B).

2. *Amphicrine mucoargyrophilic cells in the fundus ventriculi* (Table II). In the case of chronic gastritis M_1 (Figs 4—6) we found in the glands in addition to purely exocrine chief and parietal cells and mucous neck cells, and in addition to mono-endocrine cells amphicrine mucoargyrophilic cells with large light mucus granules in the basal parts of the cell. The contents are contrastr-poor and delicately cloudy and thready. On the outside, the round peptide graanules frequently lie close against the membrane, while inside, delicate roundish or elliptical lamellated profiles either stick to the membrane or are free inside the cell. Half moon-shaped united, lamellar defined vacuoles

←

Fig. 9. Pylorus: A2 Regenerating zone next to a peptic ulcer. Superficial glands. Formol *B—G.* ×360 *A:* van Gieson in the fundus of the gland, below the crosslines, hyperplasia of argyrohpobic clear cells at the base of the epithelium (curved arrows); g = goblet cells. Basa granulated EC cell (thick arrow). ×180

B: haematoxylin–PAS: four mucoargyrophobic cells of the apical subtype (arrows). * = mucus

C: haematoxylin–PAS: apical subtype of mucoargyrophobic cell (moa) with large basal vacuoles; m = mucus. Mucus droplets on the base of the epithelium belong to mucoargyrophobic cells of the basal subtype (arrows)

D: haematoxylin–PAS: mucoargyrophobic cell of the basal subtype (mob). The arrow indicates the extruding subepithelial mucus below an argyrophobic cell

E: haematoxylin–PAS: hyperplasia of argyrophobic cells, some with mucus (m). The arrows indicate subepithelial mucus droplets after expulsion into the lamina propria (lp). F and G = Case B4: intestinal type adenocarcinoma of the nose. Formol, Grimelius–Alcian blue

F. two mucoargyrophilic cells (arrows) at the base of the epithelium. The upper cell with very little mucus; g = goblet cells; L = lumen of the gland

G: narrow, slim cone-shaped basal and apical granulated mucoargyrophilic cell (my). g = goblet cell

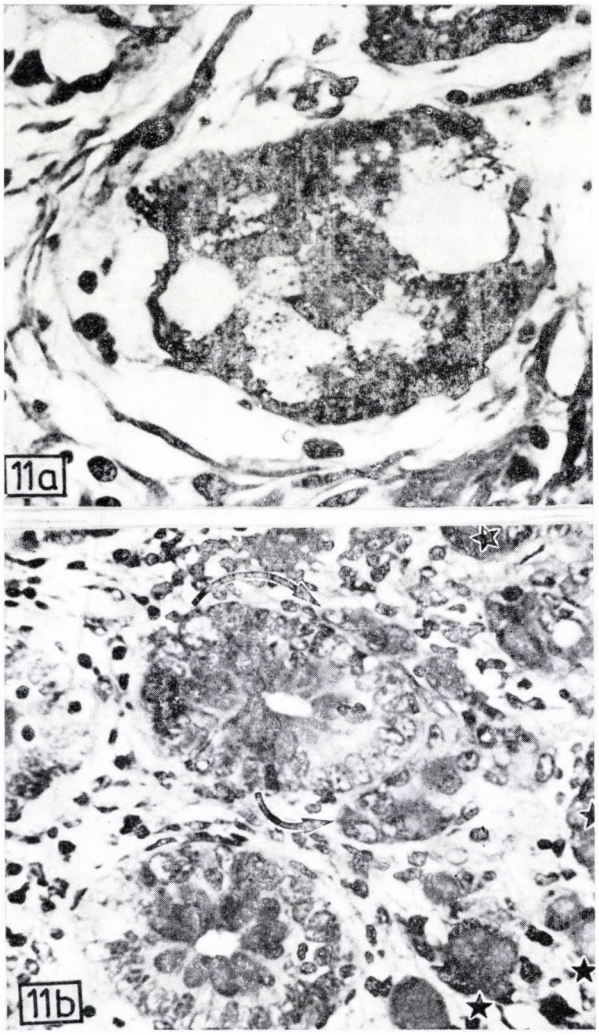

Fig. 11. A: Case $B1/A_{13}$. Amphicrine mucoargentaffine- and -argyrophilic goblet-cell carcinoid of the appendix. Sevier-Munger. Alveolus of goblet-cell-like mucoargyrophilic cells. ×420
B: Case $B1/A_{14}$. Amphicrine signet cell carcinoid of the appendix. Haematoxylin–PAS. Cross section of crypts. A crescent-shaped tumour border has developed around the upper crypt (arrows). Numerous budded-off signet tumour cells. (*) ×180

(Fig. 4) form the largest profiles in the immediate vicinity of the membrane. These adhering vacuolic processes present the question as to whether they are remnants of the membranes or endocrine granules, for at times one has the impression that endocrine granules pass from the cytoplasm into the mucus through an opening in the boundary membrane of the mucus granules (Fig. 5B). It is, however, to be stressed that the endocrine granules lying on the

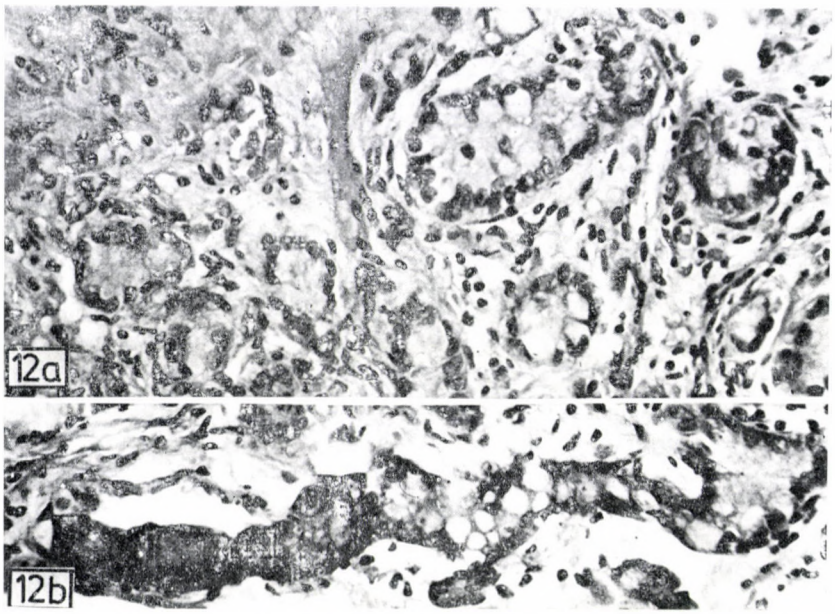

Fig. 12. Case $B1/A_{13}$. *A:* mixed argentaffine–argyrophilic mucoadenoid subtype of appendix carcinoid. HE. Crypt-like glandular formations with goblet cells. ×125
B: Sevier-Munger. Long, slightly-branched gland with argyrophilic cells (left), mucoargyrophilic cells (middle) and exocrine cells (right). ×125

outside of the enveloping membranes are generally intact. They posses the round shape of peptide granules and a highly and regularly osmiophilic core which is separated by a very small halo from the granule-enveloping membrane. There is no cytoplasmic granulolysis. The granules are 180—220 nm in diameter; they most closely resemble the A-like granules. Thus, such amphicrine cells are to be considered mucoargyrophilic A-cells. The genesis of mucus drops in the Golgi zone of normal monocrine mucous cells [3] is also clearly to be seen in the mucoargyrophilic cells (Fig. 5A).

The mucoargyrophilic cells of case M_2 (metaplasia and pernicious anaemia) were similar to those of M_1 (Fig. 6): an unstructured cytoplasm with little ergastoplasm, longish mitochondria varying in size and shape. The endocrine granules an,d the rather homogeneous mucus drops are located basally, and are usually mixed together in a thick layer. Owing to their varying osmiophily and size, G-granules come under consideration. A medium-sized mucus droplet contains a compact osmiophilic centre which is sharply defined but lacks a membrane; at present it cannot be identified (an immigrated granule or a "patch" inside the mucus?).

3. *A mucoargyrophobic cell free of granules* (empty clear cell), was detected under the electron microscope in the vermiform process A_3 (Fig. 7). The small, grey mucus granules lay above the nucleus in the Golgi area.

B. *Histological results* (see Tables I and II)

1. *Mucoargentaffine cells.* These histologically generally obscure and only seldom clearly demonstrable elements were observed years after their detection with the electron microscope in the Appendix A_9, owing to the presence of fine, free mucus droplets located subepithelially in the lamina propria, and in deeper-lying mucophages (Fig. 8B). The overlying enterochromaffine cells contained fine mucus granules only basally under the nuclei. A single argentaffine cell contained an unambiguous apical mucus plug as well, with theca and stoma as in a goblet cell (Fig. 8A), while the yellow enterochromaffine granules lay as usual under the nucleus.

2. *Mucoargyrophilic* cells could be demonstrated histologically in the two cases of chronic gastritis, M_1 and M_2. There were in part basal mixed granulations, otherwise the mucus was located as in goblet cells apically, with granulation under and next to the mucus (Fig. 8C, D). The mucoargyrophilic cells are most often seen as neoplastic elements: in amphicrine carcinoids (Figs 11, 12), in mucinous cystadenomas of the ovary [26], and in the endocrine cell-rich intestinal carcinoma of the nose [27, 28].

3. *Mucoargyrophobic* empty clear cells with supranuclear mucus were seen histologically in the sigmoid [22] and in a pylorus as well as in the clinically asymptomatic appendix A_3 (Fig. 8E), which was particularly rich in these cells (hyperplasia). The characteristics of the mucoargyrophobic cells are:

a) the pushed-up, compact nucleus, as in all endocrine enteral cells;

b) the same granule-free, para- and intranuclear clear cytoplasm, often occupied by large vacuoles, demonstrated by nuclear staining and with silver methods; it conforms to the electron-optical clear ground plasma (see appendix A_3, Fig. 8);

c) the mucus is located either as an *apical plug* (in A_6, Fig. 8E) and in the clear-celled regenerating mucous membrane of the pylorus (Fig. 9B, C), or *basally* (Fig. 9D).

In this basal type *subepithelial* PAS-positive *mucus* was found in the stroma in the form of small, flat or round drops (Fig. 9D, E), whereby mucophages reappeared in the lamina propria. In the apical subtype the Golgi apparatus is located between the nucleus and the mucus plug (Fig. 8E).

The neoplastic amphicrine cells (Table I and III)

In general, the arrangement of mucus in the apical or basal parts of the tumour cells is not pronounced and changeable according to the unregulated cell differentiation of tumour cells. The amphicrine carcinoids (B1) have different structures such as goblet [30], mucoadenoid [22] and signet cell types [23]. The tumours contain mucoargentaffine, mucoargyrophilic and mucoargyrophobic cells.

Table III

B. *The subtypes of the amphicrine cells in the organs under neoplastic conditions*

	Subtypes	Tumours
1 Appendix Cases A_{13}, A_{14}	muco-argentaffine and muco-argyrophylic (1) carcinoid cells	amphicrine carcinoids of muco-adenoid or goblet cell type
2 Colon	muco-argentaffine (11), muco-argyrophilic and muco-argyrophobic tumour cells, signet cell carcinoid*	mucoargentaffine, mucoargyrophilic, muco-argyrophobic carcinoids*
3 Ovary	*a)* secretin? subtype of muco-argyrophilic cells	mucinous cystadenomas (26)
	b) muco-argentaffine cells	5-HT-carcinoid of the ovary (15)
4 Nose	A-like subtype of muco-argyrophilic cells	enteral type carcinoma of the nose (28)

* Non published tumours of the colon

The argyrophobic carcinoids of the colon (B2) consist almost totally of non-granulated cells; only part of the cells contains mucus. The single cell mucin production ("Einzelzellverschleimung") is characteristic.

In the mucinous cystadenomas of the ovary [26] (B3) and the endo-amphicrine enteric carcinoma of the nose [28] (B4), the amphicrine cells are few and dispersed in the exo- and monoendocrine tumour epithelium. Beside the mucus globules they contain round peptide granules. As to their specia features see Table III.

Discussion

A schematic summary of the morphology of the intestinal amphicrine cells is shown in the cell diagram (Fig. 10) where they are compared to intestinal monoendocrine cells.

Basal mucus formation in *argentaffine EC cells of the appendix* was demonstrated by electron microscopy in eight cases; with the light microscope, mucoargentaffine cells with basal or apical mucus development could be shown only rarely (Fig. 8A, B). Electron microscopic evidence for apical exocrine secretion by the normal mucoargentaffine cell and for basal mucus production by the normal mucoargyrophobic cells is lacking. In the stomach, the muco-argyrophilic cell of the basal and apical subtype is the most frequent one. These cells are found above all as transformed pathological elements.

The mucoargyrophobic cell and its basic form, the empty clear cell of the endocrine (FEYRTER) system, have not been clarified electron microscopi-

cally, except for one single identification in A_3 (Fig. 7); they also require a precise ultrastructural differentiation from certain forms of the goblet cell of the colon [12].

Amphicrine cells as normal or pathological cell forms (see Tables II + III)

Seen as a whole, the amphicrine cells are probably very rare elements in the glandular epithelium of normal intestinal organs or they would have been noticed earlier.

In the endocrine cells of the cardial glands peptide cells of D_1 and A1 subtype were found [12a] having mucus vacuoles beside the round endocrine granules. In Figs 4 and 5 they conform exactly to the gastric mucoargyrophilic cell, presented in Figur 4. In man we saw them under normal conditions too, in the juvenile appendices A_5 and A_7, otherwise only under pathological conditions or as neoplastically degenerated cells in neurogenic appendicopathy with and without endophytia, in the regenerated and metaplastic epithelium of the stomach, and in amphicrine tumours (Table II and III). On the other hand, endocrine cells with exocrine secretion products (endo-exocrine cells) have also been found in normal or differentiating gastric epithelium in rodents. KATAOKA [13] reported "a new cell type" from the mouse stomach, with endocrine granules and large inclusions corresponding to the mucus grains, but he was unable to explain these inclusions. CAPELLA et al. [4] showed a mixed endo- and exocrine cell in the rat stomach; it contained ECL granules and large zymogen inclusions.

NABEYAMA [17] found in the colon and pylorus of the mouse mixed endo-exocrine cells; the inclusions were identified as mucus with their fine, grey contents and peculiar thickenings (so-called "patches"), which are known from the mucigen droplets. TAHARA [31] created artificial stomach ulcers in the mouse and found in the regenerated epithelium a) chief and parietal cells with mucus, but also b) endocrine cells with mucus and zymogen granules. In the mouse small intestine there were registered rare epithelial cells containing mucus globules and entero-endocrine granules [4a].

These findings in the rodent stomach indicate that in man the amphicrine cells are by no means always a pathological element. They probably develop and certainly increase in number in differentiation disorders within the context of regeneration, metaplastic epithelial regeneration in the stomach and above all in carcinoid growths, further in the endocrine elements of carcinomas [14, 15, 28] and in mucinous cystadenomas of the ovary [26] (Table III).

Neoplastic proliferation of amphicrine cells leads to formation of amphicrine tumours. They have only recently become known first time in the appendix and then in the colon. These tumours are built up of mucoargentaffine,

mucoargyrophilic and mucoargyrophobic cells, yet in very different relation to the secretion products. One may distinguish between

1. the goblet cell carcinoid [30] (Fig. 11B) described under electron microscope by ABT and CARTER [1];

2. the muco-adenoid carcinoid [22] (Fig. 12);

3. the signet-cell carcinoid [23] (Fig. 11A), not to be confused with the signet-cell carcinoma.

Among the commended names, WHO proposed mucocarcinoid; this is a superposed name.

The development of amphicrine tumours is another valuable indicator of the existence of non-neoplastic amphicrine cells among the rare elements of the gastrointestinal epithelium.

As to the morphogenesis of the mucus in amphicrine cells the smallest mucigenic droplets develop in the Golgi zone (Fig. 5A, 8E). This apparatus is the key location of the maturation of monoendocrine granules or, under the conditions mentioned above, of the differentiation of endo- and exocrine secretory products.

FEYRTER [5] has stressed the possibility and the potency of his clear cells to produce mucus, and to do so in carcinoids as well. He thereby meant, however, only the acid-thionine or PAS-red-stained "slimy excretions" on the cell surface, which often fill in glandular spaces in carcinoid formations. Ultrastructural examination shows that this homogeneous excretion is not mucus but a very fine, of course, granuled material with a peculiar structure which fills the spaces between the cilia of the tumour cells and may by no means be held to be ejected cytoplasmic mucus. These cell surface products thus have nothing to do with the mucus globules as a secretion product of amphicrine cells and amphicrine tumours. Appropriate mucus globules are missing in both monocrine endocrine cells and common carcinoids.

Amphicrine cells and amphicrine carcinoids offer important support to the old theory of the endodermic origin of the endocrine cells. Other clear evidence is the occurrence of argentaffine granulated enterocytes and Paneth cells in carcinoids, as well as by the varying quantities of argentaffine granulated tumour cells present in gastric (3.1%) and intestinal carcinomas (2.5%) [14]. Further proof is offered by gastrointestinal tumours showing transition from carcinoma to carcinoid. [23].

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ÜBER DIE AMPHIKRINEN (ENDO-EXOKRINEN) ZELLEN IM MENSCHLICHEN VERDAUUNGSTRAKT MIT EINEM KURZEN HINWEIS AUF DIE AMPHIKRINEN NEOPLASIEN

M. RATZENHOFER und L. AUBÖCK

Im Gastrointestinaltrakt des Menschen werden als Sonderform der endokrinen die amphikrinen Zellen näher beschrieben und hiebei — abhängig vom Verhalten bei Versilberung — 3 Untergruppen unterschieden: die mucoargentaffine, die mucoargyrophile und die mucoargyrophobe Zelle. Sie wurden 1969 von Ratzenhofer und Mitarb. zuerst elektronenmikroskopisch gesehen, aber erst 1977 histologisch verifiziert und als Schlein-sezernierende endokrine Elemente gedeutet. Solche Zellen wurden seit 1969 auch im normalen und regenerierenden Magen bei Ratte und Maus beobachtet. Das eigene menschliche Untersuchungsgut umfaßt nunmehr die Appendix vermiformis (12 Fälle), Magen (3) und Dickdarm (3) und einzelne amphikrine Wucherungen und Geschwülste. Unter den Appendices waren 2 normale (6 u. 7 Jahre) und 10 pathologisch veränderte: 7mal neurogene Appendicopathie (14 bis 58 J.), 1 lymphatische Hyperplasie, 1 Hyperplasie von mucoargyrophoben Zellen mit Mucostase. Es fanden sich ganz überwiegend mucoargentaffine, selten mucoargyrophile und mucoargyrophobe Zellen. Der Schleim kann in den Zellen entweder apical oder basal liegen, wobei in diesem Falle eine parakrine Sekretion in die subepitheliale Lamina propria und Mucophagen beobachtet wurden. 2 Fälle von chronischer Gastritis und 1 chronisches peptisches Pylorusgeschwür enthielten in den atypischen Schleimhautdrüsen mucoargyrophile Zellen mit Schleim unterhalb des Kernes. Die Möglichkeit eines Ausschleußens von endokrinen Granula in die Schleimkörner wird diskutiert. Neoplastische amphikrine Zellen bauen die seltenen Becherzell- und mucoadenoiden Karzinoide der Appendix und des Colon auf. Sehr seltene Fundstätten amphikriner Tumorzellen sind ferner ein Nasenkarzinom (enteraler Typ 27, 28), ein argentaffines Karzinoid des Ovars 15. Regelmäßig kommen neoplastische amphikrine Zellen (als bequemes Studienobjekt) vor allem in den Mucinkystomen der Ovarien vor (Schmid [26]). Die amphikrinen Zellen treten danach vor allem als *Differenzierungsstörungen der endokrinen Zellen unter folgenden pathologischen Bedingungen auf:* neurogene Appendicopathie, Hyperplasia, Metaplasie und echte Neoplasie.

ДАННЫЕ К АМФИКРИННЫМ (ЭНДО-ЭКЗОКРИННЫМ) КЛЕТКАМ, НАХОДЯЩИМСЯ В ПИЩЕВАРИТЕЛЬНОМ ТРАКТЕ ЧЕЛОВЕКА, С КОРОТКИМ УКАЗАНИЕМ НА АМФИКРИННЫЕ НОВООБРАЗОВАНИЯ

М. РАТЗЕНХОФЕР

Амфикринные клетки, находящиеся к желудочно-кишечном тракте человека, описываются как особые формы эндокринных клеток. В зависимости от их поведения при серебрении различаются 3 подгруппы: мукоаргентаффинные, мукоаргирофильные и мукоаргирофобные клетки. В 1969 г. автор впервые наблюдал в электронном микроскопе эти клетки, однако их гистологическое доказание удалось только в 1977 году, причем он рассмотрил их как слизовыделяющие эндокринне элементы. С 1969 года такие клетки наблюдали также в нормальном и регенерирующемся желудке крысъ и мыши. Человеческий материал для исследования автора охватывает теперь слепую кишку (12 случаев), желудок (3 случая) и толстую кишку (3. случая), а также отдельные амфикринные разращения и опухоли. Среди толстых кишок было 2 нормальных (6-и 7-летние больные) и 10 патологически измененных, а именно 7 случаев неврогенной аппендикопатии (больные от 14 до 58 лет), 1 случай гиперплазии и 1 случай гиперплазии мукоаргирофильных клеток с застоем слизи. В преобладающем большинстве случаев встречались мукоаргентаффинные, но изредка и мукосаргирофильные и мукоаргирофобные клетки. Слизь располагалась в апикальной или в базальной части клетки. В последнем случае наблюдали паракринную секрецию в субэпителиальную слизистую оболочку (Lamina propria) и мукофаги. В двух

случаях хронического гастрита и в одном случае хронической пептической язвы привратника атипичные железы слизистой оболочки содержали мукоаргирофильные клетки со слизой под ядром. Обсуждается возможность выделения эндокринных зернышек в слизистые зерна.

Редко встречаемые бокаловидные клетки и мукоаденоидные карциноиды слепой и толстой кишок построены неопластическими амфикринными клетками. Очень редко амфикринные клетки встречаются также при раке носа (энтеральный тип 27, 28), и аргентаффинном карциноиде яичника 15. Неопластические амфикринные клетки регулярно наблюдаются (как удобные обьекты для изучения) прежде всего в муцинкистомах аичников (Шмид 26. Следовательно, амфикринные клетки появляются прежде всего в результате расстройств дифференциации эндокринных клеток при следующих патологических состояниях: неврогенная аппендикопатия, гиперплазия, метаплазия и настоящая неоплазия.

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INTRAEPITHELIAL MAST CELLS IN THE HUMAN GASTRIC MUCOSA IN A CASE OF MICROCARCINOIDOSIS

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A 63-year-old female patient with pernicious anaemia and chronic transformation-gastritis with numerous disseminated endocrine cells of the fundus glands had developed a diffuse microcarcinoidosis. Mast cells were identified not only in the stratum proprium mucosae, but also in the epithelium of atypical glands of the basal mucosa. Electron and light microscopic investigations clearly showed that the intraepithelial mast cells emigrated into the neighbouring stroma. During the process of secretion the ultrastructure of the specific granules changes continuously, whereby four different phases can be distinguished by the electron microscope.

Introduction

Mast cells, first described as independent cell forms by Ehrlich in 1877, represent an important cellular component of the connective tissue. The function of the mast cells is essentially tied to their histamine, heparine, dopamine and serotonin content [7, 8, 9, 15, 20]. Especially their functional importance in the course of inflammatory processes of the connective tissue has been emphasized [1]. Because of their enzymatic active substances, mast cells are additionally considered to have lysosomal properties [11, 12, 19, 24], limited phagocytic activity [18] and a share in antigen–antibody-reactions. The influence of mast cells on the small blood vessels and the organ parenchyma cells as a type of neurohormonal feedback–system has also been shown [3, 4, 22].

Electron microscopic examinations of human material were restricted to the identification of mast cells in connective tissue [2, 10, 22], and only few authors reported on the presence of mast cells in the gastrointestinal tract, in particular of intra- (inter-) epithelial mastocytes [5, 23].

Therefore electron microscopic identification of numerous mast cells both in the epithelium and in the lamina propria of the gastric mucosa in a case of diffuse microcarcinoidosis seems of interest.

Materials and methods

A 63-year-old female patient with pernicious anaemia and chronic transformation gastritis whereby numerous disseminated endocrine cells of the gastric mucosa were produced had developed a diffuse microcarcinoidosis. Tissues for ultrastructural studies obtained by gastric biopsy were immediately cut into 1 mm^3 large pieces and prefixed for two hours

in 3% glutaraldehyde buffered with 0.1 *M* sodium cacodylate buffer. The specimens were dehydrated in graded ethanol, embedded in Epon 812, 1 μm thick sections were stained with toluidine blue for light microscopy. Ultrathin sections were cut at 50 to 70 nm by a Reichert ultramicrotome OmU2, stained with uranyl acetate and lead citrate, and photographed with a Philips EM-200 electron microscope.

Electron microscopic results

Mast cells in large numbers were identified not only in the stratum proprium mucosae but also in the epithelium of the gastric mucosa. On the basis of their fine structure, but particularly because of the different ultrastructure of the specific cytoplasmic granules, the mast cells could be subdivided into four different phases, representing four different secretorial stages.

Phase I — mast cell (Fig. 1) has the usual aspect of a well differentiated mature mast cell and is an approximately 10 μm — large, elongated or round cell, with numerous 400—600 Å thick and several μm long microvilli on its outer surface. The usually kidney-shaped, 5—8 μm large nucleus contains large amounts of clumped chromatin which tends to be more compact at the periphery. Distinct dense nucleoli were occasionally present. The cytoplasm is electron dense and has numerous, irregularly arranged 50—70 Å thick filaments. The endoplasmic reticulum was sparse with approximately equal amounts of smooth and rough surfaced elements. The Golgi complex is moderately prominent, consisting of flattened lamellae, vesicles and occasional vacuoles. Mitochondria are round or slighty elongated with a lenght up to 0.2 μm. They are perinuclear in position or closely adjacent to the Golgi complex, although occasionally they are present all over the cytoplasm. The usual variety of lysosome-like bodies was rarely seen. Some cells contain one or two dense bodies, presumably lipid droplets. Glycogen particles could not be identified, neither were microtubules and mast cells in mitosis. The ultrastructural appearance of the phase I — mast cell is determined by the morphology of the specific cytoplasmic granules, more precisely by the ultrastructure of the granule content.

Membrane enclosed granules, irregularly distributed in large numbers over the entire cytoplasm, average 0.3 to 0.6 μm in size and range up to 1 μm in greatest diameter. The enveloping membrane is not always continuous and sometimes difficult to delineate. The granule content consists predominantly of concentrically arranged membrane cylinders lying next to each other in groups in close proximity of parallel running at times also concentrically arranged membrane profiles, or of incomplete lamellar cylinder sections. All of these structures are stored in an electron-dense and fine granular matrix.

The phase I — mast cell is found not only in the lamina propria but also in the epithelium of the gastric mucosa (Fig. 1). These interepithelial phase I — mast cells lie at the base of the epithelium just above the basal lamina and stand in close intercellular contact with the adjacent epithelial cells by

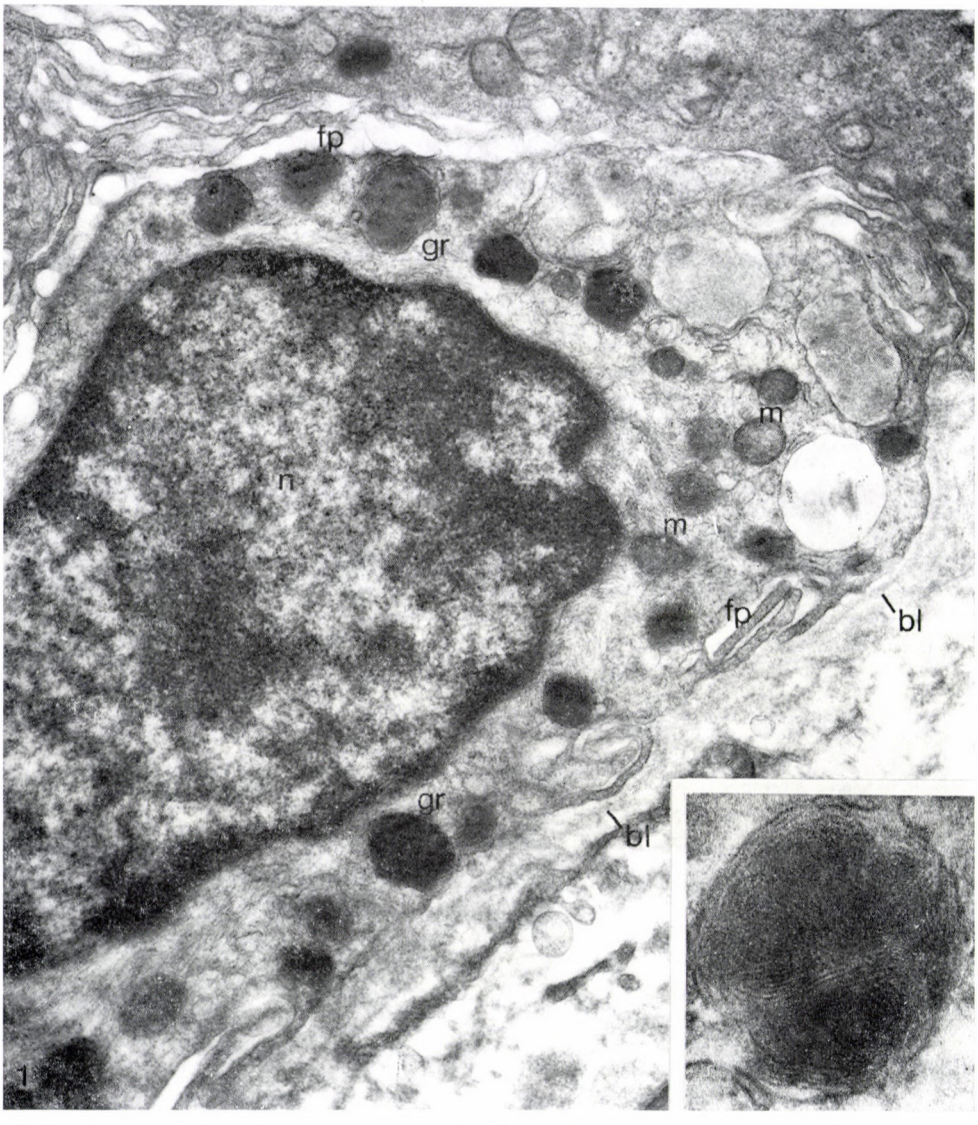

Fig. 1. Intraepithelial phase I — mast cell with large nucleus (n), cytoplasmic filaments, mitochondria (m), finger-like projections (fp) and specific granules (gr). The mast cell is situated just above the basal lamina (bl) of the gastric mucosa. Magnification ×30,200. In the inset, a specific granule at ×92,000 magnification

means of their numerous prominent cytoplasmic projections or microvilli. They do not always lie in line with the epithelial cells, but often bend over bud-shaped into the lamina propria (Fig. 2). Thereby the intercellular contacts are loosened, the fingerlike projections adjoining each other in a network-like way are untangled and the basal lamina is pushed onto the outer surface of the cell

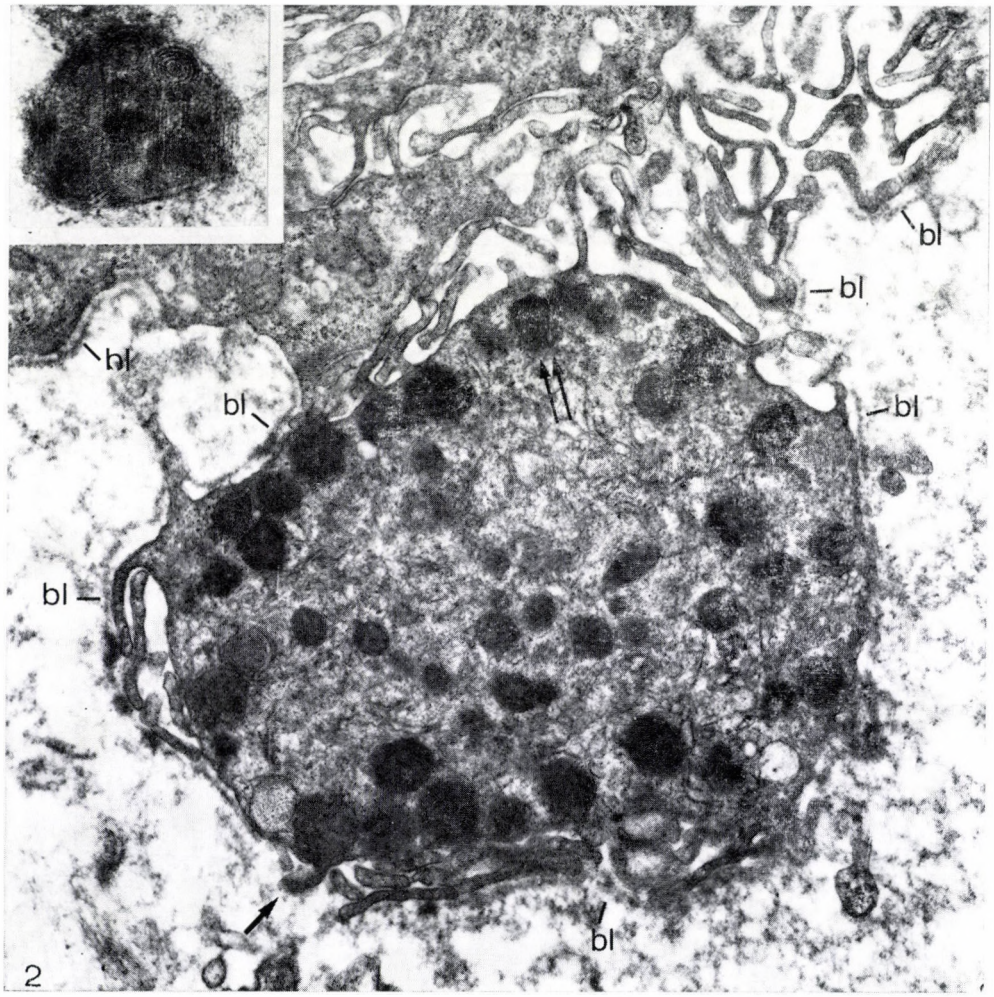

Fig. 2. Emigrating phase I — mast cell bending over bud-shaped into the lamina propria. The basal lamina (bl) is penetrated by single cytoplasmic projections (e) ×24,700

which is turned towards the lamina propria and ahead of the cytoplasmic projections which at these points closely adjoin the cell membrane. The basal lamina is in many places no longer continuous but is penetrated by single microvillous cytoplasmic processes of the mast cell. Occasionally thin cytoplasmic projections of submucosal fibroblasts in the vicinity of the epithelium are misformed by the mast cells.

Phase II — mast cell (Fig. 3) differs only slightly in respect of its subcellular structure from the cells described above. An essential difference between the two cell forms lies in the different inner structure of the specific granules. The granule content of the phase II — mast cell consists mainly

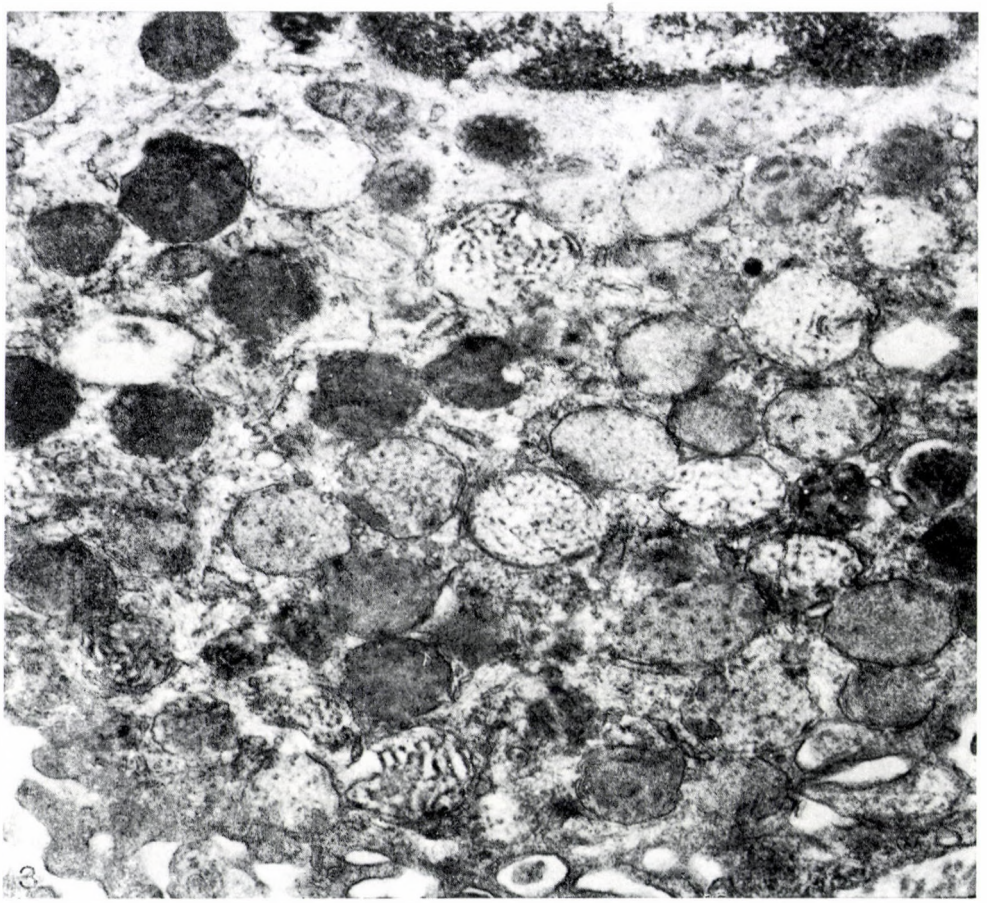

Fig. 3. Part of a secretory active phase II — mast cell with numerous specific granules. The content of the granules consists of threads or fine granular matrix. Magnification ×48,600

of threads tightly bound into balls or of a fine granular matrix with different degrees of electron density. Mast cells with this granule population are described as secretory active cell forms. Sporadically we also find granules of the phase I — mast cell, displaying a lamellar inner structure in the cytoplasm of the phase II cells.

Phase I and II cells can be identified with the light microscope in tolouidine blue-stained semi-thin control sections as lilac-granulated cells, provided that they have been seized and identified with the electron microscope. Electron microscopical identification of phase II — mast cells succeds both in the epithelium as well as in the lamina propria. They are not infrequently found near small blood vessels, nerve fibres and smooth muscle fibres.

In *phase III — mast cells* (Fig. 4) the polymorphism of the granules is somewhat more pronounced. Here the granule spectrum ranges from the forms

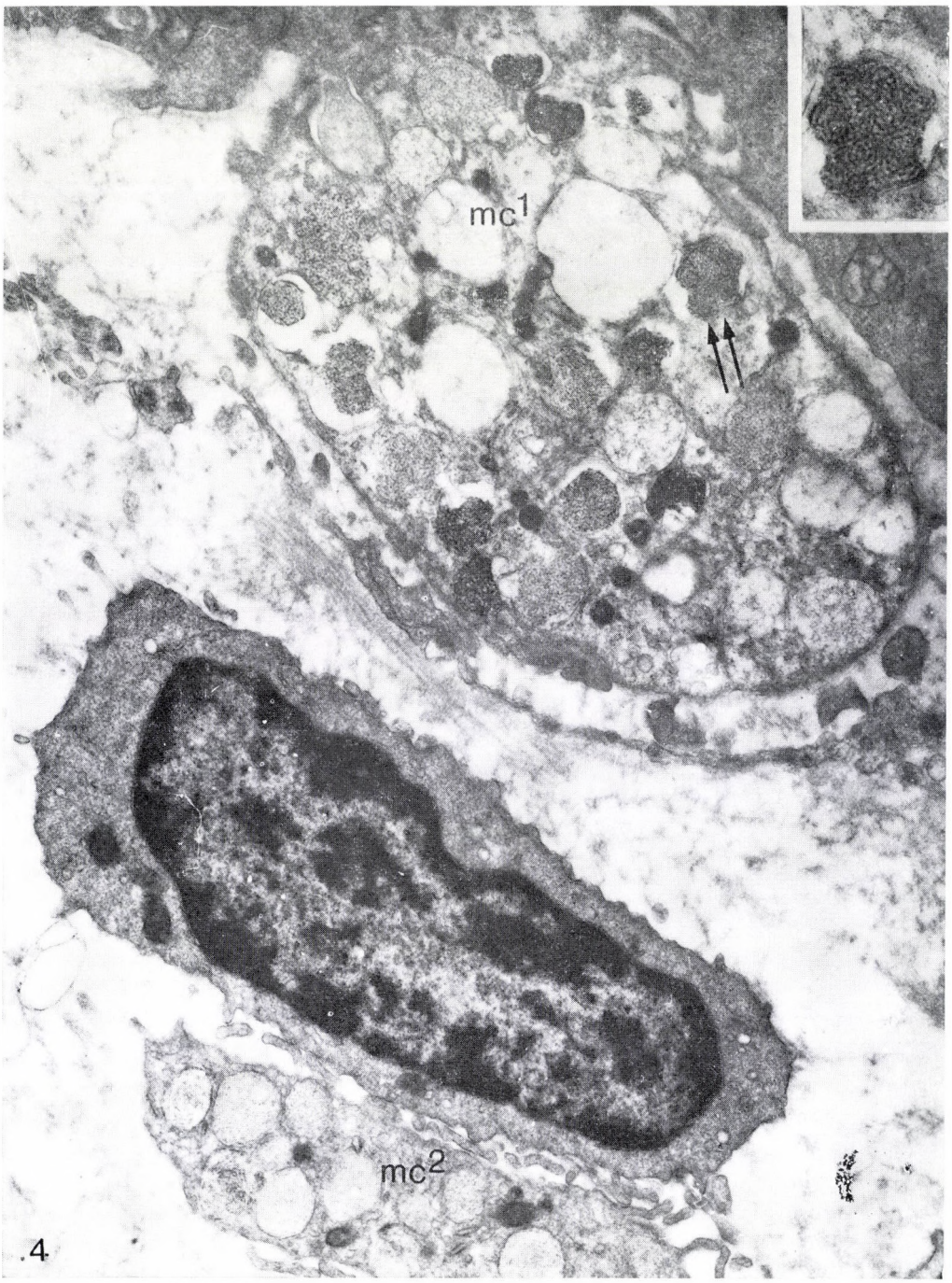

Fig. 4. Intraepithelial phase III — mast cell (mc^1) leaving the epithelium and part of an emigrated phase III — mast cell (mc^2) situated in the lamina propria. Magnification ×32,300. In the inset, a granule of the emigrating mast cell at ×54,100 magnification

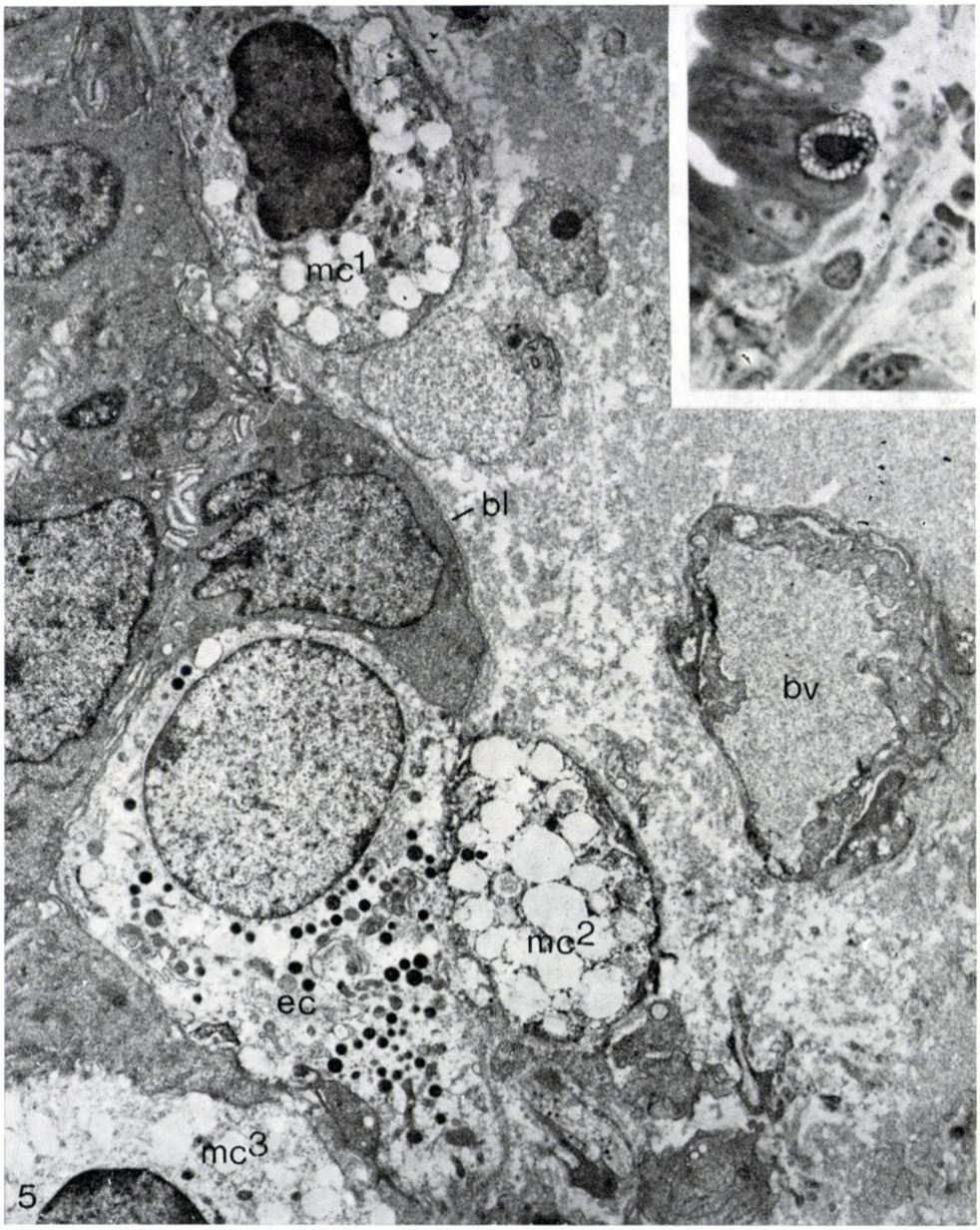

Fig. 5. Three emigrating intraepithelial phase IV — mast cells (mc^1, mc^2, mc^3) packed with large empty vacuoles; round granulated endocrine cell (ec), basal lamina (bl), small blood vessel (bv). Magnification ×9800. In the inset, a phase IV — mast cell with light-coloured vacuoles in toluidine blue-stained semi-thin section. Magnification ×1000

seen in phase I and II cells to larger granules. These now have a small amount of electron-dense, usually excentrically arranged material, or appear as empty vacuoles.

In the *phase IV — mast cell* (Fig. 5) the cytoplasm is almost completely filled with large, empty vacuoles. Under high magnification it becomes evident that the enveloping membrane is for the most part dissolved and that the existence of partial membrane residuals is only simulated by fine fibrillar, circularly arranged material. Between these vacuolar granule structures there are isolated granules of the phase III mast cells. Whereas the mitochondria exhibit no fine structural changes, the phase IV cell nucleus shows degenerative changes such as distinct clotting and occasionally a considerable extension of the space between the two membranes of the nuclear envelope. This cell is recognizable light microscopically in toluidine blue-stained semi-thin control sections as a cell with a dense, reduced nucleus and many large, empty vacuoles. While electron microscopically the cell can easily be recognized as a mast cell form with its remaining content of phase III granules, it can be mistaken for a light-coloured cell in the light microscope. In many micrographs intraepithelial mast cells of all four phases were found in very close proximity to round granulated endocrine cells (Fig. 5).

Discussion

What is the cause of the peculiar abundance of intra- (inter-) epithelial mast cells of the gastric mucosa in the particular case of microcarcinoidosis? An obvious assumption would be that the mast cells as cellular components of the recticuloendothelial system [18], similarly as the lymphocytes in intestinal enteropathy [16, 17], under the pathological conditions of the microcarcinoidosis leave the blood vessels and settle in the epithelium of the gastric mucosa [5]. On the basis of our electron microscope photographs, however, we assume that such a mast cell migration does not take place, but instead of it, an emigration of mast cells occurs out of the epithelium through the basal lamina into the lamina propria. The electron micrographs in which the still intraepithelially situated mast cells are seen to arch forward with two-thirds of their cell surface into the lamina propria, push the basal lamina ahead and break through with their finger-like projections and bend thin cytoplasmic processes of the fibroblasts in proportion to their surface, can be interpreted as a stationary phase of the dynamic emigrational process. This mast cell emigration, however, presupposes the assumption of a great increase of mast cells in the epithelium. For a histogenetic connection between intraepithelial mast cells and basophilic leucocytes [25], T lymphocytes [14] or monocytoid cells, there was no morphological support. While the light microscopical

identification of intraepithelial mast cells in toluidine blue-stained semi-thin control sections often presents difficulties, a subdivision into four mast cell forms is possible with the electron microscope, above all on the basis of differences in the fine structure of the secretory granules. In agreement with DIETERICH [2] and other authors, our phase I — mast cell can be considered a mature storage form, the phase II — mast cell as a secreting form. Apparently, the release of hormones and enzymes involves a change in the inner structure of the specific granules; this is underlined by the frequent presence of both granule — forms in one and the same mast cell. The phase III and phase IV — mast cells with their toludine blue pale and not very electron-dense or empty vacuolar granules are thus to be interpreted as stationary pictures of the advanced or already finished secretory process. On this basis a continuous transition from the presecretory storage phase I — mast cell to the exhausted, empty phase IV — mast cell should be assumed. Degranulation [2] is to be supposed as a secretory mechanism not through coalescence and incorporation of the limiting membrane of the secretory granule into the plasmalemma in the sense of exocytosis, but through membrane release (diacrine mechanism of granule release) and/or intracellular granulolysis. The process of secretion seems to involve irreversible fine structural cell changes in the final stages; chromatin clumping, concentration and marginal clotting as well as extension of the perinuclear cistern of the nuclei of the phase IV — mast cell are obviously ultrastructural signs of cell death.

Since release of the content of the specific granules takes place in the epithelium, an endocrine and/or paracrine effect of the intraepithelial mast cells (increased mucous secretion, increased membrane release in endocrine polypeptide-producing cells) cannot be ruled out. The close topographical relationship between nearly all mast cells of the lamina propria and the muscle cells of the small blood vessels and the nerve fibres may be interpreted as a type of neurohormonal feedback-regulating mechanism [4]. The frequent proximity of mast cells and smooth muscle cells could point to a similar influence on the latter cells.

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INTRAEPITHELIALE MASTZELLEN IN DER MAGENSCHLEIMHAUT BEI MIKROKARZINOIDOSE

L. AUBÖCK

Bei einer 63j. Patientin mit Perniciosa und chronischer Umbaugastritis und Hyperplasie der disseminierten endokrinen Zellen in den Drüsen der Magenschleimhaut war es zur Entwicklung einer diffusen Mikrokarzinoidose gekommen. Mastzellen fanden sich nicht nur im interglandulären Stroma, sondern auch *im Epithel* der atypischen Drüsen der Schleimhautbasis. Die elektronen- und lichtmikroskopischen Untersuchungen ergaben eindeutig, daß die intraepithelialen Mastzellen ins benachbarte Stroma auswandern. Im Rahmen der Sekretabgabe ändert sich kontinuierlich die Feinstruktur der spezifischen Granula, wobei vier verschiedene Sekretionsbilder auseinandergehalten werden können.

ВНУТРИЭПИТЕЛИАЛЬНЫЕ ТУЧНЫЕ КЛЕТКИ В СЛИЗИСТОЙ ОБОЛОЧКЕ ЖЕЛУДКА ПРИ МИКРОКАРЦИНОИДОЗЕ

Л. АУБЁК

У 63 летней больной с злокачественной анемией, хроническим гастритом и гиперплазией диссеминированных эндокринных клеток в железах слизистой оболочки желудка развился диффузный микрокарциноидоз. Тучные клетки встречались не только в интергландулярной строме, но и в эпителии атпичных желез на базе слизистой оболочки. В электронно- и светомикроскопических исследованиях недвусмысленно было доказано, что внутриэпителиальные тучные клетки эмигрируют в соседнюю строму. В процессе секретоотделения непрерывно изменяется тонкая структура специфических зернышек. При этом можно различать четыре различных секреторных картин.

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POSSIBLE ROLE OF MYOFIBROBLASTS IN THE PATHOGENESIS OF DUPUYTREN'S CONTRACTURE

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In the contracting palmar aponeurosis of patients suffering from Dupuytren's disease myofibroblasts are revealed by electron microscopical examination. These cells display abnormal foldings of the nucleus, microfilaments resembling the myofilaments in smooth muscle cells, rough endoplasmic reticulum occupying circumscribed portions of the cytoplasm, free ribosomes, well-developed Golgi apparatus, numerous mitochondria, occasionally lipid droplets, cilia-like formations, hemidesmosomes and maculae adherens (desmosomes) between the cells and formation of basement membrane. According to literary data, the myofibroblasts contain actin and have contractile properties. These myofibroblasts are supposed to contribute to the development of Dupuytren's contracture. The process is finally stabilized by the disappearance of myofibroblasts and by the simultaneous development of compact collagenous fibre bundles secernated by classical fibroblasts.

Introduction

Several theories have been offered concerning the aetiology and pathology of Dupuytren's contracture. In his comprehensive work, MILLESI [15] mentions 21 aetiologic factors that might be relevant. According to KREBS [11] the most probable factors are trauma, developmental disturbances, diabetes, alcoholism, liver cirrhosis, rheumatism, arthritis, and autosensibilization. Up to now, however, the different aspects could not be reconciled, nor was the real cause of the disease found. The majority of earlier histologic examinations points to the fact that areas rich in cells may be observed in the early stage of the disease, while later the picture is dominated by compact scar tissue with fibre and poor in cells [14, 15, 19]. In MILLESI's [15] opinion degeneration of the elastic fibres and thickening as well as a myelinic transformation of the collageneous fibres are characteristic. In the palmar aponeurosis of patients affected by Dupuytren's contracture, DAHMEN [3] found immature collagen fibres containing connective tissue in isolated areas. In his opinion the neoformation of connective tissue cannot be properly proven by mere histologic examinations. Recently, however, the presence of myofibroblasts was observed in healing wounds [13], in granulation tissue (4, 17, 18] and in hypertrophic scars [1, 2]. GABBIANI and MAJNO [5] were the first to raise the possible role of myofibroblasts in the formation of Dupuytren's contracture. By means of electron microscopic examination, three ultrastruc-

tural characteristics were revealed in these cells, *viz.* deformity of the nucleus, microfilaments in the cytoplasm and the formation of basement membrane on the cellular surface. Other authors, too, observed myofibroblasts in Dupuytren's contracture nodules and assigned a primary role to these contractile cells in the development of the disease [9, 20].

In this paper we report on light and electron microscopical findings of the removed palmar aponeurosis of patients suffering from Dupuytren's contracture.

Materials and methods

Pieces of palmar aponeurosis removed in the course of operation from different areas of 6 patients suffering from Dupuytren's contracture in different stages of the disease, were fixed in 4% glutaraldehyde and 2% osmium tetroxide, then embedded into Durcupan ACM. Ultrathin sections were stained by lead citrate and uranyl acetate. Observations were made on a JEM 100 B type electron microscope.

Other parts of the subtotally removed palmar aponeurosis, as well as pieces of palmar tissue removed in 27 cases were fixed in 10% formalin. After embedding in paraffin–celloidin the sections were stained with haematoxylin–eosin and Krutsay's trichrome method, for light microscopical examination.

Results

Light microscopy

In the early stage, areas rich in cells and arranged in islands are observed. These cells and their nuclei are mainly oval in form and contain loose chromatin. The nucleus is occasionally picnotic. Between the rich cell areas, collagenous fibre bundles running in irregular course are visible among the fibroblasts and fibrocytes. In the advanced stage scar tissue similar to a tendon can be seen, consisting compact collagenous fibre. The fibres run parallel course and among them longitudinally arranged fibrocytes are seen (Figs 1a, b; 2a, b).

Electron microscopy

Fibroblasts from early stages occasionally contact each other through their processes and form an irregular, reticular organization. Their nuclear membrane is wavy, the endoplasmic reticulum is well developed. Collagenous fibre bundles running in different directions are observable, embedded into the intranuclear substance (Fig. 3). The polymorphous nucleus of loose chromatin structure contains regularly 1 nucleolus, the cellular membrane is wavy, sometimes foldings or impressions are discernible, usually transversally to the longitudinal axis (Fig. 4). Most striking is the regular appearance of microfilaments in the cytoplasm of the presumed myofibroblasts (Figs 5—8). These fine intracytoplasmic filaments run parallel to the longitudinal axis of the cell. In some cases they are seen near the surface membrane, in other cells they

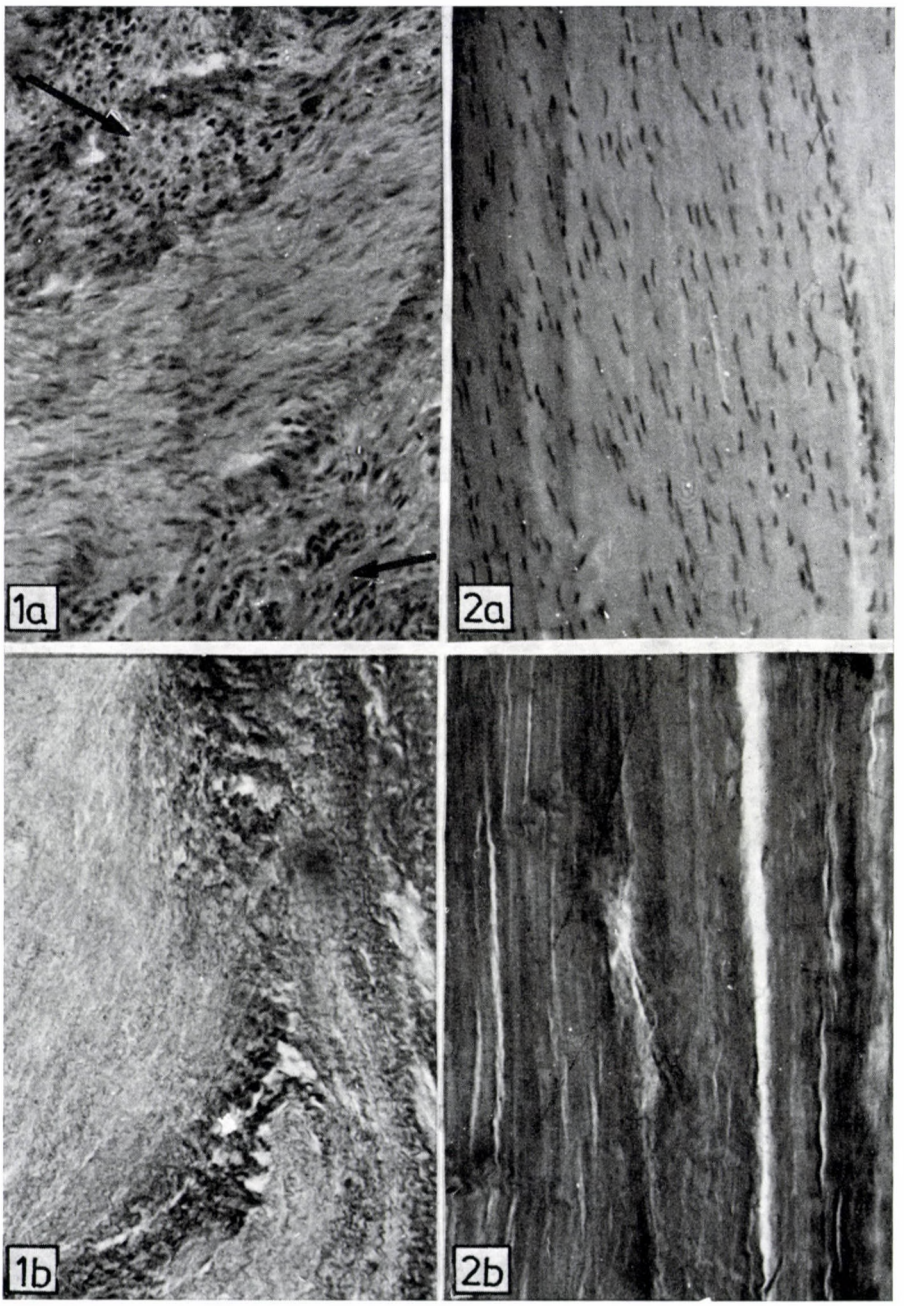


Fig. 1. Dupuytren's contracture. Light micrographs of the early stage. a) Areas infiltrated by cells with round and oval nuclei (arrows), among them loose fibres collagenous of wavy fibroblasts and fibrocytes. Haematoxylin staining, ×100. b) Darkstaining collagenous fibres running a wavy course. Krutsay's trichrome staining. ×100

Fig. 2. Light microscopic details from Dupuytren's contracture scar in advanced stage. a) Collagenous fibres parallel to each other running in longitudinal direction with fibrocytes between them. Haematoxylin–eosin staining, ×100. b) Compact scar tissue consisting of collagenous fibres. Krutsay's staining. ×100

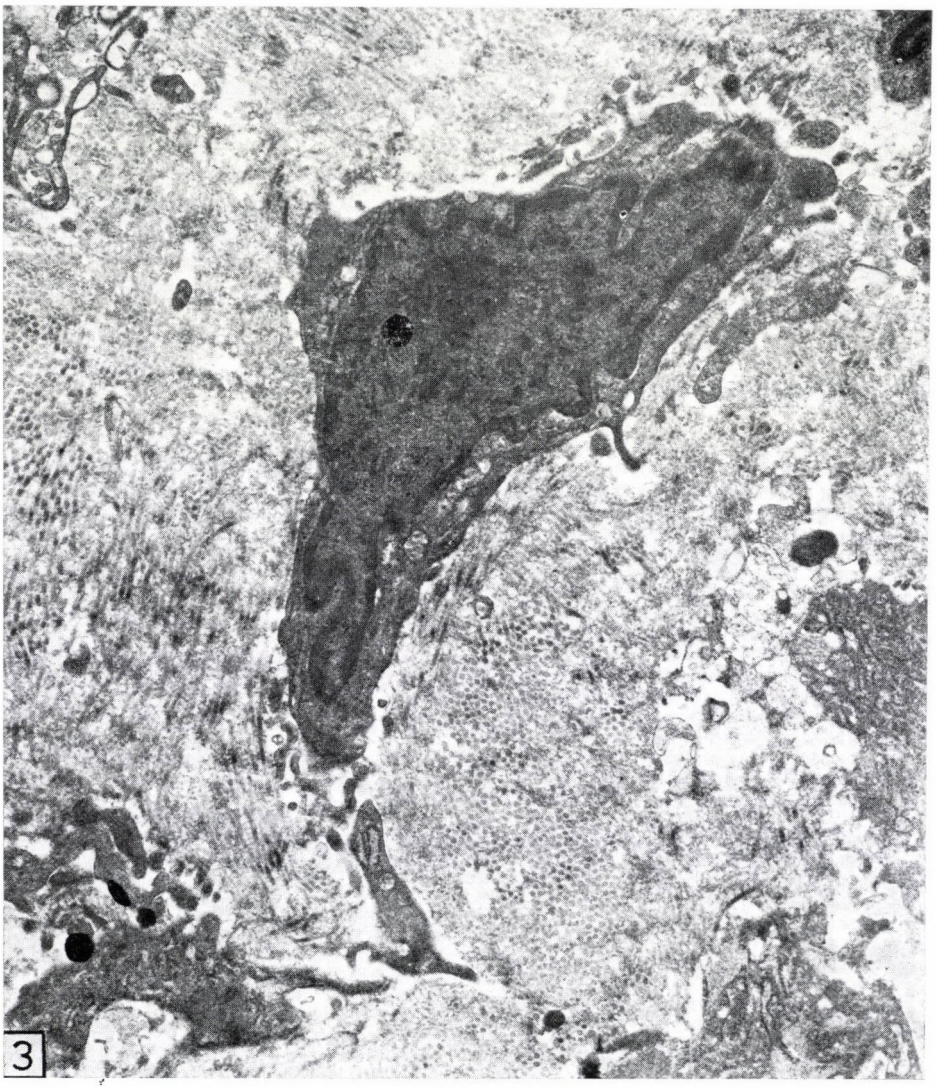


Fig. 3. Electron micrograph of Dupuytren's contracture in the initial stage. Myofibroblasts are arranged in a loose, reticular pattern. In the intercellular substance, collagenous fibre bundles run in various directions. ×12,600

occupy a major part of the cytoplasm. In these latter cases the rough endoplasmic reticulum is located in a circumscribed area of the cytoplasm. Frequently, free ribosomes also occur. Most cells are rich in oval or somewhat elongated mitochondria (Figs 4, 5). The Golgi apparatus is usually well-developed (Fig. 4a). Sometimes cilium-like organelles (Fig. 4a) and lipid droplets (Fig. 8) occur in the cytoplasm. Many myofibroblasts are in close contact (Figs 5, 6, 9).

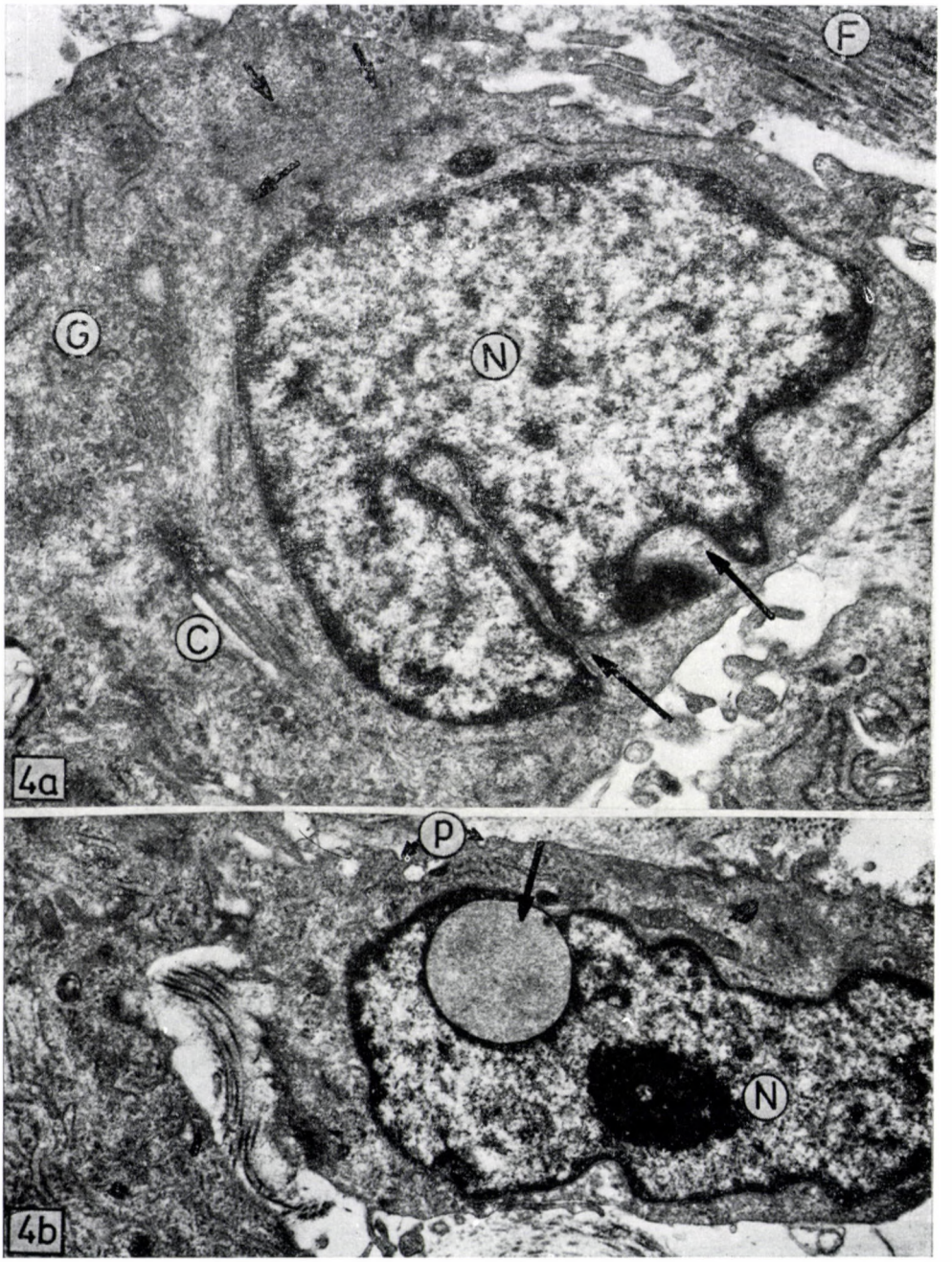


Fig. 4. Electron micrographs of myofibroblasts. a) N: nucleus. Arrows show indentations of the nuclear membrane. G: Golgi apparatus. C: cilium like organella. Short arrows: densities of myofilament bundles. F: collagenous fibres in the vicinity of the cell. ×19,000. b) N: cell-nucleus. Arrows: large impression of nuclear membrane, p: pinocytosis. ×16,000

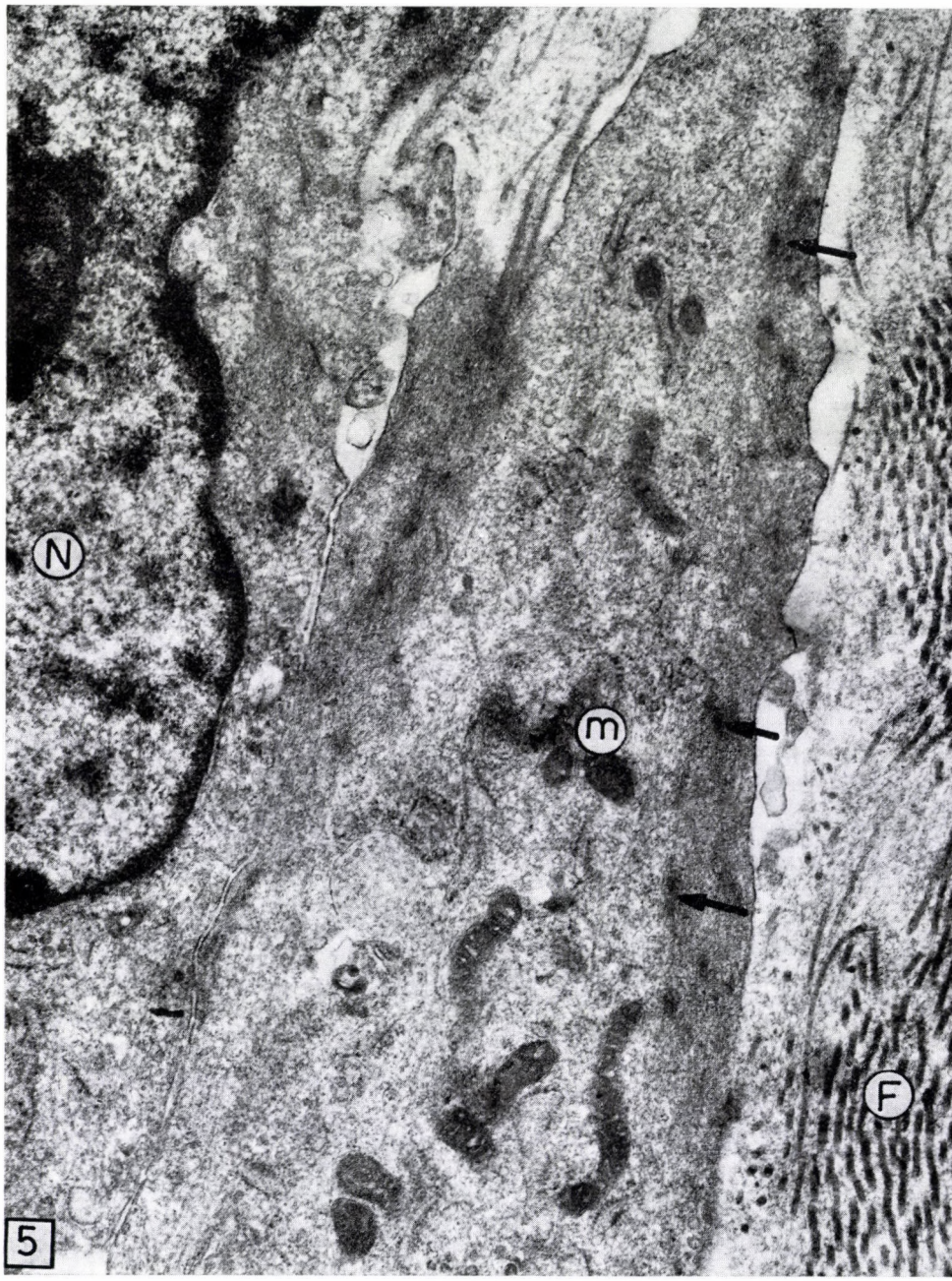


Fig. 5. Electron micrograph of two myofibroblasts contacting each other. Microfilaments are conspicious especially in the marginal areas of the cytoplasms, microfilament densities (arrows), numerous mitochondria (m), N: nucleus, F: collagenous fibres in the intercellular substance. Fine granulation among the fibres. ×27,000

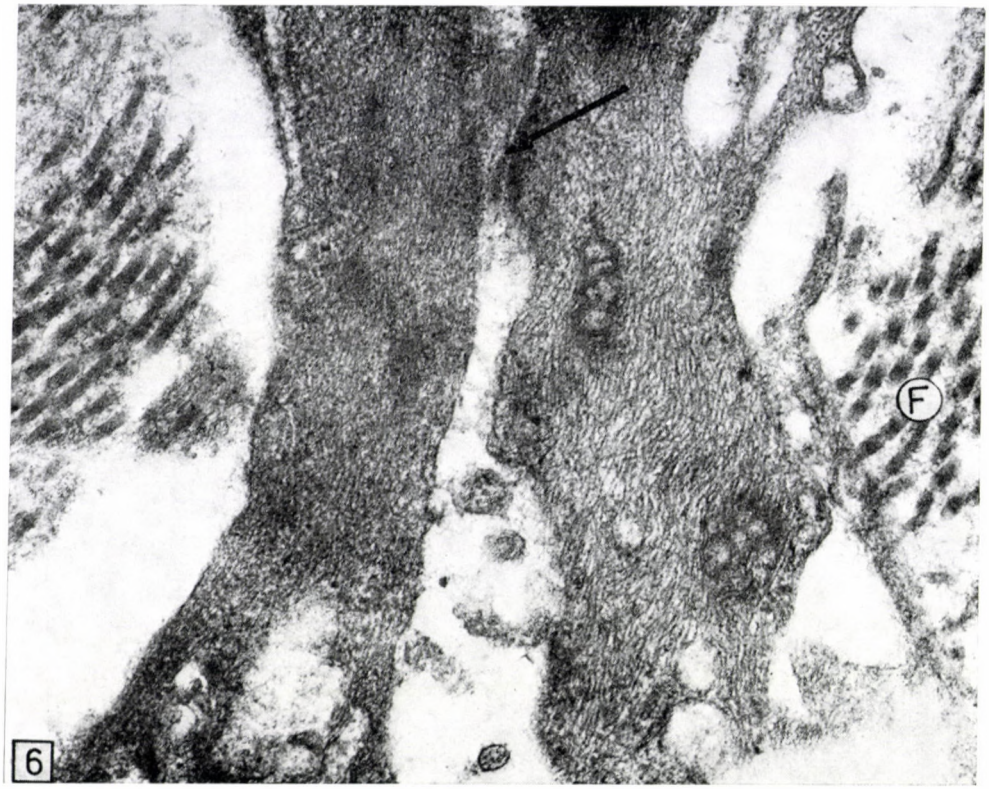


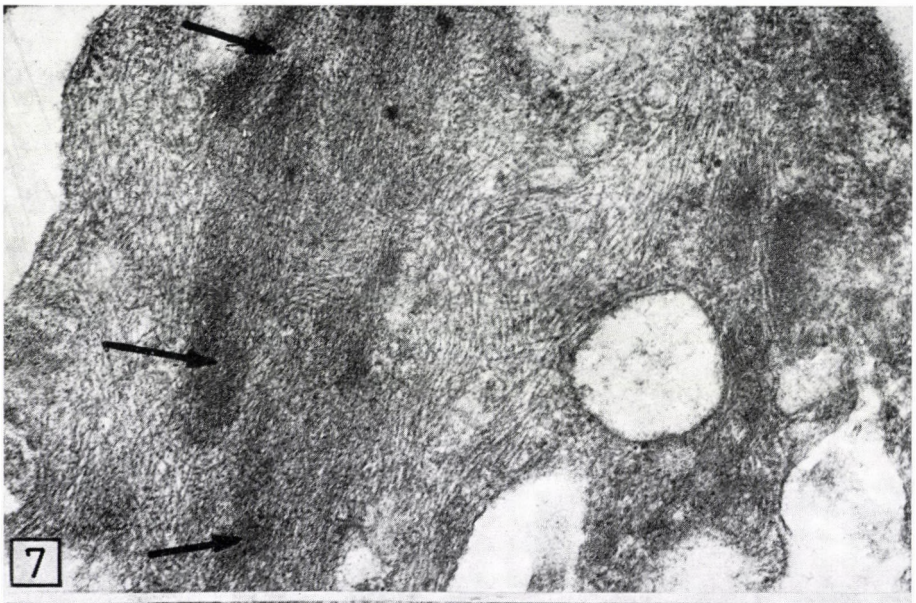
Fig. 6. Electron micrograph of two myofibroblast processes contacting each other by desmosome (arrow). The cytoplasm is filled with microfilaments. F: collagenous fibres in the intercellular space. ×48,600

These contacts may appear as hemidesmosomes, or maculae adherents (desmosomes) or long, aspecific surface contacts. On the cellular surface, as a characteristic feature of myofibroblasts, formation of a basement membrane (Figs 4a, b) and signs of pinocytosis may be observed (Figs 4b, 9).

In the advanced stage (Fig. 10), mature collageneous fibre bundles dominate the picture. The fibres show a parallel arrangement mainly in the longitudinal plane of the palm. There are few cellular elements left, which are fibroblasts or fibrocytes.

Discussion

The observations corroborate those data [5, 9, 20] according to which myofibroblasts occur in the palmar nodules of Dupuytren's contracture and these cells most possibly play an important role in the development of the disease. We could observe the contractile cells in earlier stages of the disease



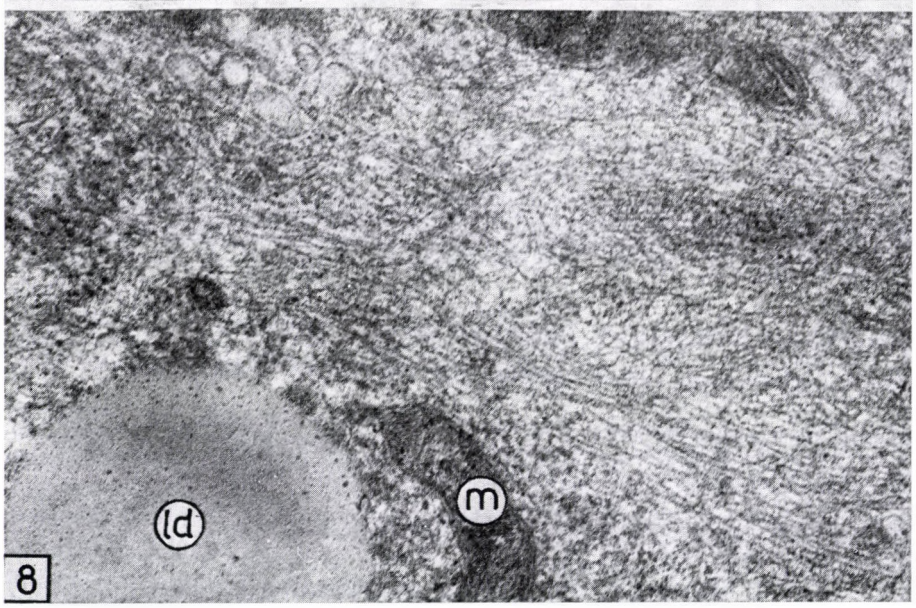


Fig. 7. Cytoplasmic detail of a myofibroblast. Numerous microfilaments; in some places they form densities (arrows). ×48,600

Fig. 8. Microfilaments in the cytoplasm of a myofibroblast; ld: lipid droplet, m: mitochondrium. ×48,600

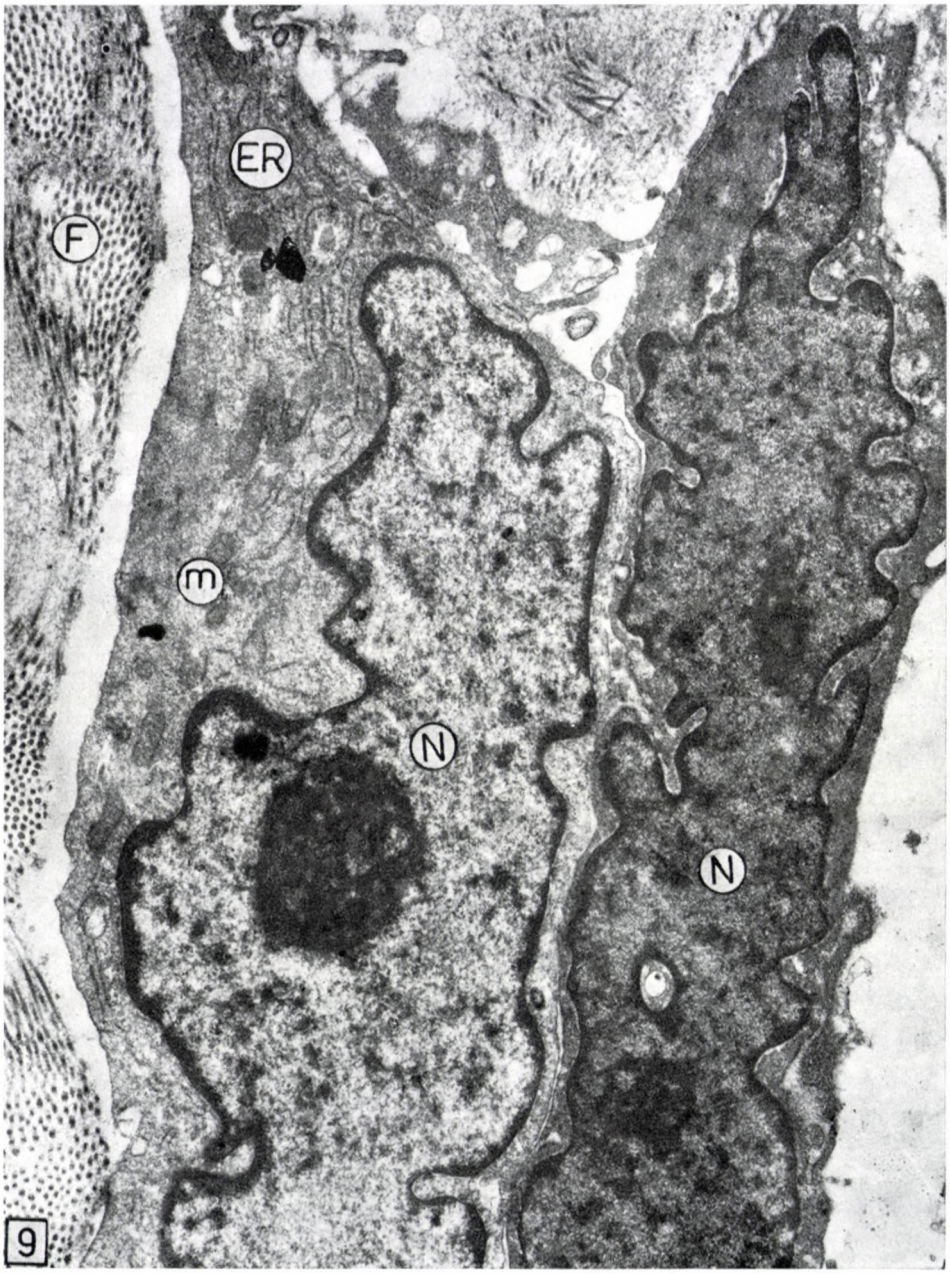


Fig. 9. Myofibroblasts in different stages of maturation contacting each other. Microfilaments in both cells. N: nucleus; ER: rough endoplasmic reticulum; m: mitochondrium. I — collagenous fibrils. ×19,440

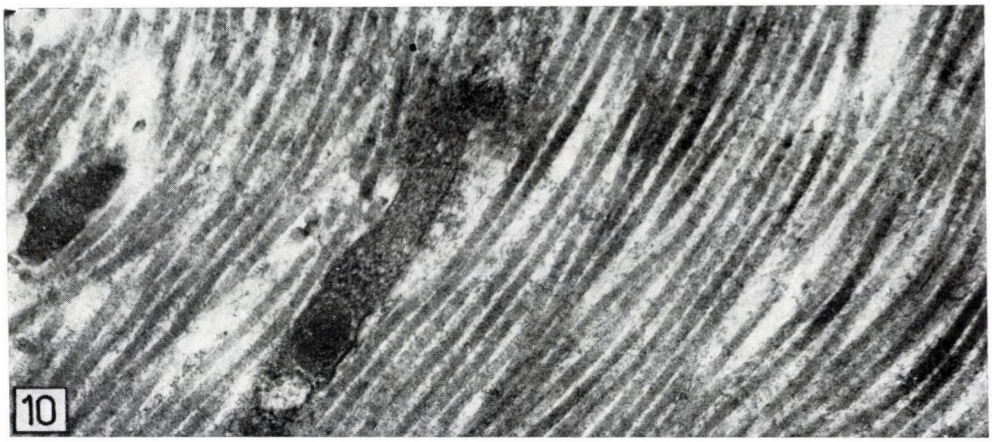


Fig. 10. Electron micrograph of Dupuytren's contracture scar in advanced stage. Collagenous fibres take a normal, tendon-like parallel course, ×40,500

when they form the majority of cellular elements in the palmar nodules. The loosely or tightly connected myofibroblasts are arranged in the longitudinal axis of the hand and of the fingers. The collagenous fibre bundles embedded into the network-like myofibroblast skeleton, exhibit an irregular arrangement and may form a network by themselves. In the basic intercellular substance fine granulation and fibre fragments are also visible. Beside the presumed myofibroblasts, classical fibroblasts and histiocytes are also seen. In contrast to earlier stages, in the mature Dupuytren's contracture the palmar aponeurosis consists mainly of mature collagenous fibre bundles and is similar structurally to a tendon.

Myofibroblasts were found beside Dupuytren's contracture also in healing wounds, granulation tissue and hypertrophic scars. Similar presumed contractile cells were observed in arteriosclerotic processes and in the scars of the cirrhotic liver, i.e. in processes related to some sort of contraction. Myofibroblasts, however, were also found in normal tissues, e.g. in the stroma of the duodenal villi [7], where they are supposed to be involved in the regulation of functions.

By their submicroscopical characteristics, the myofibroblasts may be differentiated from the classical fibroblasts as well as from the fibroblasts of histiocyte nature. The exact origin of these contractile cells is not known and the question should be raised whether they originate from the classical functioning fibroblasts, or from multipotential stem cells [6]. According to KATENKAMP et al. [10] it is theoretically possible that mesenchymal cells migrate from the perivascular space and, as fixed pericytes, develop a basement membrane. Fibroblasts transformed from pericytes were shown to play

an important role in the regeneration of tendon [10]. On the other hand, due to their actin content, the filaments located in the myofibroblasts have contractile properties [8, 12, 13]. This property is characteristic of active myofibroblasts only and cannot be observed in cells from the late stage of wound healing. In the early stage of these processes there is enhanced collagen synthesis in which both fibroblasts and myofibroblasts are involved. It was, however, suggested [5] that the myofibroblasts would produce an embryonal (Type III) collagenous fibre substance.

The most obvious function of myofibroblasts occurring in different pathalogic pracesses must be related to contraction. It appears that in the pathogenesis of Dupuytren's contracture the role of this temporary cell type is important, but aspecific. On the basis of our observations it can be suggested that due to their contractile property the myofibroblasts, play a decisive role in the development of contracture. In later stages the myofibroblasts disappear and are replaced by common fibroblasts, which by producing normal (Type I) collagenous fibre bundles will build up and stabilize the well known and non-contractile scar tissue.

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DIE BEDEUTUNG DER MYOFIBROBLASTEN IN DER PATHOGENESE DER DUPUYTREN-KONTRAKTUR

A. SALAMON, J. HÁMORI

Im Narbengewebe, das der Aponeurosis palmaris von Kranken mit Dupuytren-Kontraktur entnommen wurde, sind elektronenmikroskopisch Myofibroblasten mit den folgenden ultrastrukturellen Kennzeichen nachweisbar: Deformitäten und Fältelung der Zellkerne, Mikrofilamente im Zytoplasma, "rough endoplasmic reticulum", das einen umschriebenen Teil des Plasmas einnimmt, freie Ribosomen, ein gut ausgebildeter Golgi-Apparat, zahlreiche Mitochondrien, stellenweise Fett-Tröpfchen, zilienförmige Gebilde, Zellverklebung mittels Hemidesmosomen, Maculae adhärens (Desmosomen), Ausbildung einer Basalmembran. Die Myofibroblasten werden in den Frühstadien der Krankheit beobachtet und enthalten aufgrund von Literaturangaben Aktin. Diese kontraktilen Zellen sind vermutlich an der Pathogenese der Dupuytren-Kontraktur maßgeblich beteiligt, wobei die von den traditionellen Fibroblasten sezernierten Kollagenfaserbündel den Prozeß stabilisieren.

РОЛЬ МИОФИБРОБЛАСТОВ В ПАТОГЕНЕЗЕ КОНТРАКТУРЫ ДЮПЮЙТРЕНА

А. ШАЛАМОН и Й. ХАМОРИ

В рубцовой ткани, удаленной из сухожильного растяжения больных, страдающих контрактурой Дюпюйтрена, а электронном микроскопе выявляемы миофибробласты со следующими ультраструктурными характеристиками: деформации, вмятины клеточных ядер, микрофиламенты в цитоплазме, грубая эндоплазматическая сетчатка, занимающая ограниченную часть плазмы, свободные рибосомы, местами жировые капельки, ресничные образования, слипание клеток посредством гемидесмосом и десмосом, развитие базальной мембраны. Миофибродласты наблюдаются в ранних стадиях заболевания и на основе литературных данных они содержат актин. Эти сократимые клетки предположительно играют важную роль в развитии контрактуры Дюпюйтрена, а выделаемые традиционными фибробластами коллагенные волокна стабилизируюр патологический процесс.

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INFLUENCE OF THE PINEAL BODY ON EXPERIMENTAL TSH DOMINANCE IN RAT

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Iodide uptake of the thyroid following the joint administration of 1-methyl-2-mercapto-imidazole (MMI) and TSH was studied by autoradiography at various points of time throughout 125 days in pinealectomized and sham-operated rats. In the pinealectomized group iodide uptake decreased. The enhanced TSH effect also decreased by the end of the experiment, suggesting the existence of a mechanism antagonizing the TSH effect. It is assumed that prolonged TSH dominance stimulates the pineal which, in turn, inhibits iodide uptake by the thyroid. This mechanism would thus prevent the TSH dominant state to influence the thyroid. As this prevention occurred also in pinealectomized animals it is postulated that some other as yet unknown structures exert a similar effect. It is suggested that the pineal body acts upon the follicular cells of the thyroid, exerting thus a peripheral inhibitory effect on iodide-uptake.

Introduction

The role of the pineal body in the regulation of thyroid function has recently been demonstrated in rodents [1, 6, 7, 11, 21] although not unequivocally [18, 19]. The effect is of inhibitory nature, exerted most probably through melatonin [1, 6, 21]. The efferent part of the control system seems to be a complex one as the effect of other substances of the pineal (5-HT, 5-HIAA), and of the mediator amines (dopamine, 5-HT, histamine) of the thyroid C and mast cells must also be reckoned with. Interaction of these substances may modify individual effects [1]. The actual state of thyroid function is thus determined by a number of factors.

The other prerequisite of control, the afferent part, is rather obscure. Our experiments were undertaken to investigate the inhibitory effect of the pineal body on thyroid iodide uptake in experimentally TSH dominant rats

Materials and methods

Wistar CB rats of both sexes and of 260 g mean body weight were used. Twelve animals were pinealectomized 3 weeks before the experiment. The sham-operated and untreated control groups contained 11 and 5 animals, respectively. The sham-operated and pinealectomized animals received throughout 125 days 20 mg/animal/day 1-methyl-2-mercapto-imidazole (Metothyrin, Richter; MMI) dissolved in drinking water. Proper intake was maintained by determining the daily amount of water drunk by each animal.

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From the 41st day of treatment, the above two groups received daily 10 mU TSH (Ambinon, Organon) intraperitoneally to ensure TSH dominance. Animals were sacrificed on days 41, 89 and 125 (for the schedule of treatment, see Fig. 1). Prior to killing with chloroform, the animals were injected intraperitoneally with ^{125}I (specific activity, 10 μCi/100 g). The thyroid glands were removed, weighed and embedded in paraffin. Six μ sections were cut, covered with Ilford K5 nuclear emulsion using the dipping technique. Preparations were developed in RO9 ORWO and after 21-day exposure fixed for 5 min in an acid fixer, and counterstained with haematoxylin–eosin.

Preparations were evaluated under 600-fold magnification using an ocular square. Grain numbers over colloid and follicular cells were counted. Average grain number per unit area was determined, results were evaluated using Student's t test.

Results

1. *Animal weight during treatment* (Fig. 1)

Before TSH treatment, mean weight did not decreased substantially. Soon after the beginning of treatment, the weight and motility of animals decreased substantially to an extent that we were afraid of stronger animals killing the weaker ones. Therefore TSH treatment was continued but MMI administration was temporarily replaced by pure water. In a few days the animals reached their weight measured at the last MMI dose, and a further gradual decrease continued. By the end of the treatment (day 125), the sham-operated and pinealectomized animals showed a 12.4% and 9% weight decrease, respectively. The weight curves of the two groups were similar.

During treatment, the animals in both groups lost their motility, became dysbasic, their fur was drenched and they were indifferent to external stimuli. The pinealectomized animals were in a slightly better condition than the sham-operated ones.

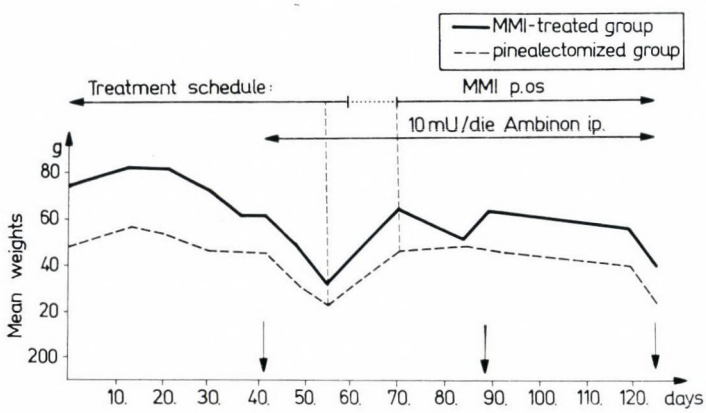


Fig. 1. Mean weight of animals during treatment. Schedule of treatment. Time of sacrifice indicated by arrows

2. *Thyroid weight during treatment* (Table I)

In the control group, thyroid weight of the animals killed on days 89 and 125 was 16 mg. In the other two groups, a 3.8—6.9-fold increase was observed. In these groups the thyroid was conspicuously vascularized. As measured on day 89, the weight increase of the thyroid of sham-operated animals surpassed

Table I

Thyroid weight (mg) during treatment

2nd sacrifice, day 89

Untreated control	Sham-operated		Pinealectomized	
		Growth (x)		Growth (x)
$\bar{x}$=16.05	$\bar{x}$=111.6	6.9	$\bar{x}$=94.36	5.9

3rd sacrifice, day 125

Untreated control	Sham-operated		Pinealectomized	
		Growth (x)		Growth (x)
$\bar{x}$=16.0	$\bar{x}$=60.53	3.8	$\bar{x}$=76.65	4.7

that of the pinealectomized animals (6.9-fold vs. 5.9-fold) but the increase was in both cases greater than on day 125 (3.8-fold and 4.7-fold). In the sham-operated group where the increase was smaller, mean weight was higher. As a result of treatment, thyroid weight showed a sharp initial increase followed by a decrease, the degree of which was lower in the pinealectomized group.

3. *Total grain number over the thyroid* (Table II)

It was included the amount of grains, counted over follicular cells and colloid, separately. Average grain number per unit was determined (8—41 $\times$ 100 squares). Considering the inhomogeneous groups, standard deviation was not counted.

Table II

Total grain number over the thyroid

	Day 41	Day 89	Day 125
Untreated control	666.4	695.5	722.8 $P>0.5$
Sham-operated	426.3	356.3 $P>0.2$	276.1 $P<0.01$
Pinealectomized	331.5	479.6 $P<0.02$	345.4

No significant changes occurred in the iodide uptake of the control group. In the sham-operated group the total grain number decreased gradually, but significantly only on day 125. At this time the degree of decrease was 35.3% as compared to the first killing. In the pinealectomized group the iodide uptake increased significantly on day 89, later on decreased on day 125, approximated the value of the first killing.

Comparing the groups, iodide uptake of the pinealectomized group was seen over the sham-operated one, excepting the first killing. Thus the degree of decrease of iodide uptake of groups having the pineal body, was more.

4. *Grain number over follicular cells and colloid* (Table III)

In the untreated controls a slight increase of iodide uptake was seen over the follicular cells on day 125, but neither this increase nor that observed over the colloid proved to be significant statistically. Grain number was 1.44—1.82-times higher over the colloid than over follicular cells. As a result of treatment, iodide uptake of follicular cells increased in both absolute and relative terms, so that their ratio (colloid/cell) tended to decrease.

Table III

Grain number/100 square units over colloid (C) and follicular cells (c)

	Untreated control	Sham-operated	Pinealectomized
	day 41		
C	860 ± 197.8	395 ± 167.6	321 ± 77.9
c	473.5 ± 121.5	452 ± 53	342 ± 57.6
C/c	1.82	0.87	0.93
	day 89		
C	869.9 ± 277	352.5 ± 138.5	402.8 ± 67
c	520.9 ± 147.4	378.9 ± 179.2	534.5 ± 159.9
C/c	1.67	0.93	0.75
	day 125		
C	836.8 ± 154.4	237.6 ± 62.2	225.1 ± 82.9
c	580.5 ± 85.4	310.8 ± 77.5	454.8 ± 121.4
C/c	1.44	0.76	0.49

In the sham-operated group, treatment brought about a gradual decrease of iodide uptake both into the follicular cells and into the colloid. Follicular cells contained consistently more grains.

The degree of decrease was compared between cells and colloid (Table IV). The total grain number in the pinealectomized group was non-significantly lower, than in sham-operated ones (666.4 versus 331.5 $P > 0.1$). Iodide uptake increased from this value till the 89th day then it decreased till day 125. The tendency of changes was similar in the follicular cells and the colloid but their amplitude was different (Table IV, Fig 2). The increase was more pronounced in the cells and even on day 125 it exceeded the day 41 value which was taken as 100%. On the contrary, colloidal iodide uptake showed a steep fall and, with advancing treatment, the colloid/cell ratio diminished gradually.

Table IV

Grain number changes over colloid and follicular cells

Sham-operated group (MMI only)

Follicular cells	Day 41	Day 89	Day 125
Mean grain count change	452	378.9	310.8
per cent		—16.2	—17.6
			resp.
Colloid			—31.3
Mean grain count change	395	352.5	237.6
per cent			—32.4
		—10.7	resp.
			—40

Follicular cells	Pinealectomized group		
Mean grain count change	342	543.5	454.8
per cent		+59	—16.2
			resp.
Colloid			+33
Mean grain count change	321	402.8	225.1
per cent		+25.5	—43.9
			resp.
			—30

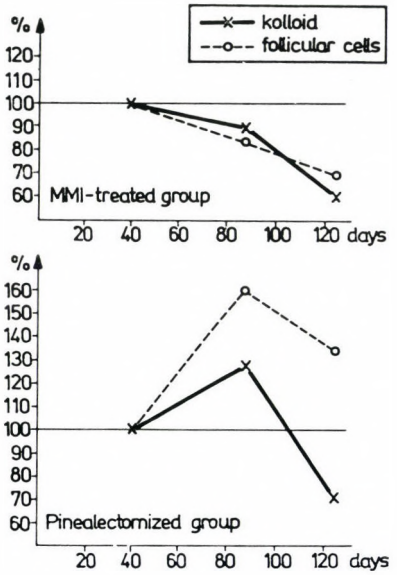


Fig. 2. Iodide uptake during treatment, in per cents of the value at the first sacrifice

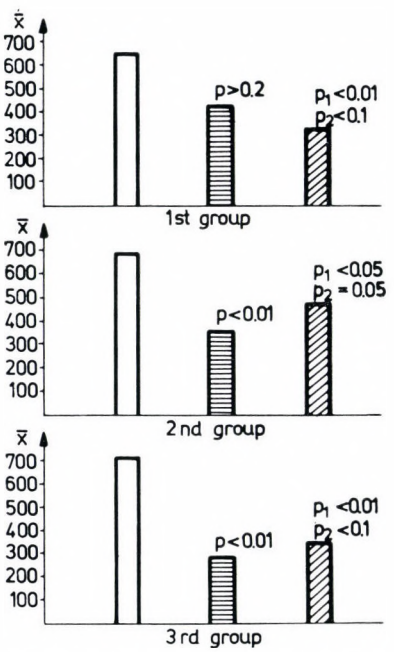


Fig. 3. Total grain numbers in the experimental groups: □ control; ▤ sham-operated; ▥ pinealectomized. P_1 = significance vs. the control; P_2 = significance vs. the sham-operated group

On day 89, iodide uptake by the follicular cells was 59% higher than on day 41, and colloidal uptake also increased although somewhat less. By day 125, total grain number nearly reached the initial value as a result of opposite trends in follicular cells and colloid. The decrease over the colloid on day 125 was almost identical in the pinealectomized and the sham-operated animals.

Discussion

The first experimental period lasted till day 41. In this period the pinealectomized and sham-operated groups received MMI only.

MMI is currently known not to affect iodide uptake of follicular cells but to inhibit the organic binding of iodide and to block the inhibitory effect of iodide on the TSH mediated cAMP response [17]. A consequence of this is centrally a hypothyroid state and peripherally an increased TSH response and enlargement of the gland.

In both groups the enhanced TSH effect was evident from the thyroid weight and histology (Figs 4a—h). It appears, on the other hand, that during treatment this effect is diminished in both groups in spite of unchanged drug administration. This argues for the existence of an anti-TSH mechanism elicited by TSH treatment.

a) In the sham-operated group iodide uptake decreased non-significantly (Fig. 3). In the follicular cells it was almost unchanged as compared to the control. Entry into the colloid was significantly blocked ($P < 0.01$). Due to suppressed thyroid hormone production, the histology was typical of the TSH effect (Fig. 4b).

b) In the pinealectomized group, iodide uptake decreased significantly while the decrease was non-significant as compared with the sham-operated group (Fig. 3). For the total decrease, the marked reduction of colloidal grain number is held responsible ($P < 0.01$). In the sham-operated group the decrease was non-significant ($P > 0.3$).

Histology showed an even more pronounced TSH effect than in the previous group (Fig. 4c).

Earlier studies have shown that pinealectomy increases iodide uptake of the thyroid [6, 7, 21]. This may be achieved through either a hypothalamo-hypophyseal (central) action or a direct peripheral counteraction of the TSH effect in follicular cells. The existence of both mechanisms is supported experimentally, but the exact mode of action is still unknown [1, 3, 7, 11, 16, 20, 22, 23]. In our pinealectomized group the increased iodide uptake was inhibited by MMI that has a direct effect on the thyroid, and the enhanced TSH effect did not increase iodide uptake of the follicular cells but, on the

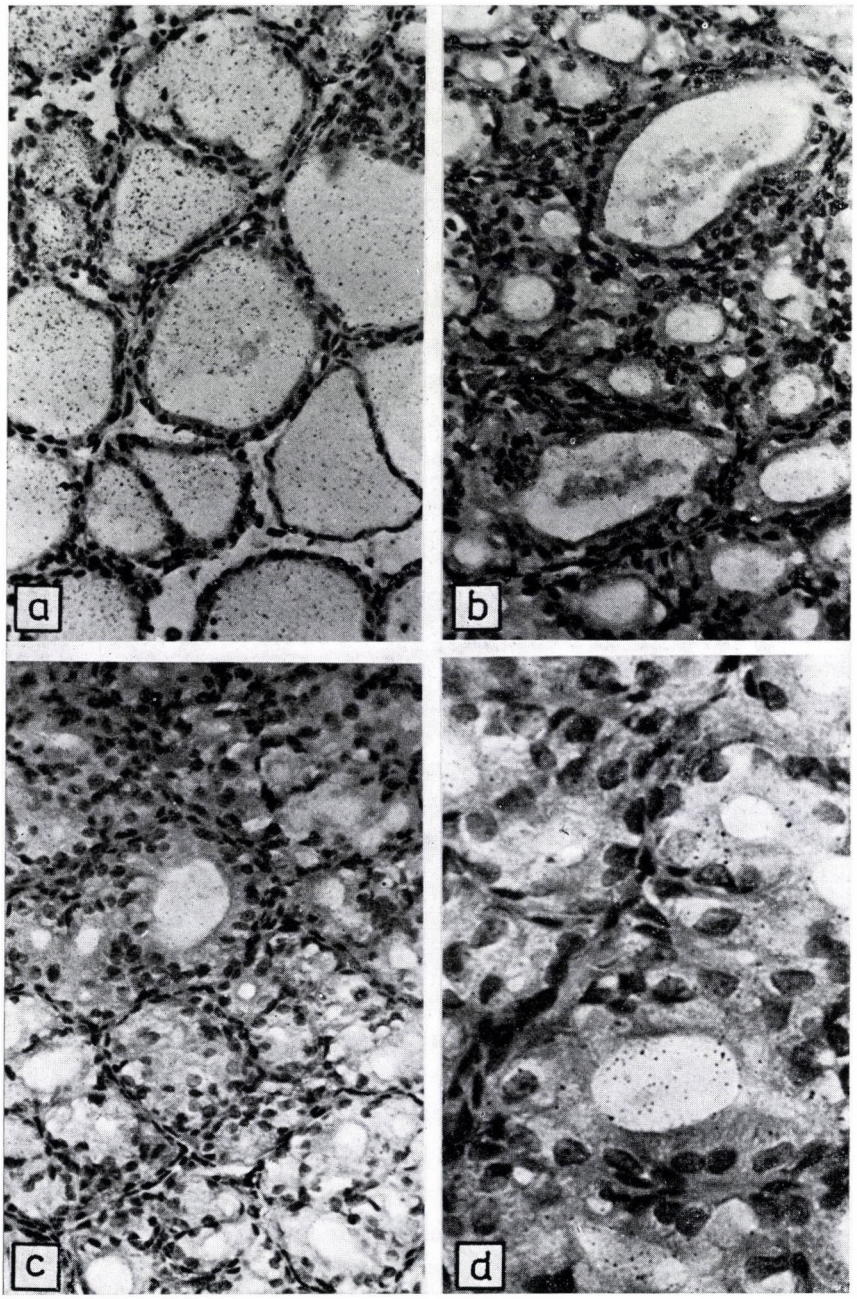
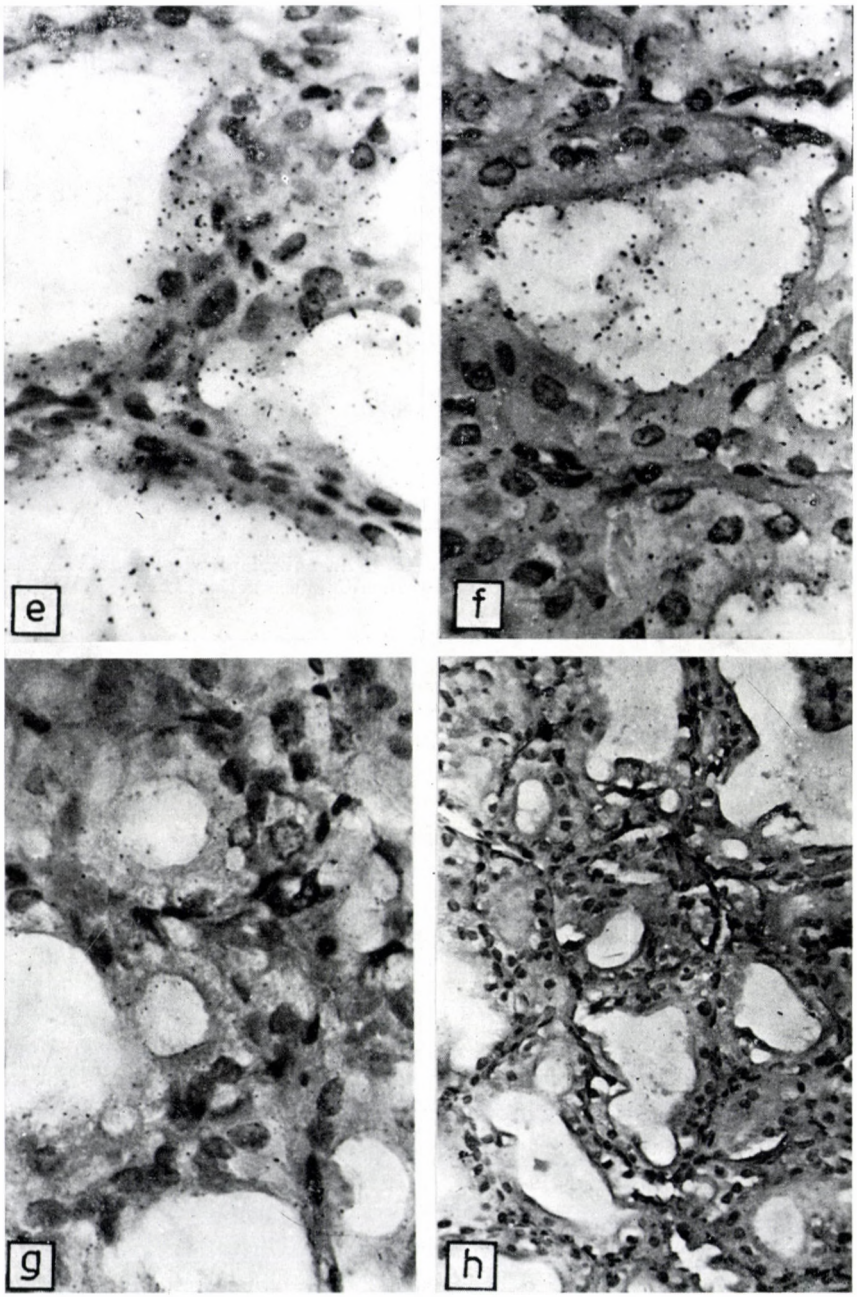


Fig. 4. Autoradiograms of the thyroid. HE counter-staining. *a* = Untreated control (×16); *b* = sham-operated group, 41 days (×16); *c* = pinealectomized group, 41 days (×16);



d = pinealectomized group, 41 days (×40); *e* = sham-operated group, 89 days (×40); *f* = pinealectomized group, 89 days (×40); *g* = sham-operated group, 125 days (×40); *h* = pinealectomized group, 125 days (×16)

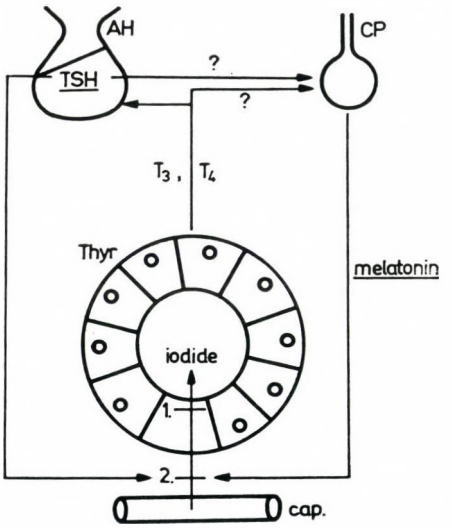


Fig. 5. Relationship between endocrine organs. AH = adenohypophysis; Thyr = thyroid follicle; CP = pineal body; cap = capillary; 1 = site of MMI action; 2 = supposed site of action of the pineal

contrary, it decreased it (Table III). These phenomena are indicative of a direct inhibitory effect of the pineal gland.

The increased iodide uptake was decreased by MMI to such an extent that due to the significant reduction of colloidal uptake, the total grain number was also reduced. The reduced hormone production further stimulated TSH release.

From the time of external TSH administration, the decrease in iodide uptake becomes strongly significant in the sham-operated animals (Fig. 3). Both colloid and follicular cells showed a reduced iodide uptake but this was more pronounced in the cells, resulting in a reduced grain number over the colloid. This means that in spite of TSH, iodide uptake did not increase. It is therefore assumed that the inhibitory effect of the pineal gland is exerted at the site of iodide uptake. This is supported by the fact that after pinealectomy, when iodide uptake by the follicular cells is markedly increased, MMI is insufficient to antagonize this process, leading to an increase of grain number over the colloid (Table III).

On day 125, iodide uptake decreased significantly in the sham-operated animals, due primarily to the decreased colloidal transport, but the decrease was substantial also in the cellular iodide uptake. In spite of TSH, cellular uptake decreased further. This anti-TSH effect was indicated also by the thyroid weights (Table I). The considerably reduced iodide uptake on day 89 represented a reduced T_3, T_4 production, which is a strong stimulus of further

TSH release. Provided that the pineal has an inhibitory effect on the iodide uptake of follicular cells, the continuously elevating TSH level is thought to stimulate the pineal. The decrease of the blood hormone level, in addition to inducing a TSH response, may after some time stimulate melatonin release which in turn would moderate the excessive TSH effect by inhibiting iodide uptake. CADY et al. [4, 5] have shown that the pineal may take up thyroid hormones and this process is enhanced by melatonin.

Our experiments suggest that the pineal body antagonizes the TSH effect at the level of follicular cells. This emphasizes the possibility of a new feedback mechanisms operating under certain circumstances involving a regulatory effect of the pineal gland.

As iodide uptake of the follicular cells was inhibited after pinealectomy, it is supposed that other structures are able to substitute pineal inhibition. Their exact site is not known.

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PINEALE WIRKUNGEN BEI EXPERIMENTELLEN TSH-ÜBERGEWICHT BEI RATTEN

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Die Aufnahme von Jod durch die Schilddrüse von pinealektomierten und scheinoperierten Ratten wurde bei gleichzeitiger Verabreichung von 1-Methyl-2-mercapto-imidazol und TSH autoradiographisch registriert. Die gesteigerte TSH-Wirkung nahm am Ende der Experimente ab, was an das Vorhandensein eines dem TSH-Effekt entgegenwirkenden Mechanismus denken läßt. Die Autoren vermuten, daß das anhaltende TSH-Überangebot einen Reiz für das Corpus pineale bedeutet, der eine Hemmung der Aufnahme von Jod durch die Schilddrüse bewirkt und so die Wirkung des exzessiven TSH-Übergewichts auf die Schilddrüse abwehrt. Da die Abnahme der TSH-Wirkung auch bei pinealektomierten Tieren beobachtet wird, ist anzunehmen, daß der die Schilddrüse hemmende Effekt des Corpus pineale auch von anderen, einstweilen noch nicht bekannten Strukturen übernommen werden kann.

Aufgrund der Befunde hat es den Anschein, daß das Corpus pineale seine die Jodaufnahme hemmende Wirkung an den follikulären Zellen der Schilddrüse mit peripherem Angriffspunkt entfaltet.

ПИНЕАЛЬНЫЕ ДЕЙСТВИЯ ПРИ ЭКСПЕРИМЕНТАЛЬНО ВЫЗВАННОМ ПЕРЕВЕСЕ TSH У КРЫСЫ

А. ЗАЛАТНАИ, ЖУЖАННА У. НАДЬ и Д. ЧАБА

У крыс удаленной шишковидной железой и у животных с ложной операцией автор регистрировали авторадиографическим методом поглощение йода щитовидным телом в ходе совместной дачи 1-метиль-2-меркаптоимидазола и TSH. К концу экспериментов повышенное действие TSH понижалось и это выдвигает возможность, что существует механизм, действующий против эффекта TSH. Авторы полагают, что длительный перевес TSH означает раздражение для щицковидного тела, задерживающее поглощение иода щитовидной железой и предотвращающее таким образом действие крайнего перевеса TSH. Ввиду того, что уменьшение действия TSH наблюдается также у животных с удаленным шишковидным телом, можно полагать, что задерживающее действие последнего, оказанное на щитовидную железу, могут вызывать и другие, еще неизвестные структуры. На основе экспериментальных данных авторов кажется, что шишковидное тело оказывает свое задерживающее действие на поглощение йода щитовидной железной на фолликулярных клетках последней с периферической точкой приложения действия.

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6TH CONFERENCE OF THE EUROPEAN TERATOLOGY SOCIETY

EDITORIAL

We have the honour to publish most of the papers presented at the 6th Conference of the European Teratology Society, held at Szentendre, 4–7 September, 1978.

We are greatly indebted to Professor Jan LANGMAN who undertook the tiresome task of reading and correcting all manuscripts and whose work was indispensable to detect mistakes and to give final stylistical touches in proper English.

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FORMS AND CAUSES OF HUMAN MALFORMATIONS

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VESALIUSWEG 12—14 INSTITUT FÜR HUMANGENETIK MÜNSTER 44, MÜNSTER, GERMANY

There is a great variety of forms and causes of human malformations, but no simple relation between cause and form. The same factor may produce different malformations in various individuals or not produce any malformation at all, while in addition various factors may produce the same anomaly. We can easily understand how the effect of an exogenous teratogen depends on the exact time of its application in relation to the development of the embryo. Thus in thalidomide embryopathy the following sequence is observed (Table I).

Table I

Thalidomide intake

Postmenstrual days	Type of malformation
35—37	No ears
39—41	No arms
41—43	No uterus
45—47	No tibia
47—49	Triphalangeal thumbs

The same line of reasoning also helps to explain why thalidomide did not invariably produce malformations. The critical period for thalidomide malformations appears to extend from the 35th to the 48th postmenstrual day if conception occurred at the 14th postmenstrual day, and to a few days thereafter if conception took place later. Five sufficiently well documented cases are on record, in which the mother apparently took thalidomide during the critical period without any damage to the child.

In the case described by Pembrey et al. (1970), the expectant mother had been prescribed thalidomide on the 45th day after her last menstrual period. Assuming that she took the tablets on the same or the following day, this was clearly within the critical period. Kajii et al. (1973) have reported four additional cases in which thalidomide was taken from the 46th to 49th, 47th to 53rd, 48th to 52nd and 49th to 55th postmenstrual days. The first case is somewhat doubtful, since the mother was suffering from nausea and vomiting and could not remember whether she took the drug. Her pregnancy terminated at the 243rd day. The authors also discuss the possibility, that in the Japanese language postmenstrual days might be understood as the days after the last day of the last menstrual period. This would, after an appropriate correction, place the prescriptions on the 49th, 53rd, 52nd and 55th day. One of the four cases, moreover, is doubtful because of a history of irregular menstrual periods. 104 Japanese mothers had been prescribed thalidomide after the critical period. Their offspring were normal, except for one infant with anal stenosis.

Similar experience has been reported by a German Obstetric Department, in which notes were available on 100 mothers who took thalidomide at some time during pregnancy. In one case thalidomide was administered in the sixth week, and the infant was born with radius aplasia and rectal stenosis; in another case thalidomide was prescribed on the 45th day, and the child had only rectal stenosis; in a third case, thalidomide was administered on the 49th or 50th day, and the child had anal stenosis. In the other 97 cases, thalidomide was given in the 10th week or later (Gagel, 1970).

Similar series have been widely quoted in the literature as supporting a comparatively low risk to the fetus exposed to thalidomide. If 4 abortuses with malformations in the Japanese series are not considered, the figures give an incidence of 1 per cent malformations in children born to mothers who have taken thalidomide during pregnancy, yet they are consistent, as all other studies, with a 100 per cent risk if the mother took thalidomide between the 40th and the 45th day. Any conclusions on the risk of teratogenicity based on the percentage of malformed infants born to mothers who took a certain drug at some time during the first trimester may be grossly misleading.

In constructing the time table for specific malformations due to thalidomide only cases of such malformations have been used. To determine the critical period, e.g., for aplasia of the radius, all such cases with documented time of intake of thalidomide have been collected and analysed. By this method one could find out the earliest and the latest as well as the most frequent day on which thalidomide would produce radius aplasia. By this method, however, the limits of the critical period are set by exceptional cases, possibly due to irregularities of menstruation, unusual early or late conception, or even errors in the data. It is important, therefore, to supply the results by an analysis of negative findings. If the cases of other thalidomide malformations but with the radius present and well documented thalidomide intake are analysed, the negative picture of the critical period showing on which days thalidomide intake might not result in radius aplasia can be obtained. The somewhat blurred contours of the critical period come into sharper focus by the second method. Critical periods for defined single malformations are shorter for the majority of cases than originally envisaged. Pooled experience from thalidomide cases from various countries is, however, in conflict with the idea, that at any given menstrual age the developmental horizon of the embryo is as variable as assumed by Nishimura et al. (1974). It remains to be seen, which method is more reliable. Obviously, both cannot be correct.

The variability of malformations due to an exogenous teratogen may be attributed to timing, dosage and genetic factors. It is much more difficult to explain why some dominant genes have variable effects in different members of the same family. We have, of course, fine words such as expressivity, specificity or penetrance, but these are not really helpful.

As an example of variable expressivity we may choose the autosomal dominant Holt-Oram syndrome. The gene may have the following consequences:

1. Triphalangeal thumbs,
2. hypoplastic thumbs in syndactyly with forefingers,
3. aplasia of thumbs and radii,
4. aplasia of thumbs, radioulnar synostosis, hypoplasia of humeri.

These various forms of the Holt-Oram syndrome are very similar to malformations caused by thalidomide given at different days between the 42nd and 48th postmenstrual day. It is difficult to see, however, how a dominant gene, which is present all the time, could be active at different times in different cases. In thalidomide cases, deafness and paralysis of the eye muscles which arise about 4 to 5 days before the limb defects are frequent. In the Holt-Oram syndrome, deafness and abducens paralysis are not seen. Three other autosomal dominant syndromes, however, have been reported, in which deafness is associated with malformations of the first ray of the hands. These are

1. acrofacial dysostosis Nager
2. lacrimo-auriculo-dentodigital syndrome (LADD)
3. Arias syndrome.

It is not clear, at present, whether these are distinct syndromes or variable expressions of the same gene, but they certainly are different from the Holt-Oram syndrome. Only by careful study and documentation of many more families can we hope to obtain more knowledge about the intrafamilial and interfamilial variability of these syndromes and thereby solid data for genetic counseling. Given the phenotypic variability within one genotype and the phenotypic similarities between different genotypes, it is difficult to decide which cases may by pooled for analysis. Morphological analysis, the study of form, has to go hand in hand with genetic analysis, i.e. the study of etiology.

Thalidomide embryopathy does not appear to be the only phenocopy of the three dominant syndromes involving the first ray and the ear. There are many sporadic cases with unilateral and ipsilateral involvement of the ear and the first ray and, in addition, variable malformations of the eye, the mandible and the cervico-thoracal spine, which show symptoms thought to be typical of such ill defined entities as the Goldenhar syndrome, or hemifacial microsomia, the Wildervanck-syndrome, or the VACTERL association. In the majority of cases of unilateral radius defects, the cervicothoracic spine is malformed. In my experience, unilateral radial defects in association with hemivertebrae and/or fusion of vertebrae are invariably sporadic. I have also seen hemifacial microsomia with hypoplasia of the right hemithorax and of the right thumb in one of monozygotic twins. Four pairs of monozygotic twins discordant for Goldenhar's syndrome have been reported (Bock, 1961; Berthelon and Cremer, 1968; Cordier, 1970; Papp et al., 1974). In the case from Debrecen, as in our case, only the twin with the lower birthweight was affected. In Papp's case, there was a history of progesterone and oestrogen injections in the sixth week. In our case progestogens were given for vaginal bleeding in the ninth week. In a case of microtia and dermoid of the conjunctiva, reported by Hollwich and Verbeck (1969) 250 mgms and 125 mgms proluton were administered for uterine bleeding in the second month.

Pruett (1965) has reported a case of the oculovertebral syndrome with hypoplasia of the first right metacarpal bone and a hypermobile right thumb. The mother was treated for about six weeks with medroxyprogesterone acetate, because of vaginal bleeding during the 9th and 10th weeks. Christiaens et al. (1965) have seen a boy with cervical hemivertebra 7, a dermoid of the right eye and a preauricular appendage, whose mother received progesterone injections starting from the first month. Mandelcorn et al. (1971) described a boy with a short right humerus, three metacarpals and two fingers on the right hand a missing thumb on the left hand. On the right side of his face he had a preauricular tag and a dermoid on the bulbar conjunctiva. The mother had been treated for threatening abortion. Weyers (1950) has described a case with microtia and mandibular hypoplasia, in which the mother received three times 50 mgms Lutocyclin and three times 10 tablets Proluton.

There was a history of hormone treatment in 4 of our 9 cases of radial defects accompanied by spine malformations. In an additional case of right side microtia, facial paralysis, hypoplasia of the jaw and preaxial polydactyly, proluton and gestanon have been given for bleeding in the second month of pregnancy.

Nora and Nora (1974, 1975, 1976) have found a history of progestogen and/or estrogen administration between the 14th and 60th day of pregnancy in 9 out of 15 cases of the VACTERL complex. In 4 different control series only 8 to 15 per cent of mothers had taken progestogens and/or estrogens. Kaufman (1973) has reported a case with vertebral anomalies, imperforate anus, absence of the left kidney, hypoplastic left thumb, and preaxial polydactyly of the right foot. The mother was given oral stilboestrol and intramuscular progesteron followed by intramuscular hydroxyprogesteron for two months beginning 47 days after the last menstrual

period. Greenberg et al. (1977) have compared records of drug intake in early pregnancy by mothers of 836 malformed and 836 normal babies. They found histories of hormonal pregnancy tests in 93 mothers of malformed infants against 55 in controls. The difference has been widely interpreted as indicating a possible teratogenic effect of pregnancy tests. As reporting by doctor, midwife, or health visitor was voluntary, and as of 2867 notifications only 836 were followed, alternative explanations are not excluded. One might speculate, e.g. that mothers who have had a pregnancy test, were more concerned about pregnancy and the child and thus more apt to cooperate. They also represent a sample of mothers who can more easily be traced, because they are likely to be more doctorminded. Moreover, it is not clear whether the doctor who attended the birth of the malformed child or the doctor who attended the mother in early pregnancy was approached. The second possibility seems to be more likely, since from the 1405 cases in which investigation was attempted the doctor could not be traced in 130 cases, had no record in 46 cases, could not cooperate in 53 cases and the remaining 130 cases failed for various reasons such as difficulty in identifying the child from the records. Only children born in the same practice were selected as controls, for which information on early pregnancy may have been less complete.

If the British figures are taken at face value, and if a basic malformation risk of 1 per cent is assumed, the risk of a malformation is increased by 0.7 per cent by a hormonal pregnancy test.

There are various reasons, however, which make me believe that the British figures do not prove a teratogenic effect of hormonal pregnancy tests. First, the same or similar finding has been reported by Greenberg et al. for barbiturates, despite the fact that in the more extensive prospective study by Heinonen et al. (1977) the overall malformation rate in babies born to 2413 pregnant women who took barbiturates in lunar months 1 to 3 was not higher than in controls; for the central nervous system the raise of malformations was only 87 per cent, and for musculoskeletal malformations 90 per cent of the rate in controls. In the same study the incidence of central nervous system malformations, respiratory malformations, including cleft lip and musculoskeletal malformations was not higher in babies born to 2327 pregnant women who took hormones, hormone antagonists or contraceptives in lunar months 1—4 than in controls. The same was true for the subgroups of 866 women who took progestational agents as well as for 614 women who took estrogenic agents.

Another argument against a causative interpretation of the British findings is, that they seem to apply likewise to central nervous system malformations, oral clefts, limb malformations and other abnormalities. It would be strange for a teratogen to have no specific effects but to cause all sorts of malformations.

Villumsen (1970) found in a retrospective study, that 383 out of 8833 expectant mothers had taken hormones such as insulin, thyroxin, corticosteroids, oestrogens and progestogens during the first trimester. This was the case in 6.1% of mothers of malformed children against 3.2% of mothers of normal children. 5.5% of the mothers who were treated with hormones gave birth to malformed children, which was not significantly different from the incidence of 3.6% in the case of mothers who did not receive medication. Of 21 malformed children born to mothers on hormones, 3 had malformations consistent with the diagnosis of VACTERL complex or Goldenhar syndrome. Unfortunately, the type of hormone treatment given in these cases is not mentioned.

Baumann et al. (1976) have described in detail 17 cases of the VACTERL complex including 8 with radius aplasia. Careful questioning revealed in no case hormonal pregnancy tests, hormone injections or ovulation inhibitors.

Shokeir (1977) has reported essentially negative findings in 24 Canadian cases of the Goldenhar syndrome. All these cases were sporadic. Pregnancy histories were unremarkable. No consistent history of specific drug ingestion or exposure to any teratogen could be revealed.

I think the balance of the evidence is against the hypothesis, that non-genital malformations, more specifically, malformations of the radius and the spine are caused by sex hormones.

There are some autosomal recessive conditions in which the radius and the thumb may be absent or hypoplastic. These are:

1. Fanconi's anemia (panmyelopathy),
2. the radius aplasia-thrombocytopenia syndrome,
3. Thomson's poikilodermia,
4. pseudothalidomide syndrome,
5. Roberts syndrome: tetraphocomelia with cleft lip and palate,
6. autosomal recessive popliteal pterygium syndrome (Bartsocas and Papas, 1972; Rosselli and Gulienetti, 1962).

In Fanconi's anemia various nonskeletal malformations such as atresia of the auditory canal, duodenal atresia, deafness, strabism, facial paralysis, aplasia of the appendix, cryptorchidism and renal dystopia may be seen, which are also part of thalidomide embryopathy. As such malformations may be lethal, some children with Fanconi's anemia die as newborns, i.e. at an age, when anemia or thrombocytopenia have not yet developed and the correct diagnosis has not been made. Only a retrospective explanation for the multiple malformations through the birth of another affected, but surviving sibling is found. The incidence of malformations of the internal organs as given in the literature is probably underestimated, as the cases surviving until school age, when anemia and thrombocytopenia are becoming apparent, are selected for having no lethal malformations. This is illustrated by a family reported by Csordás and Schneider (1955) in which the diagnosis of the proband's older sister, who was born with a birthweight of 1820 gms, aplasia of the left thumb, hypoplasia of the right thumb, aplasia of the left kidney, right ureter duplex, duodenal atresia and ventricular septal defect, and who died on the fourth day, was only retrospectively made.

The striking similarities between thalidomide embryopathy and Fanconi's anemia suggest that one should look for a common denominator in the pathogenesis of both syndromes. On the other hand, more severe defects of the arms, and those of the legs are not seen in Fanconi's anemia, and neither chromosomal breaks or reunions, nor hematological alterations, typical of Fanconi's anemia are seen in thalidomide cases. Whereas the defects of the first ray are variable in Fanconi's anemia, bilateral total aplasia of the radius is seen in all cases of thrombocytopenia radius aplasia syndrome, where, moreover, it is not accompanied by aplasia, triphalangy or severe hypoplasia of the thumb. Bilateral radius aplasia with thumbs present is highly characteristic of the TAR syndrome and may serve to distinguish it from all other hereditary syndromes involving radius aplasia as well as from thalidomide embryopathy. Radius defects are also seen in some chromosomal aberrations, notably trisomy 18, partial trisomy and partial monosomy for the long arm of chromosome 4 and partial monosomy for the long arm of chromosome 13. In the 13q-syndrome, absence, hypoplasia or duplication of the thumbs with radius present is sometimes associated with proximal synostosis of the 4th and 5th metacarpals. The same type of metacarpal synostosis is also seen in the radius aplasia thrombocytopenia syndrome and in the pseudo-thalidomide syndrome, but not in other syndromes with radial defects.

Aplasia of the thumb is seen in some patients with splithand splitfoot malformation, which in the other affected members of the family show the more usual type with thumbs present. In other families a monodactylous type of splithand, in which only the fifth finger is present, is seen as the typical defect, accompanied by monodactylous or lobster type feet. The splithand splitfoot malformation poses a most difficult problem in genetic counseling when an affected child has been born to normal parents. Though this situation is probably often due to a fresh mutation with no recurrence risk for consecutive siblings, this may not

be so. There are many families on record in which two or more affected children have been born to unaffected parents. Failing penetrance, twostep mutations, delayed mutations or single strand mutation have been suggested as explanation, but the problem is far from being solved. Variability of the phenotype in some families, probably great heterogeneity, and irregularities defying any simple mendelian analysis contribute to a confused picture. This may be overcome only by systemic collection, documentation and analysis of a large unselected material.

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IN VITRO METHODS FOR THE STUDY OF THE EFFECTS OF TERATOGENS ON PREIMPLANTATION EMBRYOS

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Introduction

Key words:

Preimplantation embryos, blastocyst, implantation, *in vitro* culture, transplantation, cyclophosphamide, UV-irradiation, steroid sex hormones.

Two methods are predominantly used in teratological research, the testing of teratogens in pregnant laboratory animals *in vivo* and epidemiological investigations on human populations. Inherent to both approaches are serious limitations and drawbacks, as has been stressed repeatedly (Karkinen-Jääskeläinen and Saxen, 1976; Neubert and Barrach, 1977). Epidemiological studies have to be based on very large numbers of exposed women, since most abnormalities occur at low frequency (1%). Even the more than 50,000 pregnancies investigated in the "Boston Study" (Heinonen et al., 1977) do not allow final conclusions. *In vivo* studies on experimental animals on the other hand often do not permit conclusions on teratogenic mechanisms, since the system (mother, placenta and embryo) is rather complex. Furthermore, the type of malformation induced in one species does not necessarily coincide with the abnormalities found in other species or in man.

Therefore, additional *in vitro* culture techniques have been developed, to study special events of differentiation and morphogenesis and their modification by teratogens (Kochhar, 1975; Neubert et al., 1977). Whole organ and tissue culture methods have been used by developmental biologists since the twenties. The method of whole embryo culture of postimplantation embryos has been established by New (1967) about a decade ago and has successfully been applied to experimental teratological problems (Cockroft and New, 1975).

Although methods to culture mammalian embryos successfully during the preimplantation period have been developed more than a decade ago (Whitten, 1957; Brinster, 1963), these techniques have hardly ever been used in teratological research, since it is generally believed that preimplantation embryos after being exposed to teratogenic agents either die before implantation or survive to term unharmed. However, the methods developed in the field of early embryology provide new possibilities to study teratological problems during preimplantation development. Information on influences of toxicological and pharmacological agents on preimplantation embryos can especially be obtained with the *in vitro* culture and transplantation of early embryos. Recent results obtained in our laboratory with the two techniques prove that the action of drugs on embryos during preimplantation development is more complex than is generally assumed (Eibs and Spielmann, 1977a; Spielmann et al., 1977; Spielmann and Eibs, 1978).

Materials and methods

Mating, handling and treatment

Female mice of the NMRI and C57BL strains weighing 30 g (Zentralinstitut für Tierzucht, Hannover, FR-Germany) and Wistar SW 72 rats weighing 200 g (Winkelmann, Kirchborchen, FR-Germany) were bred under a normal day/night cycle and placed with males

overnight. Embryos were obtained at the following times after the midpoint of the dark period: 1-cell stage of mice and rats at 12 h; 2-cell stage of mice and rats at 36 h; 8-cell stage of mice at 60 h and of rats at 84 h; morulae and early blastocysts of mice at 84 h and of rats late blastocysts of mice at 96 h and of rats at 120 h. Implantation occurs between 96 and 108 h in the mouse and between 120 and 132 h in the rat. All media and solutions were prepared from three times quartz-distilled water. During flushing from the oviduct or uterus and throughout transplantation the embryos were handled a phosphate buffered medium (PB-1) which does not require gassing with CO_2 (Spielmann and Eibs, 1977). Cumulus cells were removed from zygotes with 150 units (mice) or 400 units (rats) hyaluronidase (Boehringer, Mannheim, FR-Germany) per ml BP-1 medium.

Cyclophosphamide (CPA) and its metabolites (4-OH-peroxy-CPA = 4-HP-CPA; acrolein = Acr; phosphoramide-mustard = PAM) were gifts from Prof. N. Brock (Asta-Werke, Bielefeld, FR-Germany). CPA was dissolved in distilled water and injected subcutaneously. The stable isotope ^{13}C content of the uniformly labelled ^{13}C-glucose (U-^{13}C-glucose) was 86 atom % ^{13}C. The U-^{13}C-glucose was a gift from Dr. C. T. Gregg (Los Alamos Scientific Laboratories, New Mexico, USA). Cyproterone acetate (CA; Schering AG, Berlin, FR-Germany) and medroxyprogesterone acetate (MPA; Upjohn, Kalamazoo, US) were dissolved in benzyl benzoate (10% w/v) and diluted with castor oil to obtain a volume of 0.1 ml/33 g mouse. The steroid hormones were injected s.c., control animals received an injection with the solvent (benzyl benzoate/castor oil). To expose preimplantation mouse embryos to drugs *in vitro*, CPA and its metabolites were dissolved in the culture medium and the steroid hormones were dissolved in ethanol and added to the culture medium.

In the UV-irradiation experiments embryos of different developmental stages were irradiated in 50 μl droplets of culture medium on glass slides with a conventional germicidal low-pressure mercury lamp (Philips AG, Netherlands) having an output of 253.7 nm. The irradiation dose was varied from 8.3 to 249 erg/mm^2 by changing the time of exposure to UV light from 1 to 30 sec. In a few experiments irradiated and control embryos were cultured in medium containing caffeine (Merck AG, Darmstadt, FR-Germany) at concentrations of 0.1 or 0.5 mM.

Determination of the cell number of the embryos

The cell number of preimplantation embryos was determined according to Tarkowski's method (1966).

In vitro culture of preimplantation embryos and transplantation

Preimplantation mouse embryos from the 1-cell (zygote) to the blastocyst stage were cultured in Whitten's medium for ovum culture (WMOC; Whitten, 1971) supplemented with 0.4% bovine serum albumin (BSA; Sigma AG, München, FR-Germany). The embryos were cultured in disposable tissue culture dishes (No. 1420, Nunclon, NUNC A/S, Denmark) in a humidified 5% CO_2 in air gas mixture at 37 °C in a continuous flow gas incubator (No. KB 600 K CO_2, Heraeus AG, Göttingen, FR-Germany).

Mouse blastocysts which had developed *in vitro* from 4- or 8-cell embryos during a 48 h culture period, together with normal blastocysts which were obtained from the uteri of female mice 84 h after mating, were surgically transferred in groups of five to the uterine horns of pseudopregnant females on day 3 of pseudopregnancy. Pseudopregnancy was induced by mating normal females with vasectomized males. The foster mothers were sacrificed on day 17 of gestation and the success rate of the transplantations was determined by the percentage of resorbed and live fetuses. The fetuses were weighed, carefully inspected for growth retardations and malformations, and stained for skeletal abnormalities with Alizarin Red S.

Growth and differentiation of mouse and rat blastocysts in vitro during implantation period

Mouse and rat blastocysts that had either developed *in vivo* or *in vitro* were cultured in groups of ten in plastic culture dishes (NUNC, Nulcon, Denmark) without oil at 37 °C in a humidified 5% CO_2 in air atmosphere in medium NCTC-109 (Microbiological Associates, Frankfurt, FR-Germany) supplemented with 10% FCS according to Sherman (1975) (NCTC-109-FCS). When mouse and rat blastocysts were cultured for 96 h in medium NCTC-109-FCS, development proceeded through the following differentiation stages (Eibs and Spielmann, 1977a; Spielmann et al., 1978): hatching from the zona pellucida after 24—48 h, attachment to the surface of the culture dish after 36—60 h, and outgrowth of three characteristic cell types — a trophoblast layer with giant cells and an ICM consisting of the ectoderm and endoderm germ layers after 96 h.

Photomicrographs of the embryos were taken on a Biovert photomicroscope (Reichert AG, Austria) at a magnification of ×160 on Ilford Pan F film (Ilford Co., England).

Results and discussion

I. In vitro *culture of mouse embryos during the preimplantation period*

a) Normal development

In vitro culture of preimplantation mouse embryos in a simple defined bicarbonate buffered medium (e.g. WMOC) containing pyruvate, lactate and glucose as the only energy sources, is a standard procedure in many laboratories since Brinster (1963) had introduced the method of incubating the embryos in microdroplets of medium under oil. Besides being sometimes toxic the oil will allow lipid soluble agents that are tested for embryotoxicity (e.g. steroid hormones) to diffuse from the culture medium into the oil. This may lead to misinterpretation of the results. When, however, automatically gassed and humidified incubators were introduced, it became possible to culture embryos without any oil at the same success rate.

Table I

Differences in development of NMRI and C57BL preimplantation mouse embryos in culture

Period in culture		Culture medium	Time in culture (h)	Success rates	
Begin	End			NMRI (%)	C57BL (%)
1-cell	2-cell	WMOC	24	72	96
1-cell	Morula	WMOC	72	0	76
1-cell	Blastocyst	WMOC	96	0	24
2-cell	Blastocyst	WMOC	72	85	96
Blastula	Implanted embryo*	NCTC-109	120	89	15

* Embryos consisting of a trophoblast layer with giant cells and an inner cell mass (ICM) with 2 germ layers (entoderm and ectoderm). For abbreviations and culture conditions (without oil) see "Materials and methods" section. — At least 75 embryos were used in each group

Fertilized 1-cell embryos (zygotes) of a few inbred mouse strains (e.g. C57BL, Table I) could be cultivated to the blastocyst stage in sufficient quantities. In all other mouse strains a block in development occurred after the 2-cell stage (e.g. NMRI, Table I) which prevented a comprehensive testing of *in vitro* cultured 1-cell embryos. However, 85% of all 2-cell mouse embryos developed to the blastocyst stage in WMOC medium (Table I). Toxicological studies on preimplantation embryos in culture were therefore performed on 2-cell, 4-cell and 8-cell embryos which usually developed to the blastocyst stage at a slower rate *in vitro* than *in vivo*. Rabbit embryos showed a lower success rate, and rat embryos could only be cultured *in vitro* for one or two cleavage divisions.

b) Effects of treatment during *in vitro* culture

When the effect of *in vitro* UV-irradiation on the different developmental stages of preimplantation mouse embryos (2-cell embryo to morula) was investigated by determining the percentage of embryos reaching the blastocyst stage in culture, we were able to confirm the decreasing radiosensitivity during preimplantation development previously reported for X-irradiation (Fig. 1). However, when embryonic development during the 24 h following UV-irradiation was studied morulae showed a higher UV-sensitivity than earlier stages (Eibs and Spielmann, 1977b). The ability to form a blastocyst cavity, which is the first step of differentiation during embryonic development (Epstein, 1975), was therefore a better indicator of embryonic UV-sensitivity than development during the first 24 h after irradiation.

The *in vitro* development of UV-irradiated embryos was further impaired by the presence of caffeine in the culture medium at concentration levels which did not interfere with the development of non-irradiated embryos (0.1 and 0.5 mM). Since caffeine is a well-known inhibitor of the post-replication DNA repair mechanism, these results suggest that during preimplantation development mammalian embryos were able to repair DNA lesions by the post-replication repair mechanism (Eibs and Spielmann, 1977b).

The *in vitro* culture of preimplantation embryos can also be used to test the embryotoxicity of a teratogen and its metabolites. The alkylating drug cyclophosphamide (CPA) has

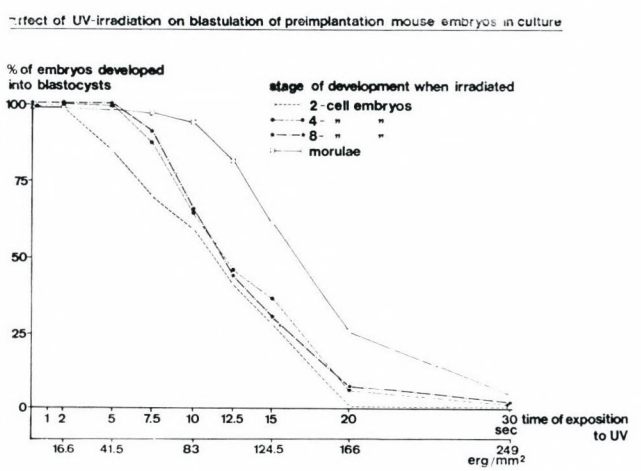

Fig. 1. Development of preimplantation mouse embryos into blastocysts following UV-irradiation. Curves represent the responses of 5 replicates. After variance analysis ratios between stages and doses were significant ($p < 0.01$)

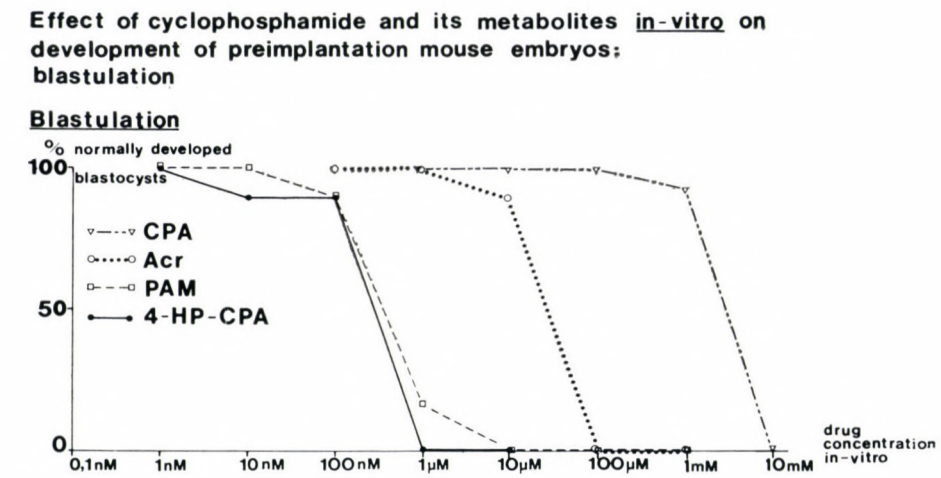

Fig. 2. CPA: cyclophosphamide; *Acr:* acrolein; *PAM:* phosphoramide mustard; *4-HP-CPA* 4-hydroxyperoxy-CPA

to be metabolized by hydroxylating liver enzymes to 4-OH-CPA to be metabolically active in cancer treatment and probably also to act as a teratogen. Incubation of 4- and 8-cell mouse embryos in the presence of increasing concentrations of CPA and its metabolites is shown in Fig. 2. Blastulation of the embryos was inhibited by concentrations of the two metabolites PAM and 4-OH-CPA (4-HP-CPA is an unstable precursor of 4-OH-CPA) that were 10,000 times lower than CPA concentrations and 100 times lower than Acr concentrations. Since 4-OH-CPA is metabolically degraded into PAM and Acr at a 1 : 1 ratio, PAM probably is the embryotoxic metabolite of CPA when the drug is applied in embryotoxic doses to the mother *in vivo.*

It is important to note that in the two examples given for treatment of embryos in culture (UV-irradiation and CPA treatment) S-shaped effect relationships were found (Figs 1 and 2) when a logarithmic transformation of the dosage was employed and the effect of treatment was scored by the percentage of embryos reaching the blastocyst stage.

c) *In vitro* culture and transplantation

Following *in vitro* development to the blastocyst stage mouse embryos can be transferred to the uterus of a foster mother and will then develop to normal foster term fetuses (McLaren and Biggers, 1958). In the same manner embryos treated with a teratogen in culture can be transplanted to a recipient (Fig. 3). An example of this type of experiment in teratological research is given in Table II.

Mouse embryos were cultured in the presence of ^{13}C-glucose from the 4-cell to the blastocyst stage to study the teratogenic effect of the stable isotope ^{13}C. During the 48 h culture period embryonic development was not inhibited as indicated by the percentage of embryos reaching the blastocyst stage and by the number of cells in these blastocysts. The ^{13}C content of these blastocysts was 20 atom % according to incorporation studies with ^{14}C glucose (Spielmann et al., 1976). To test the viability of such blastocysts they were transplanted to pseudopregnant recipients and the percentage of living and resorbed embryos was recorded at term. Table II gives the success rates of the blastocyst transplantation experiments. The percentage of implantations was identical for *in vivo* developed normal blastocysts

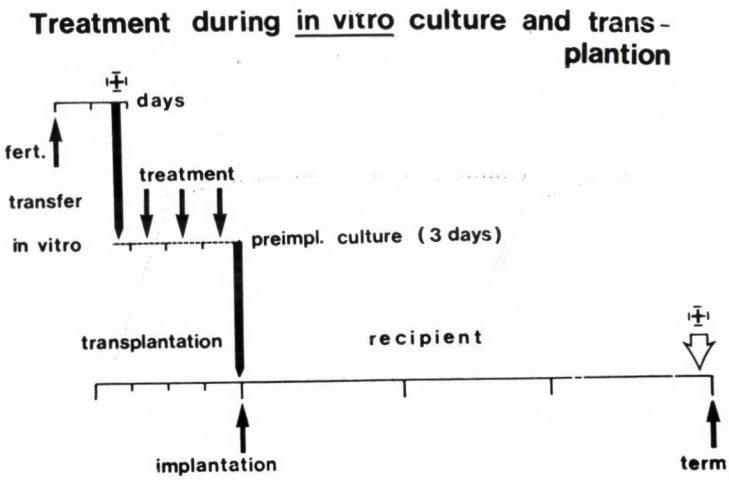

Fig. 3. Diagram showing the combination of *in vitro* culture of preimplantation mouse embryos and subsequent transfer to a foster mother

and for those cultured in WMOC medium. The difference between the two groups was the lower percentage of living term fetuses resulting from *in vitro* developed blastocysts. No difference was found in the development when blastocysts had been cultured in WMOC medium containing either normal or ^{13}C glucose. No weight reduction or malformations could be detected in any of the transferred mouse embryos (Spielmann et al., 1976). This result was not surprising, since so far only one report has appeared in the literature claiming increased malformation rate in embryos cultured in the presence of a teratogen (captane, an insecticide; Staples, 1975). Various types of manions and treatment of embryos *in vitro* have never been able to induce malformations at term after transplantation of the embryos (Spielmann, 1976; Spielmann and Eibs, 1978).

Table II

Success rate of blastocyst transfer experiments in the mouse

A Development before transfer	B Culture medium (Glucose)	C Total number of embryos	D Implantation sites (% of C)	E Living embryos (% of C)	F Resorbed embryos (% of C)
in vivo	—	400	52	38	14
in vitro	WMOC (^{12}C)	75	53	27	26
in vitro	WMOC (U-^{13}C)	75	52	25	27

Blastocysts were transferred in groups of 5 to each uterine horn of a pseudopregnant recipient. Evaluation of success rate on day 17, 24 h before term

II. *In vitro culture of mouse and rat embryos during implantation*

a) Normal development

Conditions for the *in vitro* culture of mouse blastocysts have been greatly improve during the last 5 years. Although Hsu (1973) has achieved differentiation of preimplantation mouse embryos *in vitro* to the early somite stage, his method cannot be used in toxicological studies, since the success rate is far below 10%. We prefer to use the culture conditions described by Sherman (1975; see "Materials and methods"), since about 90% of all mouse blastocysts (strain NMRI, Table I, Fig. 4) were able to proceed with differentiation during a 96 h culture period in medium NCTC-109 with FCS: they hatched from the zona pellucida, attached to the bottom of the culture dish, developed a trophoblast layer with giant cells and of an inner cell mass (ICM) with two germ layers (ectoderm and entoderm) (Eibs and Spielmann, 1977a; Spielmann et al., 1978). A typical example of a well-developed ICM with at least two germ layers is given in Fig. 5.

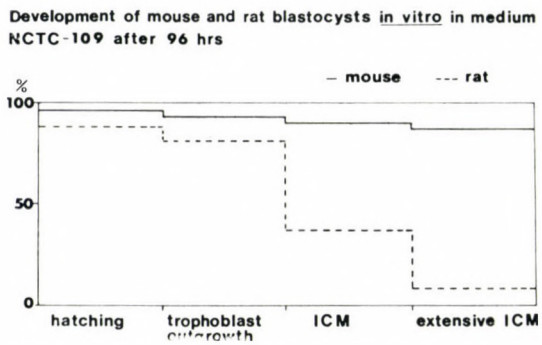

Fig. 4. In vitro development of rat and mouse blastocysts after 96 h in medium NCTC-109. Original data are given by control values in Table 3. ICM = inner cell mass extensive ICM = ICM with 2 germ layers (entoderm and ectoderm)

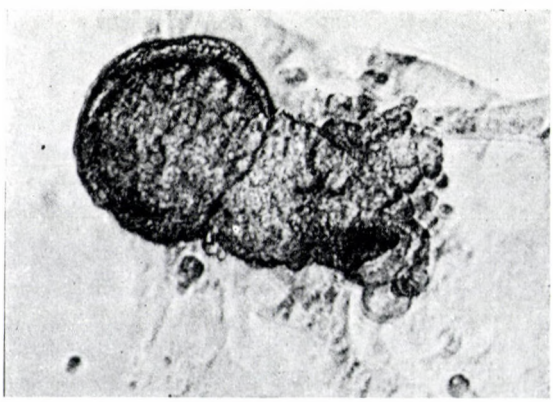

Fig. 5. Well-developed ICM of a mouse embryo cultured for 96 h in medium NCTC-109 with 10% FCS. The egg cylinder-like structure consists of at least two germ layers. It is growing on top of the trophoblast layer (which contains giant cells) like a mushroom. ×160

Rat embryos cannot routinely be cultured during the whole preimplantation period, and no attempts have been published to culture rat embryos beyond the time of implantation. We have achieved a moderate success rate with rat blastocysts in medium NCTC-109. Fig. 4 and Table III show that less than 50% of the rat blastocysts developed an ICM and between 5 and 10% an ICM with 2 germ layers. Biochemical differentiation as indicated by the appearance of the LDH isozyme LDH-5 in implanting mouse blastocysts (Spielmann et al., 1978) was also found in rat blastocysts cultured during implantation. It has to be stressed that blastocysts of some mouse strains like C57BL did not develop any better than rat blastocysts under identical conditions (Table I).

b) Development after treatment of the mother

Studies on the effects of CPA treatment 36 h before implantation on the development of rat embryos before and after implantation (Spielmann et al., 1977) showed that the early treatment interfered with the development of the embryo before as well as after implantation. In identically treated pregnant rats and mice there was no reduction of the number of embryos per mother and the ratio blastocysts/morulae 24 h after maternal treatment was the same. The cell number of the morphologically normal blastocysts, however, was reduced dose-dependently (Eibs and Spielmann, 1977a). Blastocysts from CPA treated mothers were transferred to medium NCTC-109 and cultured for 96 h. The results are given in Table III. Differentiation of CPA treated mouse embryos was inhibited dose-dependently for all stages of differentiation. The development of the 2 germ layers of the ICM was the most sensitive

Table III

Effect of cyclophosphamide (CPA) treatment in vivo on differentiation of mouse and rat blastocysts in vitro

CPA dose (mg kg^{-1})	No. of blastocysts (100%)	Hatching, %	Trophoblast outgrowth, %	ICM, %	Extensive ICM, %
Mouse					
0	300	96	93	90	87
20	119	89*	82*	73**	61***
40	73	81	71*	48**	25**
60	184	62**	50**	31*	11**
80	62	30*	22***	8**	0**
Rat					
0	260	88	81	37	7
30	139	81	79	5***	0**
60	131	40**	32**	0**	0

Treatment 36 h before implantation (mouse = day 2; rat = day 3). Begin of culture 12 h before implantation (mouse = day 3; rat = day 4). Culture period 96 h. Significance levels were determined by the X^2 test separately for each step of differentiation by comparing the growth rate (as percentage of blastocysts cultured) at every CPA dose with the growth rate at the next lower CPA dose. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

parameter. It was already inhibited significantly at the lowest CPA dose studied (20 mg/kg), a dose which did not affect the cell number of treated blastocysts (Eibs and Spielmann, 1977a). The same table demonstrates that development of rat blastocysts from CPA treated mothers was inhibited dose-dependently in the same manner. Similar to preimplantation embryos developing in the presence of teratogens S-shaped dose–response curves were obtained with blastocysts cultured during implantation after maternal CPA treatment. The slopes of the dose–response curves were, of course, different for the various developmental parameters tested (hatching, trophoblast outgrowth, ICM development with 1 or 2 germ layers).

In identical experiments on embryos treated *in vivo* 36 h before implantation with the sex steroid cyproterone acetate (CA) and medoxyprogesterone acetate (MPA) *in vitro* development of mouse blastocysts was inhibited at doses that not at all influenced the cell numbers of treated blastocysts but only the malformation rate of treated embryos at term (Eibs et al., 1978). This result again stresses the importance of the *in vitro* culture method in studies on teratogenic effects of drug treatment during the preimplantation period which cannot be detected by morphological methods (Spielmann and Eibs, 1978).

c) Development after treatment *in vitro* during the preimplantation period

As described elsewhere in more detail the *in vitro* culture of mouse embryos beyond implantation has been used by several investigators to test the viability of blastocysts treated during *in vitro* preimplantation culture (Eibs and Spielmann, 1977a; Spielmann and Eibs, 1978). In investigations on teratogenic effects of ^{13}C enrichment in preimplantation mouse embryos we could not detect any differences in the viability of mouse blastocysts cultured during implantation after development from the 4-cell stage in WMOC medium containing either ^{12}C or ^{13}C glucose (Spielmann and Eibs, 1978). This result confirmed the earlier data obtained with the embryo transplantation technique on effects of ^{13}C incorporation into cultured mouse blastocysts (Table II). We were furthermore able to find a dose-dependent inhibition of embryonic development beyond implantation after *in vitro* incubation of 4-cell embryos in the presence of the steroid sex hormones CA and MPA at concentrations comparable to those occurring *in vivo* during hormone treatment (Eibs et al., 1978).

Comments

The results obtained with *in vitro* cultured preimplantation embryos treated *in vivo* or *in vitro* demonstrate that clear-cut dose response relations exist for the effects of teratogens even before the embryos have implanted in the uterine mucosa. The inhibited *in vitro* differentiation of blastocysts from pregnant mice and rats, previously treated with teratogenic doses of CPA or with steroid sex hormones (CA and MPA), proved that these drugs had penetrated into the embryo already before implantation. However, the early treatment had not influenced the morphology of the preimplantation embryos. Embryo culture beyond implantation therefore is the most sensitive method to detect effects of treatment during the preimplantation period. The most sensitive parameter of the system is the development of the two germ layers of the ICM. Inhibited development of embryos treated *in vivo* with CPA before implantation as detected by this culture technique is also of importance for *in vivo* development since embryos in treated mothers die during organogenesis (Spielmann et al., 1977). Treatment with the steroid sex hormones CA and MPA on the first days of pregnancy seems to increase the incidence of malformations in term fetuses (Eibs et al., 1978). The *in*

vitro culture of blastocysts from treated mothers, therefore, is of importance in testing putative teratogens during early pregnancy.

Inconsistent success rates obtained even by experienced investigators are the general disadvantage of the embryo transplantation experiments (Spielmann, 1976). Treated and subsequently transplanted embryos have so far not been found malformed but either resorbed and dead or alive and normal. The success rate of transfer experiments can, therefore, only be determined with the two parameters dead or alive. It consequently takes a long time and many pregnant animals to test the effect of a single dose of a teratogen on preimplantation embryos in transplantation experiments.

For the evaluation of the effects of teratogens on preimplantation embryos, the *in vitro* culture of treated embryos beyond implantation, as described in this and a previous report (Eibs and Spielmann, 1977a), has several advantages when compared to the embryo transfer technique. The *in vitro* system requires fewer embryos and it is faster and more precise because maternal factors and individual variations are not involved. It also gives clear-cut dose response relations which are difficult to obtain when treated embryos are transferred.

However, the *in vitro* approach can only analyze the direct effect of the teratogen on the embryo up to the early egg cylinder stage and does only indirectly allow an assessment of a disturbed maternal physiology. The detection of maternal effects of the early treatment still requires transplantation experiments. Transplantation of embryos is also necessary in experiments to test whether treatment of preimplantation embryos during *in vitro* culture is able to induce malformations at term.

Summary

In vitro culture methods are described for mouse embryos during the preimplantation period (Whitten's medium) and for mouse and rat blastocysts during the time of implantation (NCTC-109 medium). Examples are given for the influence of the genetic background (strain differences) on *in vitro* development and for the detection of a DNA repair mechanism in cultured preimplantation embryos. S-shaped dose effect curves were obtained for the influence of UV-irradiation and cyclophosphamide (CPA) and its metabolites on development of preimplantation mouse embryos treated during *in vitro* culture. Similar curves were obtained for mouse blastocysts cultured at implantation after treatment of the mother with either CPA or the steroid sex hormones cyproterone acetate (CA) or medroxyprogesterone acetate (MPA). The embryo transplantation technique is described as a teratological method to test the viability of preimplantation mouse embryos which have incorporated the stable isotope ^{13}C during *in vitro* culture. The importance of the *in vitro* culture techniques and of the transplantation method in experimental teratology during the earliest period of pregnancy is discussed.

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APPLICATION OF POST-IMPLANTATION EMBRYO CULTURE TO PROBLEMS IN TERATOLOGY

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Key words:

Post-implantation embryo culture; hyperthermia; diabetes; hyperglycaemia; carbon monoxide.

Introduction

Culture methods for post-implantation mammalian embryos have found application in many fields, as they allow close control of the embryos' environment, without the complications that may be introduced by maternal effects (Reviewed by New, 1978). The methods are applicable to rat embryos explanted at any age between 7 1/2 days (i.e. early egg-cylinder stage, Buckley et al., 1978) and 13 1/2 days of gestation (i.e. 50—55 somite foetuses, Cockroft, 1973, 1976). The middle of this range (i.e. 9 1/2—11 1/2d) is of particular interest to teratologists, since during this period the main processes of organogenesis are taking place.

The culture methods themselves are of a number of types. Static-medium systems have been used successfully in a number of studies, but better development can usually be obtained with circulation or agitation of the culture medium. In the New-type circulator (New, 1967) bubbles of gas are used both to oxygenate the culture medium and to propel it past the embryos, which are anchored in an embryo chamber. This type of circulator was used for the initial studies on carbon monoxide effects on early rat embryos (Robkin et al., 1976) which will be discussed more fully later. Another culture system, in which embryos are similarly anchored in a current of oxygenated medium in an embryo chamber, is the 'Plasmom' (Robkin et al., 1972). In this case however, the medium is oxygenated in a silicone membrane 'lung' and the flow is provided by a peristaltic pump. One feature of this design is the smooth flow of medium past the embryos, which was used to advantage in a study on the effects of cardioactive drugs on mid-term rat embryos (Robkin et al., 1974); the heart rate was monitored with a laser beam and photoelectric cell, and plotted on a chart recorder.

Although each system has its particular advantages which may dictate its use in the study of a particular problem, the one which has found greatest application in the field of teratology is culture in rotating bottles (New et al., 1973; Kochhar, 1975; Cockroft, 1977) — the embryos are incubated in bottles or tubes containing culture medium and a gas phase, and the vessels are rotated to facilitate interchange of gases and nutrients between the embryos and their environment. This paper describes the system in use in our laboratory and some of the ways we have applied it to the study of problems in teratology.

Materials and methods

Two types of culture bottle are illustrated in Fig. 1; 60 ml Pyrex reagent bottles with ground glass stoppers are very satisfactory. A little silicone grease on the stopper allows it to form a gas-tight seal. Alternatively, bottles may be purpose made in any size to suit requirements. The type illustrated in Fig. 1(b) is closed with a silicone rubber bung and has a capacity of 30 ml, which is convenient for small numbers of embryos.

The bottles are placed on horizontal rollers about 30 mm in diameter and 250 mm long, and rotated at 30—60 rpm in an incubator at 37—38 °C.

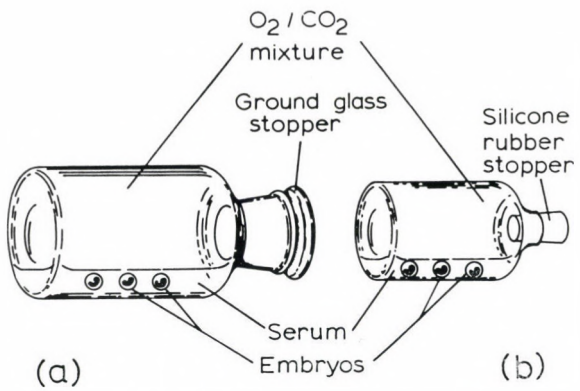

Fig. 1. Two types of culture bottle containing embryos and culture medium. a) 60 ml Pyrex reagent bottle with glass stopper; b) 30 ml bottle with silicone rubber stopper

The culture medium is usually pure rat serum, and rather than allowing the clot to form before centrifugation, it has been found that better development is obtained if the blood is centrifuged immediately after withdrawal from the dorsal aorta of the ether-anaesthetized donor rat (Steele and New, 1974). The clot then forms in the supernatant and this is squeezed to release contained serum and discarded. After re-centrifugation the serum is decanted and stored at −10 °C until required. Before use the serum is thawed, heat inactivated at 56 °C for 30 min, gassed to drive off residual ether and centrifuged to bring down any further fibrin clot.

The explantation procedure for embryos to be cultured in roller bottles is simple: the pregnant uterus is removed, opened in saline along the antimesometrial side and the conceptuses cut away with a cataract knife. After transfer to fresh saline the maternal decidua is removed an Reichert's membrane is torn open. The ectoplacental cane, or in older embryos the allantoic placenta, is left attached to the explant which is now ready to be placed in a roller bottle.

At 9 1/2 days of gestation (day of sperm-positive vaginal smear = day 1/2) about 6 embryos may be placed in 6 ml of serum per 60 ml bottle. For optimum development of 11 1/2 day embryos, the same size bottle will accommodate 3 embryos in 9 ml serum.

The air in the bottle is replaced with a suitable gas mixture which for the 9 1/2-day rat embryo consists of 5% O_2, 5% CO_2, 90% N_2 which is changed to 20% O_2, 5% CO_2, 75% N_2 at 24 h of culture (New et al., 1976a). Embryos explanted at 11 1/2 days require 95% O_2, 5% CO_2 throughout their culture period.

Results and discussion

At 9 1/2 days of gestation, the embryos are pre-somite egg cylinders with the beginning of the head-fold and the allantoic bud, and measuring about 1.5 mm long and 0.6 mm in diameter. The embryonic portion contains less than 5 μg protein. After 48 h in culture, and depending on the time of day they were explanted, they have developed 25—28 somites, with a crown rump length of 3.0—3.8 mm and a protein content of 120—240 μg. Development of these embryos is very close to that obtained in vivo over the same period (New et al., 1976b) and equally important for teratological studies, a very high proportion of control embryos (usually 100%) develop normally in culture.

Embryos explanted at 11 1/2 days of gestation develop for 18—24 h at slightly slower rates than in vivo. At explantation they have 27—31 somites, a crown rump length of 3.5—4.5 mm and 200—500 μg of protein. After 18—24 h in culture the somite number has reached 39—42 the crown-rump length has increased to 5.5—6.5 mm, and the protein content to 700—1200 μg.

In this laboratory embryos cultured through these periods have been used to study effects of hyperthermia, excess glucose and carbon monoxide.

Hyperthermia

Temporary elevation of deep body temperature, resulting from fever or climatic conditions, may be teratogenic in the pregnant female. Abortions, resorptions and malformations have been found in a number of species in which hyperthermia has been induced experimentally (reviewed by Edwards, 1974 and Edwards and Wanner, 1977), usually by heating the whole pregnant animal. This obviously causes severe distress to the mother; hence the effects on the embryo may be a result of maternal disturbance rather than a direct effect of heat on the embryo. A further complication is introduced by the mother's thermoregulation which makes precise control of the embryos' temperature impossible. One way of eliminating these maternal effects is to remove the embryos from the mother and graw them in culture.

In collaboration with Denis New I have been studying the effects of temperature elevation on rat embryos in vitro.

The embryos were explanted at 9 1/2 days of gestation and cultured for 2 days in the roller-bottle system. In an initial series of experiments (Cockroft and New, 1975) embryos were cultured at control temperatures of 37—38 °C, at 40 °C and at 41 °C. Embryos cultured at 40 °C were very similar to controls, but at 41 °C development was very abnormal and growth seriously retarded. Exposure to 41 °C for only part of the culture period showed that hyperthermia during the first half of culture had a greater effect than in the second half, and exposure for various 12 h periods suggested that the early parts of the 11th day might be particularly sensitive to increased temperature.

More recent work has been directed towards specifying the critical temperature range more closely, and to identifying less dramatic but potentially serious effects of hyperthermia (Cockroft & New, 1978). The first was studied by including 40.5 °C in the range of temperatures so that development at 38, 40, 40.5 and 41 °C was compared. As before, little difference was found in protein content, somite number or crown-rump length at 38 and 40 °C. At 40.5 °C however, there was significant reduction in all these parameters and in addition the majority of the embryos failed to undergo axial rotation. At 41 °C, protein content and crown-rump length were significantly lower than at 40.5 °C, and somite development was so poor at 41 °C that no counts were possible.

The malformations in these embryos followed the same trends. With 18 embryos in each group, no abnormalities were found in the control embryos cultured at 38 °C, and at 40 °C only one embryo showed obvious microcephaly. At 40.5 °C over half the embryos showed severe microcephaly and one had expansion of the pericardial cavity in addition. The embryos cultured at 41 °C were all abnormal, mostly with microcephaly and/or expansion of the pericardium; in one embryo the neural folds had failed to fuse and in three the anterior and posterior folds had fused to each other (Fig. 2).

Although only one of the embryos cultured at 40 °C was obviously microcephalic, it was our impression that in several others the brain might be smaller than normal. To measure this impression more objectively a series of experiments was performed in which the anterior part of the head was cut off after culture, and the head and body protein contents were measured

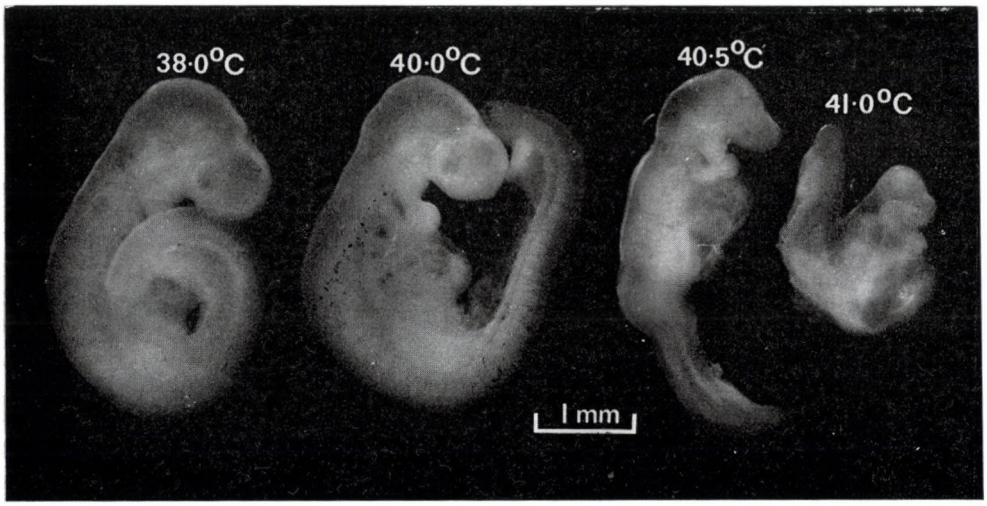

Fig. 2. Rat embryos explanted at 9½ days of gestation and cultured for 48 hours at temperatures of 38—41 °C

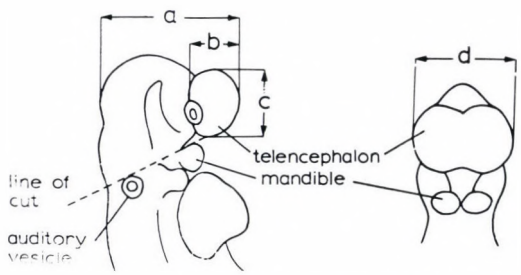

Fig. 3. Side view (left) and front view (right) of head region of rat embryo explanted at 9½ days of gestation and cultured for 48 hours. The diagrams show the position of the cut made for separate head and body protein determination and the four head dimensions that were measured: the length of the head (a) and the length (b), height (c) and width (d) of the telencephalon

separately. The line of separation was along the anterior edges of the auditory vesicle and the mandible (Fig. 3). For each embryo the head/embryo protein ratio was calculated, and to eliminate any effects arising from variation in the size of the embryos, each embryo was paired with a control embryo with a similar total protein content — the maximum discrepancy allowed being 5 μg. In each group, 18 out of 30 embryos could be closely matched in this way and the remainder were excluded from the results. The mean embryo protein content was thus the same in each group (167 $\pm$ 9 μg = mean $\pm$ S.E.) but the head/embryo protein ratio at 40 °C (0.278 $\pm$ 0.005) was significantly lower than at 38 °C (0.295 $\pm$ 0.003).

As a further way of investigating the microcephaly induced at 40°, measurements were made of the length of the head (a), and the length (b), height (c) and width (d) of the telencephalon (Fig. 3). The embryos cultured at 38 °C and 40 °C were again paired, this time on the basis of their crown rump length, and 22 out of 30 embryos were matched with a maximum discrepancy of 0.1 mm. It was found that the length of the head (a) and the length (b) and

height (c) of the telencephalon were all significantly reduced at 40 °C, but that the width (d) of the telencephalon was the same at the two temperatures.

Hence small but significant defects in brain development have been shown to occur as a direct result of a temperature elevation of only 2 °C. This type of deficit in the early foetus could well be responsible for the permanently reduced brain size found by Edwards and his co-workers (1976) in guinea pigs subjected to hyperthermia during pregnancy, and also the cause of the reduced learning ability of neonatal guinea-pigs similarly treated in mid gestation (Edwards et al., 1974).

Hyperglycaemia

A number of clinical studies have shown that pregnant diabetics are more likely to give birth to abnormal children than normal mothers (e.g. Pedersen et al., 1964; Kucera, 1971). A higher incidence of malformations is also found in rodents with experimentally induced diabetes (Horii et al., 1966; Deuchar, 1977). Since a number of factors may be involved in diabetes (e.g. hyperglycaemia, hyperosmolarity, insulin, toxemia etc.) the cause of these malformations is not clear. The present study was made in collaboration with Pat Coppola, and was aimed at isolating the effects of hyperglycaemia from the other concomitants of diabetes (Cockroft and Coppola, 1977).

The experiments were done on embryos explanted at 9 1/2 days of gestation and cultured for 48 h in rotating bottles. To the culture serum was added D-glucose at concentrations of 6—15 mg/ml. In some cultures, the osmolarity rise caused by the glucose was corrected by addition of distilled water. As a further osmolarity control, and to see whether it had any teratogenic action, L-glucose, the non-metabolisable isomer of D-glucose, was also tested, again with and without osmolarity correction.

It was found that concentrations of 12 mg/ml of D-glucose caused abnormalities in about one fifth of the embryos even when osmolarity correction was applied, but that lower concentrations of D-glucose had no effect. At 15 mg/ml D-glucose, half the embryos showed severe malformations when the osmolarity was corrected, and without osmolarity correction all the embryos were abnormal and the severity of the abnormalities was greater (Fig. 4).

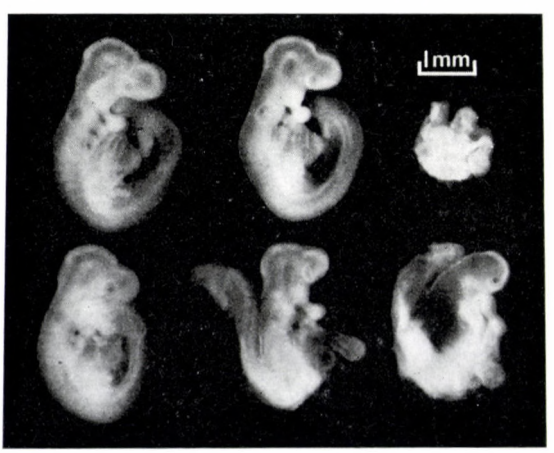

Fig. 4. Rat embryos explanted at head fold stage (9½ days) and cultured for 48 h in control medium (left), medium containing 15 mg/ml exogenous D-glucose with correction to normal osmolarity (centre) and 15 mg/ml D-glucose without osmolarity correction (right)

The most common malformations in these embryos were abnormal fusion of the neural folds, microcephaly and eye defects. L-glucose, even at the highest concentrations and without osmolarity correction, did not cause abnormalities, though some growth retardation was evident.

We have therefore demonstrated that 12—15 mg/ml D-glucose has a direct teratogenic effect which is distinct from, but exacerbated by, its osmotic effect. The concentrations we have used are high, but such levels have been recorded in severely diabetic patients (Fulop et. al., 1975).

Recently studies have been made with embryos explanted 12 h earlier (i.e. at 9 days of gestation) and cultured for 66 h (Cockroft, in preparation). These embryos are much more sensitive to hyperglycaemia: all the control embryos developed normally but one quarter of the embryos cultured with 6 mg/ml D-glucose with osmolarity correction had brain or eye defects, and at 9 mg/ml, nearly half the embryos showed these anomalies.

Carbon monoxide

As a result of atmospheric pollution or cigarette smoking, carbon monoxide may accumulate in the blood stream of pregnant women where it combines preferentially with both maternal and fetal haemoglobin to form carboxyhaemoglobin. This may drastically reduce the amount of haemoglobin available for oxygen transport.

Although concentrations of only 0.1% in the inspired air are rapidly lethal to adults, a study by Robkin et al. (1976), showed that concentrations of 10% CO in the perfusing gas had no effect on the heart rate of rat embryos cultured at 10 1/2, 11 1/2 or 12 1/2 days of gestation. This unexpected finding raises the question of whether CO has any physiological affects on rat embryos in culture.

The study described here was carried out in collaboration with Maurice Robkin from Seattle and we have tried to relate growth in culture to the changing patterns of energy metabolism in the presence of CO and of suboptimal oxygen levels.

Rat embryos were explanted at 11 1/2 days of gestation and cultured for 18 h in rotating bottles. Control embryos were cultured in 85% O_2, 5% CO_2 with 10% N_2. The experimental embryos were exposed either to 10% CO in place of the nitrogen, or to a reduced oxygen level of 40% but without CO. Assays were made of the glucose and lactate in the culture medium so that the glucose consumption and lactate production of the embryos could be calculated.

Neither 10% CO nor reduction to 40% O_2 had a significant effect on somite production or crown-rump increase and no abnormalities were produced. However, both treatments caused a significant reduction in the growth rate (as measured by protein increment). The lactate production was significantly increased by CO or hypoxia, and glucose consumption increased slightly in the presence of 10% CO and significantly in the presence of 40% O_2. The ratio of lactate produced to glucose consumed increased significantly in the presence of both 10% CO and the reduced oxygen level of 40% O_2, but there was no significant difference between the two treatments.

These results suggest that the embryos respond to interference with oxygen transport by a relative increase in anaerobic metabolism and a concomitant decrease in the more efficient oxidative metabolism of glucose via the citric acid cycle. This results in a net energy loss which could account for the observed reduction in growth rate. (Robkin and Cockroft, 1978). The finding that the overall effect of CO is small suggests that in our culture system relatively little oxygen is being carried by the red blood cells, and that physical solution is the dominant mechanism of oxygen transport.

In vivo, where prevailing oxygen levels are lower, haemoglobin presumably plays a bigger role in oxygen transport: hence the effects on the foetus of carbon monoxide would probably be more severe.

Summary

Rat embryos explanted at 9 1/2 or 11 1/2 days of gestation and cultured in rotating bottles were subjected to a number of agents.

Embryos explanted at 9 1/2 days (head-fold stage) and incubated for 48 h at 40.5 °C were retarded and abnormal and these effects were more seevre in embryos cultured at 41 °C. Embryos exposed to 40° over this period were superficially normal, but separate head and body protein determinations and careful measurement of the head dimensions showed them to be microcephalic.

Addition of 12—15 mg/ml D-glucose to the culture medium of embryos grown in vitro over the same period also produces malformations. This teratogenic action of D-glucose is distinct from but exacerbated by its osmotic effect. Embryos explanted 12 h earlier (i.e. at 9 days of gestation) and cultured for 66 h were more sensitive to excess glucose — abnormalities were produced by 6—9 mg/ml exogenous D-glucose.

Embryos explanted at 11 1/2 days of gestation and cultured for 18 h responded to both 10% CO and suboptimal oxygen levels with a reduction in growth rate and an increase in the ratio of lactate produced to glucose consumed, suggesting a shift to less efficient anaerobic energy metabolism.

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EXAMINATION OF NORMAL AND ABNORMAL LIP DEVELOPMENT IN MOUSE EMBRYOS USING *IN VIVO* AND *IN VITRO* TECHNIQUES

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Key words:

Fusion facial swellings; surface coat; cleft lip; nasal processes.

Fusion of epithelial ridges is a commonly observed process during embryogenesis, occurring in development of the neural tube, nasal folds, palate, eye, heart and other regions. These regions are also common sites for congenital malformations such as exencephaly and spina bifida, cleft lip and palate, and bulbus and conus lesions respectively (Langman, 1976). The relationship between factors responsible for normal epithelial fusion and the occurrence of abnormalities is not clear. However, at least two processes — increased production of surface coats and formation or elevation of ridges — appear to be essential for normal fusion. In some instances interference with these events in laboratory animals has resulted in congenital deformities.

The presence of surface coats has been observed during palatal shelf fusion (Pratt et al., 1973; Greene and Kochhar, 1974; Pratt and Hassell, 1975; Souchon, 1975; Greene and Pratt, 1976), and neural tube closure (Moran and Rice, 1975; Lee et al., 1976; Sadler, 1978). Surface coat material is increased over prospective fusion areas immediately prior to contact and is thought to provide a means of temporary adhesion between opposing ridges, folds or shelves until more permanent cell to cell contacts can be established (Greene and Pratt, 1976). Inhibition of surface coat synthesis has been shown to prevent fusion between palatal shelves *in vitro*, thus providing evidence that the coat is essential for normal closure (Greene and Pratt, 1977). However, no clefts or fusion defects have been produced in the intact organism by inhibition of surface coat production.

Formation and elevation of ridges and folds is also essential for normal closure in all of these fusing areas. The ridges, folds or shelves must be elevated into a position of apposition or a severe defect will result. Many factors are involved in formation and apposition of the ridges and folds, including the underlying mesenchyme. This tissue appears to play a significant role in neural fold elevation (Schroeder, 1969; Myrianthopoulos, 1978). Substances known to affect mesodermal cells, such as vitamin A, disrupt normal mesenchymal development and in this way inhibit closure of the folds (Morriss and Steele, 1977). Thus embryos treated with vitamin A during neurulation have insufficient mesodermal tissue in the neural folds and exhibit closure malformations such as exencephaly. These results suggest a cause and effect relationship between interference with the underlying mesenchyme and the defect. Direct effects on the neuroepithelium itself, however, cannot be excluded.

Although the appearance of surface coats at the time of fusion and the disruption of mesenchyme in malformed embryos suggest an important role for these events during closure processes, it is still not clear how these processes are involved in the production of malformations. One difficulty in assessing the importance of these events has been the inaccessability of the mammalian embryo to the investigator. However, recent developments, primarily by New and his co-workers (1978) and by Kochhar (1975) and Sadler (1978), have led to the successful maintenance of intact rat and mouse embryos *in vitro* throughout organogenesis.

Using the technique of whole embryo culture, our laboratory has been involved in analyzing processes responsible for normal and abnormal lip development. Cleft lip is one of the most common human malformations (1 : 1000) (Fraser, 1971), but one of the least understood. However, it is known that the nasal folds must elevate and fuse if development is to proceed normal. Thus, based on results from investigations in the palate and neural tube, the nasal processes provide an excellent opportunity to further assess the importance of surface coats and underlying mesenchyme for normal closure of the nose and lip regions. Furthermore, the use of the whole embryo culture technique offers a unique opportunity to manipulate the developing lip region at crucial stages of its development.

Materials and methods

In all experiments we used random bred mice of the ICR strain which were mated from 8:00 A.M. to noon. Plug day was considered to be the 1st day of gestation.

Whole embryo culture

These techniques involved adaptations of those originally designed by New (1978) and modified by Kochhar (1975). Eleven day embryos were stripped of most placental tissue and Reichert's membrane, leaving the yolk sac and a small ectoplacental cone. Tears were made in the cephalic and caudal ends of the yolk sac taking care not to damage major blood vessels. The embryos were then placed in 25 ml round bottom flasks, containing 2 ml of 50% Waymouth's medium, 50% fetal calf serum, and gassed with 95% O_2 and 5% CO_2. The flasks were placed on a rotator wheel revolving at 30 rpm. Cultures were maintained at 38 °C and regassed approximately 12 h after their initiation.

Localization of surface coat material (in vivo)

The presence of a surface coat over the epithelial linings of the nasal swellings was examined by using a modification of the ruthenium red (RR) technique developed by Luft (1971). Twelve day embryos were fixed for 1 h in 0.25 ml of 50% glutaraldehyde, 5 ml of 0.2 M Na-cacodylate buffer (pH 7.3), 1.5 ml of distilled H_2O and 3.3 ml of 4500 ppm RR (4.5 mg RR/ml distilled H_2O). Fixation was followed by rinsing the embryos twice for 20 min in 5 ml of 0.2 M Na-cacodylate buffer, 2 ml of 4500 ppm RR and 3 ml of distilled H_2O. The tissues were subsequently post-fixed in 2.2 ml of 0.1 M Na-cacodylate buffer containing 1% OsO_4 and 0.3 ml of 4500 ppm RR. Post-fixation was followed by dehydration, embedding in araldite and sectioning for observation with an RCA EMU-3H electron microscope.

Role of underlying mesenchyme (in vitro)

In these experiments emphasis was directed toward manipulating the extracellular matrix in the nasal folds. Hyaluronidase, an enzyme known to digest certain glycosaminoglycans (GAG) in the extracellular matrix, and DON, a known inhibitor of GAG synthesis, were employed to disrupt normal mesenchymal integrity. Using a microneedle and *in vitro* techniques, 5 μl of hyaluronidase (2—4 μg/μl) or 5 μl of diazo-oxo-norleucine (DON, 1 μg/μl) was injected into the area of the fusing nasal folds. Controls received only sterile saline. Following treatment, embryos were cultured for 12—24 h and then embedded in araldite, sectioned, and examined with the light microscope.

In addition to *in vitro* studies, DON was also employed *in vivo*. Pregnant mice were injected intramuscularly with 0.5 mg/kg DON on the 11th day of gestation. On the 18th day the fetuses were removed and examined for external malformations. Several fetuses were then embedded in paraffin and sectioned for light microscopic examination, while others were cleared in KOH and stained with alizarin red S for observation of the skeleton.

Results and discussion

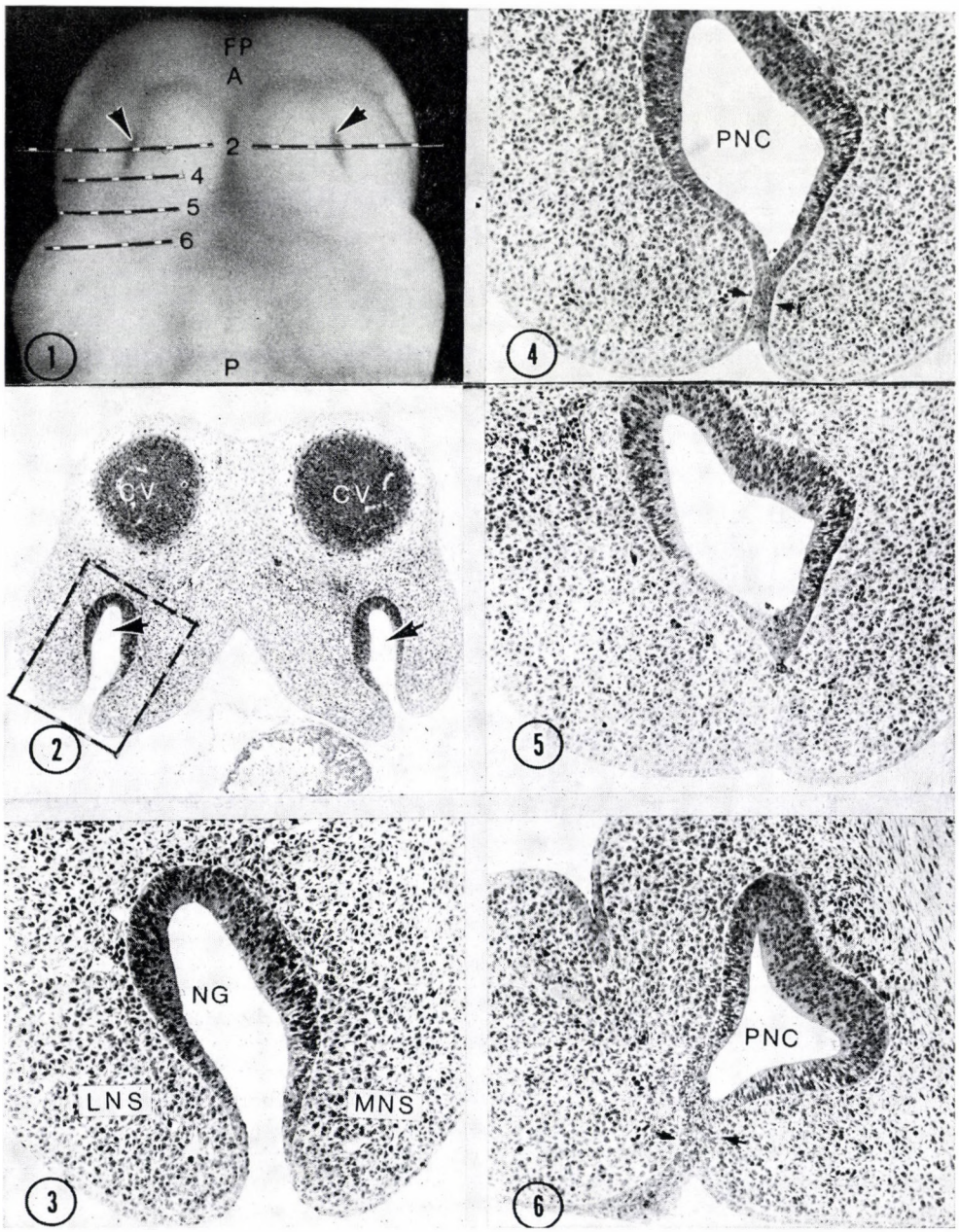
Studying the development of cleft lip in mice, Trasler (1968) found that the roof of the primitive oral cavity in 11 day old mouse embryos is characterized by two deep bilateral depressions, the nasal grooves. Each nasal groove is flanked by two prominences, the medial and lateral nasal processes. As development proceeds, the epithelial linings of the opposing nasal processes make contact (Trasler, 1968; Lejour, 1970; Pourtois, 1972). Fusion initially occurs in the posterior part of the nasal groove and proceeds in an anterior direction. The result of this postero-anterior zippering process is the formation of the primitive nasal cavity (Fig. 1).

At about the time that the medial and lateral nasal processes fuse mesenchyme of the two medial nasal processes merges in the midline region. This merging completes the formation of the prolabium which represents the middle third of the upper lip (DeMyer, 1975). The end of the fusion and merging of the nasal processes is the formation of the upper lip and the primary palate.

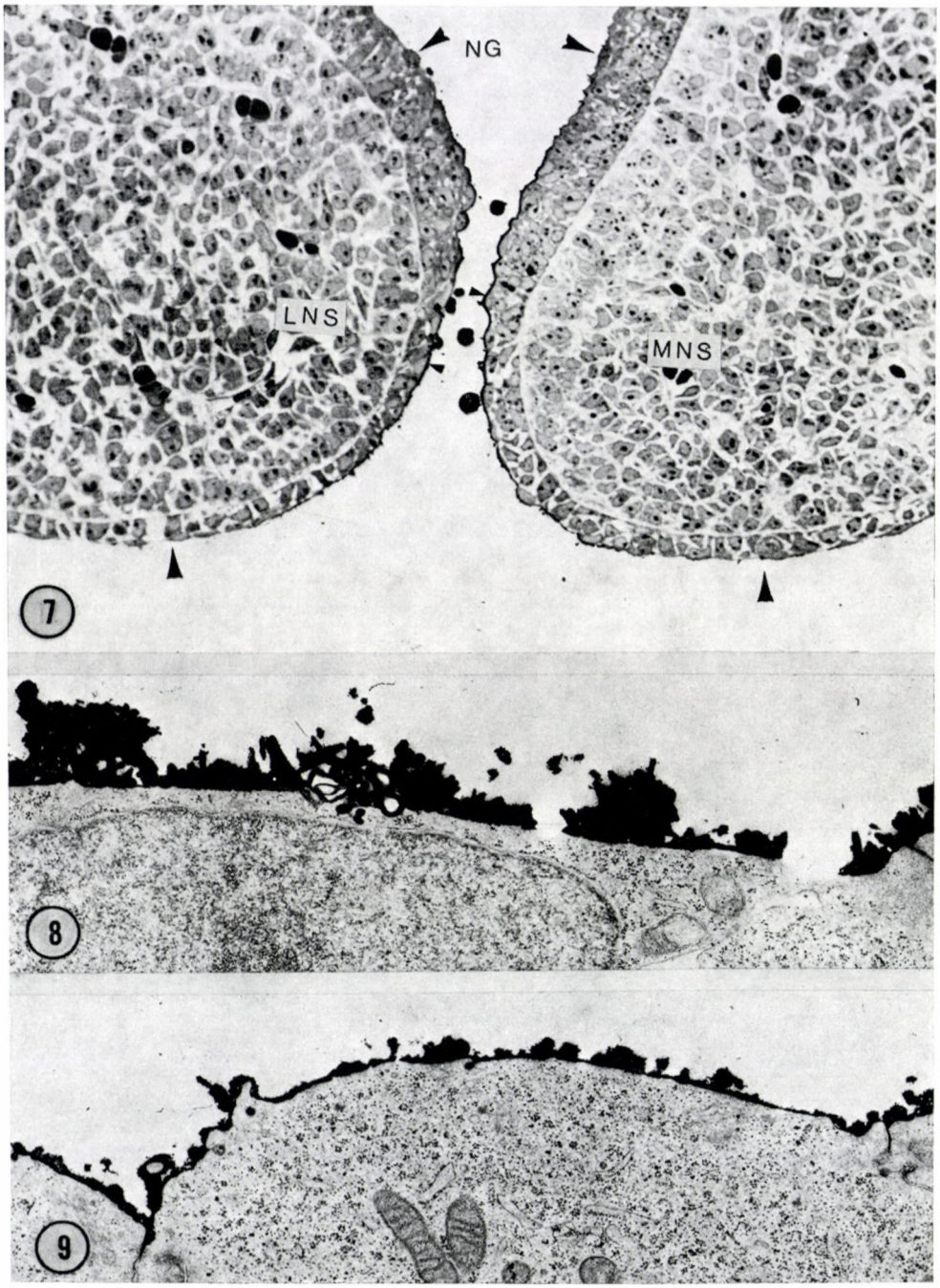
Serial coronal sections through the heads of 11 to 12 day old embryos demonstrated a separation between the medial and lateral nasal folds, anteriorly (Figs 2 and 3). In more posterior sections, where the epithelial linings of the nasal swellings had fused, the primitive nasal cavity and an epithelial plate (nasal fin) were observed (Fig. 4). In sections further posteriorly the nasal fin was absent, having been replaced by mesenchyme (Fig. 5). In the most posterior part of the primitive nasal cavity, the nasal fin had not regressed, but persisted as the bucconasal membrane (Fig. 6).

When the ruthenium red procedure was applied to 12 day embryos a surface coat, indicated by a black precipitate, was observed over epithelia in presumptive zones of fusion (Fig. 7). However, some variation existed in the coat distribution. In some embryos the coat was thickest over epithelia of presumptive fusion areas (Fig. 8), with lesser amounts of ruthenium red observed over epithelial cells in deeper areas of the nasal groove and mouth (Fig. 9). In other embryos this difference in thickness was not seen and an even distribution of surface coat material was observed over all these areas. At the point of initial contact between medial and lateral nasal swellings, the coat existed as a thin black film between cellular projections (Fig. 10). However, the precipitate was absent in regions further posteriorly where epithelial contact was firmly established (Fig. 11). Further investigations by our laboratory on surface coat formation of the nasal swellings using ^{3}H-concanavalin A and quantifiable techniques, have shown conclusively that a greater amount of surface coat material exists over prospective regions of fusion immediately prior to contact than over non-fusing regions (Burk et al., 1978).

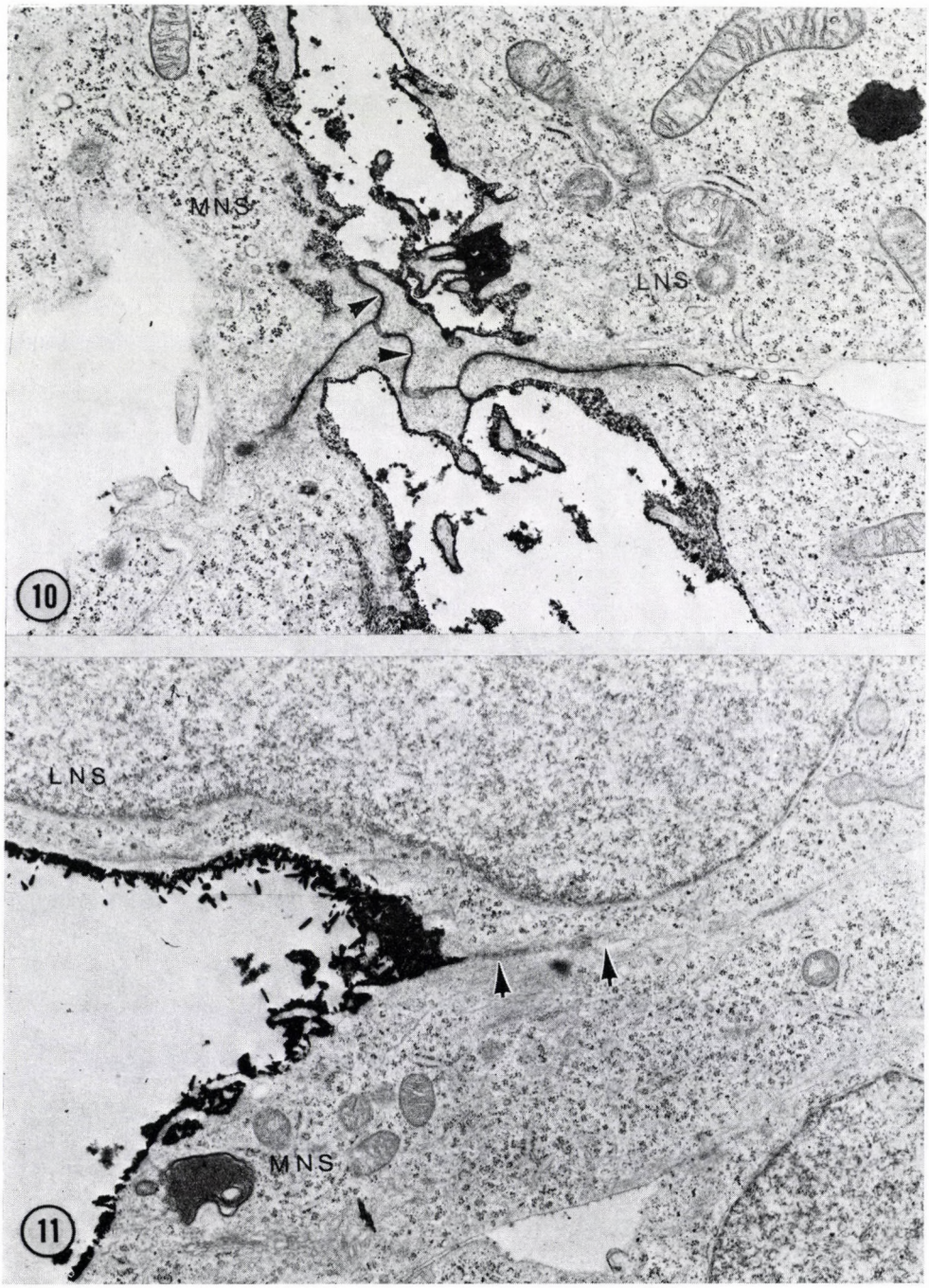
The observation that surface coat material is thickest over prospective fusion areas immediately prior to contact is in agreement with the work of other investigators (Pratt et al., 1973; Greene and Kochhar, 1974; Pratt and Hassel, 1975; Souchon, 1975; Greene and Pratt, 1976) who found that the cell coat on palatal shelves was heaviest over epithelial linings at prospective contact surfaces. Similarly, Moran and Rice (1975) and Sadler (1978) found the surface coat of closing neural folds in amphibia and mice, respectively, to be thickest over the regions of initial contact.



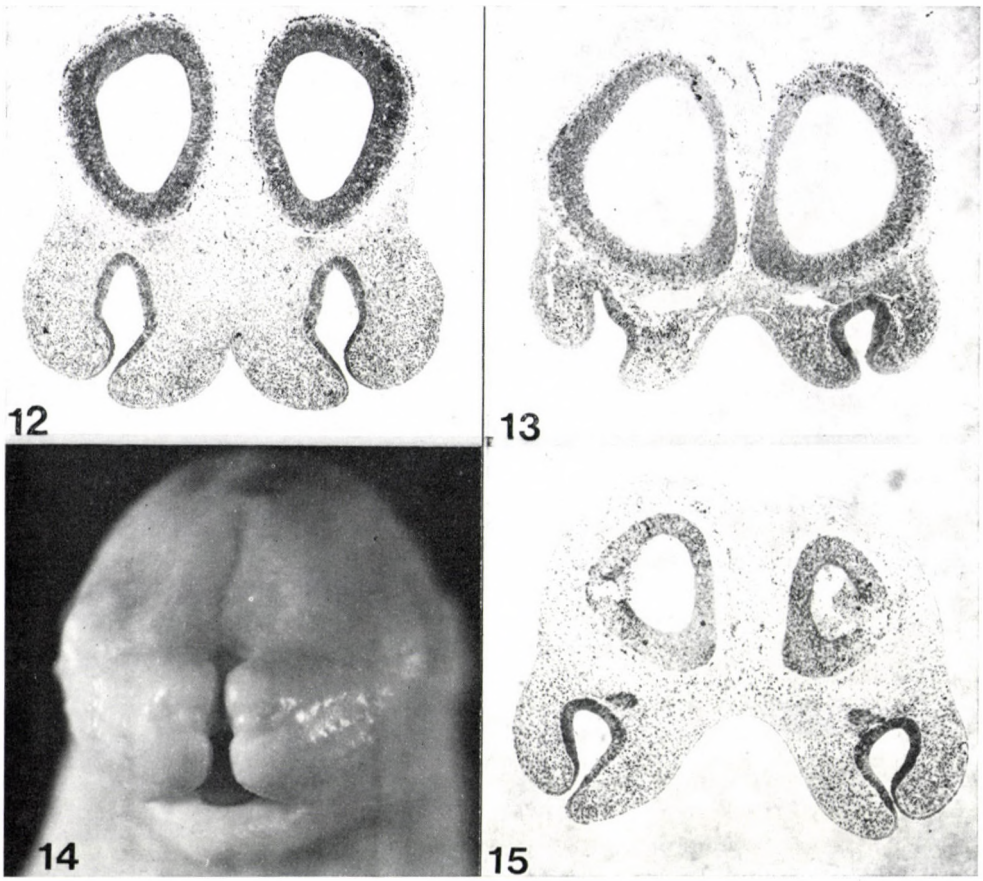
Figs 1—6. (1) Surface view of the roof of the primitive oral cavity. The bilateral nasal grooves (arrows) are situated anteriorly in the roof of the primitive oral cavity and are flanked by the nasal swellings. A, anterior; P, posterior; FP, frontonasal prominence; ×27. (2) Coronal 1 μm section through the nasal grooves at the level of line 2 in figure 1. Note the two cerebral vesicles (CV) cranial to the nasal grooves (arrows). ×36. (3) Higher magnification of Fig. 2. The nasal groove (NG) is flanked by the lateral (LNS) and the medial (MNS) nasal swellings. ×100. (4) Coronal 1 μm section at the level of line 4 in Fig. 1. The primitive nasal cavity (PNC) and the epithelial nasal fin (arrows) are visible. ×100. (5) Section at the level of line 5. The nasal fin is absent and the space between the primitive nasal and oral cavities is filled up by mesenchyme. ×100. (6) Section at the level of line 6 to show the epithelial bucco-nasal membrane (arrows). Slightly posterior to this section the primitive nasal cavity (PNC) end. ×100



Figs 7—9. (7) Coronal 1 μm section through the nasal groove (NG) of an embryo stained with ruthenium red. A black precipitate is seen over the epithelial cells in the region of prospective contact (small arrows); this precipitate is not seen above or below the presumptive fusing regions (large arrows). ×370. (8) Epithelial surface in the region of prospective fusion shown in Fig. 7. The thick surface coat is continuous with the cell membrane. ×16,500. (9) Epithelial surface below prospective fusion in Fig. 7. Note that the coat is considerably thinner than in the prospective fusion area. ×16,500



Figs 10—11. (10) Point of initial contact between the superficial cells from opposing nasal swellings. Note the thin black film at the interface between the contacting cytoplasmic projections (arrows). MNS, medial nasal swelling; LNS, lateral nasal swelling. ×16,500. (11) Contact area between the medial nasal swelling (MNS) and lateral nasal swelling (LNS). Note that the coat is present over epithelial cells with a free surface, but it is absent where contact is firmly established (arrows). ×12,000



Figs 12—15. (12) Coronal 1 μm section through the nasal grooves of a 12-day embryo (in vivo). $\times 40$. (13) Coronal 1 μm section through the nasal grooves of an embryo treated with hyaluronidase on day 11 of gestation and cultured for 18 h. $\times 40$. (14) Face of an 18-day fetus recovered from a mouse treated with DON on the 11th day of gestation. Note the median cleft of the upper lip and nose. $\times 12$. (15) Coronal 1 μm section through the nasal grooves of an embryo treated with DON on day 11 of gestation and cultured for 18 h. $\times 40$

Greene and Pratt (1977) suggested that in the secondary palate the surface coat is important in mediating adhesion between opposing palatal shelves. When these investigators inhibited surface coat production by means of DON *in vitro*, the palatal shelves failed to fuse. Our findings that the coat is present as a thin film between contacting epithelial cells of the nasal swellings also suggests that it may be important in mediating epithelial fusion during lip development.

In addition to the surface phenomena, the underlying mesenchyme of both the medial and lateral nasal processes appears to play a major role in normal lip development. Embryos in which hyaluronidase was selectively injected into the area of one nasal groove, showed a severe affect primarily on the mesenchyme of the treated site. Mesenchyme from the medial and lateral nasal processes of these unilaterally treated embryos contained large amounts of pyknotic debris characteristic of cell necrosis (Figs 12 and 13). Little effect was observed

in the overlying epithelium of these processes where only occasional pyknotic cells were observed. Fusion of the nasal processes on the treated side was inhibited and appeared to be due to a lack of fold elevation. The lateral nasal fold was affected more severely than the medial one and failed to make contact with the medial fold throughout its entire length (Fig. 13). Although presently control and treated embryos cannot be cultured long enough to envisage complete lip development, the lack of fold fusion due to mesenchymal collapse in the treated embryos would probably lead to a lateral cleft of the lip.

Merging of mesenchyme between the medial nasal processes also appears to be important for normal lip formation. For example, *in vivo* studies using DON, a glutamine analogue which may affect either matrix production and/or cell proliferation, produced a variety of midline defects in embryos treated on day 11 of development. In some instances the entire lip and palate were clefted. Embryos having the most severe defect showed clefting of all midline structures including the nasal septum (Fig. 14).

In vitro studies using whole embryo culture and injection of DON into the region of the nasal processes showed serious damage of the mesenchyme in this region. The amount of mesenchyme was decreased and a wider distance was observed between medial nasal processes in treated embryos than in controls (Fig. 15). Further investigations are being conducted to substantiate this effect, but our preliminary results demonstrate the importance of normal mesenchymal development in the region of the medial nasal processes and suggest a possible mechanism for the median clefts observed in embryos treated with DON *in vivo*.

In summary, surface coat synthesis and mesenchymal cell proliferation and matrix production appear to play an important role in normal upper lip development. The time of appearance of increased surface coat material in prospective fusion areas may suggest that the coat has an adhesive function in normal closure of the medial and lateral nasal processes. However, experimental manipulation of coat synthesis will be necessary before conclusive results can be obtained. The importance of the mesenchyme in the nasal processes has been clearly demonstrated and suggests a possible mechanism for the origin of clefts in the lip and nasal region.

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CAN WE MAKE A SAFE WORKPLACE?

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I am honored to chair the Symposium on Teratology of Occupational Health at the 6th Conference of our Society. It is ti timely and pertinent to discuss these practical matters within the European Teratology Society since many countries, several scientific disciplines and various professional groups are represented. Issues relevant to today's symposium have recently been debated at several conferences in the USA. It will be of great interest for us to compare how teratological hazards from occupational exposure have been dealt with within the various European countries.

Physical and *biological agents* such as ionizing radiation or rubella virus were early identified as risk factors for the developing embryo and measures were taken to protect radiologists and nurses at maternity and pediatric wards from undue occupational exposure. *Chemical agents* are now of concern with regard to exposure of operating room personnel and battery workers and I am restricting my presentation to chemical exposure when discussing if we can create a safe workplace.

In addition to *birth defects*, the adverse effects of occupational exposure to be considered are *transplacental cancer* and *fertility impairment*. Birth defects suggested as being caused by various environmental factors including occupational exposure have received much public attention after the thalidomide tragedy (Lenz, 1961; McBride, 1961) and particular attention has been focused on the risks for child-bearing women. One of the recent and most dramatic events in human teratology was the first report of transplacental chemical carcinogenesis (Herbst et al., 1971). Eight cases of adenocarcinoma of the vagina in women of 14—22 years of age were described in which seven of the mothers had been treated with diethylstilbestrol. The increased interest in the risk of intrauterine exposure is reflected in the IARC monographs, evaluating various chemicals as potential carcinogens. More recently exposure of male workers to 1,2-dibromo-3-chloropropane has been reported to cause impaired fertility (Whorton et al., 1977). This observation, in addition to earlier reports on lead- and Kepone-induced infertility in men, has had a great influence in balancing the discussion on the special safety problems for women and men at the workplace.

To evaluate the possibilities for establishing a safe workplace the *conditions* of exposure have to be considered as illustrated in the following scheme:

Occupational exposure

Origin	Target group
Workplace use	
Restricted area	workers
Open area	workers, public
Emission	
Controlled	public, workers
Uncontrolled	public, workers

Most attention has been given to strict occupational exposure when a chemical is used in a classical workplace, i.e. a restricted area such as a chemical industry or a laboratory. Those exposed at the restricted workplace are the employees and the amount of exposure should be monitored so as not to exceed "the safe levels". The exposed person or a group of people are included in various medical programs to detect adverse effects early. If in Sweden a person has suffered an occupational related harm, it has to be reported to the Swedish OSHA. The adverse effects discussed today should be reported. Since many issues of occupational hazards are mainly related to strict occupational exposure, I will discuss this category in more detail after first reviewing the other conditions for exposure.

When true occupational exposure occurs in an open area workplace — such as spraying with pesticides on crops in farming or in forestry — protective measures can be taken to control the exposure of the workers. Assuming that non workers are not directly exposed, delayed exposure of the public is however still possible if the chemicals persist in the soil, in water or in edible wild berries and thus contribute to environmental pollution. This latter of exposure has been a hot side issue to occupational use of chemicals in Sweden where birth defects were claimed to be caused by phenoxy acids. An old tradition, "Allemansrätten" ("All Men's Right"), allows everyone to walk free in the forest and to collect wild berries. Due to the growing demand of wood-products, it has been proposed that parts of the forests at certain periods of time will be considered as "industrial workplaces" with restricted access to the public. I would like to stress that the degree of exposure to the public by residues in berries or by strolling in a fenoxy-sprayed area has been judged negligible. More potent chemicals, however, may be used in the future and improvements will have to be made with regard to efficiency and obediency to the warning systems. To make these types of workplaces safe without causing panic, educational programs on environmental risks have to be more effective.

When a chemical is used in various occupational processes some emission from the factories will occur. Safety standards can be set by control agencies and the amount of released chemical can be controlled. However, those living in the vicinity of certain factories can be exposed to noxious agents suspect to cause birth defects or abortions. A discussion on the increase of birth defects in the vicinity of PVC factories has recently been reported (Edmonds et al., 1978).

Not only airborn exposure from chimneys of chemical factories but also illegal dumping of waste chemicals from industry could create a hazardous exposure of the public. Since it will take time to detect harmful exogenous agents emanating from a workplace, this type of exposure could represent a greater risk than the regular daily emission-exposure via chimneys or vents. While writing this presentation, I read a newspaper article concerning the leakage of waste chemicals dumped some thirty years ago in a swamp in Niagara Falls. The potential risk from this type of occupationally related exposure is always present.

However, the most dangerous type of exposure from a workplace is probably when a manufacturing process goes out of control as in the Seveso plant. In such a case the amount of exposure to outsiders can be considerable. Even if the exposure is of a short duration, the teratogenic threshold levels can more easily be reached than at a longer but more moderate exposure. To make workplaces safe in this respect, it is important to institute emergency alarm systems and in advance — from the type of production — to make up lists of the possible chemicals to be found outside the plant at an explosion or fire. The various health hazards could then be predicted and dealt with without delay.

Although occupational exposure may include a risk to people outside plants in residential areas, it is however pertinent to review in more detail the exposure risk of workers in restricted areas. The issue at stake is women at the workplace. In June 1976, an important conference was organized by the Society for Occupational and Environmental Health, the

National Institute for Occupational Safety and Health and the National Foundation — March of Dimes. I would recommend to read this conference report for additional information.

The following factors which have contributed to bringing this problem to an emotionally loaded issue, will be briefly commented upon:

Increased interest and knowledge in chemically induced prenatal damage.

Increased number of women occupationally exposed to chemicals.

The question of job restriction — sex discrimination.

Since the thalidomide disaster in 1961, it has become evident to laymen and scientists that chemicals as drugs can cause severe birth defects if the mother used the drugs at special sensitive stages in the embryo-development. It has also been recognized that intrauterine chemical exposure can result in cancer later in life. Several other chemicals besides drugs, such as food additives, have been claimed to cause congenital malformations, but fortunately very few new human teratogens have been found. Some of the chemicals highly suspected in causing birth defects or abortions such as anesthetic gases or solvents are used in professions which have a considerable number of female employees such as nurses and lab technicians.

The increased number of women facing the risk of chemical exposure during pregnancy can be illustrated by some figures from the USA. In 1975, 1 million out of 25 million women before the age of 15 to 44 years worked in occupations with a potential exposure to chemical substances which may cause birth defects and/or miscarriages. Of the total workforce, two fifths or 36 million were women. Women continue to take more new jobs than men — in 1976, 1.6 million women, twice as many as men, obtained new jobs. The same year, half of the American female population was employed or seeking work. In 1977, 1 million American children were born of women who worked during pregnancy. According to a recent ILO-report, new jobs in Europe go to women even more so than in the USA and Japan.

Job restriction — sex discrimination has mainly been discussed in the USA. Some employers refuse to hire women for work in certain parts of their plants where toxic chemicals are used — unless the women can prove that they no longer can bear children. These companies claim that their policies are based on the fact that the toxic substances may possibly damage the unborn children of pregnant workers.

After this short review of the problem, the following points are proposed for discussion on how to make a workplace safe:

1. *The reduction of exposure to occupationally used chemicals*

A further mechanization of workplaces will reduce the exposure at the plants. However, discussion on safe exposure levels for the workers will remain an important area for investigation. As experimental biologists many teratologists may be able to contribute to improvement and evaluation of animal test methods to predict teratological effects, transplacental cancer and other delayed effects and reduced fertility. The exposure level leading to these various types of injury may be quite different. To predict when a special workplace is safe for a given individual — a man or a pregnant or non pregnant woman — more information has to be obtained about these basic risk potentials.

2. *The role of occupational exposure to simultaneous non occupational chemicals or other unfavourable conditions resulting in health hazards*

When analyzing the special risk of occupational exposure, all types of environmental influences have to be considered. According to my view, the known adverse effects of alcohol and smoking seem to be neglected as co-factors in harmful occupational exposure. If for any

reason chemical industries would move to developing countries with less safety regulations than e.g. in industrialized countries, nutritional deficiencies among the workers might be another contributing factor to occupationally induced hazards in fertility and reproduction. Teratologists working in the area of epidemiology will continue to contribute in creating safer workplaces. It can however be assumed that it will be difficult to collect and store all the data needed on the various types of exposure in the most efficient way. Employees, unions as well as employers, share a responsibility in disclosing *full* information.

3. *Socio-legal aspects of the problem*

As well-educated citizens, all of us should keep abreast of the discussion. In the capacity of specialists, we might be able to take part in the debate and in informational/educational programs to the workers. Close contact with the problems at the workplace could initiate meaningful scientific work to create a safe workplace. Teratologists in administrative bodies in various countries could also contribute to improved dissemination of information to their fellow scientists. ETS by keeping up with safety at the workplace, could also be of assistance in these matters to countries in which this type of problem will occur in the future.

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EXPERIENCES FROM THE ACCIDENT OF SEVESO

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REGIONE LOMBARDIA, UFFICIO SPECIALE PER I PROGRAMMI DELLA L. R. 17. 1. 1977, NO. 2

I. Introduction

On July 10 1976, at noon, a run-away reaction started in a plant for the synthesis of trichlorophenol at the ICMESA chemical factory in Meda, a city 15 miles north of Milano. The safety valve of the reactor was not equipped with protective devices, so the hot vapours blew into the atmosphere and the resulting cloud, moved by a weak wind, began to fall to the ground covering an area south east of the plant. A few days later, lesions of the burning type began to appear on the skin of several people, mainly youngsters; grazing animals, mainly rabbits, began to die. It was only 9 days later that ICMESA admitted that TCDD, and not only TCP, sodium hydroxide, sodium trichlorophenate and ethylene glycol, could have been expelled from the reactor, so that for about two weeks the people were not protected to any extent against the damages caused by TCDD.

Immediately, the County Health Laboratory went to work trying to define the extent and the intensity of the TCDD pollution by means of GC-MS determinations in superficial soil samples taken at 50—100 m intervals.

Three zones were then established, as seen in Fig. 1. Zone A was subdivided in 7 subzones A1 to A7, with A1 the closest to the factory and the most polluted. Zone B is located south–east of zone A, while zone R ("Respect"), which surrounds both zones A and B, was originally judged free from contamination. Subsequently — as the sensitivity of the tests improved — it was found to be slightly and unevenly contaminated.

Table I

Distribution of TCDD contamination ($\mu g/m^2$) in the A, B, R areas on the basis of GC-MS analysis of soil samples (data provided by Regione Lombardia, Piano Operativo No. 1)

Zone	Size (ha)	$\mu g/m^2$ TCDD values		No. samples	Negative samples (1)		Estimated amount of TCDD on the area (g)
		average	top		No.	%	
A tot	80.3			306	12	*3.9*	147.5
A 1	10.7	580.4	5447	51	1	1.9	62.1
A 2	5.1	421.1	1700	19	0	0	26.5
A 3	9.2	350.5	2015	34	3	8.8	32.2
A 4	7.2	134.9	902	26	3	11.5	9.7
A 5	16.3	62.8	427	50	2	4.0	10.02
A 6	14.0	29.9	270	61	2	3.2	4.1
A 7	17.8	15.5	91.7	65	1	1.5	2.7
B	269.4	3.0	43.8	106	26	*24.5*	8.0
R	1430.0 (2)	0.9	9.7	449	308	*68.6*	8.5

(1) less than 0.75 $\mu g/m^2$; (2) only 950 ha mapped

Table I shows the size of the area involved, the average and top concentrations of TCDD for all zones and subzones. Average TCDD concentrations, highest in subzone A1 (580.4 $\mu g/m^2$), are lowest in zone B (3 $\mu g/m^2$) and R (0.9 $\mu g/m^2$); but an uneven distribution of TCDD, particularly in zones B and R, is suggested both by the high ranges of individual measurements and by the percentage of points, for each zone, found "free" (threshold concentration: 0.75 $\mu g/m^2$) from contamination (from 24.5% in zone B up to 68.6% in zone R).

Immediately after preliminary data had been obtained, people were evacuated from zone A; in contrast, inhabitants of zones B and R were left on the spot and invited to follow a number of hygienic rules, including a temporary advice neither to start pregnancies nor to breast-feed these babies. Only evacuation of children and pregnant women was provided. Prohibition to grow vegetables and to breed animals was enforced, orchards were destroyed and all edible animals were taken away or killed after reimbursement.

Therefore, inhabitants of zone A are to be considered as a group exposed directly to the toxic cloud and thus to the most polluted area, but only for as long as 19 days. Could the inhabitants of zone B, who were left in their homes, be considered chronically exposed to a much lower amount of TCDD?

Whether such chronic exposure is real or only theoretical depends on:

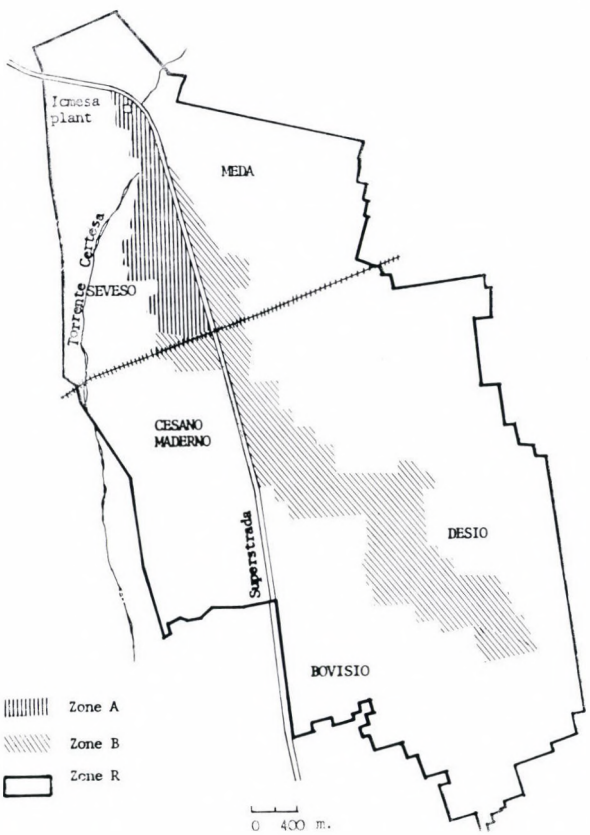

Fig. 1. Schematic map of Seveso area by pollution zones

1. whether TCDD in the soil can reach their organisms, and whether they followed all precautions suggested by the Health Authorities. This is a point of great debate, which nobody has clarified yet;

2. how carefully they follow the precautions suggested. This is very hard to state. For instance, owing to the lack of grossly detectable health consequences, many people have never stopped or have resumed illegally to grow vegetables and to breed animals. By the end of August 1978, a hundred of them were charged in front of the Court with disobedience of the Mayors' ordinances not to resume breeding animals and growing vegetables (Corriere della Sera, Sept. 1 1978).

In addition, owing to too many political and ideological debates that ravaged the area for many months, the exposure questionnaires administered to the people of zone A and B who accepted to cooperate, are often hard to trust.

The health surveillance plan, which had been published two years ago in Gargnano (Fara, 1976) and slightly modified thereafter, included:

1. clinical follow-up of each individual living in the polluted area. This was not mainly for epidemiological purposes, but also because of humanitarian reasons, and on a request by the population;

2. longitudinal control of groups at higher risk;

3. special projects, searching for damages of the neurological, immunological, chromosomal type as suggested by previous experience with accidents in humans and by experiments in animals;

4. surveillance of general health indicators.

The objectives of the program and the importance of the task were in contrast to the reality of the situation, namely an almost complete lack of local public health organization.

In spite of that, the decision of the Regional Council was not to give direct responsibility of the plan to external organizations, but to improve the local offices and to complete their staff. This decision, though democratically correct and in agreement with the health philosophy of the Region, took a tremendous lot of time to get the plan started and well at work. Despite the generous help given by many on a voluntary basis during the initial months, things, proceeded too slowly. For instance, the epidemiological working team only got started in January 1978 !

Therefore, data collection and elaboration of available data are still far from complete.

According to the prevailing interests of the participants in this meeting, data will be presented about birth defects, abortions, births and deaths in the area. We shall also present data on the only clinically ascertained consequence of the accident, chloracne, and give a summary of the results of some special projects under way.

Table II shows the distribution of the population involved in the accident by zone. Zone A includes segments of the population of 2 cities, Seveso and Meda; Zone B includes the segments of three: Seveso, Cesano, Desio; Zone R, 31,800 people, the segments of six (the four quoted, plus Seregno and Barlassina).

According to table II, the 11 cities can be divided into three groups. The first group, Seveso and Cesano, has more than 50% of its population included in the contaminated area (A + B + R); the second group (Meda and Desio) has an involvement of around 20%; the third group (seven cities) is only for 0.2% involved.

Table II

Distribution of inhabitants (No. and %) by city and pollution zone. The 6 cities whose territory was partially classified in one or more of the pollution zones (A, B, R) are members, with other cities, of three Health Administrative Departments (Consorzi Sanitari di Zona or C.S.Z. in Italian). Therefore, under zone "non (A, B, R)", in last column, 5 cities are listed which belong to the 3 C.S.Z. but are 100% outside the pollution zones (e.g.: Seregno, Lentate, etc.)

Cities	No. (and %) inhabitants					
	Total	Zone A	Zone B	Zone R	Zone A+B+R	Zone non A, B, R
Seveso	16975	668 (3.93)	628 (3.69)	7945 (46.79)	9241 (54.45)	7734 (45.55)
Cesano M.	33799	—	2736 (8.09)	14991 (44.35)	17727 (52.44)	16072 (47.56)
Total 1	50774	668 (1.31)	3364 (6.62)	22936 (45.17)	26968 (53.11)	23806 (46.89)
Meda	19571	62 (0.31)	—	4017 (20.52)	4079 (20.83)	15492 (79.17)
Desio	33011	—	1373 (4.15)	4608 (13.95)	5981 (18.10)	27030 (81.90)
Total 2	52582	62 (0.11)	1373 (2.61)	8625 (16.40)	10060 (19.14)	42522 (80.86)
Bovisio M.	11225	—	—	167 (1.48)	167 (1.48)	11058 (98.52)
Barlassina	5656	—	—	72 (1.28)	72 (1.28)	5584 (98.72)
Seregno	36838	—	—	—	—	36838 (100)
Lentate	13037	—	—		—	13037 (100)
Varedo	11841	—	—	—	—	11841 (100)
Nova M.	19467	—	—	—	—	19467 (100)
Muggio'	18690	—	—	—	—	18690 (100)
Total 3	116754	—	—	239 (0.20)	239 (0.20)	116515 (99.80)
Total 1+2+3	220110	730 (0.33)	4737 (2.15)	31800 (14.47)	37267 (17.84)	182843 (82.06)

II. Chloracne

During the first month after the accident, only lesions due to the burning components of the cloud were observed among the inhabitants of the area; afterwards, up to 187 cases of chloracne were diagnosed, 164 in children 0—14 years old, the other 23 in adults.

A first wave of chloracne, 50 cases (34 of them children 0 to 14 years) was detected from September to December 1976; 137 cases more (only 7 among adults) were diagnosed between February and April 1977, including both people referred by physicians and those found during an *ad hoc* school screening.

Distribution of cases and rates by pollution zones is shown in Table III. Rates for zone A of both (1) "early" and (2) "late" chloracne are by far the highest. But for "late" chloracne the rates for Seveso and Meda, zone R, are higher than for the whole zone B.

Table III

Chloracne cases (No. and ×1000 inhabitants) by pollution zones

Pollution zone		(1) Chloracne Sept.—Dec. 1976 "Early"		(2) Chloracne Feb.—Apr. 1977 "Late"		Chloracne (1) + (2)	
		No.	×1000	No.	×1000	No.	×1000
Zone A		46	63.01	15	20.55	61	83.56
Zone B		0	—	9	1.90	9	1.90
Seveso	Zone R	1	0.13	28	3.52	29	3.65
Meda	Zone R	0	—	20	4.98	20	4.98
Cesano M.	Zone R	0	—	13	0.87	13	0.87
Desio	Zone R	0	—	2	0.43	2	0.43
Seveso	Zone non (A,B,R)	0	—	13	1.68	13	1.68
Meda	Zone non (A,B,R)	0	—	14	0.90	14	0.90
Cesano M.	Zone non (A,B,R)	0	—	8	0.50	8	0.50
Desio	Zone non (A,B,R)	0	—	5	0.18	5	0.18
Other cities		3		10		13	
Total		50		137		187	

Presence of some chloracne cases in areas where soil pollution is poor, scattered or apparently absent, is hard to explain, also because answers to exposure questionnaires are often reticent or hard to trust. Remembering that zones R of Seveso and Meda are by closer to zone A than many parts of southern zone B, it is likely that people from such zones could have had direct or indirect contact with zone A before it was evacuated and fenced off.

Table IV shows the distribution by pollution zone of cases (and rates) of "early" and "late" chloracne and also of "other lesions" (which include atrophodermy and dermatological lesions which were referred to as dating back before July 10, 1976) in children 0—14 years. "Early" chloracne apparently affected only children from zone A;[1] the highest rate of "late"

[1] Only 31 out of 34 cases of "early" chloracne reported in children 0—14 years are considered here. Three more children, usually living in other cities (2 in Milano, 1 in Mariano Comense) were involved while spending their vacations in zone A.

chloracne is in zone A and the lowest in zone non (A, B, R) while in zone B and R the "late" chloracne rates are low and almost the same. Speculations reported for the previous table can be repeated. In addition, we must consider the "other lesions", whose distribution in zone A, B, R is not different from that of chloracne. The significance of those lesions and their distribution by zone, taking into account that production of TCP by ICMESA dates back to 1975, is now under careful consideration.

Table IV

No. and percentage of subjects 0—14 years showing dermatological lesions [zone A, B, R, non (A, B, R)]

Dermatological lesions	Zone A		Zone B		Zone R		Zone non (A, B, R)	
	No.	%	No.	%	No.	%	No.	%
1. Chloracne, Sept.–Dec. 1976 ("Early") to	31	14.5	0	0	0	0	0	0
2. Chloracne, Feb.–Apr. 1977 ("Late") to	11	5.1	8	0.5	63	0.7	46	0.1
3. Chloracne 1.+2.	42	19.6	8	0.5	63	0.7	46	0.1
4. Other lesions*	2	0.9	7	0.5	38	0.4	44	0.1
5. Total of subjects 0—14 y**	214	—	1468		8680		48263	

* Atrophodermy Feb.–Apr. 1977 + dermatological lesions before July 10, 1976 + + atrophodermy before July 10, 1976

** Population 0—14 years at December 31st, 1976

Table V shows the relative risks of chloracne in children by pairs of zones. Relative risks of zone A *vs.* B, R and non R are highest, relative risks of B and R *vs.* non (A, B, R) are still around 7, relative risk of B *vs.* R is, on the contrary, close to 1.

Table V

Chloracne (1) + (2) in children 0—14 years, relative risk for pairs of groups (zones), standard error and confidence limits at 95% probability level

Relative risks (RR)	S. E. (In RR)	Confidence limits 95%	
		Lower	Upper
A *vs.* B = 44.6	0.39	20.6	96.5
A *vs.* R = 33.4	0.21	22.0	50.8
A *vs.* non (A,B,R) = 255.9	0.23	164.1	39.1
R *vs.* B = 1.33	0.38	0.6	2.8
B *vs.* non (A,B,R) = 5.7	0.38	2.7	12.2
R *vs.* non (A,B,R) = 7.7	0.19	5.2	11.2

Other studies are in progress on people affected by chloracne.

From clinical and dermatological reports it appears that those people had no other signs of pathology until now, and also that the immune system of a group of children with chloracne showed no signs of alterations within two years from the accident, compared with the controls (Sirchia, 1978).

III. Birth defects (BD's)

Fearing that TCDD could be responsible for a rise in BD rates (as suggested by the animal literature), special recommendations to local physicians were issued just after the accident of July 10 1976, stressing the importance to comply with notification of BD's, which is mandatory. This was done because it was well known that BD's in Italy are usually under-reported: Table VI shows that only 1 or 2 BD out of 1600—1800 births were notified, per year, between 1972 and 1975, in the four cities which were to become the most polluted by TCDD. The corresponding rates (0.55 to 1.24 × 1000) were not too far away from the national rates, 1.6—1.9 × 1000; it has also been ascertained that before the ICMESA accident many physicians had agreed not to report several malformations, because such a notification was felt by the parents to be a socially negative mark. (A retrospective study is now under way to retrieve BD's born in previous years by means of children screening and examination of death notifications by cause.)

During 1976 only 4 BD's were reported, 3 of which before July and 1 in August, obviously all of them unrelated to TCDD. The expectation of a possible BD rise was for the beginning of January 1977, when women, 3 months or less pregnant at the date of the accident, were to deliver their babies.

During 1977 (with no clustering in any particular month) 38 BD's were reported: 7 in Seveso and Cesano, 16 in Meda and Desio, 15 in the other seven cities. During the first semester of 1978 additional 22 cases of BD were reported. Rates for the three groups of cities are, respectively, 12.72, 23.02 and 9.81 in 1977; 16.7, 21.8 and 13.97 in 1978 (Table VI).

Table VI

Birth defects in the areas involved by the ICMESA accident and in Italy, 1972 to 1978 (I—VI)

Year	Seveso + Cesano	Meda + Desio	Other 7 cities	Italy
1972	1/1712 or 0.58‰		N.A.	1.8
1973	1/1812 or 0.55‰		N.A.	1.6
1974	1/1721 or 0.58‰		N.A.	N.A.
1975	2/1603 or 1.24‰		N.A.	N.A.
1976	0/754	1/825 (1.21‰)	3/1630 (1.84‰)	N.A.
1977	7/550 (12.72)	16/695 (23.02)	15/1529 (9.81)	N.A.
1978 (I—VI)	5/298 (16.7)	7/321 (21.8)	10/716 (13.97)	N.A.

N.A. = not available yet

Therefore, a dramatic increase in BD in the whole area has been observed, suggesting that "something has happened". But several points conflict with the possibility to associate this rise with the accident.

1. In 1977 the rate of BD in Meda and Desio, the less polluted area was twice as much as in the more polluted Seveso and Cesano; again, in 1978, the highest rate was in the 7 cities whose population lived in the polluted area less than 2%;

2. a simultaneous increase in notified BD's took place in several counties of Lombardy (Table VII): it has doubled in Mantova and Varese, tripled in Sondrio, increased in other four counties, possibly due to the improvement in notification accuracy (according to the recommendations issued by the Health Authorities);

Table VII

BD rates (‰) in Lombardy and its counties. Data for 1970—1975: Carreri and Buratta (1976). Years 1976—1977: courtesy of Dr. V. Carreri, Assessorato alla Sanità, Regione Lombardia (unpublished data)

Counties	1970	1971	1972	1973	1974	1975	1976	1977
Brescia	4.01	4.75	4.33	4.91	4.40	3.62	3.70	3.14
Bergamo	3.08	3.37	2.41	1.97	3.44	5.02	5.14	3.62
Como	3.82	4.11	5.64	5.52	4.56	5.61	6.39	6.50
Cremona	7.21	6.54	4.17	6.20	2.09	5.60	6.44	6.93
Mantova	1.16	3.97	0.59	1.75	2.22	1.94	1.38	3.84
Milano	0.74	2.00	3.08	3.49	3.44	3.86	4.13	4.99
Pavia	6.03	3.73	N.A.	4.01	5.46	4.38	5.59	7.48
Sondrio	3.09	3.02	3.12	2.09	1.79	3.31	2.07	6.02
Varese	1.80	1.73	1.51	1.92	2.20	2.82	1.74	4.45
Lombardy	2.25	2.94	3.01	3.52	3.50	4.01	4.15	4.85
Italy	1.9	1.8	1.8	1.6				

3. the distribution of BD's by type shows no clustering and there exists a great variety in the BD's compared to their total number (Table VIII);

4. the rates observed in 1977 and 1978 in the different groups of cities, up to 23.0 /1000 were never higher than the ones usually observed in the western countries where notification systems work. This is also confirmed by a longitudinal study in our country, sponsored by the National Research Council: 23.2 BD's per thousand on 2409 newborns in 4 Maternity Centers is of the same magnitude observed now in the Seveso area (Fara and Marubini, 1974).

The BD rates for 1977, computed by pollution zone (Table IX), are 12.45 (non R) and 23.93 (Zone R). No BD's were observed in Zone B (out of 66 births) and in Zone A (out of 4 births).

It is interesting to know whether the lack of BD's in zones A and B is only because of the small number of births, and whether the hypothesis of an increased rate is still compatible with such observed negative results.

In Table X, probabilities have been calculated that no BD's are observed among newborns of zone A and B for several assumed BD incidence levels.

Little can be concluded for zone A: probability that the incidence has risen up to 50% is only 6%, but the hypothesis that the incidence has reached up 5 or 10 or even 20% cannot be rejected, because the probability of no BD's out of 4 births is still, 81.5, 66 and 41%, respectively.

There is still a 20% probability that no BD's are observed in zone B out of 66 births, should the incidence be equal to the one in zone R (namely 2.4%), however there is only a 3.4% probability should the incidence have risen up to 5%.

Table VIII

BD observed in the polluted area, 1976 to 1978 (I—VI)

A. Single defect	ICD (1975)	No. in 1976	No. in 1977	No. in 1978 (I—VI)
Inguinal hernia	550.9			1
Meningocele	741.9			1
Hydrocephalus	742.3		1	
Bulbus cordis anomaly	745		1	4
Interventricular septal defect	745.4		3	1
Stenosis of pulm. valve	746.0		2	
Cleft palate	749.0			1
Stenosis of small intestine	751.1		1	
Anomaly intest. fixation	751.4		1	
Ectopic anus	751.5		1	
Hypospadia	752.6	2	2	1
Cong. anomaly of scrotum	752.9			1
Club foot	754.7		9	3
Syndactyly	755.1		2	3
Osteogenesis imperfecta	756.5		1	
Diaphragmatic hernia	756.6			1
Down's syndrome	758.0	2	2	1
Hamartoblastoma cordis	759.6		1	
Neuroblastoma	M/9500/3		1	
B. Multiple defects				
Interventricular septal defect and ostium secundum type atrial	745.4			
septal defect	745.5		1	1
Interventricular septal defect and agenesis of lung	745.4			
	748.5		1	
Bulbus cordis anomaly and epispadia	745			
	752.6		1	
Meningocele and hydrocephalus	741.9			
	742.3		1	
Atresia of auditory canal and anomaly N.O.S. of ear	744.0			
	744.3		1	
Clubfoot and syndactyly	754.7			
	755.1		1	
Exotrophy of urinary bladder and hypospadia	753.5			
	752.6		1	
Omphalocele and gastroschisis	553.1			
	756.7		1	
Anencephalus and omphalocele and spina bifida	740.0			
	553.1			
	756.1		1	
Clubfoot and clubhand omphalocele	754.7			
	754.8			
	553.1		1	
Bulbus cordis anomaly and Down's syndrome	745			
	758.0			1
Down's syndrome and polydactyly	758.0			
	755.0			1
Polydactyly and ectopic anus and cyst of urachus	755.0			
	751.5			
	753.7			1

Table IX

BD/births by pollution zones and year
[1976 to 1978 (I—VI)]

Year (months)	Zone A	Zone B	Zone R	3 C.S.Z. (⁻ A; B; R)
1976 (VII—XII)	0/2	0/29	0/221	1/1280 (7.8‰)
1977	0/4	0/66	9/376 (23.93‰)	29/2328 (12.45‰)
1978 (I—VI)	0/..	1/..	5/..	16/..

Admitting a BD rate for zone B as for zone R (23.93‰), 1.5 $\pm$ 0.7 or 0.8 to 2.2 malformed children out of 66 births should be expected ($p < 0.05$).

Table X

Probability of no cases of Birth Defects in 1977 in zone A and zone B, given several assumed incidence levels (adapted from Modlin et al., 1976)

1977	Observed BD	Zone A	Zone B
		0	0
	No. births	4	66

Assumed BD incidence levels (×100)	Zone A		Zone B	
	No. cases of BD expected	Probability of no BD	No. cases of BD expected	Probability of no BD
2.4	0.096	0.907	1.6	0.201
5.0	0.2	0.815	3.3	0.034
10.0	0.4	0.660	6.6	0.001
20.0	0.8	0.410	13.2	4×10^{-7}
50.0	2.0	0.060		

Therefore, in spite of the low number of births in zone B, among which no BD's have occurred, it can be concluded that the incidence in zone B is not higher than in zone R. Nothing can be said for zone A, with only 4 births, except that, should an increase have taken place, this would have not been higher than 20% !

In summary it has been noted that the lack of increase in BD's in the area could be due to the fact that several women, fearing the fate, underwent a kind of "therapeutic" abortion; nothing can be said for the ones who aborted secretly or abroad. But for the 30 official abortions, which were all studied by Prof. Gropp, 4 were women of zone A, three of them exposed as long as 14 days before evacuation. No malformations were found in their embryos (Rehder et al., 1978). Therefore, in spite of some incertitudes intrinsic to embryological diagnosis of BD's, it can be concluded that at least 8 pregnancies in zone A did not involve any BD.

IV. Spontaneous abortions (SA's)

To evaluate the possible effects of TCDD pullution on the frequency of SA's, two lines of investigations are being considered: the first one consists of the registration of SA's reported by physicians to County Medical Officer; the second one integrates the notifications with a special search on admission/discharge hospital forms. The first study allows comparisons with regional and national statistics, when available, while the second one is an *ad hoc* project.

In Table XI SA rates per 100 pregnancies per year are shown. Rates are slightly fluctuating from 1973 to 1977. In 1977, rates are always higher than in 1976: neverthe-

Table XI

Spontaneous abortion rates (×100 pregnancies) from 1973 to 1977

Years / Cities	1973	1974	1975	1976	1977
Cesano M. Seveso	9.55	9.76	10.04	10.13	10.19
Desio Meda	12.34	10.57	10.81	9.01	12.31
Total 4 cities	10.91	10.15	10.45	9.54	11.38
Other 7 cities	10.88	10.90	9.64	10.11	11.81
Total 11 cities	10.89	10.55	10.01	9.74	11.63

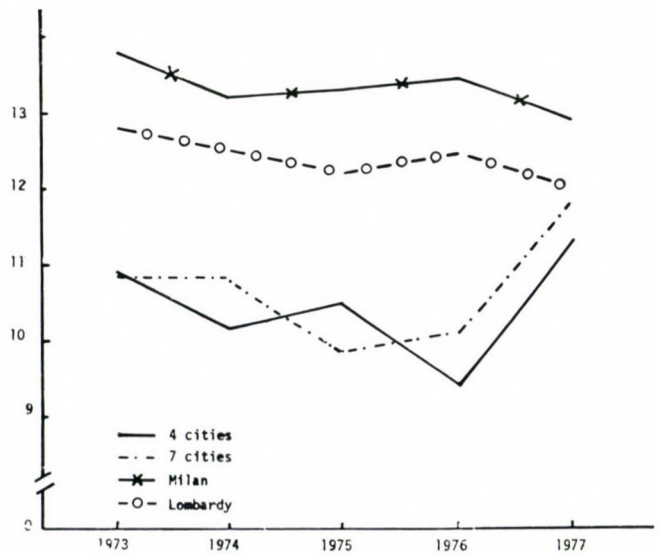

Fig. 2. Abortion rates (×100 pregnancies) from 1973 to 1977

less, they are lower or rather similar to the ones of previous years. The rise is not easy to explain. It could be affected by improved physicians' care in the reporting. In fact, such a rise is common in all the 11 cities and it is not a very large one.

Figure 2 compares polluted areas with those in Milano and Lombardy. The trend of polluted area rates is very similar to Milano and Lombardy rates, which are always higher, until 1976. In 1977 there is a rise in the polluted area rates, but even the improvement of SA notifications does not indicate that the rate reaches the level of Milano and Lombardy.

As to the second line of investigation, the aim is to refer SA's to total conceivements of the same period.

SA's of a given trimester, collected by the County Medical Officer, and the hospital forms constitute the numerator. At the denominator there are the same SA's plus births and stillbirths with the same concievement time.

$$\text{Pregnancy loss rate (PLR)} = \frac{\text{Abortions } (J_a + F + M)}{\text{Births} + \text{Stillbirths}\,(J_u + A + S) + \text{Abortions}\,(J_a + F + M)} \times 100$$

Data on births and stillbirths are collected by Census Bureau and Hospital forms. Induced abortions were excluded both from numerator and denominator.

Such a system of collecting data became possibly only after July 10 1976, when ICMESA accident happened: in fact, after this date, hospital forms related to people living in the polluted area were separately filed from all the others. Data were processed by hand because in the regional computer, working then for administrative rather than for epidemiological purposes, data were fed in without names.

Table XII shows PLR by trimester from July 1976 to December 1977. In the cities with more than 50% of inhabitants in the polluted area, the rates show a rise in the 4th trimester of 1976; then they decrease in the following trimesters. In the cities with about 20% of inhabitants in the polluted area, the rates are increasing until the first trimester 1977; then they decrease. A similar trend is visible in the other 7 cities.

Table XII

Pregnancy loss rates (PLR) by trimester from July 1976 to December 1977

Cities		Jul.–Sep. 1976	Oct.–Dec. 1976	Jan.–Mar. 1977	Apr.–Jun. 1977	Jul.–Sep. 1977	Oct.–Dec. 1977
Cesano M. Seveso	A	27	30	21	21	24	25
	P	164	141	188	171	186	186
	R	16.5	21.3	11.2	12.3	12.9	13.4
Desio Meda	A	18	26	33	27	25	32
	P	200	187	208	197	198	237
	R	9.0	13.9	15.9	13.7	12.6	13.5
Other 7 cities	A	50	58	84	59	44	66
	P	427	414	462	418	423	443
	R	11.7	14.0	18.2	14.1	10.4	14.8

A = abortions; P = preguancies; R = PLR

Such a trend is very hard to understand, because data before the accident are not available. It will be difficult to obtain these data because of hospital form information system criteria. Moreover, should difficulties be overcome, the earliest comparison period would go back no further than January 1976, the date of the beginning of routine hospital form collection in Lombardy.

A more satisfactory definition of exposure levels is in progress. Table XIII shows PLR by pollution area (zone B, R and other zones): zone A data were excluded because of the small number of pregnancies. PLR for zone B are always higher than in other zones. Only in the third trimester of 1977 rates show statistically significant differences ($P < 0.005$). Nevertheless, such a difference does not exist among various rates in zone B.

Table XIII

Pregnancy loss rates (PLR) by trimester from July 1976 to December 1977

Cities		Jul.–Sep. 1976	Oct.–Dec. 1976	Jan.–Mar. 1977	Apr.–Jun. 1977	Jul.–Sep. 1977	Oct.–Dec. 1977
Zone B	A	3	4	5	8	10	4
	P	27	18	29	28	32	29
	R	11.1	22.2	17.2	28.5	31.2	13.7
Zone R	A	19	17	15	17	16	20
	P	138	104	118	135	140	144
	R	13.7	16.3	12.7	12.5	11.4	13.8
3 C.S.Z. $\bar{S}$ A,B,R	A	74	94	119	81	67	99
	P	670	632	713	621	634	691
	R	11.0	14.8	16.6	13.0	10.5	14.3

A = abortions; P = pregnancies; R = PLR

V. Crude birth and death rates (BR, DR)

Data on births and deaths were provided by the Census Bureau. BR and DR were calculated per year and per thousand inhabitants. Table XIV shows birth rates from 1973 to 1977: they are provided for 3 groups of cities differing by the amount of inhabitants in polluted areas, as previously shown. BR are generally decreasing from 1973 to 1977.

In Figure 3 such a decrease is quite evident until 1976, mainly in the 2 cities with more than 50% of inhabitants in polluted areas. A reduction of BR occurs with more evidence in 1977 in the 4 most polluted cities, probably because of birth control which authorities advised for about one year since July 1976.

Figure 4 shows BR by 4 cities, 7 cities, Lombary and Italy. In the polluted area BR are generally higher than in Italy and in Lombardy. The most interesting phenomenon can be seen in 1977 when BR for the more polluted area has a steeper decrease in comparison with previous years.

About mortality, we can provide only crude DR. It has been impossible so far to calculate specific DR per cause of death, age and sex because of the lack of information on

Table XIV

Birth rates (×1000) from 1973 to 1977

Cities \ Years	1973	1974	1975	1976	1977
Cesano M. Seveso	18.78	17.51	14.86	13.62	10.94
Desio Meda	17.44	16.63	16.54	15.77	13.20
Total 4 cities	18.21	17.07	15.71	15.36	12.10
Other 7 cities	17.68	17.15	16.68	14.14	13.22
Total 11 cities	17.93	17.11	16.22	14.72	12.66

this matter. Nevertheless, we are studying a specific research program to obtain DR per age and sex, while rates per cause of death will be possible only from now onward.

Table XV shows crude DR per year for the 3 groups of cities. We can observe differences in the DR trend at Cesano and Seveso which have more than 50% of inhabitants in the polluted area. Rates are increasing at Cesano until 1975, in contrast to this, they are decreasing in 1976—77. At Seveso MR decreases until 1975 and has a rise in 1976; then it decreases again in 1977. In the cities with about 20% of inhabitants in the polluted area (Desio and Meda) rates are irregular with little variations. In the other 7 cities crude DR are stable except a rise in 1976.

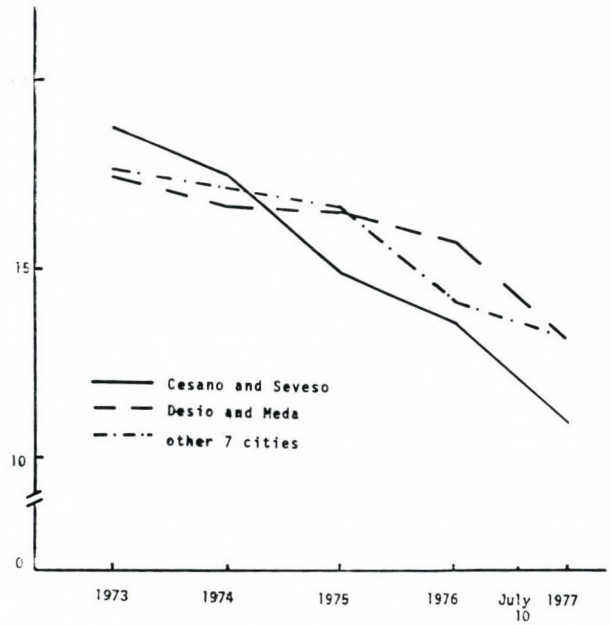

Fig. 3. Birth rates (×1000) from 1973 to 1977

Table XV

Crude mortality rates (×1000) from 1973 to 1977

Cities \ Years	1973	1974	1975	1976	1977
Cesano M.	6.28	6.65	7.13	7.07	6.51
Seveso	9.18	7.50	6.83	9.55	7.72
Desio	8.92	8.27	8.86	9.05	7.69
Meda	8.48	7.72	8.33	8.25	8.71
Total 4 cities	8.00	7.51	7.86	8.33	7.51
Other 7 cities	7.91	7.94	7.80	8.36	7.69
Total 11 cities	7.95	7.73	7.83	8.35	7.61

Figure 5 shows the same data for the 4 cities and the 7 cities: an increasing DR is registered in 1976. Because of this rise we suspected a possible association with the TCDD pollution. Nevertheless, a six-months period disaggregation of DR in 1976 (Table XVI) shows that rates are generally higher in the first half-year, before the July 10 accident. This phenomenon occurs in all the 11 cities and particularly in Seveso. The same phenomenon appears also in 1977, except in Seveso where the rate is higher in the second half-year.

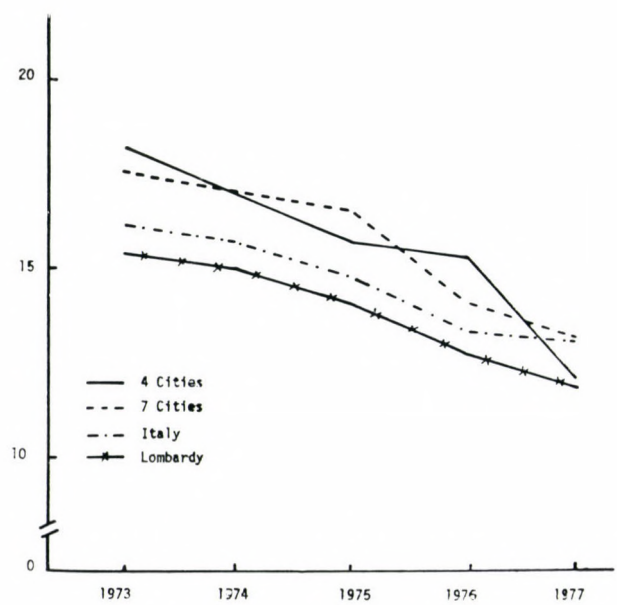

Fig. 4. Birth rates (×1000) from 1973 to 1977

Table XVI

Crude mortality rates (×1000) by six-months periods in 1976 and 1977

Years / Cities	1976			1977		
	Jan.–Jun.	Jul.–Dec.	Jan.–Dec.	Jan.–Jun.	Jul.–Dec.	Jan.–Dec
Cesano M.	3.64	3.43	7.07	3.57	2.94	6.51
Seveso	5.66	3.89	9.55	3.65	4.06	7.72
Desio	5.16	3.88	9.05	4.14	3.54	7.69
Meda	4.38	3.86	8.25	4.43	4.28	8.71
Total 4 cities	4.60	3.73	8.33	3.93	3.58	7.51
Other 7 cities	4.45	3.91	8.36	3.88	3.81	7.69
Total 11 cities	4.52	3.83	8.35	3.91	3.70	7.61

Figure 6 shows crude DR for the 4 and 7 cities vs Italy and Lombardy. DR in Italy and Lombardy are higher than in the polluted area. Moreover, Lombardy shows the same very little rise of crude MR observed in the polluted area.

In summary, birth rates in the area show a steeper decrease in 1977 probably due to birth control, and crude DR do not seem to have been affected by the TCDD pollution as yet.

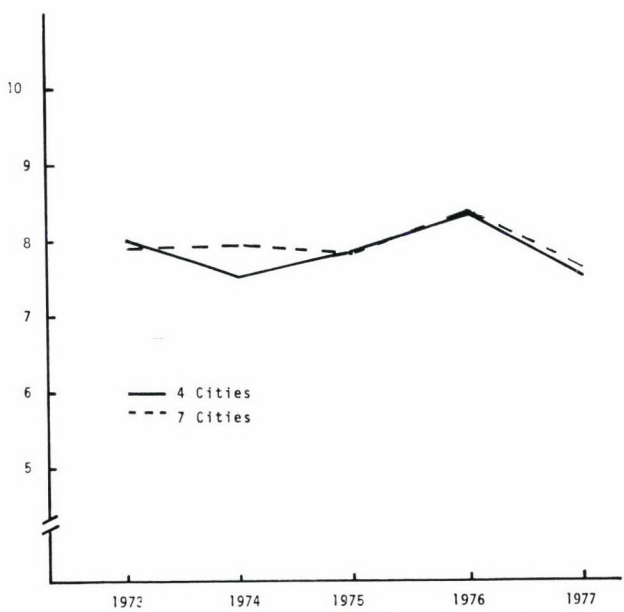

Fig. 5. Crude mortality rates (×1000) from 1973 to 1977

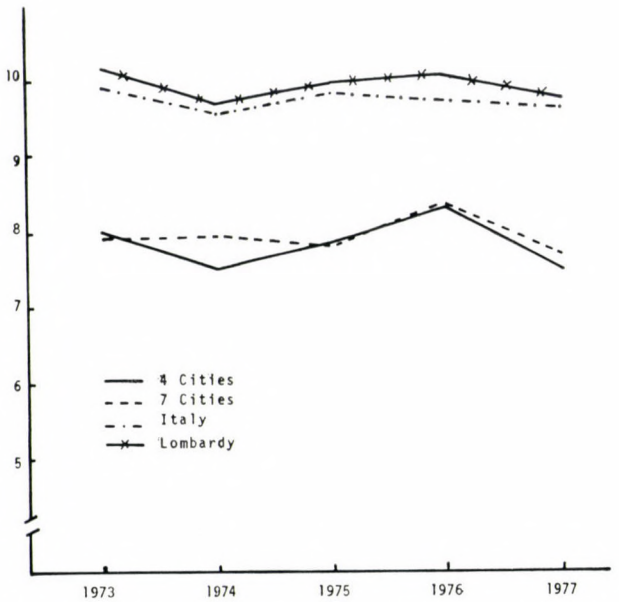

Fig. 6. Crude mortality rates ($\times 1000$) from 1973 to 1977

VI. Specific research programs

Specific research programs dealing with immunology, neurology and cytogenetics are being developed by *ad hoc* teams, and only progress reports have been released and will be quoted.

I m m u n o l o g y: 45 children previously living in zone A, 21 of them with chloracne, of the "early" type, together with as many age-matched unexposed controls, were submitted to a battery of tests to investigate their immune response. This was started in September 1976, and tests were repeated 3 times at 4 months intervals. According to a statistical evaluation of the results, no significant differences between exposed children and controls could be demonstrated (Sirchia, 1978).

N e u r o l o g y: instrumental examination of groups of persons have shown higher rates of subclinical neurological damages in zone A than in B and R, i.e. reduced nerve conduction velocity, which could not be related to any known cause; this was confirmed also during another screening in 1978. No association exists between the occurrence of the skin lesions and the neurological findings. A careful evaluation of these findings is in progress (Boeri et al., 1978).

C y t o g e n i c t e s t s: they were conducted on peripheral blood lymphocytes stimulated by PHA and obtained from 125 people of zone A, 59 ICMESA workers and 69 controls. Frequency of gaps, breaks and structural rearrangements did not differ greatly from group to group, neither was there a significant difference between people with and without chloracne. Nevertheless, a higher dispersion of total cell aberration frequency was observed in the ICMESA workers, zone A and chloracne people (Morganti et al., 1978).

Neurological, immunologic and cytogenic studies are still in progress to check whether any alteration will appear in the long run.

Conclusions

This paper represents the first report on selected topics prepared by the working team appointed by the regional government of Lombardy. The joint efforts, started at the end of July 1976, and based on a plan prepared to cope not only with the need of epidemiological knowledge but also with the urgent demand for sanitary assistance, help and prevention emerging from the population involved, have immediately met with great difficulties.

Obstacles were many and great, beginning with the availability of poor quality demographic data and with the lack of a local public health organization; progressing with the sudden political struggle in favour and against the abortion policy to be applied to local women, and with a lot of nonsense solutions proposed by many soi-disant scientists, also from abroad, in order to terminate the pollution; and ending up with the progressively spreading resistance of the population to cooperate in the surveillance plan.

Therefore not all data are available which could help in preparing a final picture of all the health events which followed the 10 of July 1976 accident. Part of them are missing forever, others could be collected and accumulated, but their elaboration was delayed because of lack of operators and call for more urgent activities.

Systematic analysis and elaboration of available data could only start at the beginning of 1978, when the working team in Seveso was completed.

Interpretation of data presented here does not seem to indicate that any major event has happened in the field of birth defects, abortions, births and deaths (crude data.) It is difficult to say whether this has been attributable to original limitations of TCDD exposure or to the efficacy of health measures immediately including the evacuation of zone A residents. As far as chloracne is concerned most all serious cases ("early" cases) were detected in people of zone A (or living there during the accident); in spite of the general recommendation to call the physician, no case was reported in zone B and only one in zone R, who had a history of contact with zone A. "Late" cases (by far the least serious) were mostly detected through screening, but their distribution is not confined to zones A or B: it covers also zones R and non (A, B, R), with higher prevalence in zones R of Seveso and Meda, closest to zone A, than in zone B. Such lack of overlapping of the chloracne map with the map of TCDD in soil is now under consideration, employing the exposure questionnaires administered to the chloracne patients. Apart from the evidence that the Seveso and Meda residents of zone R, during the days after the accident and before its evacuation, had easy access to and contact with the area classified later as A, a possibility exists that the 10 of July cloud has involved the atmosphere of a larger area around the factory than is now suspected according to the detection of TCDD deposited on the ground. This will only be clarified after the ICMESA reactor, now under sequestration by the Court, will be studied for its contents.

Additional studies will be needed to clarify the significance of atrophodermy and other lesions resembling healed chloracne which were diagnosed and classified as dating back earlier than July 10.

Additional data will also be needed to interpret the functional indications of neurological involvement found with higher prevalence in zone A residents than in other groups; and to explain their clinical significance and the apparent lack of correlation with chloracne lesions.

Finally, although the immunological and cytogenetic studies, carried on subjects both with and without chloracne, have not shown any significant alteration so far, there is a wide agreement as to the opportunity of their continuation, because it cannot be excluded that some abnormality could appear in the long run.

Summary

Provisional data on selected sanitary events which took place at Seveso after July 10 1976 are reported. 187 cases of chloracne, mostly in children, were detected, 50 just after the accident, the others within a year. Most polluted area (zone A) provided almost all "early" and most severe cases, but the territorial distribution of chloracne prevalence rates showed some inconsistencies with the soil TCDD pollution map; interpretations for such findings are discussed. Thirty-eight birth defects were detected in 1977 (none in zones A and B), more than in previous years, but still less than expected in a well controlled "normal" population: no clustering around a given type was observed. Spontaneous abortions, evaluated both as abortion rates and as pregnancy loss rates, showed scattered and statistically non-significant variations, inconsistent with the pollution map. No differences in birth and death rates compared to surrounding areas were observed. Data on ad hoc cytogenetic, neurological and immunological surveys are commented. Limitations of the presently available data are discussed and further research lines are anticipated.

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STUDIES ON THE TERATOGENICITY OF PVC

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Vinyl chloride (VC) is ranking 23rd among the 50 most widely used industrial chemicals, and has an annual production of approximately 8 billion kgs in the world (Reynolds et al., 1975). To give an idea of the considerable industrial, economic and social importance of polyvinyl chloride (PVC) manufactured from VC I quote from the Chem. Eng. News in 1975: "If the polyvinyl chloride products industry were to be shut down before substitutes could be found and their manufacture begun, perhaps 2 million jobs and 75 billion dollars worth of production would be lost" (Boden, 1976).

The importance of the chemical explains that once the suspicion of the oncogenic effect of VC — thought of as an inert gas sofar — was established, a series of studies were started on the carcinogenic, mutagenic and teratogenic effects of VC. Viola et al. (1971), then Maltoni (1973), Maltoni and Lefemine (1974, 1975) confirmed the oncogenic effects of VC in mice, rats and hamsters, by showing that VC exposure induced malignant neoplasms in different tissues in a dose-dependent fashion, more or less often, sooner or later after exposure. Malignant tumours could be found in any of the organs but most of them were seen in the liver, brain and lymphatic nodes. It was recognized that the VC-induced liver tumours in experimental animals have the structure of an angiosarcoma. The first human case of angiosarcoma with a probable causal relationship with VC exposure was found in 1973 (Creech and Johnson, 1974) suggesting an increased incidence in VC workers of the angiosarcoma of the liver — an otherwise rare tumour in men. The internal report of the WHO's International Agency for Research on Cancer gave account of 14 VC-induced liver angiosarcoma cases in June 1974, while 43 cases were reported on in the 1975 January issue. The increased incidence of VC-induced angiosarcoma of the liver in men has been confirmed by a number of studies (Thomas and Popper, 1975; Makk et al., 1976; Smith et al., 1976). A higher incidence of other tumours than in control populations has also been reported (Tabershaw and Gaffey, 1974; Lange et al., 1975; Infante et al., 1976c; Waxweiler et al., 1976).

The finding of the oncogenic effect of VC was shortly followed by reports on the mutagenic effects of its metabolites chloroethylene oxide and chloroacetaldehyde in microbial assay systems (Rannug et al., 1974; Bartsch et al., 1975; Malaveille et al., 1975; Loprieno et al., 1976) and on mammalian cells (Huberman et al., 1975). At the same time an increased incidence of chromosome aberrations in PVC workers was described simultaneously in several countries (Ducatman et al., 1975; Purchase et al., 1975; Funes-Cravioto et al., 1976; Hausteen et al., 1976; Szentesi et al., 1976).

These findings led to studies on the possible teratogenic and embryotoxic effects of the agent. Studies in human epidemiology were reported by Infante et al. in 1976 (a, b, c, d). They followed the incidence of birth defects from 1954 in 3 Ohio communities, with 12—24 000 inhabitants, where PVC polymerization plants are located. In one of them — Painesville — there are 2 plants; the second began operation in 1967. The incidence of malignant tumours of the CNS and lymphatic system in the 3 communities between 1958 and 1973 was significantly higher than that in the entire Ohio State. Similarly the incidence of congenital defects and malformations in the 3 communities was significantly above that in Ohio. Malformations of the CNS, lip and palate clefts, abnormal genital organs and clubfoot were leading but the number of other defects was increased too. With knowledge of the data reported by Infante et al. (1976a, b, c, d) Edmonds et al. (1975) reinvestigated the question. They compared the incidence of conatal CNS malformations in two hospitals located in cities with PVC poly-

merization plants, one in Pennsylvania, the other in Painesville, Ohio (the one studied by Infante et al.). No increase in CNS malformations was seen in the Pennsylvania hospital, but an increase was noted in the Painesville hospital. A detailed analysis of the Painesville data revealed that there was no correlation between the increase in CNS malformation rate and VC exposure. None of the parents of the affected infants had ever worked at the VC polymerization plants at Painesville and control mothers worked closer to the plant than those of the affected infants. In Hungary Czeizel et al. (1978) studied the incidence of congenital malformations among the offspring of 86 workers (62 males, 24 females) employed in VC production and polymerization in the Borsodi Chemical Works at Kazincbarcika, Hungary, with the interview-questionnaire method. They found one case of spina bifida among 3 cases of malformations.

Recently Edmonds et al. (1978) published the results of a new epidemiological study. For the period between 1970 and 1974 they found an increase in CNS malformations in Charleston, Kanawha County, West Virginia, where a PVC manufacturing plant is working. They studied the relationship of CNS defects and occupational or residential VC exposure of the parents. The incidence of malformations and the occupation of the parents were not related. There was an accumulation of cases at one location, but the increased VC contamination of the place could not be proved. (This observation did not entirely correlate with existing data on local patterns of wind direction and air pollution.) No correlation between the annual change of incidence of CNS malformations and the amount of annual VCM emissions was found.

Another potential noxious effect of VC exposure on the offspring is the increase in intrauterine mortality and stillbirth ratio. Based on an interview-questionnaire investigation including all polymerization workers Infante et al. (1976c) calculated the fetal mortality rates in the pregnancies of the wives. Prior to husbands' exposure, the lethal mortality rates for the control and study group were 6.9 and 6.1 per cent respectively, while subsequent to husbands' exposure the rates were 8.8 and 15.8, respectively. The difference is significant. A similar tendency was seen even when mothers with 2, 3 or 4, spontaneous abortions were not

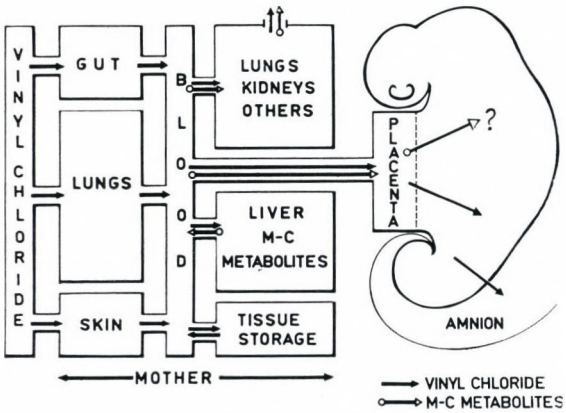

Fig. 1. Diagram of the maternal and fetal organisms exposed to VC (→). Through the lungs, gut and skin of the mother the VC is absorbed into the maternal blood stream; then VC crosses the placenta and enters the fetus as well as the amniotic fluid. In the maternal liver the VC is metabolized into mutagenic and carcinogenic metabolites (M-C, o→) — chloroethylene oxide and chloroacetaldehyde — which reach the fetus and are excreted by the maternal organism via the lungs, bile, kidneys. The VC concentration in the maternal as well as fetal blood samples depends on the atmospheric VC concentration inhaled by the pregnant mother. In spite of the fact that VC and its metabolites enter the fetus, no teratogenic effect can be observed

included. The authors suggest that the deleterious effect of VC exposition on the germ cells of the fathers is responsible for the increased fetal mortality.

Among wives of workers employed in VC production Selikoff (1974) found a fetal loss of 7—14 per 100 pregnancies, — a value higher than expected.

Czeizel et al. (1978) conducted a study in the Borsodi Chemical Works at Kazincbarcika, Hungary, where a VC as well as a PVC producing plant are operated. Fetal mortality was higher after, than before the start of employment (taken as the start of exposition). They found a twofold increase in spontaneous abortions and stillbirth rate among the wives of male workers and a 15-fold increase in the female workers. The increase in age alone does not explain the differences. The stillbirth rate and duration of exposure were correlated. In an earlier study at the same plant they found correlation between the frequency of chromosome aberrations and the duration of VC exposition (Szentesi et al., 1976).

Purchase et al. (1975) and Anderson et al. (1976) studied the VC for dominant lethal effects in male CD-1 fertile mice. No mutagenic effects of VC exposure on any maturation stages of spermatogenesis in the treated males were detected. No increase was found in postimplantation and early fetal deaths, in preimpantation egg losses and no reduction was found in fertility, i.e. VC did not produce dominlant lethal effect in mice. Short et al. (1977) reported on a similar finding with CD male rats — where also no change in postimplantation fetal losses were noted.

The mutagenic effect of VC exposure of the *Drosophila* species was studied by Verburgt and Vogel (1977). The dominant lethal test gave negative, while the recessive lethal test in short and long term experiments gave positive results. They argued that the point mutations are brought about at lower, while chromosome breaks only at higher levels of exposure to the mutagen.

In an experiment by John et al. (1977) pregnant CF-1 mice were exposed to 50 or 500 ppm of VC and pregnant SD rats and NZ white rabbits were exposed to 500 or 2500 ppm of VC for 7 hours a day during the entire period of organogenesis. Other groups exposed to the above VC concentrations for the same period were given ethanol in their drinking water. In spite of the toxic effects on the mothers produced by the exposure to higher concentrations of VC, no sign of embryonic or fetal toxic or teratogenic effects were seen in any of the three species. Alcohol treatment brought about a substantial increase in maternal and a slight fetal toxicity, but no teratogenic effect was observed after combined VC and alcohol treatment.

Our results can be summarized as follows. Pregnant CFLP mice were exposed to VC at 1000 ppm concentration for 2 hours, four times a day, for the entire period of organogenesis. An increase in fetal losses was found, but no teratogenic effect could be observed.

When pregnant CFY rats were inhaling VC for 24 hours a daye during the entire perio of organogenesis at 1000 ppm concentration, an increase in relativ reweight of the maternal liver, indicating maternal toxicity was found, but no embryotoxic orateratogenic effects were observed (Ungváry et al., 1977). A similar VC exposure of pregnant ru ts during the last third of pregnancy had no effect on the offspring; when a similar exposaın was applied during the first third of pregnancy an increase in fetal mortality watunoted as well as an embryotoxic effect indicated by the weight retard ation of the feses. When a group of pregnant rats was exposed to 1000 ppm of VC during the 1—9 days of pregnancy, but was given trypan blue injections on the 7th and 8th days of gestation, the trypan blue induced increase in fetal losses and incidence of malformations were not potentiated by VC exposure (Ungváry et al., 1978). For 3 weeks before mating and during the entire pregnancy a group of rats kept on DeCarli and Lieber's alcoholic liquid diet was exposed to 1000 ppm of VC continuously for 24 hours a day, during the neurulation. No congenital malformation was seen in the offspring, but an increase in the incidence of skeletal retardation cases was found after the combined VC ethanol treatment.

One could say, that epidemiological studies in men have caused considerable concern, but that these concerns in some way are counterbalanced by the negative results of experimental studies. *One may well seek the difference between fetal losses and congenital malformations.* Most of the epidemiological studies in men including those of Edmonds et al. (1978), indicate that VC does not possess a teratogenic effect. Animal studies in three species argue also against the teratogenic effect of the agent (John et al., 1977; Ungváry et al., 1977, 1978). Though the straight extrapolation of the results from animal studies in experimental teratology to the human conditions raises considerable difficulties, careful consideration of the experimental design might substantially reduce these difficulties. One may recall, that the animal studies of Maltoni and Lefemine (1974) revealed not only the oncogenic effect of VC exposure, but indicated the induction of a particular tumor, hemangiosarcoma of the liver. Indeed, the carcinogenic property of VC in men has just been identified by the finding of the hemangiosarcoma of the liver, a very rare tumour in the human.

Moreover, considerable amounts of VC cross the placenta. The VC concentrations of the maternal as well as embryonic blood samples are correlated with the atmospheric VC concentration inhaled by the pregnant mother. VC can be found also in the amniotic fluid (Ungváry et al., 1977, 1978). Although thus far no correlation has been shown between the mutagenic and oncogenic VC metabolite concentration of maternal origin in the maternal and fetal blood (Fig. 1), it is known that VC exposure of the mother may induce transplacental tumours though these metabolites (Maltoni, 1975). Taking our finding into consideration, that VC exposure of pregnant rats for 24 hours a day, during the entire period of organogenesis did not result in an increased incidence of congenital malformations, *one may conclude, that the VC and its metabolites do not possess a teratogenic effect, or if they do, they do not reach the effective teratogenic concentration even in the case of continuous exposure of the mothers at high atmospheric concentration.*

It is different, however, with the fetal losses. The data of Selikoff (1974), Infante et al. (1976a, b, c, d) and Czeizel et al. (1978) leave no doubt about the increase in fetal losses subsequent to VC exposure. Still no positive response was seen in dominant lethal tests in mice, rats and the *Drosophila* species. The finding of Czeizel et al. (1978) of an increased fetal mortality in the pregnancies of female VC polymerization workers is in good agreement with our finding of increased fetal losses seen after the continuous exposure of rats to VC during the first 3rd of pregnancy, or after a frequent exposure of pregnant mice to the agent during organogenesis (Ungváry et al., 1978).

In summary, one may conclude, that the substance VC of high industrial and socioeconomic importance is most probably not a teratogenic agent. Taking, however, its carcinogenic and mutagenic effects into consideration the lack of teratogenicity does not reduce the concern raised by VC for the industrial hygiene. *The increase in embryonic and fetal mortality after exposure of the parents is a further hazard.* Since the induction of carcinogenic as well as mutagenic effects are most probably highly dependent on the duration and the level of exposure (Maltoni and Lefemine, 1974, 1975, Szentesi et al. 1976, Waxweiler et al., 1976, Picciano et al., 1977), the reduction of occupational exposure might considerably contribute to reduction of the hazards of VC exposure. The enormous increase in fetal mortality in the human and the finding of a high incidence of fetal losses after VC exposure of rats and mice indicate that with women in the fertile age a further hazard of VC exposition on intrauterine fetal life must be taken into account.

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INTRODUCTION TO SYMPOSIUM ON EPIDEMIOLOGY IN TERATOLOGY

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Epidemiology is defined in the shorter Oxford English Dictionary as "that branch of medicine which treats epidemics" and an epidemic is defined as a disease "prevalent among a people or a community at a special time and produced by some special causes not generally present in the affected locality". It is usual to consider "epidemic" disease as superimposed on "endemic diseases" which may be defined as "due to permanent local causes" and "peculiar to a people or to a district . . .".

The first step in epidemiology might therefore be said to be the establishing of normal levels of the various congenital malformations which occur. As systems for recording malformed children at birth have been set up in various parts of the world it has become apparent that many malformations occur more frequently than others and there is surprisingly little fluctuation in numbers of malformations reported for large groups of births. This indicates that the malformations which are being counted are probably occurring at a fairly steady rate in the observed population of births.

The second step is the establishing of an epidemic. This involves validating that any increase in reporting is in fact due to new cases and not merely due to the better reporting of cases which have formerly not been discovered in the population.

Further stages in an epidemiologist's work consist of observing the factors occurring in and around the affected population to see if any group of factors occur more commonly among persons affected with the epidemic disease than among unaffected persons.

Finally when associated factors have been displayed it is desirable to set up some system or experiment to validate the association and to establish a causal connection. Such an investigation might involve a search for other groups of births which may have had similar epidemics of the same disease. Or it may involve setting up an experiment where the factor or factors, suspected to predispose to the disease, are excluded from one section of the population. If the hypothesis is correct, the group excluded from the factor will not get the disease.

Various epidemiological techniques are used in studying teratogens in man. When planning to make observations, standard methods should be used which should not be affected by the particular outcome which is being observed. Suppose one plans to make an investigation about events during pregnancy which might cause malformations. If the history of events is obtained after the outcome of the pregnancy is known to the informant, one cannot eliminate the possibility that the response of the informant is biassed by the outcome. It will be evident that since one doesn't know in advance about which mothers will give birth to malformed infants, results for all mothers must be recorded during pregnancy in order to trap the near 5% of slightly and seriously malformed children. This may prove an expensive way of recording the data about the 5%. The method is a prospective study, and the whole of the data is analysed to study the distribution of all factors in the population studied.

A variation of this method is to record events for all pregnancies and their outcome but to process and analyse data after birth for only the malformed children and for matched or randomly selected control babies and their mothers. This is a more economic method, especially when the data recorded is needed for good care of the mother.

Further methods may involve matched pair studies: in which children with specified anomalies, are identified and a suitable control child for each malformed child is matched. Data is best collected retrospectively from records made routinely during the pregnancy for every mother. Such a method has been used in looking for effects of drugs prescribed in pregnancy, and is a variant on the routine recording of data method.

As the outcome of pregnancy is not usually known until after delivery or miscarriage, or sometimes following amniocentesis, it is of great saving if prospective studies can be conducted on a group of women who are likely to produce more than the normal 5% of malformed children. The identification of groups of such women who are at higher than normal risk allows the group to be used for testing hypotheses by intervention studies: exposure to a suspected teratogenic substance is avoided in one randomly selected mother of each pair of women during pregnancy. This method of identifying high risk groups has been successfully employed among women coming to genetic counselling units who identify themselves by so coming. There is little difference in this method from that used by Reed in identifying the vector of yellow fever when he shut himself up with sufferers of yellow fever in a mosquito free environment.

Sometimes an outbreak of an unusual malformation may allow an analysis of the circumstances relating to the mothers. This was done eventually for the mothers of infants with phocomelia and other major reduction deformities during the thalidomide epidemic, and is a method which has been used in classical epidemiology for many years. For example Snow used this method when he recognised water as the vector for epidemic cholera long before the cholera vibrio organism was demonstrated.

Epidemiologists concerned with human teratology are from time to time practising all these methods and I hope that the following lectures will illustrate some of these approaches.

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PROBLEMS IN CONDUCTING PROSPECTIVE SURVEYS

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The search for an association between etiological factors and diseases is one of the major purposes of analytic epidemiology. Prospective surveys or cohort surveys are especially designed to test the hypotheses developed by clinical observations or descriptive surveys.

Problems met in conducting cohort surveys on congenital malformations will be described according to our personal experience in conducting 2 French prospective surveys, the first one from 1963 to 1969, the second one from 1975 to 1977.

I. Objectives of the survey

Prospective surveys on congenital malformations were initiated in different countries in 1962, to study the prevalence of congenital malformations in relation with environmental experience of pregnant women: infectious diseases, drugs, physical hazards . . . Because of the teratological effect of thalidomide, the questions on drug usage were very detailed.

The first difficulties met in the first French survey (1963—1969) were in the *formulation of the hypothesis.* At that time, every drug was suspected; the clinical or epidemiological observations were limited to thalidomide and antitumor drugs; the animal experimentations were refuted by many specialists. So it was decided by our team to investigate every type of drug. The same decision was taken in other prospective surveys conducted in England and Scotland by the Royal College of General Practitioners, US Collaborative Perinatal Study, German prospective survey and the Swedish prospective survey.

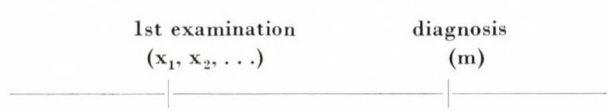
The same problem was met for infectious diseases and the women were interviewed about every disease, but serological examinations were limited to rubella, measles, influenza and to some others specific viruses.

In the second French prospective survey (1975—1977) the investigations were limited to a small number of drugs, after taking into consideration the results of our first survey and those of recent literature. We now think that it is necessary to focus the objectives of new prospective surveys on a precise hypothesis.

II. Problems in conducting the survey

1. *The choice of the population and the sampling methods*

A cohort survey begins with the observation of a sample to determine the distribution of etiological factors (x_1, x_2, . . .) and then exposed and non-exposed people are followed up to the diagnosis of the disease (m).



But there is a big difference between cohort surveys performed on a representative sample of the general population and those in which it is possible to identify a priori exposed people.

If the purpose of the survey is well limited, it is possible to use the second method: for example the follow-up study of epileptic women during pregnancy, the follow-up study of women receiving abdominal irradiation . . . In these cases the number of exposed people is generally small, but the major problem is the identification of a control group comparable with the exposed group.

In the first French survey, as in many other surveys, it was possible to identify exposed groups before the beginning of the survey, the purpose of the study being not limited. The only solution was to observe the general population of pregnant women or a more accessible part of the general population.

The first difficulty was the large number of persons who had to be followed to gather a sufficient number of malformed babies.

Our first survey was carried out in 12 University hospitals in Paris and involved 12,724 pregnant women registered during the 1st trimester of the pregnancy. The number of malformed infants at birth on a total of 12,895 infants (including twins) is indicated in Table I. In spite of the large number of cases included in the survey, the number of specific malformations was very low. The only solution is to include a larger number of pregnant women, but this is very expensive, or must be undertaken with international collaboration. This solution seems the best one, but it requires some fundamental conditions on the homogeneity of the investigations.

The second problem concerning the sampling methods is the choice of the population when it is not possible to involve the whole community. As explained, the French survey was made in University Hospitals in Paris. The choice was the same in USA, Federal Republic of Germany

Table I

Malformed infants registered at birth and type of malformations. First French Prospective Survey

Type of malformations	No. of cases	Prevalence % in the survey
Single malformations	153	1.19
Central nervous system defects	15	0.12
Congenital heart defects	30	0.23
Pharynx defects	1	0.01
Cleft lip and/or palate	13	0.10
Digestive system defects	12	0.09
Urinary system defects	2	0.02
Genital system defects	29	0.22
Skeletal defects	41	0.32
Skeletal muscle defects	7	0.05
Sense organ defects	3	0.02
Chromosomal anomalies	23	0.17
Multiple malformations	37	0.29
Hereditary malformations	4	0.03

and Sweden. In England and Scotland, it was possible to obtain the collaboration of general practitioners.

In our survey, the following criteria were required: the women lived in Paris or its suburbs, were French speaking and wished to cooperate. When it was not possible to interview all the women visiting the clinic, they were not randomized and we did not give any new criteria of selection, but we indicated precisely that the choice of women to interview had to be made before the beginning of the examination. The sample studied in our survey was not representative of pregnant women who consulted in University Hospitals. In this respect,

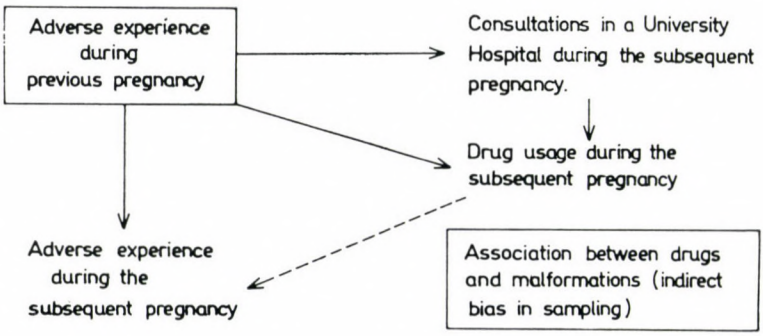


Fig. 1. Indirect bias in hospital selection

the prevalence of malformations and the rate of drugs used were not representative. But it is well known that in prospective surveys these biases are not so important in the study of association between etiological factors and disease, because the selection of the population is made before the diagnosis of the disease. Only indirect sampling errors can be found in this prospective survey: women with an adverse experience in previous pregnancies may more frequently attend University Hospitals in a subsequent pregnancy. If there was a relationship between previous adverse experience, drug usage and a subsequent adverse experience, it might have been possible to find an association between drug usage and malformation resulting of an indirect bias in the selection of cases (Fig. 1). In studying the correlation between phenothiazine and malformation, we have found 2 cases in which such an explanation could be proposed (Rumeau-Rouquette et al., 1977):

Case 1: during previous pregnancies were registered 2 premature children, 1 induced abortion, 1 spontaneous abortion and 1 extrauterine pregnancy; during the subsequent pregnancy the mother was very anxious and received chlorpromazine and pipamazine; she consulted with a University Hospital and was included in the survey. She delivered a malformed baby with endocardial fibroelastosis, brachymesophalangy and clinodactyly.

Case 2: in previous pregnancies were 2 microcephalies and 3 normal children; during the subsequent pregnancy the mother had a depression syndrome and received chlorpromazine and promethazine. The baby had microcephaly.

It is possible to eliminate this cause of error by taking into account the outcome of previous pregnancies by different methods of adjustment.

The third problem has been that people dropped out during the follow up. In our first survey (1963—1969) the initial sample included 18,275 pregnant women; 12,764 delivered in the Hospitals of the survey and 5511 women dropped out during the follow-up. In this last group, there was a significant number of women born in a foreign country, single women,

younger women, manual workers . . . This new selection was also made before the diagnosis of malformation and could not introduce a direct bias in the study of the correlation between environmental experiences and malformations.

To summarize: many prospective surveys have been done on self-selected groups; the self-selection is very dangerous in retrospective survey, because it is made after the diagnosis of the disease. It is less danegrous in prospective surveys, when it is made before the diagnosis of the disease.

2. *The collecting of data on exposure to risk factors*

a) *First interview*

The recording of data was very difficult in our study. Each woman was interviewed by a young physician appointed by the INSERM using a standardized questionnaire (Etienne et al., 1967). The interview took place during the 3rd month of the pregnancy and began with questions concerning the pregnancy. A detailed history of illness and drug use during the 3 months before and after the last menstrual period was obtained: after some general questions about the principal events, detailed questions were asked about morbidity (date of first symptoms, examinations, treatments, hospitalization . . .). Information about intake of drugs was completely recorded with a detailed description of the drug (name, description of the box . . .), of the duration and of the dose of the treatment. The description of the drugs was very useful to detect errors and to complete information. The great variety of drugs offered in our country helped us in this research.

The second part of the interview was related to previous pregnancies. No particular difficulty was encountered and it was possible to verify most of the information with official and medical records.

The third part of the questionnaire covered social aspects, alcohol consumption and smoking habits. This informations is often difficult to obtain, but after the detailed interview on the pregnancy, the women accepted willingly these questions.

Each interview required about one hour, included more than 200 items and involved about 30 punched cards.

The first difficulty was to find good interviewers, to educate them and to find money to pay them.

The second one was the attitude of pregnant women. Generally they did not refuse to answer and sometimes it was difficult to limit the time of the interview, because they were happy to speak of their problems of hygiene and benign diseases which do not generally interest medical doctors. But they had great difficulties to remember the name of some drugs and to give a good description.

The third difficulty was the detection and correction of errors. But it must be mentioned that in prospective surveys errors in the data on exposure cannot be biased by the diagnosis of the illness, this is a great advantage in comparison with retrospective surveys.

b) *Serological examination*

Blood samples were collected at 3, 6 and 8 months of pregnancy and the serological analyses were done after the birth of the malformed babies and a randomized control group.

It was very difficult to obtain the 3 blood samples for each woman involved in the survey. This was another selection of cases, but again it was made before the diagnosis of malformations.

c) *Other investigations*

In the first French survey, it was not possible to verify the drug usage by biological tests. Now, we begin to take blood samples when the women describe usage of some specific drugs such as diazepam and anticonvulsant drugs.

The blood samples are analysed by a laboratory which performs pharmacokinetic investigations. With this procedure, it was possible to verify the answers of the women.

3. *The examination of the newborn infants*

In our survey pediatricians of the INSERM examined the infants during the first five days of life, according to a standardized procedure. If there was a suspected malformation, further examinations were performed during the hospitalization. The pediatricians did not receive any information concerning the drug history obtained during the interviews in order to have unbiased records. This point is very important, because in prospective surveys *the diagnosis of the disease is made after the determination of the exposure and can be influenced by it.*

Another difficulty was the definition of malformations and the choice of the border line between minor and major malformations. Finally we decided to include in the analysis all clear-cut malformations defined as an abnormality of appearance or function evident at birth.

III. Problems in conducting statistical analysis

1. *Analysis of the association drugs/malformations*

a) *Strategy of the analysis*

If we consider the problem of drugs, a strategy had to be defined regarding the statistical analysis.

First, we decided to compare the infants with malformations to the control group, including all live born infants without any apparent or suspected malformations.

Secondly, the analysis proceeded in several stages. Drugs were classified in large groups such as phenothiazines, barbiturates, antibiotics . . . And we compared the proportion of malformed infants in the 2 groups of users and non-users during the 1st trimester of the pregnancy. Then these groups of drugs where subdivided into different chemical subgroups such as phenothiazines 3-carbon side chain, 2-carbon side chain . . . When the number of cases was sufficient it was possible to subdivide these subgroups and to consider each drug such as chlorpromazine or promethazine.

The third problem concerned significance tests (Rumeau-Rouquette et al., 1978). Generally one is interested in "null" hypothesis (H_0): if P_1 = probability to have a malformed baby in users and P_0 = probability to have a malformed baby in non-users in the total population.

1. $H_0 : P_1 = P_0$

If P_1 is estimated by p_1 determined on n_1 users,
if P_0 is estimated by p_0 determined on n_0 non-users,
one can use the test of significance calculating

2. $$\frac{p_1 - p_0}{\sqrt{\frac{p_1 q_1}{n_1} + \frac{p_0 q_0}{n_0}}}$$

* If the number of cases is large enough.

If (2) $\geq$ 1.96, the difference is significant at a 5 per cent level. We can reject the "null" hypothesis with a risk $\alpha = 5$ per cent to say that there is a difference between P_1 and P_0 when there is no difference.

If (2) $<$ 1.96 the difference is non significant, we cannot conclude that the drug is not teratogenic and we do not know the risk β to say that a drug is not teratogenic when it is.

This point is very important when we study the association between drugs and malformations, because we are interested in 2 conclusions:

1. The drug had a teratogenic effect: $P_1 > P_0$
2. The drug had no teratogenic effect: $P_1 < P_0$.

So we have taken as "null" hypothesis:

3. $H_0 : P_1 \leq P_0$

And for "alternative" hypothesis:

4. $H_1 : P_1 > P_0$

We have constructed a one sided significance test with a given risk α to conclude that the drug has a teratogenic effect when it has not, but when the test is not significant, it gives an estimation of the risk β conditionally to P_1/P_0 and the number of cases. Table II gives an example of the results.

b) *Results: association drugs-malformations*

In the first prospective survey, when one considers only the first stage of drug classification (largest groups), there were almost 25 tests of comparison and about 10 relative to the central nervous system; there were 2 significant associations, one for phenothazines ($0.001 < \alpha < 0.01$, Rumeau-Rouquette et al., 1977) the other for carbamates (Crombie et al., 1975). Table III indicates the results for groups of drugs taken by more than 30 women.

The first question is: are these results due to chance ?

According to the number of comparisons (less than 25 for this level) it is difficult to consider that the 2 significant differences ($\alpha = 0.01$ et $\alpha = 0.05$) are due to chance. We think that it is very important in these large prospective surveys to give the total number of comparisons at each stage of the analysis.

At the second stage of the analysis, the groups of drugs were subdivided in subgroups, the test significance were more numerous and the possibility of chance association could not be rejected.

The second question is: may the association be explained by maternal factors ?

The association may be related to the reason for which phenothiazines and carbamates were taken. An abnormal outcome of a previous pregnancy such as a malformed baby may

Table II

Outcome of pregnancies according to consumption of phenothiazines during the first trimester

Maternal history of phenothiazine consumption	Mothers with normal infants No.	Mothers with malformed infants		
		No.	%	
No phenothiazine	10,921	178	1.6	$\alpha < 0.05$
Chlorpromazine	53	4	7.0	
No phenothiazine	10,921	178	1.6	$\alpha > 0.05$
Trimeprazine	48	1	2.0	β see the note

* The risk $\beta < 10$ per cent if $P_1/P_0 > 8$

Table III

Results for groups of drugs taken by more than 30 pregnant women

Drug	Mothers with normal infants No.	Mothers with malformed infants		α	$\beta < 10\%$ if $P_1/P_0 =$
		No.	%		
Mothers not exposed	about 10,000		1.6		
Phenothiazines	304	11	3.5	0.01	
Butyrophenones	40	2	4.7		12
Carbamates	77	4	4.9	0.05	
Benzodiazepines	132	2	1.5		7
Amphetamines	42	1	2.2		8
Phenobarbital	362	11	3.0		3
Antiemetics: antihistamines	78	3	3.7		6
Progestatives derived from testosterone	31	2	6.1		12
Oestroprogestatives (testosterone derivative)	330	5	1.5		3
Progesterone	162	5	3.0		4
Progesterone derivative	237	6	2.5		4
Oestroprogestatives (progesterone derivative)	815	15	1.8		2.5
Oestrogens	78	3	3.7		6
Synthetic oestrogens	223	5	2.2		6
Corticoids	139	3	2.1		5
Penicillin	170	3	1.7		4
Tetracycline	161	4	2.4		4
Antibio-aminoglucosidics	69	1	1.4		7
Sulfonamides antimicrobial	182	3	1.6		4
Acetyl salicylic acid	2217	35	1.6		2

be a determinant of drug intake in a subsequent pregnancy; if this abnormal issue tends to increase the risk of another one, an association between drug usage and malformations may be found. Among the reasons to prescribe a phenothiazine or a carbamate are anxiety, insomnia, vomiting. We considered more specific cases with vaginal hemorrhage. This last symptom is related to malformation (C. Rumeau-Rouquette et al., 1971), and the association with a drug will appear more as a result than as a cause of a malformation. It would be necessary to take into account the factors related to malformation to compare the group of users and the group of non-users. This adjustment was made in the american collaborative survey, here the number of cases was not sufficient.

A third question relates to the association of drugs.

In our survey, women who received phenothiazine and carbamate received other drugs. For example one woman treated with phenothiazine was also treated with oxomemazine and hydrocortisone ointment and delivered a baby with cleft lip.

In this survey it was not possible to study the association of drugs and the possible teratogenic effect of their interactions.

A last question relates to the specific teratogenic effect of a given drug increasing the risk of a specific malformation. This has been proposed for thalidomide and anticonvulsants. In our

statistical analysis it was not possible to take into account the type of malformation; the number of significance tests would have been too important for the number of cases. We have only made the comparisons for some malformations (cleft lip, cardiac malformations), in relation to some drugs (anticonvulsants, corticoids, progestogen/oestrogen).

2. *Other analysis*

The cost-efficacy analysis of prospective surveys must be considered, since they are very expensive. We have refused therefore to limit our survey to the problem of congenital malformations.

An important part of the analysis was devoted to the study of prematurity, hypotrophy, mortality. We established a list of risk factors and a classification by different methods such as discriminant analysis. A special study was made for two important risk factors: tobacco and alcohol. There was no relationship with congenital malformations, but they increased the mortality rate (stillbirth and neonatal mortality) and the proportion of small for date babies.

We cannot enter into the details of these results, but we want to mention them, because they are sometimes more important in their practical application than results of malformations.

IV. Perspectives

It seems now necessary to focus the research on some specific aetiological factors. Regarding drugs, the last prospective and retrospective surveys have reported an increase in congenital malformations among children exposed in utero to drugs used for treatment of central nervous system diseases, namely anticonvulsants (Meadow, 1970; South, 1972; Speidel and Meadow, 1972; Fedrick, 1973; Millar and Nevin, 1973; Monson et al., 1973; Niswander and Wertelecki, 1973; Goujard et al., 1974), carbamates (Milkovich and van den Berg, 1974; Crombie et al., 1975), benzodiazepines (Milkovich and van den Berg, 1974; Safra and Oakley, 1975; Saxén, 1975), tricyclic antidepressants (Idänpään-Heikkilä and Saxén, 1973), amphetamines (Nora et al., 1970; Levin, 1971; Nelson and Forfar, 1971).

Progestagen/oestrogen drugs represent also a good way for focused research (Gal et al., 1967; Aarskog, 1971; Levy et al., 1973; Nora et al., 1973; Robertson, 1974; Harlap et al., 1975; Janerich et al., 1974; Heinonen et al., 1977).

Prospective surveys limited to a small number of drugs are easier. In our 2nd survey, it was possible to interview 4000 pregnant women in 2 hospitals during 2 years. This number of cases however was not sufficient to give conclusions. Our purpose was to complete the results obtained in the first prospective survey; they confirmed them for phenothiazines. There was also an excess of congenital malformations when the fetus was exposed to progestagen/oestrogen (testosterone derivative) at the beginning of the pregnancy.

Even if the research project is well focused, it is sometimes necessary to consider a great number of pregnancies, if the usage of the drug is very unfrequent as for tricyclic derivate; in this case an international cooperative survey is required.

It seems also necessary to complete the interviews with pharmacological investigations to verify the data and to understand possible aetiological mechanisms.

The last point is about the choice between prospective and retrospective surveys. Retrospective surveys are often less expensive but risks of errors in the sampling and in the collection of the data is larger, while in addition pharmacological investigations are not possible.

In spite of these problems, it is sometimes better to perform a retrospective survey. In two cases retrospective studies are favored:

— the first is the use of retrospective surveys to complete the data collected in malformation registers;

— the second concerns exposure to a rare factor for which one may have objective proofs such as prescription, medical records . . ., this is possible for many drugs (anticonvulsants, antidiabetic, neuroleptic . . .) and other factors as radiations . . .

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ARE CONTRACEPTIVE PILLS TERATOGENIC?

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Oral contraception is one of the most controversial topics of the second part of this century. As evidence it is enough to cite two sentences from the Editorials of the Lancet and the New England Journal of Medicine: "Posterity may decide that the greatest contribution of the twentieth century to the health of mankind was not a vaccine, an antibiotic or a transplant but the contraception pill" (Leader article, 1974) and "The pill is a double edged sword, it prevents ovulation but produces diseases" (Small, 1976).

Actually at present the pill is the most effective method of contraception having many advantageous and some disadvantageous effects (Fig. 1). Let us consider the *benefits* first: control of the population explosion, reliable family planning and unalloyed sexual life, improvement of e.g. menstrual disturbances, and reduced risk of e.g. benign breast tumor; the *risks* are: rare major adverse effects e.g. thromboembolic disorders, frequent side effects e.g. increased blood pressure, subjective symptome e.g. weight gain, and finally the potential hazard, that is teratogenic and mutagenic effects.

The conclusion seems to be obvious: the benefits are far exceeding the risks and the problem of a possible teratogenic effect is relatively small and dubious.

Results

In monitoring of congenital malformations in Hungary (Table I), we became interested in the possible teratogenic effect of the pill.

Table I

The number and occurrence of congenital limb reduction malformations (CLRMs) in Hungary, 1970—1975

Years	Number of total births	Number of CLRMs	Occurrence of CLRMs per 1000 total births
1970	153,339	34	0.22
1971	152,159	50	0.32
1972	154,652	48	0.31
1973	157,623	64	0.40
1974	187,957	79	0.42
1975	195,847	103	0.52

In 1973 and 1974 a somewhat higher incidence of congenital limb reduction malformations (CLRMs) was recorded than in the previous years. In 1975 a further increase was seen and the level reached the so-called "*alarm-situation*" (Czeizel and Pazonyi, 1976). The point prevalence at birth of CLRMs remained at the higher levels in 1976 and 1977. First of all the possible technical biases had to be excluded:

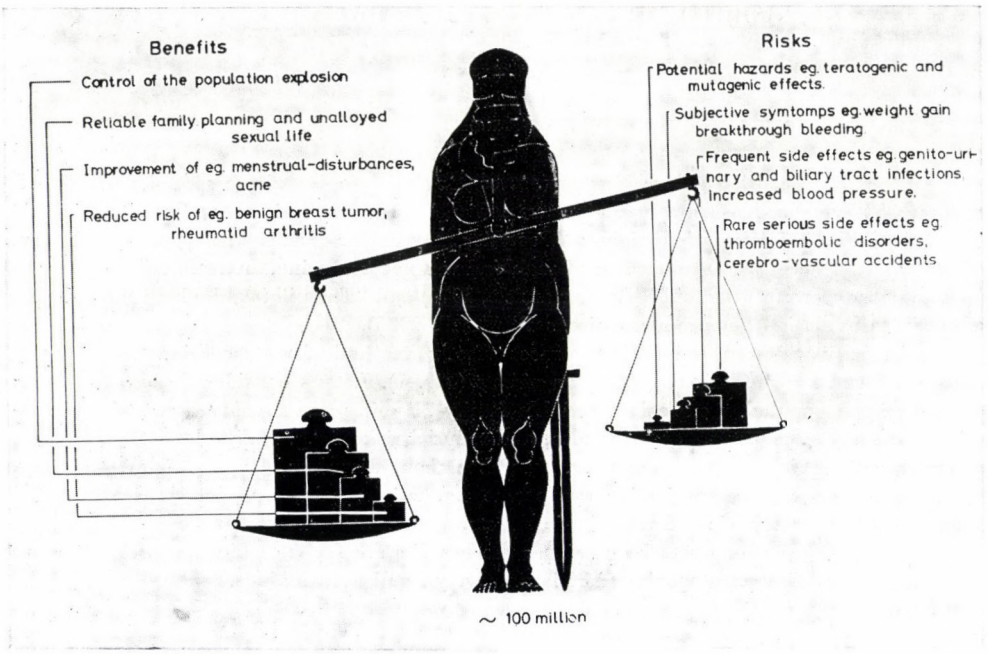


Fig. 1

(i) The cluster as a whole cannot be explained by a *better reporting system* (Fig. 2). In 1975 as compared to 1971—1974 the reporting of all congenital malformations increased by nearly 20 per cent, but the recorded CLRMs increased by about 50 per cent, that is more than twice that of all other congenital malformations. A similar increasing trend was not found for other types of congenital malformations.

THE ABSOLUTE NUMBER OF LIVE BIRTHS, INDUCED ABORTIONS, CONTRACEPTIVE PILL USERS (PACKAGES IN MILLIONS) AND CLRMs IN HUNGARY

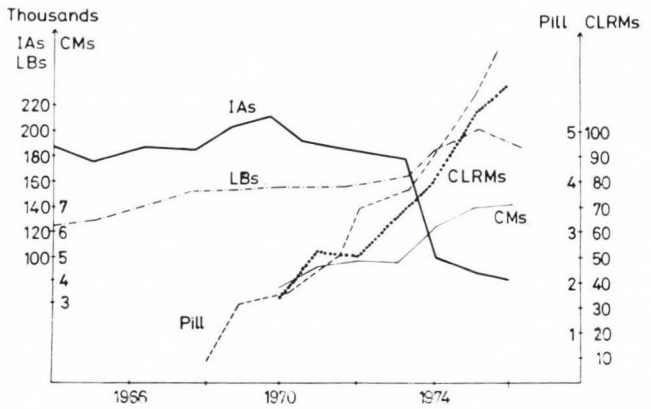


Fig. 2

(ii) The cluster cannot be ascribed to *improved diagnoses*. The per cent of mild cases was not higher in 1975 than in previous years and a great number of severe cases was found (Fig. 3).

(iii) The cluster cannot be explained by a change in the evaluation and registration of congenital malformations.

Checking personally on the reported cases born in 1975, 95 out of 103 patients (92.2 per cent) had CLRM (Czeizel, 1978). Our purpose was to study the etiology of these 95 cases

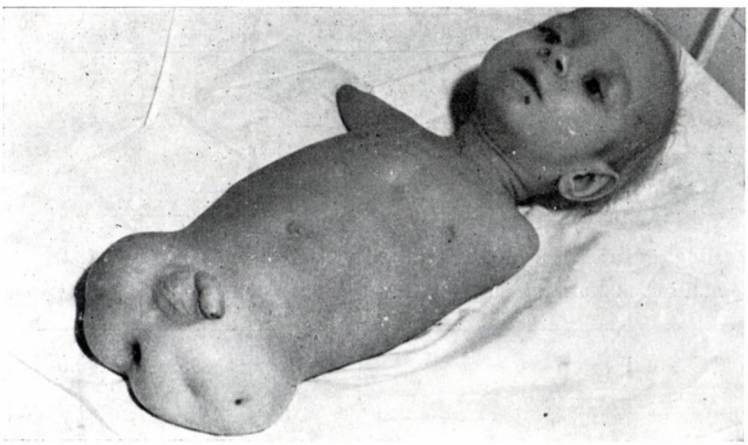
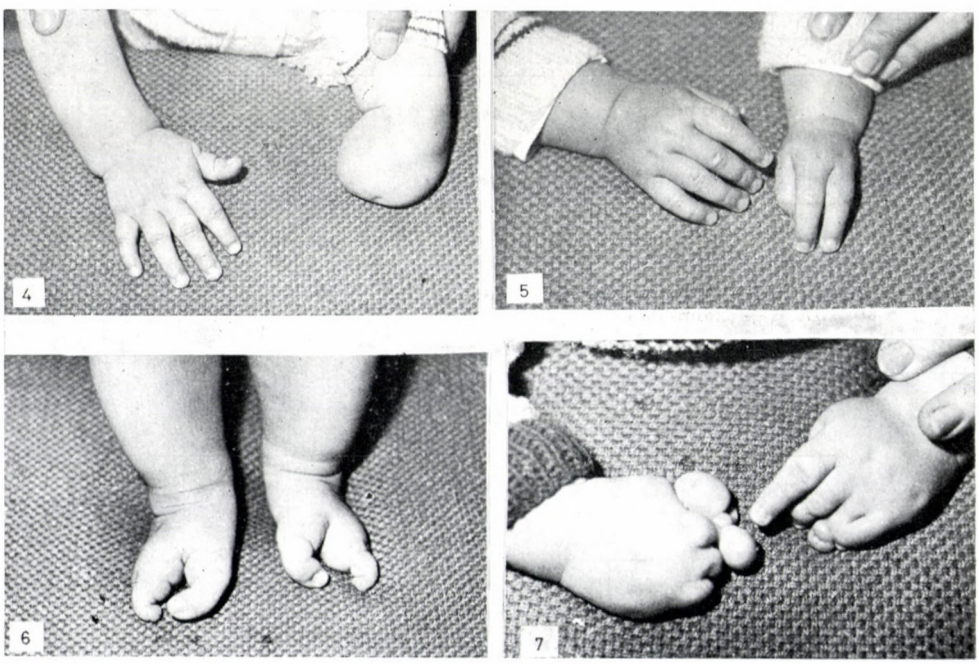


Fig. 3



Figs 4—7

by separating all cases in 5 probably different nosological types (Lenz, 1969; Tentamy and McKusick, 1969; Working Group, 1975; Bergsma and Lenz, 1977): 1. terminal transverse (Fig. 4), 2. longitudinal (Fig. 5), 3. split hand and/or foot (Fig. 6), 4. ring constriction (Fig. 7) groups (Table II) and those cases where the CLRM are associated with other non-limb malformations (Table III).

Table II

The different nosological types of CLRMs

Type	Abbreviation	Number	Per cent
Terminal transverse	T	29	30.5
Longitudinal	L	16	16.8
radial-tibial			
ulnar-fibular			
Split hand and/or foot	S	7	7.4
Ring constrictions	R	22	23.2
Multiple malformations	M	21	22.1
Total	CLRM	95	100.0

Table III

The distribution of multiple cases

Type	Number of cases
Holt–Oram syndrome	2
EEC syndrome	1
Roberts syndrome	1
FFU syndrome	1
VACTERL association	7
CLRM + neural tube defect	5
CLRM + exomphalos	1
CLRM + exomphalos + renal malformations*	1
CLRM + polycystic kidney	1
CLRM + microcephaly + cryptorchism	1
Total	21

* Cases with sirenomelia were excluded

Several noxae were suspected to play a role in the etiology of CLRM, but based on the data of the new Hungarian population policy introduced on January 1, 1974, the female *sex hormones* got particular attention (Fig. 2). As a result of the population policy the number

of live births increased and the number of induced abortions decreased significantly. Parallel with these events the use of the pill has become widespread (22.5 per cent of reproductive aged women used them in 1975). Furthermore on account of restrictions in the abortion law a growing trend of the use of estrogens for inducing abortion was suspected. (It is an old "custom" in our country.)

The number of previous *pill users* (Table IV) did not show a remarkable difference between the CLRM, and matched (time and place of births, sex) control groups. The number of women, however, using the pill within three months prior to conception was nearly twice as high in the CLRM group as in the controls. The number of women with pill use in early pregnancy was the same in both groups.

Table IV

The number of pill-users in CLRM and in matched control groups

Type	Number	Previous pill users	Pill use for 3 months prior to conception	Pill use during pregnancy
T	29	10	4	1
L	16	5	3	—
S	7	3	3	—
R	22	6	2	1
M	21	7	5	—
CLRM	95	31 32.6%	17 17.9%	2 2.1%
Control	95	27 28.4%	9 9.5%	2 2.1%
		—	$0.10 > p > 0.05$	—

Only one attempt of *hormonal abortion induction* was mentioned in the CLRM group and this case was in the ring constriction group. It is possible, however, that such attempts were concealed, and therefore the features of family planning were studied. In the CLRM group only 69.5 per cent of the women had planned pregnancies, but in the control group 87.4 per cent. The difference is significant ($p < 0.01$). On the other hand 15 extra-marital conceptions occurred in the CLRM group whereas only 3 among the controls.

In our opinion a possible correlation betwen CLRM and the more frequent use of the pill immediately before conception and the use of abortive estrogens cannot be excluded. A further positive correlation was the significantly higher rate of threatened abortions with supportive hormone (progestational drugs) treatments in the women of CLRM group. These three etiological factors occurred mainly in the transverse and the multiple types of CLRMs.

Discussion

The *direct* teratogenic effect of the pill may be become manifest when used during pregnancy. At the first moment this seems to be utter nonsense since the pill is an antifertility drug. (It may remind one of the old joke: "my father was a famous eunuch.") The pill use failure, however, may occur in *inadvertent* pregnancies when the pill use instructions were

not followed, in the so-called *breakthrough* pregnancies when the effect of the pill is blocked or modified by other drugs and diseases, and in cases when *pregnancy already existed* at the beginning of pill use. These cases are relatively rare, e.g. in Hungary the estimated yearly occurrence is about 0.3 per cent of all pregnancies. Additionally a certain percentage of these pregnancies is terminated.

The direct teratogenic effect of the pill, apart from the *masculinization* of female fetuses (Fig. 8), has not been proved. The masculinization was caused by some metabolites of the synthetic norsteroid progestational component of the pill in a well-defined critical period and

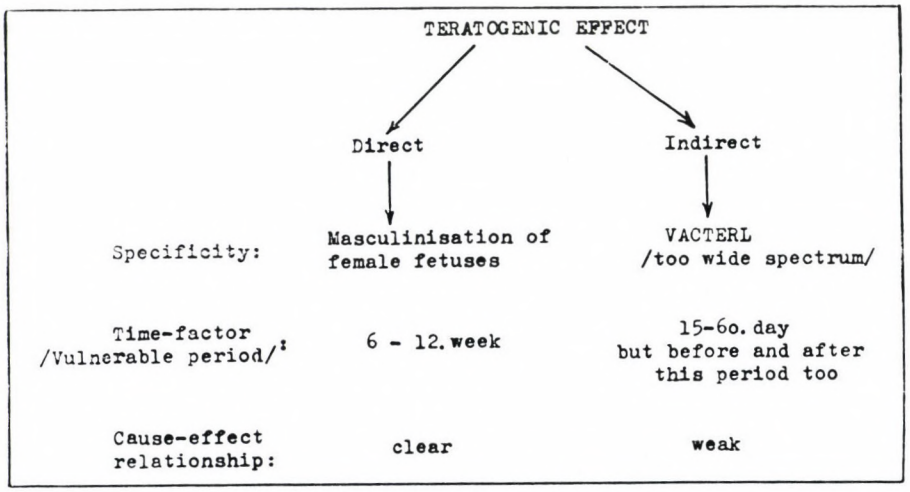


Fig. 8. The evaluation of teratogenic effect of contraceptive pills

the cause–effect relationship manifested itself in a specific malformation pattern, that is in various degrees of clitoral hypertrophy (in general it is slight and transient), more rarely in the labioscrotal fusion as well as in an advancement of bone maturation. Here the use of the past tense is justified because the recent types and smaller doses of the pill have fortunately lost this side effect.

According to some recent publications (Table V), however, the pill and in general the female sex hormones may increase the occurrence of *cardiovascular*, *limb reduction* and *tracheo–oesophageal* malformations or a more or less characteristic association of these and other congenital malformations at a low frequency rate. The suggested name for this association is the VACTERL syndrome.

These data, however, have to be handled with caution. On the one hand in a number of other studies these correlations were not found. On the other hand the female sex hormones may probably operate *indirectly* (or secondarily) and only in predisposed pregnants. The main argument for the indirect effect (Fig. 8) is that the teratogenic effect was confirmed not only for the sensitive or critical period of the embryogenesis but also before this period, e.g. during the preimplantation stage as well as *after* this period. Furthermore the great diversity of the symptoms of the VACTERL syndrome makes a single etiological agent unlikely and the cause–effect relationship weak or dubious. The *predisposition* may be a maternal endocrine dysfunc-

Table V

Some congenital malformations seem to occur more frequently in the pregnancies of pill users

Congenital malformation		Authors	
V	*V*ertebra		
A	*A*nal atresia	Heinemen, 1977 2.3x	Nora, 1975
C	*C*ardiovascular	Janerich, 1977 8.5x	
TE	*T*racheo-oesophageal	Nora, 1976 2—4x	
R	*R*enal		
L	*L*imb	Janerich, 1974 4.7x	

tion (previous menstrual and other hormonal disturbances), which may need supportive hormone therapy during pregnancy because of threatened abortions or which may exhibit a breakthrough pregnancy owing to a reduction of the antifertility effect of the pill. As far as I have realised this predisposition may be closely related to the oversuppression syndrome (Horowitz et al., 1968). The cause of this syndrome is the effect of synthetic sex hormones, particularly the estrogens, on the hypothalamus (Fig. 9). The direct consequences of the oversuppression syndrome may be a transient hormonal imbalance causing partially a delay in

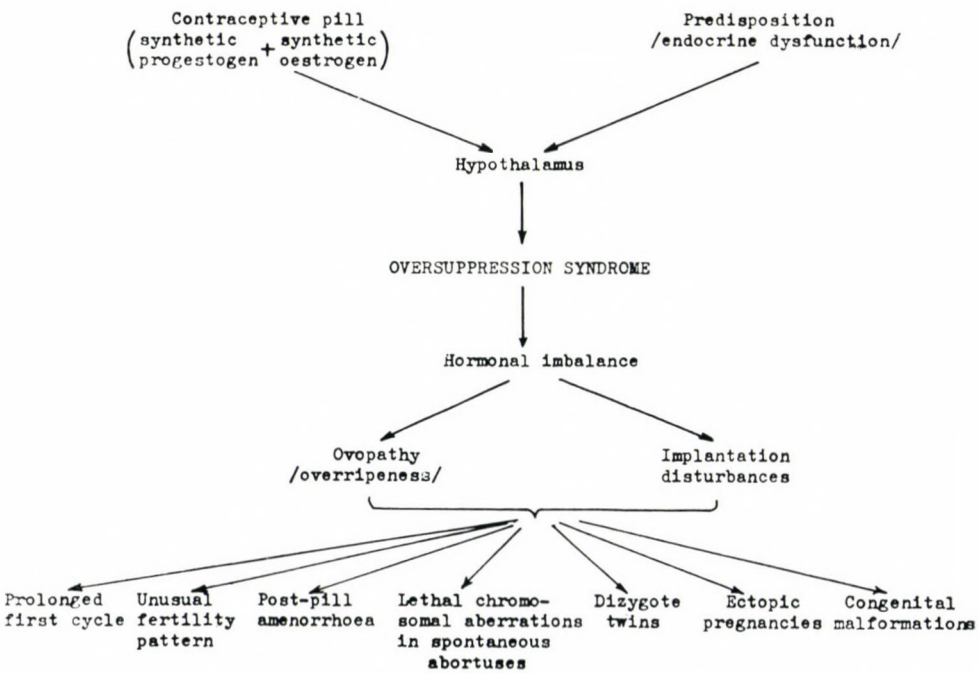


Fig. 9. The genesis and possible consequences of oversuppression syndrome

the preovulatory phase inducing an overripeness (that is ovopathy) and partially implantation disturbances. The indirect consequences of this process may be:

(i) *The prolonged first cycle after discontinuation of the pill.* The cause of this delay may be a protraction of the pre-ovulatory phase in the first menstrual cycle (Larsson-Cohn, 1969; Homesley and Goss, 1970). Consequently the first menses occurs 6—14 days later than after the use of other e.g. mechanical contraceptive methods.

(ii) *The unusual fertility pattern.* In about 20 per cent of pill-users there is delay of either two or three months before their capacity to conceive is restored [i.e. it occurs only in the predisposed women (Wolfers, 1970; RCGP, 1974)], in others discontinuation of the use of the pill causes an oscillatory pattern of fertility approximately four menstrual cycles in lenght (RCGP, 1974; Janerich et al., 1976). Several mechanisms (e.g. internal seasonal biological rhythm, early intrauterine mortality) were suggested to explain this phenomenon (Jongbloet and Zuets, 1976). Considering that coital frequency was nearly 40 per cent higher among those who used the pill than those using other methods of contraception (Westoff et al., 1969), a slightly and probably transient reduced fertility could be expected after withdrawal of oral contraceptives. Pinkerton and Carey (1976) estimated the reduced fertility discontinuation of the pill by 9 women at six months and by 6 at one year after.

(iii) *The low incidence (0.8—1.0 per cent) of secondary amenorrhea.* A number of authors reported secondary amenorrhea (which persist for at least 6 months after stopping the pill) and galactorrhea occurring separately or together (Chiary—Frommel syndrome) following the use of oral contraceptives (Taylor, 1964; Whitelaw et al., 1966; Shearman, 1966; Gregg, 1966; Schachner, 1966; Dodek and Kotz, 1967). The so-called post-pill amenorrhea is due to prolonged hypothalamic—pituitary suppression of gonadotropin secretion through the gonadotropin-releasing factors induced by the various components of the pill. At the beginning the incidence of post-pill amenorrhea was exaggerated, because the 2—7 per cent occurrence of spontaneous amenorrhea in women at risk (e.g. exposed to certain stressful situations) (Drew and Stifel, 1968) was not taken into consideration. Thus the excess of secondary amenorrhea induced by pill usage may be about 1 per cent. Additionally these amenorrheas occur mainly in women with preexisting menstrual irregularities and fertility problems (Fries, 1974). There is no specific relation between the duration of pill usage or type and the occurrence of amenorrhea.

(iv) *A small increase in the occurrence of lethal chromosomal aberrations in spontaneous abortuses.* Carr (1967, 1970) was the first to report an increased frequency of triploidy in a small number of spontaneous abortuses from women having previously used the pill. Other authors (e.g. Dhadial et al., 1970; Kajii and Ohama, 1972; Klinger et al., 1973; Lazar et al., 1973; Boue, 1973a, 1973b; Lauritsen, 1975) could not confirm this correlation. Alberman et al. (1976), however, found a 10 per cent increase in the proportion of chromosome aberrations in a relative big material of spontaneous abortuses of former pill users. (An accumulation of specific types of chromosome aberrations was not found.) This finding could be due to an absolute increase in chromosome aberrations or to a decrease in the total number of recognized spontaneous abortions. [The rates of fetal death e.i. spontaneous abortions and still births were observed to be lower for former pill users than for non-users (Robinson, 1971; Vessey et al., 1976; RCGP, 1976; Rothman, 1977).]

(v) *A bit higher frequency of dizygotic twins* (Janerich, 1974; Rothman, 1977). In Hungary the decreasing trend of multiple births showed a significant increase (about 10 per cent) in 1970 and so far has remained on this higher level (Fig. 10). This change could not be explained by the usual factors such as maternal age and parity. The use of oral contraceptives has been spreading in Hungary since 1967—1968. Our first study of 292 twins born in Budapest in 1970 seemed to demonstrate a positive correlation between former pill-usage and twin births (Vajda et al., 1972). Our second study involving 734 twins born in Budapest (1970—

1974) proved only such an effect of pill use immediately (within 1 month) before conception (Sárkány et al., 1975). The third study including 661 twins born in Budapest (1975—1977) could not confirm this correlation either. The change in the effect of the pill on twinning may be explained by the regrouping of pill types. In the early seventies the norethynodrel type was the common pill but later the lynestrenol type has became more popular. According to this argument the rate of twin births in the third study was higher after the use of the norethynodrel pill type immediately before conception. The higher occurrence of multiple births in the previous pill users, however, was not found in the majority of studies (RCGP, 1976; Vessey et al., 1976). Rothman (1977) also found a similar positive correlation.

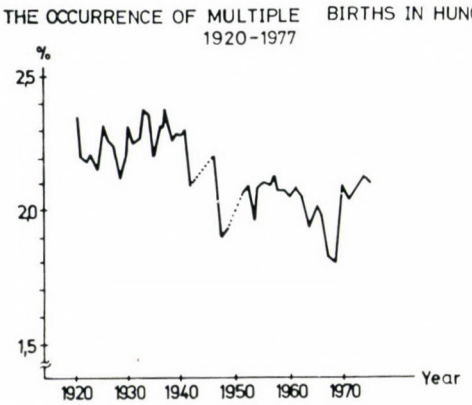


Fig. 10

(vi) *Somewhat higher occurrence of ectopic pregnancies* (Smith et al., 1974; Weiss et al., 1976; Bonnar, 1974). The explanation for this phenomenon may be partially a slower tubal transport of the ovum due to the transient hormonal imbalance, partially by the fact that the pill being much more effective at preventing uterine than extrauterine implantation. Some other authors (e.g. Berger et al., 1976; RCGP, 1976) could not confirm this observation.

(vii) *The different congenital malformations at a low frequency rate.* The positive findings were summarised in Table V. Several papers, however, are known in which the relationship between the previous pill usage and the higher occurrence of congenital malformations in subsequent pregnancies was not found. Here only five published important follow-up studies are mentioned. In the USA (Washington) Peterson (1969) examined the outcome of pregnancy among 442 patients who became pregnant after stopping pill usage and the outcome of pregnancies of a similar series of 699 patients who had employed other contraceptive methods. The number of malformed babies in previous pill users and non-users was 15 (3.7 per cent) and 30 (4.8 per cent), respectively. Canada (Nova Scotia) Robinson (1971) conducted a well-controlled study of the outcome of pregnancies among 1250 previous pill users and 1250 non-pill users. Each offspring was individually examined by very careful clinical methods. The unusual high occurrence of abnormal births: 36.2 and 31.1 per cent in previous pill takers and non-pill-takers, respectively, are attributed to these procedures. The difference was not significant. The number of minor, major and life-threatening congenital malformations was 26 (2.08 per cent), 13 (1.04 per cent) and 12 (0.96 per cent) in previous pill takers and 16 (1.28 per cent), 8 (0.64 per cent) and 5 (0.40 per cent) in non-pill-takers. The differences were not significant either but a trend is obvious. Additionally 7 versus 2 cases of congenital heart diseases occurred in study and control groups. In England (Oxford area) Vessey et al. (1976) evaluated 56,000 women-years of over 17,000 white British married women aged between

25—39 who voluntarily agreed to participate by the help of 17 clinics. 56 per cent were using the pill while 25 and 19 per cent were using a diaphragm and IUD, respectively. 107 congenital malformations were found altogether and the rates were 1.62, 3.29 and 2.12 in the pill, diaphragm and IUD groups, respectively. The English Oral Contraception Study of the Royal College of General Practitioners (RCGP, 1976) reported the outcome of about 5500 pregnancies in pill users and 11,000 pregnancies in controls. Eighty-six babies of former pill users (1.90 per cent) had congenital malformations while 177 control babies (1.84 per cent) were affected. In the USA (Boston) Rothman and Louik (1978) reviewed the birth certificates and hospital records of 7723 infants whose mothers had been reported using the pill. The overall frequency of congenital malformations was 4.3 per cent for infants whose mothers terminated the use of the pill shortly before conception while the occurrence of congenital malformations was 3.3 per cent in the infants whose mothers did not take pills for three years before conception.

The outcome of pregnancies which occurred accidentally where the pill was used during early pregnancy are interesting. Rutensköld (1971) reported on 90 Swedish pregnancies. 45 children were born before the investigation was ended. One baby had polydactyly, one had congenital dislocation of the hip and one had PKU. Any abnormal development of the external genitalia was not seen. The Oral Contraception Study of RCGP (1976) had 136 pregnancies, of which 102 went to term. Only two babies had congenital malformations (one multiple malformation involving imperforated anus, hydro-ureter, renal agenesis, rudimentary bladder and one congenital dislocation of the hip). None of these congenital malformations were obviously attributable to exogenous hormone ingestion during pregnancy.

Summary and recommendations

It now seems very unlikely that a period of pill usage has any direct harmful effects on subsequent desired pregnancies. Hence pill usage immediately before and after conception is no indication for induced abortion. A slight indirect teratogenic effect of the pill in some predisposed females cannot be excluded for the time being. The risk is obviously small, but there is a potential risk is another important point. The estimated percentage of pregnant women taking the pill accidentally during pregnancy is 0.3 per cent in Hungary. On account of the so-called "residual" effect of the pill, the use of oral contraception immediately before conception should also be taken into consideration. In Hungary 20 per cent of babies are delivered by previous pill users. In nearly half of them the interval between pill use and pregnancy is shorter than 3 months. Let us estimate that the rate of predisposed women for the oversuppression syndrome is per 3 cent, than the indirect teratogenic effect of the pill may operate in 0.3 per cent of pregnancies. Thus both the potential (suggestive but not conclusive) risk and the number of probably affected newborns will be small. Three interim ecommendations, however, seem to be reasonable:

1. A meticulous confirmation of the absence of pregnancy before the use of oral contraception is important. [According to the FDA (1978): "It is recommended that for any patient who has missed two consecutive periods, pregnancy should be ruled out before continuing the contraceptive regimen. If the patient has not adhered to the prescribed schedule, the possibility of pregnancy should be considered at the time of the first missed period (or after 45 days from the last menstrual period if the progestogen only oral contraceptives are used), and further use of oral contraceptives should be withheld until pregnancy has been ruled out."]

2. It is worthwhile suggesting a 3-month interval between the discontinuation of the pill and the planned conception. (An alternative form e.g. condom, diaphragm with spermicidal products of contraception should be advised for a period of time before attempting to conceive.)

3. It is necessary to study the method of diagnosing the predisposed women and the process of a possible indirect residual effect of the pill on the subsequent pregnancy.

After establishing the best method of pill use it is hoped that the suggested small risk for subsequent pregnancies will be reduced to practically zero in the future.

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THE IMPORTANCE OF EPIDEMIOLOGY IN IDENTIFYING DRUGS WHICH MAY CAUSE MALFORMATIONS — WITH PARTICULAR REFERENCE TO DRUGS CONTAINING SEX HORMONES

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Key words:

Epidemiology; teratology; sex hormones; food and drug a dministration; diethylstilbestrol; estrogens; progestins; oral contraceptives; post-marketing drug surveillance; patient drug labeling.

Before a new drug can enter the market in the United States, evidence of its safety and effectiveness for its proposed use or uses must be presented to and approved by the Food and Drug Administration. Before initiating tests in humans, the manufacturer must present safety data, including chemical control information, results of animal toxicity studies, and any clinical experience with the drug outside of the USA, together with the proposed protocols(s) for the initial trials. This information is reviewed promptly by pharmacologists, chemists, and medical officers in the Food and Drug Administration and if within 30 days, the Agency has not notified the manufacturer of deficiencies, clinical trials may begin. The Agency must be notified whenever investigators are added to the clinical trials or when other protocols are to be followed. In most cases additional animal safety data are required as clinical trials progress.

One requirement is that, with few exceptions, animal reproduction studies in at least one species and teratology studies in at least two species must be carried out before a drug may be given to women of childbearing potential. Pharmacokinetic studies in pregnant animals may also be requested especially if the drug is to be used during the period of organogenesis. The shortcomings of currently recommended procedures for toxicity tests in animals are readily apparent. Species and strain differences in drug metabolism are well established. Additionally, the animal dosage is considerably higher than those expected to be used clinically to compensate for the limited number of animals used.

Adverse effects in animal reproduction studies do not necessarily preclude the initiation of clinical trials in women of childbearing age if warranted by the early safety and effectiveness data in males. Nevertheless, even comparatively minor adverse effects in animal reproduction studies are usually sufficient to discourage continued investigation of a drug that does not offer advantages over currently marketed products with regard either to safety or effectiveness.

If animal reproduction studies show no adverse effects on reproduction, embryogenesis, parturition or early development, women of childbearing potential may be included in clinical trials of drugs of potential therapeutic benefit. It is recognized that absence of adverse effects in animal studies does not establish safety in human beings. Therefore, informed consent, a requirement of all investigational drug studies, should include information gained from animal reproduction studies and must make it clear that the safety to the human fetus has not been determined. Fetal well-being should be assessed throughout pregnancy. The infant should be carefully examined at birth for anatomical malformations and follow-up evaluations of physiological and psychological development should be made to detect late appearing effects.

If the drug is proposed for use during pregnancy, controlled clinical trials are required to determine safety to the mother and her unborn child as well as to establish efficacy. Requiring such trials in a drug *not* specificially indicated for use during pregnancy or not offering unique therapeutic potential to the pregnant woman of the fetus, raises ethical questions yet unresolved, since once the drug has been approved for marketing it may well be prescribed in an indiscriminate fashion to women of childbearing capacity.

The investigational phases of a new drug may involve some 2000 to 3000 patients. Thus, even in the unlikely event that the trials involve pregnant women only, the number of subjects would be insufficient to detect in a statistically significant fashion a weak teratogen, affecting only a very small proportion of the exposed fetuses, particularly if the sensitive period should be of short duration and the deformity not particularly unusual. It should be emphasized that this applies to a teratogen with a low degree of activity. A clinical trial involving only 40 patients suffering from nausea and vomiting of early pregnancy enabled McBride (1963) to detect the teratogenicity of thalidomide when some 35% of the mothers gave birth to deformed infants. A similar study might have detected the masculinizing effects of certain synthetic progestins since such defects have been estimated to occur in from 2% to 18% of female infants of mothers receiving these drugs in early pregnancy for threatened abortion. This effect was not reported however until 1958, some 15 years after these agents had been introduced for this indication (Wilkins et al., 1958).

Once a drug is approved for marketing the manufacturer must make reports at prescribed intervals of time of all adverse reactions reported to him. Additionally, FDA solicits drug experience reports from physicians and other health practitioners. Miller (1974) has pointed out the role of the alert practitioner in initially calling attention to an unexpected association between a drug or other environmental factors and manifestations of toxicity, including teratogenicity. However, while spontaneous reports may alert, albeit often belatedly, to a presumptive harm, epidemiologic studies must follow not only to strengthen the cause and effect relationship of the association but also to give an idea of the magnitude of risk.

To get the necessary epidemiologic information, in addition to the open literature, the Food and Drug Administration may contract to have appropriate studies performed or may reap the benefit of studies executed or funded by other agencies such as the National Institutes of Health. Epidemiologic and statistical consultants are available in-house and as members of FDA Advisory Committees to assist the agency in evaluation of epidemiologic data or in the design of appropriate studies.

An almost essential requirement for large scale drug epidemiology studies is a record linkage system that permits surveillance of a population receiving the drug. While the United States does not have a national health insurance scheme, a number of privately funded systems have provided epidemiologic data with regard to drugs and birth defects such as the Kaiser Foundation Health Plan System, and the Mayo Clinic Registry which contains data based on the medical records for virtually the entire 90,000 population of Olmsted County, Minnesota. Other sources for epidemiology studies include birth defects monitoring programs such as those maintained by the Center for Disease Control of the United States Public Health Service in Atlanta, Georgia and by the New York State Department of Health in upstate New York. Information with regard to the mother's exposure to drugs during pregnancy may be obtained retrospectively through the attending physician.

The importance of epidemiologic investigations in providing information on which regulatory decisions with regard to the safety of drugs in pregnancy can be based, is illustrated by recent actions with regard to products containing estrogens or progestational agents or mixtures of the two.

Many such preparations were introduced in the USA prior to 1962, when the requirement for efficacy was first introduced, and were therefore approved for marketing on the basis

of safety only. Under the 1962 New Drug Amendments, the Food and Drug Administration was committed to review these drugs to determine whether adequate evidence of efficacy existed to support labeling claims. In reassessing these drugs, to reach a decision whether the expected benefits outweighed the possible risks, safety data, including information of possible teratogenicity, were also reviewed.

In determining what steps to take after an unexpected adverse effect of a drug is reported, the Food and Drug Administration must reasses the benefit to risk ratio of that drug for a particular use in the light of the new information. If the risk clearly outweighs any benefit, the drug may be removed from the market. More usual is to require changes in the labeling of the drug to point out the new evidence of toxicity often with letters to all physicians acquainting them with this information. Additionally, contraindications to the use of the drug may be introduced depending on the nature of the effect.

Pregnant women may be exposed to drugs containing sex hormones in a number of ways. Currently, the greatest exposure comes from the use of oral contraceptive agents. Most such agents contain both an estrogen and a progestin but some consist of a progestin only. Estrogen–progestin combinations or progestins alone have been used in the diagnosis of pregnancy and both progestins and synthetic estrogens have been used in the treatment of threatened or habitual abortion, dosages for these indications being usually considerably higher than those used for contraception. Recently, diethylstilbestrol in high doses has been advocated as a "morning after" pill. Because of the wide variety of estrogenic and progestational agents marketed and the range of dosages used, and because estrogens and progestins are often prescribed simultaneously, most epidemiology studies do not provide sufficiently specific data with regard to the safety of any given compound.

Two prospective studies, the University of Chicago diethylstilbestrol study conducted between 1951 and 1952 and the Collaborative Perinatal Study of the National Institutes of Neurological and Communicative Disorders and Stroke conducted between 1959 and 1965 have recently provided important data with regard to the teratogenicity of sex hormone preparations. The former was a randomized trial undertaken by Dieckmann and his coworkers (1953) at the Chicago Lying-In Hospital. It involved 840 pregnant women who received graduated amounts of diethylstilbestrol during pregnancy, starting between the sixth to the twentieth week and 820 who received placebo tablest.

Diethylstilbestrol, a non-steroidal compound with estrogenic activity was widely used during the 1940's and early 1950's for threatened and habitual abortion and for other complications of pregnancy following favorable reports by the Smiths (Smith, 1948; Smith and Smith, 1948). The Dieckmann study failed to show any advantages of diethylstilbestrol over the placebo with regard to the outcome of pregnancy and thus may have prompted the reduction, though not total elimination, of the use of this and other synthetic estrogenic preparations for complications of pregnancy.

The finding, first reported in a case-control study by Herbst et al. (1971) that female offspring of mothers receiving diethylstilbestrol during pregnancy may develop an unusual type of cancer of the genital tract (clear-cell adenocarcinoma) as well as benign vaginal adenosis after the age of puberty prompted a review of the Dieckmann study and a tracing of the various mother-child subjects. No cases of cervical or vaginal cancers have been found in the 346 live born female offspring exposed in utero to diethylstilbestrol that have been examined to date. The review has however provided important epidemiological information with regard to teratological effects of diethylstilbestrol in both female and male offspring of mothers receiving the drug in early pregnancy. Findings in male offspring include epididymal cysts, hypoplastic testes and reduced sperm counts (Bibbo et al., 1977).

The report of vaginal cancer in offspring of mothers receiving diethylstilbestrol also prompted a follow-up of 1719 persons born in hospitals affiliated with the Mayo Clinic from

1943 through 1959 who were exposed to estrogens (predominantly diethylstilbestrol) *in utero*. There was no evidence of vaginal or cervical adenocarcinomas in the 803 live born females.

Following the 1971 report by Herbst and his associates and in the absence of evidence of effectiveness for spontaneous and habitual abortion, FDA concluded that DES should be contraindicated during pregnancy.

From the University of Chicago and Mayo Clinic studies and some additional studies of a similar nature, a task force appointed by the Secretary of the Department of Health, Education and Welfare to review all aspects of diethylstilbestrol use during pregnancy has tentatively concluded that the risk of cancer to females exposed to the drug *in utero* is at least less than 1 in 1000. The incidence of vaginal adenosis reported varied from 30% to 80% or more depending on the population examined. Detailed breakdown of the Chicago series with regard to the months in which the diethylstilbestrol was started suggests that the earlier the drug is given during pregnancy, the higher the likelihood of the daughter developing this condition at or about the time of puberty.

The second prospective study, the Collaborative Perinatal Project was designed to study factors in pregnancy related to cerebral palsy and other forms of CNS damage. It involved 50,282 mother-child pairs recruited in 12 centers. On entry, the mothers were questioned on present drug use as well as drug use extending back beyond the last missed menstrual period. At subsequent visits, drug intake information was also obtained. A complete analysis of drug intake during the first four months of pregnancy and pregnancy outcome has recently been published (Heinonen et al., 1977a) though preliminary reports on certain classes of drugs have appeared from time to time. Many drugs were not prescribed often enough to permit statistically significant conclusions to be drawn with regard to their safety or hazard. Additionally, the study is necessarily confined to drugs introduced prior to 1965.

The Perinatal Study included 1042 children exposed to sex hormones during the first four months of pregnancy. The distribution amongst the 1042 mother-child pairs were as follows: 438 mothers received exclusively progestational agents, 176 received exclusively estrogens while 438 received both progestational and estrogenic agents. Of these 438, 278 received oral contraceptives.

Among the 1042 children, there were 19 (18.2 per thousand) with cardiovascular defects. This rate compares to one of only 7.8 per thousand amongst the rest of the mothers whose exposure to sex hormones was either non-existent or unknown. Estrogens and progestins when used in combination had a statistically significant association with cardiovascular birth defects. Such an association was not significant when each of these agents was considered strictly by itself probably because of an insufficient number of subjects in each class. However, if the analysis is made for each agent (alone or in combination with the other) the results for each of the two agents is statistically significant (Heinonen et al., 1977b).

The above findings are supported by other epidemiological studies. Thus Nora and Nora (1973) in a retrospective study found 20 of 224 patients with congenital heart disease compared with 4 of 262 controls had been exposed *in utero* to estrogens and/or progestins. Levy et al. (1973) reported that 7 of 76 mothers giving birth to babies with transposition of the great vessels, had received sex steroids during pregnancy: 6 mothers had received the hormones for threatened abortion and one as a pregnancy test. None of the 76 mothers of normal babies had received sex hormones during early pregnancy. Janerich et al. (1973) in a case control study involving 104 infants with congenital heart disease identified by birth certificate, found that 18 of the infants had been exposed to sex steroids. Ten of the mothers had hormone pregnancy tests; six had supportive hormone treatment and two had inadvertent oral contraceptive use after pregnancy had commenced. There were 3 exposures amongst the 104 infants free of anomalies, two of the mothers having had pregnancy tests and one supportive treatment. Janerich (1974) in a similarly designed study reported an association

between limb defects and exposure to exogenous sex hormones. Gal et al. (1967) reported that 19 of 100 mothers giving birth to infants with meningomyelocele or hydrocephalus had a history of receiving hormones for the diagnosis of pregnancy while only 4 of 100 matched control mothers of normal babies had a similar history. However, this report could not be substantiated by either Laurence et al. (1971) or Oakley and Flynt (1973).

In addition to studies involving exposure of the fetus through the mother inadvertently continuing oral contraceptive use in early pregnancy, the question as to whether such agents, even if discontinued prior to pregnancy may nevertheless affect the offspring has also been addressed. Carr (1970) has reported evidence that triploidy and possibly other types of polyploidy are increased in women who become pregnant shortly after discontinuing oral contraceptives. Such chromosomal defects, however, appear to be incompatible with life.

On the basis of such epidemiological studies, the Food and Drug Administration has concluded the risk of teratogenicity following inadvertant use of oral contraceptives in early pregnancy is negligible in comparison with the large scale benefits of effective contraception. The physician labeling provided with these products must list known or suspected pregnancy as a contraindication. There must be a warning of an increased risk of heart defects and limb defects if sex hormones are taken during early pregnancy, and the possibility of increased chromosomal defects, usually ending in spontaneous abortion, if conception occurs soon after ceasing the use of oral contraceptives. Also, to be emphasized is the need to rule out pregnancy if a period has been missed and the patient has not been adhering to the prescribed schedule.

On the other hand, it was concluded that sex hormone pregnancy tests represented needless exposure to risk since more effective chemical methods are now available. Products introduced specifically for this purpose are no longer allowed on the market and physician labeling for progestins must include as a contraindication their use as a diagnostic test for pregnancy.

Additionally, progestational agents may no longer be recommended for threatened or habitual abortion during the first four months of pregnancy. This too, is based not only on teratogenicity reports including the earlier reports of masculinization, but also because there is no adequate evidence that such use is effective. Early abortion is usually considered to be due to a defective ovum and progestins would only serve to delay the expulsion of the products of conception.

The Food and Drug Administration also requires that all oral contraceptive drug products and all estrogenic and progestational drug products have patient labeling to be distributed to the patient at the time her prescription is filled. The labeling for estrogens and oral contraceptives must bear a warning that these drugs are contraindicated in pregnancy and must indicate the need for the patient to inform her physician should she become pregnant while taking the drug. The increased risk of birth defects, including heart defects and limb defects that have been associated with the use of sex hormones in pregnancy must be described. Also to be included are the hazards associated with the use of diethylstilbestrol during pregnancy with the acknowledgement that while there is presently no data with regard to other estrogens in this respect, it cannot be presumed that they would not induce similar changes. Oral contraceptive labeling must also make reference to an apparent increased risk of "miscarriages" in women who become pregnant soon after stopping oral contraceptives.

Patient labeling for progestational drug products must warn of an increased risk of birth defects in children whose mother have taken the drug during the first four months of pregnancy; must describe the nature of the risks; and must state that the patient should inform her physician as soon as possible if she discovers that she was pregnant when she took the drug. The labeling must also state the drugs are no longer considered safe as a test for pregnancy.

The need for effective post-marketing surveillance of drugs has been long recognized by the Food and Drug Administration. A step in this direction is provided by the so-called Phase IV studies, when the manufacturer agrees to conduct an agreed upon number of well-controlled studies after the drug has been released for marketing. However, to date Phase IV studies have been used to a limited extent, usually for seemingly unique drugs that the agency feels should be available to the public as rapidly as possible. Approval therefore, may be based on fewer studies with the proviso that additional studies will be supplied during the early post-marketing period.

Recognition of the unsatisfactory current status of drug monitoring prompted the FDA to sign an interagency agreement with the National Bureau of Standards in 1976. Under this agreement a total of $ 1.1 million can be reimbursed to FDA for the conduct of an experiment in post-marketing surveillance. Phase I of the experiment is being conducted under a contract with IMS America, a firm in Ambler, Pennsylvania, which will result in the design of several methods of collection and processing of severe and low incidence adverse effects and unanticipated beneficial effects. In Phase II of the experiment one or more of these methods will be pilot-tested and evaluated for future use. Representatives from the Joint Commission for Prescription Drug Use, a private, non-profit group funded primarily by the Pharmaceutical Manufacturer's Association for the purpose of studying various drug utilization problems have joined the FDA and the NBS in developing and directing this contract.

In conclusion, despite occasional genetically determined differences in drug metabolism and occasional national differences in patterns of drug usage, drug toxicity is a universal problem that merits the highest degree of international cooperation. Exchange of information between the various drug regulatory agencies has expanded greatly since drug regulation in many countries was strengthened after the thalidomide episode. The International Clearinghouse for Birth Defects Monitoring currently maintained by the National Foundation collates information from birth defect monitoring systems in some twelve different countries permitting the rapid dissemination of epidemiological information that may well lead to early identification of unsuspected teratogens. Finally, the three teratology societies, the European Teratology Society, the Teratology Society, and the Congenital Anomalies Research Association of Japan encourage through meetings and publications the exchange of information between scientists both regarding suspected or proven teratogens and regarding the possible mode(s) of action of such agents.

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AN EPIZOOTIC OF AKABANE DISEASE IN BOVINES, OVINES AND CAPRINES IN ISRAEL, 1969—70: EPIDEMIOLOGICAL ASSESSMENT

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Introduction

"Akabane disease" (Inaba et al., 1975) is an epizootic virus disease causing abortion, premature birth, stillbirth and congenital arthrogryposis–hydranencephaly (A.H.) in cattle, sheep and goats. Recent epizootics have been described in Israel, Japan and Australia, and the aetiology was established by serological surveys (Miura at al., 1974; Hartley and Wanner, 1975; Kalmar et al., 1975), by virus isolations from bovine (Kurogi et al., 1976) and ovine (Della Porta et al., 1977) fetuses, and by experimentally inducing AH in a calf, a kid (Inaba, 1975) and a lamb (Parsonson et al., 1975).

Retrospective similar epizootics occurred periodically in Japan since 1949 (Inaba et al., 1975) and in New South Wales, Australia at least since 1940 (Hungerford, 1970). In Israel only one epizootic has been experienced so far.

Epidemiological investigation

At the end of 1969 and the beginning of 1970 a mass outbreak of A.H. occurred in the northern parts of Israel involving some 3000 dairy calves, 677 lambs and 563 kids. The pathology of the syndrome was described (Nobel et al., 1971) and data on the epidemiology and possible aetiology were published in the case of sheep and goats (Shimshony, 1970) and cattle (Markusfeld and Mayer, 1971).

The main conclusions of the epidemiological investigations in sheep and goats were:

a) The epizootic was caused by an infective agent, possibly insect-born, which was active in the northern part of Israel, mainly in lower altitudes, during the months October—December 1969.

b) The agent caused abortions in sheep and goats.

c) The agent caused malformations, mainly hydranencephaly, in fetuses between 30—50 days of gestation. No malformations were caused in fetuses during the last 100 days of pregnancy.

d) Peak months of the syndrome were February, March. In badly affected flocks 50% of all lambs and kids were abnormal during these months.

e) No clinical symptoms were seen in pregnant animals.

f) Males, twins and goats were more affected than females, singles and sheep.

g) The vector could be similar to the one transmitting bluetongue.

The main conclusions concerning cattle were:

a) The epizootic was caused by an infective agent, and might have been transmitted by a vector which was active for a peak period of 2—3 months.

b) The agent was new in this part of the world.

c) Damage to the spinal cord (arthrogryposis) occurred during 4th—6th months, of pregnancy, and to the brain (hydranencephaly) during the third month of pregnancy.

d) The vector was not similar to the one transmitting bluetongue.

e) No clinical symptoms were seen in pregnant cows.

f) Among abnormal calves males outnumbered females to a significant degree.

The assumption that the pathological changes were due to an infective agent, active during the autumn months, received support from the finding of high levels of immunoglobulins in the pre-colostral sera of affected calves (Trainin and Meirom, 1973). A retrospective examination of pre-colostral sera of affected fetusis from the described epizootic was performed 4 years later, revealing antibodies to Akabane virus. Sera were collected in 1975 from 239 cows on 8 farms which had been affected in 1969—70, and from 119 cows on 4 farms which had not been affected (Kalmar et al., 1975). All the younger animals, born after the epizootic, were negative to Akabane virus, in contrast to the high percentage of positive sera among the older cows. The percentage was significantly higher among cows from affected farms than cows from non-affected farms (60.7% v. 23.3%). Similar results were obtained with sheep and goats.

Fig. 1. Lamb: arthrogryposis, peromelia, torticollis, kyphosis

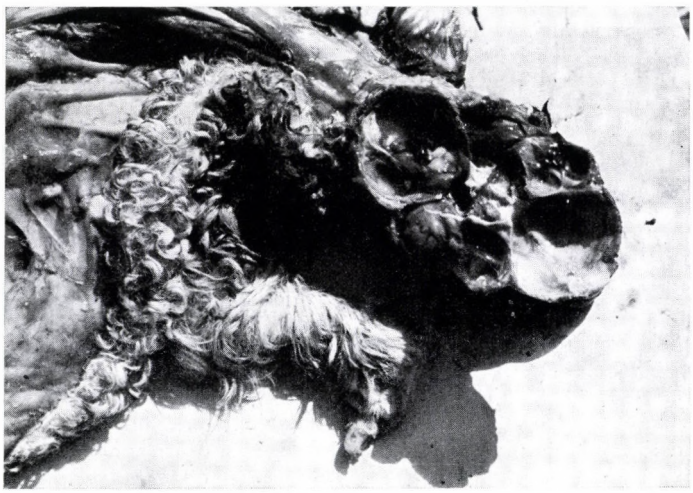

Fig. 2. Same lamb as Fig. 1: hydranencephaly. Note asymmetry of skelaton, including cranium

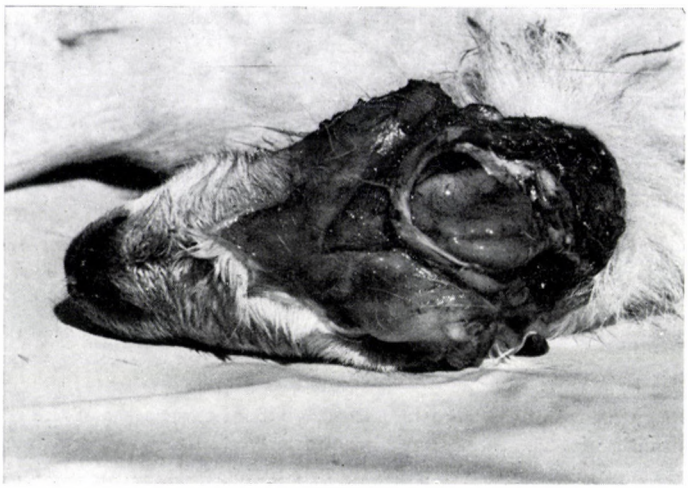

Fig. 3. Kid: micrencephaly, hydrocephalus

Fig. 4. Kid: amelia, right hind limb

These results supported earlier findings of Japanese and Australian workers and strongly indicated that the epizootic of A.H. in bovines, ovines and caprines in Israel in 1969—70 had been caused by Akabane virus.

Discussion

Akabane virus is a virus of the Simbu group that was first isolated in Japan from *Aedes vexans* and later from *Culex tritaeniorhynchus* mosquitos (Oya et al., 1961; Oya, 1975). The virus was isolated in Queensland in Australia from *Culicoides brevitarsis* (Doherty et al., 1972) and in Kenya from a pool of *Anopheles funestus* mosquitoes (Metselaar and

Robin, 1976). Antibodies against the virus have been found in pigs, horses and a few monkeys (Oya, 1971, 1972), in cattle, horses and sheep (Doherty et al., 1972, 1973) and in goats (Kalmar and Peleg, 1975). Very few human sera were positive when tested for antibodies (Oya, cited by Metselaer, 1976; Doherty et al., 1972). The aetiological role of Akabane virus in outbreaks of A.H. has been established in three countries concerning bovines, ovines and caprines, and it seems necessary to investigate the situation in other species.

The geographical distribution of the biting midge *Culicoides brevitarsis*, which is regarded as a major vector of Akabane in Australia, has been correlated with the area of endemicity of the virus as established by serological survey (Della-Porta et al., 1976). They suggest that Akabane disease occurs as an epizootic disease along the margin of the distribution of the vector, and is caused by movement of the virus from an endemic situation into populations of susceptible animals, due to unusual environmental conditions. Possibly most domestic ruminants in endemic areas become infected early in life, and therefore no clinical outbreaks occur in these areas.

In this context it is interesting to observe that all three areas of the recent epizootics are situated on latitude 32°, and that Akabane virus presence or activity have been demonstrated in areas closer to the equatorial, namely North Australia and Rhodesia, which therefore could be considered as endemic areas. Data concerning prevalence of the virus in various species, possible vectors and their geographical distribution, prevalence of positive reactors and the susceptibility of additional species should be collected to enable better assessment of this syndrome.

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ABSTRACTS OF THE THE POSTERS PRESENTED AT THE 6TH CONFERENCE OF THE EUROPEAN TERATOLOGY SOCIETY

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EMBRYOTOXIC EFFECT OF CITRAL IN CHICK AND RAT EMBRYOS AS RELATED TO THEIR DETOXIFICATIVE CAPACITY

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Citral (3,7-dimethyl-2,6-octodienal) administered intraamniotically (150 μg/embryo) to chick embryos on the 3rd day of incubation induced malformations at a rate of 55.8% and had a lethal effect upon 24.6% of the embryos. This compound when injected intraperitoneally to pregnant rats (360 mg/kg b. w) on 10th—12 th days was devoid of any embryotoxic effect.

In a previous study we found that alcohol dehydrogenase (ADH), known to be involved in the metabolism of various alcohols and aldehydes such as steroids, retinol and other aromatic compounds, could also reduce citral in vitro. During the early stages of organogenesis there was no ADH activity found. This appeared in the livers of chick and rat embryos at the 12th and 16th days of development respectively and increased progressively, at term reaching 20—25% of the enzyme activity in the adult liver. Interspecies enzyme affinity to citral was also noted.

It seems that the resistance of the rat embryos to citral is dependent on the maternal capacity to reduce citral to a harmless compound. The end product of ADH activity, geraniol, was devoid of any embryotoxic effect in the studied species. Rat embryos (10—12 days) could also be rendered susceptible even at low doses of citral (15 μg/embryo) when the maternal protective detoxifying system was bypassed by an intraamniotic injection. Lethality was 78.8% in the treated embryos as opposed to 5.8% in those sham injected.

THE REGENERATING LIMB OF THE CRESTED NEWT AS AN EXPERIMENTAL MODEL FOR ASSESSING THE TERATOGENIC EFFECTS OF THE FUNGICIDE MANGANESE ETHYLENEBISDITHIOCARBAMATE

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Effects of manganese dithiocarbamate (maneb) on the regenerating limb of the adult newt, *Triturus cristatus*, have been studied. A total of 72 animals were used. They were divided in two groups (a control and an experimental group) of 36 newts, 18 males and 18 females each. Experimental animals were exposed percutaneously to maneb 80 (80% active component) at a concentration level of 5 ppm.

Maneb treatment resulted in delayed growth, reduced melanogenesis and malformations of regenerating limbs. Most often malformations involved distal phalanges, which were hook-shaped and kinky. Incidence of skeletal abnormalities was 100%. In the females this incidence was statistically significant (at 0.1% level) when compared with the control newts. Conversely, results obtained in the males were more difficult to interprete due to the high frequency of malformations in the controls. However, these malformations were quite different from those of the treated newts.

EFFECT OF CARBON DISULPHIDE ON OXYGEN CONSUMPTION OF MATERNAL AND FOETAL TISSUES AFTER EXPOSURE DURING GESTATION

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Oxygen consumption of tissue homogenates is one of the main indices of energy metabolism, commonly used as a screening test for evaluation of the effect of chronic experimental exposure to low CS_2 levels. In this investigation Warburg's manometric method has been used for determination of oxygen consumption of maternal and foetal tissues after exposure of pregnant albino rats to 50—200 mg/m^3 CS_2, 8 hours per day throughout gestation. Oxygen consumption has been studied in mothers (liver, kidney, placenta) and progeny at birth (liver) and after reaching sexual maturity, separately in males and females (liver, kidney, brain, placenta). Oxygen consumption proved to be significantly inhibited in maternal liver, kidney and placenta, the degree of inhibition ranging between 56 and 63%. No inhibition has been noted in foetal liver. At the age of 3 months however, pregnant F_1 females exhibited 37% inhibition of oxygen consumption of placenta, although no further CS_2 treatment has been applied. At the same age F_1 males did not differ from control values. The data point out the lasting effect of carbon disulphide on oxygen consumption of progeny and the higher sensitivity of pregnant F_1 females in comparison to F_1 males.

DIAPLACENTAL TRANSFER AND EMBRYOTOXIC EFFECTS OF THIAMPHENICOL IN RAT AND HUMAN PREGNANCY

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The purpose of the investigations presented is to study the possible interference of the chemotherapeutic agent thiamphenicol (TAP) with normal early human pregnancy. In order to extend conclusions drawn from embryotoxic effects induced in experimental animals to humans the following parameters were tested: 1. Susceptibility of *in vitro* protein synthesis of mitochondrial fractions obtained from rat and human embryos and placentas to TAP, 2. dose-response relationship between the effects of inhibition of mitochondrial protein synthesis at the mitochondrial level (energy generation) and the effects on overall embryonic development (embryolethality) after application of TAP *in vivo* to pregnant rats, 3. diaplacental transfer of TAP in rat and human pregnancy.

TAP *in vitro* inhibits mitochondrial protein synthesis (inhibition of 55S ribosomal peptidyl transferase activity) at the same concentrations that are necessary to suppress mitochondrial protein synthesis *in vivo*. This is followed by impaired mitochondrial respiration and decreased ATP synthesis and results in embryolethality (rats). The concentrations of TAP measured under these conditions in rat embryos are equivalent to those obtained in human embryos after application of therapeutic amounts of the drug (4—6 μg/g embryo 4 h after drug application). A prolonged exposure to TAP is, therefore, expected to be harmful to the human embryo.

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THE ANALYSIS OF THE INCREASE OF CONGENITAL LIMB REDUCTION MALFORMATIONS IN HUNGARY, 1975—1977

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The Hungarian Congenital Malformation Monitor indicated a significant, 48.5%, increase in the congenital limb reduction malformations in 1975. The completeness of the notification of all congenital malformations increased only by 18.4%. The notified cases were checked by personal medical examinations: 92.2% proved to be limb reduction malformation. Five types were separated. The use of oral contraceptives immediately before the conceptions, unwanted pregnancies, threatened abortions, toxic effect of chemicals, salicylate treatment and familial cluster were significantly more frequent in the pregnancy of women delivered babies with limb reduction malformations than in matched controls. In 1976 and 1977 the occurrence of congenital limb reduction malformations remained on this high level. The aetiological study is in progress.

HORMONAL REGULATION OF MORPHOLOGICAL AND FUNCTIONAL DIFFERENTIATION OF THE ADRENAL CORTEX IN THE RAT

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Hypophysectomy of mothers on the eighth day of pregnancy promotes and treatment of them with corticosterone daily from tenth day of pregnancy retards the differentiation of fine structure of the adrenocortical cells of rat embryos from the fifteenth day of foetal life on. The adrenalectomy of rats on the sixth day of pregnancy had influence on the fine structure of cortical cells in the adrenal and ACTH-producing cells in the pituitary, and the plasma corticosterone level of their embryos. It has been shown that the fine structure of endoplasmic reticulum were more differentiated as soon as on the fifteenth day of pregnancy than in the corresponding controls. The level of plasma corticosterone of adrenalectomized mother on eighteenth day of pregnancy was lower than that of their embryos.

The fine structure of ACTH-producing cells in the pituitary appeared to be more differentiated in general and they occured more frequently and their immunohistochemical stainability seemed to be more intensive.

It could be supposed the hypophyseoadrenocortical axis of rat embryos has capability for functioning on the fifteenth day of pregnancy.

THE EFFECT OF PRENATALLY APPLIED STEROID HORMONES ON THE POSTNATAL DEVELOPMENT OF THE MOUSE

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On days 14—18 of gestation the animals received a subcutaneous injection of 10 mg/kg cyproterone acetate. This steroid hormone prevents spontaneous delivery, therefore, the fetuses have to be obtained by Caesarean section and are bred by foster mothers. Postnatal development is controlled by the following criteria:

a) number of fetuses per litter, birth weight and weight development of the newborn animals;

b) general development, i.e. opening of eyes and ears;

c) mortality rate;

d) motility measured using an ANIMEX apparatus;

e) fertility and sterility of the offspring.

The number of fetuses per litter as well as the birth weight of the fetuses treated in utero were reduced as compared with controls. The weight retardation corrected itself during postnatal development. The mortality rate of the animals treated in utero was almost twice as high as that of controls. Motility of the treated animals was reduced whereas general development was "normal".

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DOUBLE STAINING TECHNIQUE FOR RABBIT FOETUS SKELETONS

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The staining of rabbit foetuses for teratological examination of the skeleton is described in which both cartilage and bone are differentiated. This method is a modification of the method described by Inouye for the mouse foetus skeleton (Inouye, M.: Cong. Anom. 16: 171—173; 1976). Rabbit foetuses, after evisceration, removal of eyes, adipose tissue and skin, are fixed in ethyl alcohol. They are then stained in solutions of alcian blue and alizarin red S, in ethyl alcohol and acetic acid at 37 °C for 24 hours. This is followed by washing with water and maceration of the soft tissues in potassium hydroxide. After further washing in water they are cleared in glycerine and alcohol and then are stored in glycerine. Cartilage is stained blue and bone is stained red, and therefore anomalies of both the cartilaginous and bony skeleton can be examined in these specimens.

STAGE EFFECT IN CYCLOPHOSPHAMIDE-INDUCED CLEFT PALATE

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It is generally agreed that secondary palate closure is the result of coordinated growth of the palatal shelves, maxilla, premaxilla, glossomandibular complex and skull base. The aim of our study was to find out if any changes in growth rates of these components were present in fetuses with cleft palate. The length of the secondary palate and the size of the cleft were measured on day 18 of prenatal development in fetuses with cleft palate induced by administering single doses of cyclophosphamide to pregnant ICR mice on days 11, 12 or 13 of pregnancy. Sgnificantly shorter secondary palate of fetuses with clefts induced on day 11, and different size and shape of clefts induced on day 13 were observed.

STRUCTURE RELATED TERATOGENICITY IN RATS

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Phenothiazine derivations containing piperazine side-chain induce generally cleft palate and micromelia in rats. Similar effect of benzhydrilpiperazines was published by King et al. and by other authors. The effect of equimolar doses of six piperazine-ring containing compounds were studied on the 14th and 15th gestational days in Wistar/Riop rats. Four compounds (perphenazine, fluphenazine, chlorcyclizine and xanthen-9-carbonyldipiperazine derivative) contained one piperazine-alkyl, while the others (haloanisone and oxypertine) one piperazinephenyl or -methoxyphenyl side chain, respectively. The results suggest that piperazine-alkyl compounds induce only cleft palate and micromelia with a very good dose-dependency. The other substituents (-Cl, CF_3) modify these effects. The other two compounds did not induce cleft palate and the number of micromelia was moderate, too with a slight dose-related occurrence. The altered response to the two different types of compounds may be attributable to metabolic differences in the mothers.

TERATOGENIC EFFECTS OF GESTAGENE TREATMENT DURING EARLY PREGNANCY IN MICE

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The intention of our studies is to investigate the teratogenic potency of sex steroid hormone treatment during early pregnancy especially after maternal treatment during the preimplantation period. Pregnant NMRI mice were treated on day 1 or 2 of pregnancy with gestagens especially Cyproterone acetate 5—900 mg/kg.

Teratological investigations were performed (1) by in vitro culture of mouse blastocysts after maternal treatment (2) by teratological investigations at the end of pregnancy (Wilson's technique). 24 hours after CA treatment of the mother on day 2 the number of embryos/mother and the cell number/embryo were not affected as compared with control embryos. However, at the end of the blastocyst culture period differentiation of the inner cell mass into two germ layers was inhibited irrespective of the dose. The additional in vivo and in vitro application of estrogen could not compensate for the inhibitory effect. At the end of pregnancy neither an increase in the resorption rate nor a reduced average fetal weight was observed. However, teratological investigations of the fetuses revealed a significant increase in various malformations: exencephaly (irrespective of the dose), cleft palate (dose dependent), kidney and bladder malformations (dose dependent), heart malformations (doubtful dose response).

Because of the lack of clear dose response the possibility of co-teratogenicity was tested.

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HOW DO HORMONAL PREGNANCY TESTS CAUSE FETAL MALFORMATIONS?

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Our earlier observations (Lancet *i*, 753, 1971) suggest that the use of hormonal pregnancy tests may have undesirable consequences including fetal malformations, but the assumption of a causal relationship requires some explanation. We base our conclusion primarily on the

observation, well-known to most obstetricians, that haemorrhages are common in the chorio-decidua following injections of oestrogen-progesterone preparations in the early stages of pregnancy. This can be explained by supposing that the raising and rapid lowering of hormone levels "mimics" the non-pregnant state and therefore results in increased interstitial hyperaemia followed by withdrawal bleeding. If there is no abortion and the ovum develops further, the haemorrhages in the choriodecidua may lead to a hypoxic disturbance that interferes with the current phase of embryogenesis. In our opinion the main teratogenic factor is hypoxia rather than the abnormal hormone levels *per se*. In our department between 1965 and 1970 four infants with congenital upper limb reduction defects (dysmelia, phocomelia) were born. In three of the cases we know that injections of Limovan (an oestrogen-progesterone preparation) were given to the mother in the first weeks of pregnancy and in each case this was followed by bleeding (J. Pediat. *88*, 524, 1976). After we had warned doctors in our locality of the dangers of this preparation (which has been used in large doses to terminate unwanted pregnancies) no cases of upper limb reduction defects have been found here during the last 8 years, while these cases increased in other parts of Hungary.

CARDIOVASCULAR MALFORMATIONS INDUCED IN THE RAT

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Glycerol formal, a drugs solvent, used in toxicological studies, was administered to pregnant Sprague Dawley and Wistar rats during the organogenetic period at 0.25, 0.5 and 1.0 ml/kg dose level (i. m., s. c. and per os).

This treatment produced a dose-related embryotoxic and teratogenic effect. Interventricular septal defects (IVSD) and anomalies of the great vessels (right and double aortic arc, vascular ring) were the most characteristic malformations observed in the offspring. The highest teratogenic effect was obtained in Sprague Dawley rats with 0.5 ml/kg (IVSD were found in about 50 per cent of fetuses). The specific cardiovascular malformations induced by the solvent are interpreted as due to an alteration of the hemodynamic in the embryos.

SOME ASPECTS OF TERATOGENESIS PRODUCED BY SODIUM SALICYLATE IN FERRET AND RAT

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A single injection of 400 mg/kg sodium salicylate on day 13 and 18 of gestation in the ferret produced a high resorption rate; 91% on day 13 and 66% on day 18. The majority of the surviving fetuses of animals injected on day 18 had limb and tail defects. Rats injected at corresponding stages (8 1/2 and 11 1/2 day) of gestation produced a relatively lower rate of resorption; 23% on day 8 1/2 and 9% on day 11 as well as external and internal malformations.

In order to determine whether sodium salicylate has a direct action on the developing embryo or whether maternally mediated metabolic factors are important, explanted rat embryos at day 9 1/2 of gestation were grown for 48 hours *in vitro* using the New technique. Explanted embryos were grown in homologous serum from animals treated with a single 450 mg/kg dose of salicylate or serum to which salicylate was added. The presence of vitelline

circulation, yolk sac diameter, somite number, normal folding and neural tube closure were among the parameters used to examine the embryos. In all cases, treated embryos were affected more than the controls, and the anomalies in embryos grown in serum from a treated animal were comparable to those produced in embryos grown in serum to which salicylate had been added.

GENITAL TUMOURS IN THE FEMALE PROGENY OF MICE RECEIVING OESTROGEN DURING PREGNANCY

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Pregnant AB(Jena × DBA)2Jena hybrid mice were given orally the potent antifertilizer 17β-phenylaminocarbonyloxyoestra-1,3,5(10)-triene-3-methyl ether (1, 10 or 20 mg/kg/d) on days 12 to 16. In adult female offspring hypoplasia of ovaries and uterus horns, hyperplasia of the cervix uteri, uterine squamous epithel metaplasia, and vaginal acanthosis were observed. Administration of high doses resulted in the induction of some tumours (cervical sarcomas, vaginal carcinomas) which are not to be found in untreated controls. In contrast to diethylstilbestrol (Nomura, T. and T. Kanzaki, Cancer Res. *37* (1977), 1099) the oestrogen tested seems to have carcinogenic action on target organs.

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FUNCTIONAL ACTIVITY OF CRF-ACTH AXIS IN FETAL LIFE

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ACTH secretion in cultured hypophyses taken from different periods of gestation and their responsiveness to hypothalamic extracts prepared from adult animals were measured by radioimmunoassay. It was established that 1. the amount of the ACTH secreted by the cultures were age-dependent, the younger the embryos were the less ACTH was detected in the media; 2. if we compared the amount of ACTH released by the cultures with the total ACTH content measured in the intact gland we found the older the embryos were the less ACTH was released spontaneously; 3. hypophyseal cells originating from different periods of gestation display different sensitivity to adult hypothalamic extract (HE): hypophyseal cultures prepared from 15 and/or 18 days old rat embryos were unresponsive to HE. In accordance with this hypothalamic extracts prepared either from 15 or 18 days old embryos did not increase the ACTH secretion in the hypophyseal cultures prepared from adult rats.

NEUROBEHAVIOURAL EFFECTS OF PRENATAL EXPOSURE TO PARATHION-METHYL ON RATS

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Pregnant rats were given a daily dose of 2 mg parathion-methyl per kg b.w. through the entire gestation period. The influence of this treatment on the F_1-generation up to five weeks of age was investigated applying crossfostering. Mortality, littersize, weightgain and developmental milestones were recorded. Test's for auditory and visual function and locomotor co-ordination were performed.

A significant decreased weight gain was observed in the dosed females. A lower birth weight was recorded in pups from dosed mothers. No difference in duration of pregnancy between the two groups was observed. A lower weight gain was seen in the group dosed mother/dosed young in the entire period, in the group dosed mother/control young in the first 14 days and in the group control mother/dosed young in the last 7 days. Significant effect on static righting reflex, eye opening and auditory function was recorded in the dosed youngs nursed by the dosed mothers. Other developmental parameters indicated a slightly slower development in the same group only. Relative brain weight was significantly increased in dosed young nursed by both control and dosed mothers.

The decrease in weight gain in the dosed mothers is in agreement with previous reports of the effects of exposure to organophosphate compounds. The lower birth weight of pups from dosed animals might be a consequence of the toxic effect on the mother, a direct effect on the fetus or both. The reduction in weight gain is found not only to be of prenatal origin, as control young nursed by dosed mother showed a similar effect in the first 14 days. However, the dosed youngs crossfostered to control mothers show a lasting effect of prenatal exposure.

ROLE OF CONUS RIDGES IN NORMAL AND ABNORMAL MORPHOGENESIS OF THE RAT HEART

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It has been reported previously that transposition complexes are frequently induced in the rat by fast neutron irradiation on the 8th day of conception.

Okamoto found that the inversion of truncus swellings on anterior-posterior relationship resulted in complete transposition of great vessels.

For the purpose of clarifying morphogenesis of the double outlet right ventricle (DORV), embryonic rat hearts were examined by serial sectioning and reconstruction on 14th day of conception when conus ridges appeared most characteristically. In controls, conus ridges consisted of a sinistroventral ridge attached to the inferior anterior margin of the bulboventricular foramen or right anterior edge of muscular portion of intraventricular septum, and a dextrodorsal ridge connecting with the right side of the superior endocardial cushion and bulboventricular flange.

In experimented hearts, besides the inverted position of the truncoconal ridges, conus ridges were an increase or a decrease in volume and length, and the aortic intercalated valve swelling had elongated to the position of the dextrodorsal conus ridge.

Such morphologic changes and positional abnormalities of aortic intercalated swelling and conus ridges during early development appear to produce DORV.

EMBRYOTOXIC EFFECTS OF O-, M-, P-XYLENES

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Xylenes are on the list of chemicals suspected to have teratogenic effect. Known to have different toxicologic properties the embryotoxic effects of inhalation of the three different types of xylenes were studied separately in doses of 150, 1500 and 3000 mg/m^3, in rats, on days of 9—14 of pregnancy, for 24 h/day.

O-xylene caused weight retardation of the fetuses in doses of 1500 and 3000 mg/m^3; and a dose dependent increase of skeletal retardation signs.

M-xylene caused weight and skeletal retardation only in the dosis of 3000 mg/m^3, but also caused an increase in the incidence of extra ribs.

P-xylene increased fetal loss and weight retardation in the dosis of 3000 mg/m^3 and caused a dose dependent increase of extra ribs and skeletal retardation signs.

ONE HUNDRED SUBSTANCES TESTED WITH CHEST

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Over 100 substances comprising drugs from different pharmacological groups, pesticides, industrial poisons, food additives, cosmetics, etc. were subjected to the Chick Embryotoxicity Screening Test (CHEST). CHEST is a rapid and inexpensive screening procedure which allows to detect quantitatively adverse effects of a substance (and, if needed, also of its specific metabolites) both on the basic morphogenetic processes and on embryophysiological sphere. The procedure is capable of demonstrating of not only an embryotoxicity effect level, but also of positive dose-response relationships as well as of dependence of the effect upon stage of administration. According to the latter, the substances can be assorted into two principial groups. The first group, comprising classical cytotoxic agents, exhibits the decreasing deleterious effect along the time axis. The second group is characterized by the reversed relations, at least within some limited period of development. Significance of such a type of an effect is discussed with regard to embryotoxicity testing.

INTERSPECIES DIFFERENCES IN EMBRYOLETHAL ACTIVITY OF STEROIDS

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It is generally recognized that sex steroids can interfere with pregnancy performance in various ways. Partial or total litter loss can result if sufficiently large doses are given to laboratory animals.

The present study was undertaken to investigate the relationship between the pharmacological properties and the embryolethal potential of steroids. For that purpose repre-

sentative steroids were selected from four classes: highly and moderately active oestrogens, and progestogens with and without oestrogenic activity. All compounds were administered orally to Sprague Dawley CD rats from day 6 to day 15, to Golden hamsters from day 5 to 12 and to Dutch rabbits from day 6 to 18 of pregnancy. In each species a wide range of ELD50 values was established, e.g. in the rat from 0.015 to more than 128 mg/kg/day. The results demonstrate that the rat takes an intermediate position between the highly sensitive rabbit and the relatively insensitive hamster. A positive correlation between oestrogenic and embryoethal activity was found. The possible relationship with other pharmacological properties ls currently investigated.

TERATOEPIDEMIOLOGICAL STUDY ON PESTICIDE WORKERS

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The frequency of chromosome aberrations is higher in the worker producing organophosphate insecticides and herbicides. This poster demonstrates the reproductive data of 1377 workers in the Budapest Chemical Works where the above mentioned pesticides are produced. On one hand the data of workers (direct exposition) and industrial controls (only indirect exposition) on the other hand the data before and after starting work were compared. A significant increase in the fetal death was found in made workers after exposition. The occurrence of congenital malformations was significantly higher in the female workers the latter finding may raise the possible teratogenic effect of the working site in pesticides producing works.

THE ACTION OF RETINOIC ACID ON FETAL RAT BONE CULTURES

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The teratogenic effect of excessive doses of retinoic acid on limb development has been well documented (cf. D. M. Kochhar, Teratology **7**, 289—298, 1973).

In order to investigate the mechanism of action of retinoic acid on bone cartilage, humeri of late fetal rats were cultured in vitro. A time- and dose-dependent resorption of epiphysal cartilage was induced by all-trans-β-retinoic acid and was correlated with the loss of DNA, RNA and protein. Release of proteoglycan into the medium was coincident and quantitatively correlated with the loss of metachromatic staining with toluidin blue.

Using inhibitors of RNA and protein synthesis it has been shown that the retinoic acid-induced release of proteoglycan is dependent upon continous RNA and protein synthesis. In addition, it was found that the inducing effect of retinoic acid persists even after its withdrawal. These results suggest that enhanced synthesis of hydrolytic enzymes is required for cartilage degradation and argue against a direct action of retinoic acid involving labilization of lysosomal membranes.

POPULATION TERATOLOGY THROWS SOME LIGHT ON ISCHEMIC HEART DISEASE

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Population teratology, analysing large and for a long period collected series of congenital anomalies, contributes to better understanding of relations between mutagenesis and teratogenesis.

Recently unexpected findings made in a series of 99 cases of congenital coronary anomalies led us to the ischemic heart disease. The phenotype of 81 men having myocardial infarction (MI) was investigated including *fingerprints*. Two distinct dermatoglyphic patterns were found:

1. A *high* proportion of *arches* (18.6%) in men between 40 and 44 years of age at the time of MI, and

2. A *high* number of *true whorls* and a *drop* of *ulnar loops* in persons whose actual age at the time of MI was over 55 years.

The above findings — statistically significantly different from the Czech standard of 148,000 healthy men — seem to indicate *two types of MI*, at least the "*Arch type* of MI" is assumed to be of *prenatal origin*, based on *congenital coronaropathy*, whereas the *type* + *W/-Lu* represents probably *MI of advanced age* only.

The study continues for obtaining further data *re* prevention of *MI-type A*.

DEGENERATION AND REPAIR OF THE BRAIN AFTER PRENATAL INSULT

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When 5-FUdR (a DNA synthesis inhibitor) was injected in 12 day pregnant mice and the fetal brains examined at hourly intervals, mitotic activity was found to cease after two hours. In addition, some cells with a normal interphase nucleus lost their normal polysomal configuration probably reflecting a disturbed messenger RNA system. With time, 50% of the matrix cells degenerated according to the condensation-fragmentation pattern.

The degenerating cell debris was partially phagocytosed by healthy neuroepithelial cells and partially by macrophages. Most of the degenerated fragments remained free. These unphagocytosed fragments lysed and became ghosts, thereby giving the tissue a vacuolated appearance.

When the repair of the matrix population was examined, it was noticed that twelve hours after treatment mitotic activity started again and that this activity was almost twice that of controls between 24—48 h, indicating high cell proliferation to compensate for cell losses suffered. When the neuroepithelial cells were counted during the repair phase, the increased mitotic activity was found to be used mainly to increase the depleted neuroepithelial population and only little to produce neurons. As evidence of the insufficient production of neurons, behavioral tests showed that the postnatal animals had serious behavioral abnormalities.

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TESTING OF SITES FOR EXPERIMENTAL PRODUCTION OF SITUS INVERSUS VISCERUM CERVICALIS IN 4-DAY-OLD CHICK EMBRYO S

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In the 2nd half of incubation paired cervical organs show a marked topographic asymmetry, the right ones being situated more caudally and laterally. This is caused by the position of caudal oesophagus and of crop on the right side of neck, as already experimentally proved.

The branchial and hypobranchial region was tested by a mechanical noxa, viz. by tiny silver bands introduced into the 2nd, 3rd or 4th branchial clefts on the right and/or left side single or in combinations; a band was also applied caudal to the 6th aortic arch or caudal to the ductus Cuvieri on each side (altogether 10 sites, 21 combinations of sites tested; 11 embryos dissected per combination on day 14 of incubation).

Bands introduced into the right 3rd or 4th branchial clefts produced in 20% or 40% of cases oesophageal and crop inversions when applied single and 60% when applied simultaneously (cumulative effect). Both clefts represent an optimum for experimental production of situs i.v. cervicalis.

Bands introduced into both and clefts or into the left 4th cleft or caudal to the left 6th aortic arch cause merely an inversion of crop towards the left side of neck in 10% of cases.

TERATOGENIC ACTIVITY OF THE GLYCEROL FORMAL ON THE CHICK EMBRYO

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The glycerol formal, a fluid currently used as a drug solvent in toxicity testing has proved to be teratogenic when injected into pregnant rats.

On chick embryos, employed *in ovo* at a final concentration of 0.001%, it induces more severe and generalized malformations: exencephaly, anophthalmia or microphthalmia, coelosomia, ectopia viscerorum; beak and limbs are also frequently malformed and the heart is enlarged. Chick blastoderms, developed *in vitro* (New's technique) and treated with the same substance show open nerve tube, irregular somites, hyperemic blood islets and a greatly enlarged heart tube.

Morphological expressions of defects induced by the glycerol formal treatment *in ovo* closely resemble most of those found by us in the chick embryos treated with thalidomide.

INTERACTION OF GLYCOSAMINOGLYCANS (GAG) AND COLLAGEN TYPE I AND TYPE II *IN VITRO*

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Proteoglycans (PG) and glycosaminoglycans (GAG) alter cell proliferation, differentiation and migration during morphogenesis. In addition, GAG influence the aggregation of collagen and thus the formation of all connective tissues. For the understanding of the development of connective tissue malformations (e.g. limb defects) the GAG—collagen interaction is essential.

Collagen type I and type II were aggregated *in vitro* in the presence of various GAG and observed electron microscopically. Addition of synthetic GAG, e.g. the highly sulfated pentosan polysulfoester SP54, the mucopolysaccharide polysulfoester Arteparon and the mucopolysaccharide polysulfoester SMC, both types of collagen resulted in segment-long spacing (SLS)-collagen, fibril-long spacing (FLS) collagen and thin filaments after 3 h incubation at 4 °C. However, if the natural GAG, chondroitin-4-sulfate (C-4-S), chondroitin-6-sulfate (C-6-S), chondroitin-4-6-sulfate (C-4-6-S) or hyaluronic acid (HA) were used, only thin filaments were formed. After dialysis against phosphate buffer high doses of SP54, Arteparon, C-4-S, C-6-S, and C-4-6-S showed (SLS)-, (FLS)-collagen and a few native collagen fibrils, while low doses formed tick fibrils with a native cross-striation pattern. High doses of SMC and HA resulted in native-like collagen fibrils. These results show that under special conditions *in vitro* the highly sulfated GAG can induce the formation of collagen structures that *in vivo* are seen only under pathological conditions.

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PROPOSAL OF AN EXPERIMENTAL DESIGN TO ASSESS THE POSTNATAL FUNCTIONAL, PHYSICAL AND BEHAVIOURAL MATURATION IN MICE EXPOSED TO CHEMICALS AND DRUGS DURING THE PRENATAL PERIOD

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Exposition during pregnancy of the products of conception to drugs or other environmental toxic agents may induce anatomical or functional lesions not discernible at birth.

Recent researches have evidenced that the central nervous system during the prenatal life is sensitive to some toxic agents and that the adverse effect may manifest itself as retardation in the physical development and in functional maturation or as behavioural disturbances.

To make possible an evaluation of these alterations, which may manifest themselves during different periods of the postnatal life, a longitudinal-long term experimental design in the mouse is proposed. The time schedule of the postnatal maturation of non treated Swiss mice has been determined in standard conditions for some specific physical and behavioural parameters.

Signs of deficient physical development, modifications to the chronology of the functional maturation or abnormal behaviours would indicate a possible untoward effect of the chemical tested on the CNS development.

THE IMPORTANCE OF "GUIDING STRUCTURES" IN THE STRATIGENESIS OF THE CENTRAL NERVOUS SYSTEM

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Stratigenesis and differentiation of the cortical plate are determined by several genetic and epigenetic, partially unknown factors. From these factors, the appearance of a "guiding structure" involving ultrastructural elements, the vascular pattern and the differentiated ependymal cells have been identified. In the opinion of the authors, together with the "emigration corridors" of the avian optic tectum the vascular connectives and the peripheric processes of the ependymal cells may be considered as a morphological support having a guiding role. Its function is manifest during the second period of differentiation — the embryo-fetal transition period — preparing the next stages of differentiation in the integration centers.

DETECTION OF NECROTIC AREAS WITHIN THE EMBRYONIC ORGANISM BY FLUOROCHROMES

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Fluorochromes (acridin orange, quinakrin, koriphosin, auramin) introduced into the embryonic or foetal blood stream induce in the normal areas of cell death an elective fluorescence, as attested by experiments carried out on chick and rat embryos and foetuses. This vital method may serve as an experimental model for investigations concerning the transfer from the mother to the embryo of foetus of teratogenic substances as well as of their persistance and distribution — under certain experimental conditions — within the organism of the embryo or foetus.

DISTURBANCES OF COLLAGEN- AND PROTEOGLYCAN SYNTHESIS AND THE MORPHOLOGY OF CARTILAGE

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Collagen and proteoglycans (PG) as well as glycosaminoglycans (GAG) are of great morphogenetic significance. This is above all true of the differentiation processes and the functional maturation of cartilage. Lathyrogenic substances (D-penicillamine and β-aminopropionitrile) are e.g. able to inhibit the aggregation of collagen. β-xyloside, however, impedes the extension of the disaccharide chains which rest on the protein core or acts as a primer for the attachment of such chains.

As observed in the electron microscopical picture, in limb bud cultures the lathyrogenic substances inhibit the occurrence of collagen structures without disturbing the histiotypical structure and pattern formation of the cartilage skeleton. β-xyloside, however, leads to a decrease in the number and an alteration of the ruthenium-red-positive PG-granula. Hereby the histiotypical picture of the cartilage disappears due to a reduction of the intercellular spaces.

In addition, PG/GAG influence the aggregation of collagen. After application of β-xyloside or highly sulfated GAG untypical collagen structures (fibril-long-spacing collagen as well as giant fibrils with so-called continuous cross-striation) occur in the cartilage culture. Moreover, the highly sulfated GAG disturb cartilage and pattern formation.

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THE TWIN OCCURRENCE AFTER THE USE OF CONTRACEPTIVE PILLS

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There is an assumption concerning the connection between the use of contraceptive pills before conception and the more frequent occurrence of multiple births. In Hungary after the widespread use of pills the occurrence of twins increased (in 1970) and has remained on this high level. By the help of the Budapest Twin Register the above-mentioned correlation was

studied on three different time periods. In the material of 1970 (292 evaluated cases) a positive correlation was found. In 734 twins born 1970—1974 the correlations was confirmed in the pregnancies which occurred immediately after the use of pills. The evaluation of the material of 1975—1977 is in progress. It is worthwhile mentioning that the occurrence of conjoined twins was more than twice as high as the expected value in 1977.

TERATOGENIC EFFECTS CAUSED BY A SINGLE INJECTION OF TRITIATED WATER

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A single iinjection of tritiated water (i.p.) in mated NMRI mice revealed different damage pattern in relation to dose and time of application. Injection of 1.35 mCi tritium per g body weight, which corresponds to a dose rate of 429 Rad on the first day of application leads to a total resorption of the germs. Doses, ten times smaller did not affect the birth of the litters. Gross malformations could not be detected in this group. Doses in between show non-characteristic malformations like stunting, skeleton-deformations. — Mating of F_1-generation irradiated by the smaller doses in uteri exhibited a reduced productivity among the litters. Histological examinations show retardation of the gonads and changes at the ovary. The production of spermatogonia and oogonia is reduced. The "dosis minimalis" seems to be < 0.067 mCi/g body weight.

CONGENITAL MALFORMATIONS AND PERINATAL MORTALITY

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The perinatal mortality rate (stillbirths and neonatal deaths) was investigated on the ground of analysis of birth reports and autopsy documentations in Vilnius for the period 1961—75. The autopsies were carried out in all the cases. Congenital anomalies were registered in 21.25% of perinatally dead neonates (342 cases: 162 stillbirths and 180 neonatal deaths). Anomalies were observed in 17.9% of stillbirths and 25.56% of the deads. Sex ratio of malformations was correspondingly 1.22 and 1.07. The majority of malformations was serious. They were the cause of death of 82% cases in this group. Multiple malformations and the anomalies of the central nervous system predominated. Their dependence on the age of mothers and the seasonal variations of birth rate was determined.

TERATOGENIC EFFECT OF DITHIOCARBAMATE FUNGICIDE BASFUNGIN

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Propylene-bis-dithiocarbamate fungicide basfungin manifests a clearly expressed embryotoxic and teratogenic effect in experiment with rats. In unrepeated introduction of the formulation during separate phases of embryogenesis in doses 1/2—1/25 LD_{50} a dependence

of the embryonal reaction specificity to the quantity of the applied dose and the period of effect is established. The strongest teratogenic effect is registered when the formulation is uptaken during the period of late organogenesis (11—16 day), when structure anomalies of the CNS, jaw-facial apparatus, extremities, inner organs and bones are induced. During the critical period of embryogenesis under the effect of basfungin appear incompatible with the foetus viability malformations of all organs and systems. Threshold teratogenis dose in unrepeated introduction of basfungin (1/13 LD_{50}) is determined.

DIFFERENTIATION OF THE ORDERED MATRIX STRUCTURE IN THE DEVELOPING HUMAN CORNEA

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The spatial orientation of collagen and glycosaminoglycans was studied with polarization microscopical stainings during the development of the corneal stroma. At about the 7th week, bundles of collagen fibrils begin to take on a regular array in the juxtaendothelial region. In the same places sulfated and non-sulfated chondroitin molecules are arranged parallel to the fibrils. Subsequently, regular collagen lamellae develop and gradually fill the posterior two-thirds of the stroma by the 7th month. Keratan sulfate molecules are simultaneously built into the lamellae in increasing amount, and they are arranged with their long axes parallel to the fibrils. It is suggested that the highly organized lamellar structure of the secondary corneal stroma is a prerequisite of the transparency.

CLOFIBRATE AND THE DEVELOPMENT OF RATS

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Clofibrate (CPIB) given continuously to Wistar/Riop rats from the 16th gestational day to the end of lactation produced a decrease in birth-weight and an increase in the perinatal mortality as well as in the liver weight of 22-day-old rats. In further studies we intended to analyse this phenomenon. Mothers received daily oral doses of 150 mg/kg in the following schedules: 1. from 16th day to delivery; 2. from delivery to 8th postnatal day; 3. from 8th to 15th postnatal day; 4. from 15th to 22nd postnatal day.

Youngs were dissected on the 22nd day and the livers were weighed. No changes were found in the relative liver weight in the 1st and 4th groups, while a moderate increase was observed in the other two groups. In further investigations mothers were treated in the last week of pregnancy and 2—3 youngs from each litter were dissected on 1st, 8th, 15th and 22nd postnatal days. Increased liver weight was found in newborns but it disappeared later. The above transitorily increased liver weight in newborn and young rats might be related to enzyme induction and not to hepatotoxicity.

TERATOGENIC EFFECTS OF DIABETES IN GENETICALLY SELECTED SUCROSE FED DIABETIC RATS

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In order to study the teratogenic effects of diabetes and of high sucrose diet in rats, pregnant rats of the regular Sabra strain and diabetic rats developed by genetic selection and sucrose feeding (by A. M. Cohen) were studied. Six pregnant rats of the diabetic strain and 13 control rats were fed a high sucrose (72%) diet during gestation and four pregnant rats of the diabetic strain together with other 13 control rats were fed a normal diet. On day 16 of gestation uteri were opened, resorptions counted and fetuses studied for malformation. It was found that in the diabetic rats fed a high sucrose diet, the incidence of congenital anomalies was 11% and the resorption rate was 52%. In the sucrose fed controls the malformation rate was 6% and resorption rate was 15%. The abnormalities found were exencephaly and caudal regression defects. In the rats from the diabetic strain fed a regular diet, malformations were found in 12% and the resorption rate was 17%. In the controls, no anomalies were found, and the resorption rate was 6%. In the rats with the diabetic genetic background, the metabolic derangement in carbohydrate metabolism caused by the high sucrose diet increased the teratogenicity of diabetes by in reasing the resorption rate, thus the control of the metabolic derangement in diabetes may reduce significantly the rate of fetal death, resembling the situation in human diabetes. High sucrose diet seems to be teratogenic even in normal animals, suggesting a possible teratogenic effect of hyperglycaemia or other carbohydrate metabolic derangement caused by this diet.

EFFECT OF ALCOHOL AND ACETALDEHYDE ON MAMMALIAN CELLS

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Acetaldehyde at 40—400 μM concentrations elevated sister chromatid exchange (SCE) values in human lymphocytes and inhibited DNS synthesis in Chinese hamster ovarian cells, while alcohol was ineffective. Alcohol addicts under acute influence of alcohol with 0.1—0.36 p.c. blood level did not show any change in SCE in lymphocytes. Contrarily, SCE values elevated when blood level of acetaldehyde was high, 117—340 μM. On the basis of our data we suppose that alcohol can cause a damage at the cellular level due to the action of acetaldehyde at increased concentration. An augmented blood acetaldehyde concentration due to a defect of acetaldehyde dehydrogenase in drinking pregnant women can lead to malformed fetus. In this way, alcohol develops into a teratogenic agent.

MICROANGIOGRAPHY IN THE EXPLANTED CHICK EMBRYO FOR TERATOLOGY STUDIES

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A factor not commonly considered in teratology is the early appearance of blood circulation in embryonic development. At stages when the later malformed regions are still undifferentiated structures the embryonic circulation is an integrated physiological system.

This can be demonstrated in the embryo of the chick. A method to explant 2 and 3 day embryos has been developed which allows the study of the early circulatory development by transluminent microscopy. After intravascular injection of fluorescein-labelled dextran the early embryonic vascular bed can be clearly demonstrated and the central as well as the peripheral circulation studied in detail.

This method has been used to study circulatory disturbances following teratogenic conditions such as hyperthermia and salicylate administration. The findings support the concept that vascular insufficiency may play an essential role in teratogenic mechanisms.

VASCULAR CHANGES IN THE VITELLINE MEMBRANE OF THE CHICK FOLLOWING INCREASED INCUBATION TEMPERATURES

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Increasing the incubation temperatures is a useful method of studying the effects of a teratogen in the chick embryo in direct relation to the exposure.

The microcirculation in the pellucid area was studied after explantation and injection of fluorescein-labelled dextran in embryos exposed to 41 °C (normal 38 °C) during the first three days of incubation. A variety of vascular changes were observed which included thrombosis and haemorrhage. The general finding of an irregular vascular pattern is shown to be due to perivascular oedema which may indicate vascular insufficiency. This pathologic permeability has been demonstrated physiologically by dextran leakage and morphologically by electron microscopy. Further studies to link these vascular changes to teratogenic mechanisms are in progress.

THE OCCURRENCES OF DIFFERENT COMBINATIONS OF SUBGROUPS OF VACTERL ASSOCIATION

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The combinations of each component of VACTERL association were studied on the material of the Hungarian Congenital Malformation Register, 1970—1975. The preliminary analysis showed a spositive correlation between the components of VACTERL and the malformations of the genital organ therefore as a seventh component the latter was being included in this association. The criteria of this association was that four or more components of "VACTERL-G" occurred in the same baby. Thus the number of "VACTERL-G" was 25, three of the components occurred in 24 babies and two of them in 180 babies. The observed occurrences of two and three combinations were significantly higher than the expected values. The detailed figures will be shown in tables.

EMBRYOTOXIC EFFECTS OF BENZENE INHALATION

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Benzene is known to have mutagenic and carcinogenic effects and is suspected to have teratogenic effect, too.

CFY rats were exposed to inhalation of 150, 450, 1500 or 3000 mg/m^3 benzene on days 9—14 of pregnancy, for 24 h/day. High maternal and fetal mortality, marked fetal weight retardation and increased incidence of skeletal retardation signs were observed in all doses applied, with the exception of the lowest dose. The incidence of extra ribs increased at 1500 and 3000 mg/m^3 doses. None of these parameters showed dose-response relationship. The incidence of malformations did not increase at any of the doses.

It was concluded, that although benzene caused severe maternal and fetal toxicity above a certain concentration, it did not prove to be teratogenic.

THE JAPANESE QUAIL AS A MODEL FOR MUTAGENICITY STUDIES

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Few mutagenicity studies are carried out in avian species which is somewhat surprising considering the extent of the exposure of poultry and wild birds to large numbers of different chemical agents e.g., food additives, agrochemicals etc. This demonstration outlines a method for studying mutagenic potential of compounds in the male Japanese quail. The advantages to the research laboratory of this species include:

1. Inexpensive, low feeding costs, small size, rapid onset of sexual maturity, high egg production and extended laying period.

2. Normal development well documented.

Method: Groups of male quail of proven fertility were dosed by oral gavage with TMP at 500 or 1000 mg/kg/day for five days. Following treatment each male was penned with three virgin females each week for nine consecutive weeks. Eggs produced during the first three days were discarded, and only those produced during the following seven days were collected and incubated. Half of each batch of eggs was examined after five days of incubation, whilst the remainder were examined at twelve days. The fertility and normality of development of each egg was recorded.

Results: The results obtained indicated an initial decrease in fertility and an associated increase in mortality of developing chicks. Subsequently fertility and development returned to normal.

STUDIES ON THE GENESIS OF LIMB DEFECTS (AMELIA, HEMIMELIA) INDUCED BY NITROGEN MUSTARD

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The treatment with nitrogen mustard of 2- to 2.5-day-old embryos (stages 10 to 14 H.H.) induced 2 types of limb defects, either amelia or hemimelia.

In order to determine the first effect of the teratogenic substance, reciprocal combinations were carried out, using the method of Kieny (1960). The mesodermal component of the prospective limb area of a nitrogen mustard treated embryo was grafted under the flank of a normal embryo and inversely the prospective leg mesoderm of a healthy embryo was implanted under the flank of a treated embryo. Results: no recombination produced a normal limb. But the type of malformation obtained in these experiments was dependent on the treated component. The lack of distal parts in the case of hemimelia may be due to a deficiency of the apical crest.

CONTRIBUTIONS TO THE STUDY OF EYE ANOMALIES

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Morphological pictures obtained by the dynamical pursue of ocular changes induced by bisazo dyes and 6-AN in albino rats are presented. (Trypan-blue and Niagara-Sky-blue 6B were administered on day 9 i.p., 6-AN was administered on days 9—17 i.p. and on day 15 i.a.) Our observations allowed the following main statements:

Ocular anomalies may develop by two pathogenetic pathways: early disturbances of some morphogenetic events; late cytotoxic effect (6-AN reveals to be able to induce anomalies by both pathways mentioned).

In the case of 6-AN-induced ocular anomalies direct and indirect late effect does not differ essentially.

Some marked lesions induced by 6-AN show a striking reversibility.

The laterality of anomalies induced during various stages of development suggests a working hypothesis concerning the laterality of anomalies induced by experimental environmental factors.

A new experimental model, grafting of rat ocular primordia into the developing chick embryo is shortly presented.

SEX DEPENDENT MORTALITY RATE OF NEONATAL MICE AFTER FRACTIONATED X-IRRADIATION PRIOR TO SEX DIFFERENTIATION

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Fractionated X-irradiation at gestational days 11—13 induced dose independently more severe telencephalic lesions in female fetuses than in male ones. At 3×110 rad only, concomitant alterations of the shape of the female thalami were observed. Female neonatal mortality was consequently elevated, thus until weaning three times more males survived than females. In contrast to the controls, the body weights of the surviving irradiated males from postnatal day 16 on did not surpass those of the females. There were also no sex differences neither in the brain weights, nor in the activities of two brain enzymes, acetylcholinesterase and Na, K-ATPase. As X-irradiation occurred prior to sexual differentiation these results may be explained only by a dose dependent preferential radiation injury to X-linked functions.

A RARE CONGENITAL CARDIAC MALFORMATION IN A DICEPHALUS FETUS WITH MULTIPLE MALFORMATIONS

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A rare case of congenital cardiac malformation in a dicephalus fetus is described. The mother was a 26-year-old pluripara who in the ninth month of gestation, in 1976, gave birth via Cesarean section to a female, intrauterin dead fetus. The fetus weighed 3300 grams, with

a crown to heal length of 46 cm and with a single umbilical cord and a single placenta. Morphologic examinations revealed: two heads (dicephalus), left occipital meningoencephalocele, two superior limbs (dibrachius), and two inferior limbs. The vertebral column was doubled until the sacrum. The spinal cord showed an asymmetric architectura, with cornual hypoplasia of the grey substance on the side of the missing limbs. The single chest had two mediastinums, two hypoplastic (anectasic) lungs, and median diaphragmatic hernia with "Spiegel" lobes. Imperfect rotation of the viscera with subhepatic median position of the cecum, vermiform appendix, and Meckel's diverticulum was present. The enlarged heart was in median position (mesocardia). It was uniatrial and triventricular without septal defects or transposition of vessels, and was shared by the two fetuses. The common atrium was dilated with attached auricles, one auricles on each side. On the posterior-inferior wall of the common atrium the two venae cavae entered through a single orifice, while the pulmonary veins of each lung entered through the posterio-lateral walls. The common atrium had three atrio-ventricular openings, one for each of the two anterior ventricles and one for the single posterior ventricle. From the anterior ventricles originated the pulmonary arterial trunks, the right one from the right anterior ventricle and the left from the left anterior ventricle. The two aortas, left and right, originated from the single posterior ventricle.

The dermatoglyphogical aspect is more complex, with numerous bideltic patterns of radial orientation the triradius of the left palm in the position.

CONSIDERATIONS REGARDING A RARE CONGENITAL MALFORMATION (CYCLOPIA)

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A rare case of congenital malformation is described (cyclopia). The cause was probably a medicamentos teratogenic factor. The mother, 23 years old, took Codenal from the first two weeks of pregnancy (197 days). She gave birth spontaneously at 7 months to a still-born foetus with multiple malformations (cyclopia astomata otocephalica).

EFFECTS OF CHOLINERGIC AGENTS ON THE DEVELOPMENT OF THE AVIAN AXIAL SKELETON

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After having demonstrated the control mechanism exerted by the periaxial extracellular material on the differentiation processes of the somitic mesenchyme, we showed the existence of biogenic amines in the chick notochord. With the fluorescence method of Falck and Owman and by qualitative and quantitative analysis with labelled dansyl derivates we showed the transitory secretion and storage of E, NE, DA and 5-HT in 2 to 5 day old chick notochords. The injection of a cholinergic agonist modifies the biogenic amine content of the notochord and causes morphogenetic defects and abnormal biosynthesis. The cervical notochord and neural tube are twisted, the cervical somites are telescoped. In older embryos the spinal column is distorted. The chemical composition of the periaxial extracellular material

is altered and the vertebral cartilage is abnormal. The effects of the cholinergic agents are inhibited by cholinergic antagonists, cholinergic receptor ligands and by β-adrenergic antagonists. Neurotransmitters seem to be involved in the axial skeleton embryogenesis.

METHOD FOR DETECTION OF MICROMELIA IN RATS

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The detection of micromelia in fetuses is frequently very difficult.

The limb development can be well characterized by the length and thickness of long bones and the ratio of these parameters (femur index) is in good correlation with the body-weight of the fetuses. 20 to 21.5 days old Wistar/Riop fetuses were weighed and after KOH alizarin red S staining the length and thickness (at the distal epiphysis) of femurs were measured. Fetuses were divided in groups with half gr difference in body weight and the normal femur indexes of each weight-group were established by computer.

This method was tested on the fetuses of perphenazine-treated mothers. This compound induces micromelia of different degree when given on 13th, 14th or 15th days of pregnancy.

According to the results this method is very useful for detecting even moderate micromelia.

TERATOEPIDEMIOLOGICAL STUDY ON PVC WORKERS

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The higher occurrence of chromosome aberrations in PVC workers was proved. After this an epidemiological study was established in 231 PVC workers for the analysis of reproduction. The so-called industrial control (without direct exposition) PVC (polymer) and VC (monomer) groups were comparing and the reproduction data before and after the work in this chemical factory within each group were studied. A significant increase of fetal death and congenital malformations as well as the industrial diseases were found in the VC group.

AGGRAVATION OF CS_2 TERATOGENICITY AFTER EXPOSURE OF PROGENY OF CS_2 TREATED MOTHERS

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Our previous observations on CS_2 effect on rat embryo development, employing exposure levels of 50, 100 and 200 mg/m^3 throughout gestation, revealed marked dose-related effect on pre- and postnatal development of the generation, the threshold level being 50 mg/m^3. Further, a second generation study has been performed, which is the subject of this investigation. After reaching sexual maturity F_1, progeny has been mated within experimental groups.

Pregnant F_1 females from each group have been divided into two subgroups: the first received no further CS_2 treatment and the second was treated with the same dose levels. Embryonal lethality, weight, gross external malformations and certain indices of lipid and energy metabolism, DNA and RNA liver levels were studied. Comparisons between subgroups and with originally treated F_1 generation testify the fact that repeated CS_2 exposure results in marked aggravation of CS_2 teratogenicity in F_2, demonstrated by a two fold increase of the frequency of malformations, detectable even at the lowest dose level and a more pronounced impairment of lipid and energy metabolism. It is worth mentioning that such an approach is appropriate for teratogenicity testing of environmental pollutants as the repeated exposure of several subsequent generations is a common fact in the case of environmental pollutants.

TERATOGENIC AND EMBRYOTOXIC EFFECTS OF SALICYLIC ACID IN RATS EXPOSED TO TOLUENE

E. Tátrai, A. Hudák and Gy. Ungváry

STATE INSTITUTE OF OCCUPATIONAL HEALTH, BUDAPEST, HUNGARY

The known teratogenic effect of salicylic acid can be increased by enhancing the glycine utilization. Metabolization of salicylic acid requires glycine. Toluene detoxication also utilizes glycine, but toluene is not teratogenic. The combined teratogenic and embryotoxic effects of these two chemicals were investigated. CFY rats were exposed to toluene or air inhalation on days 10—12, and a single dosis of 50 mg/100 g b.w. salicylic acid was given p.o. on day 12 of pregnancy. Toluene inhalation caused high maternal mortality and increased fetal mortality, retardation as well, and also the incidence of anomalies and malformations in the salicylic acid treated rats as compared with the air inhaling animals. It means that toluene potentiates the embryotoxic and teratogenic effects of salicylic acid.

EXPERIENCES WITH METHODS TESTING THE TERATOGENIC EFFECTS OF OESTRATRIENS

G. Tenner, H. Lemke and K. H. Chemnitius

VEB JENAPHARM-RANGE RESEARCH AND DEVELOPMENT, DEPARTMENT OF PHARMACOLOGY, JENA, G.D.R.

It has been worked out a specifical testing programme for teratological effects of oestrogenes. Experiments have been made on laboratory animals — WISTAR-rats (stock Jenapharm).

Drugs have been applicated during embryogenesis (6th—10th day p.c.; 10th—14th day p.c.) and during fetogenesis (15th—19th day p.c.) in a dosage of 0.01; 0.1 and 1.0 mg/kg i.p.

Testing parameters have been:

- reproductional potential (i.e. quota of conception, implantation and resorption);
- weight of the litter;
- retardation of weight and squelett
- type and quota of malformations.

TERATOLOGICAL STUDIES OF ALBENDAZOLE IN SHEEP

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Only occasionally does the opportunity arise in teratological studies of a new therapeutic agent to be able to use the species for which the agent is designed. Such an opportunity presented itself in the investigation of a new wide-spectrum antihelmintic. Albendazole (SK & F62979) developed for use in sheep and other domesticated animals.

A flock of approximately 360 ewes, Dorset Horn Cross and Clun breeds were used, they ware mated naturally with rams of proven fertility. Albendazole was administered orally as a single dose on day 17 of gestation at levels of 7.5, 10, 15 or 20 mg/kg. Ewes were allowed to lamb naturally and to rear their young. Lambs found dead, killed in extremis or considered to be not commercially viable, were subjected to detailed macroscopic examination including, where indicated, radiographic examination of the skeleton.

There were no adverse effects upon maternal condition, bodyweight or parturition. The total number of lambs born was similar in all groups, but the viability at 20 mg/kg was significantly reduced. Examination of lambs indicated that at 20 mg/kg renal, cranial and spinal defects were produced, whilst at 15 mg/kg, defects were limited to the kidneys. A level of 7.5 mg/kg had no teratogenic or embryopathic effects.

SOME EFFECTS OF THIOTEPA ON RABBIT SPERM MORPHOLOGY

J. M. Tesh and F. W. Ross

DEPARTMENT OF REPRODUCTIVE STUDIES, LIFE SCIENCE RESEARCH, STOCK, ESSEX, U.K.

In the continuing search for new methods to screen compounds for mutagenic activity, a sperm abnormality assay has been proposed using mice (Wyrobek and Bruce, 1977). In the present demonstration an alternative species is suggested, viz. the rabbit, the advantages for its use are as follows:

1. Repeated collections of semen can be obtained using an artificial vagina, thus facilitating the study of semen quality before and after treatment with a test compound.

2. Semen characteristics can be assessed using a number of criteria e.g., sample volume, colour and mobility, sperm concentration, live/dead ratio and morphological characteristics.

3. If required, the functional capacity of semen samples of known quality can be tested.

Method: Following an initial training period to an artificial vagina, male rabbits were selected on the basis of their production of high quality semen. Test males received a single intraperitoneal injection of 10 mg/kg Thiotepa, and following this twice weekly semen samples were obtained for a period of ten consecutive weeks. Semen quality was assessed as indicated above.

Results: An increased incidence of sperm abnormalities were recorded from Week 2 post-treatment, whilst a decrease in total sperm numbers was observed from Week 7. The significance of these results will be discussed in relation to the use of the method in screening for mutagenicity.

LEAD — A BEHAVIOURAL TERATOGEN?

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The increasing awareness amongst teratologists that pre-natal exposure to chemicals may affect post-natal development, and the endorsement of this view by many Governmental safety committees, has necessitated the development of suitable screening procedures. However, the choice of methods has been left to individual laboratories and little information is yet available on the value of the various test systems. We therefore decided to investigate, in the developmental screening profile used in our laboratories (Tesh '77), an agent known to cause damage to the central nervous system when administered postnatally, in an attempt to obtain positive control data on the effects of pre-natal treatment.

Lead nitrate was administered intravenously to female Sprague Dawley rats on day 17 of gestation at dose levels between 15 to 50 mg/kg. A proportion of the females were killed on day 21 of gestation to assess the effects in utero. The remainder were allowed to give birth and to rear their young naturally. Offspring were monitored for both physical and behavioural development. At the higher levels, offspring survival was reduced, but growth and physical development were unaffected. Learning ability and locomotor coordination of offspring from treated females were inferior to the controls.

It is suggested that lead might prove to be a useful positive control in future studies of post-natal development.

INHIBITION OF TOOTH DIFFERENTIATION *IN VITRO* BY DIAZO-OXO-NORLEUCINE (DON)

I. Thesleff, K. Hurmerinta and L. Saxén

III. DEPARTMENT OF PATHOLOGY, UNIVERSITY OF HELSINKI, SF-00290 HELSINKI 29, FINLAND

Molar tooth germs from mouse embryos were cultivated in a Trowell type organ culture in BGJb medium supplemented with 20% horse serum and 10% chick embryo extract. After 5 days of cultivation the odontoblasts had secreted predentin and the ameloblasts had differentiated. When cultivated in the presence of 10—50 μm diazo-oxo-L-norleucine (DON), a glutamine analogue, the differentiation of odontoblasts was inhibited, but the teeth looked otherwise healthy. When DON was added after two days of culture in control medium (at this time the odontoblasts at the cuspal tips were already differentiated), it did not inhibit predentin secretion, ameloblast differentiation, nor enamel secretion. However, the boundary between the differentiated cuspal area and the undifferentiated, more cervical cells was distinct.

The results support the concept that the mechanism of the differentiation of odontoblasts is different from that of the ameloblasts. Concerning the mechanism of odontoblast differentiation we have shown earlier that a close association between the basement membrane and the differentiating mesenchymal cells is required (Thesleff et al., Differentiation 10: 71, 1978). Because DON interferes with glycosaminoglycan and glycoprotein synthesis (Greene and Pratt, Exp. Cell Res. 105:71, 1977), we suggest that DON inhibited odontoblast differentiation by affecting the mesenchymal cell surface and/or the basement membrane.

RELATIONSHIP BETWEEN TERATOGENIC POTENTIAL AND PHARMACOLOGICAL ACTIVITY OF GLUCOCORTICOIDS IN THE RAT

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CENTRE DE RECHERCHES ROUSSEL-UCLAF, 93230 ROMAINVILLE, FRANCE

The teratogenic effects of corticoids are well known in rodents. The present study in the rat treated orally was undertaken to determine whether these effects are related to their glucocorticoid activity. On the basis of their teratogenicity, estimated by cleft-palate induction after treatment from days 13 to 16 of gestation, the tested drugs were classified in the following decreasing order: triamcinolone acetonide, dexamethasone, triamcinolone, 16 α-methyl prednisolone, hydrocortisone. For these compounds the threshold dose leading to a teratogenic effect ranged from 0.5 mg/kg to 2 g/kg. Corticosterone and cortisone were not teratogenic at the dose of 2 g/kg. A similar classification of the tested drugs was obtained on the basis of antiinflammatory activity evaluated on the cotton granuloma test (range: 0.05 to 100 mg/kg), thymolytic activity (range: 0.03 to 50 mg/kg) and neoglycogenic activity (range: 0.01 to 5 mg/kg) suggesting that the teratogenic potential of glucocorticoids is highly correlated with their pharmacological activity, but that it occurs at much higher doses. Previous experiments on the effects of non steroidal anti-inflammatory drugs such as aspirin, indomethacin and phenylbutazone have not been able to establish such a correlation since their teratological study is limited by their gastrointestinal toxicity.

SENSITIVE PERIODS AFTER APPLICATION OF LATHYROGENS DURING THE DEVELOPMENT

D. WENDLER

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We investigated the action of the lathyrogens aminoacetonitrile (AAN), β-aminopropionitrile (BAPN), and D-penicillamine (PEN) upon the blastogenesis, embryogenesis, and fetogenesis of the rat. In the chicken the substances were proved only on day 7. 300 mg AAN/kg produced in the rat 2 peaks of lethality: on days 11 and 17. The malformation rates were low in blastogenesis and embryogenesis but rose in fetogenesis to 100% (ectocardia, wristdrop, humpback, hydronephrosis). 1 g PEN/kg daily given from day 15 until term did not produce any malformations in the rat. This substance has also no effect on the developing chicken embryos, but AAN and BAPN caused maldevelopment of the lower peak and bending of the tibia.

RECENSIONES

Prof. Dr. B. GERHARDT: Cell Biology Monographs. Vol. 5: *Microbodies/Peroxisomen pflanzlicher Zellen.* Morphologie Funktion und Entwicklung eines Zellorganells. With an English assessment. 223 pages, 59 figures. Springer Verlag, New York 1978.

Price: DM 139.20

As a result of multidisciplinary investigations carried out on plant cell microbodies, more light has been thrown on the function of this organelle.

This new monograph gives a comprehensive account of almost all fields of plant microbody research. A special value of the book is that it aims at guiding workers in the field particularly in pending questions such as the origin and biochemistry of peroxisomes.

The book consists of 12 chapters and concludes with a summary. A detailed list of references is added.

The first chapter deals with the definition and functional significance of the peroxisome, the second with its morphological features and with criteria helping classification. A detailed account is given of the formation of microbody inclusions and their possible functions. The third and fourth chapters consider connections between peroxisomes and other organelles and their occurrence in various plants. The fifth chapter covers the enzyme cytochemistry of microbodies with critical comments on the results. A conclusion emerging from these chapters is that cytochemistry is suitable to localize peroxisomal enzymes of the ultrastructural level. The sixth chapter describes means of isolation of peroxisomes from the plant cell, the seventh enlarges on the peroxisome membrane and its protein and lipid composition, enzyme biochemistry and function. Chapter eight deals with peroxisome function in higher plants, the role of glycosomes in lipid and carbohydrate metabolism and in the glycocyclate cycle. Chapters nine and ten contain data on the role of peroxisomes in algae and fungi. The elventh chapter is decoted to the biogenesis and development of peroxisomes. In chapter twelve a summary is given of the effects of light, phytohormones and metabolites on the regulation of peroxisomal function.

This very readable book is richly illustrated. It may be useful not only for workers in basic research but for all who would like to read a comprehensive summary of current knowledge about this organelle.

P. SÓTONYI

H. M. DUVERNOY: *Human Brain Stem Vessels.* 188 pages, 108 figures, and 2 folding plates. Springer Verlag Berlin, Heidelberg, New York, 1978.

Price: DM 200.—

This book gives a detailed description of the vessels and angio-architectonics of the human brain stem. India-ink filling was used to demonstrate the cerebral vessels. Further techniques employed were the preparation of vessels under the stereomicroscope, examination of supply territories in cleared (Spalteholz technique) serial sections, description of the vascular structure of the nuclei and tracts in impregnated (Bodian's silver impregnation) serial sections

The first part deals with the arteries and veins of the medulla, pons and mesencephalon. The supply territories of individual arteries are defined and the supply pattern of nuclei is described.

The second part is concerned with the angio-architectonics of brain stem nuclei and tracts.

The descriptions are concise, clear and didactic, the list of references is comprehensive and goes back to the last century. Illustrations are photographs of excellent quality constituting a most useful atlas. Figures 1—3 depict brain stem surfaces. They are followed by 13 large diagrammatic colour pictures on 2 folding plates showing the surface and inner vessels of the brain stem. Figures show also the nuclei and tracts found in the corresponding frontal section. Separate black-and-white schemes illustrate the supply territories of vessels. Surface vessels are also shown in original preparations, and 76 ink-filled and cleared sections document the vascularization of all brain stem areas.

The book concludes with a carefully composed subject index.

The work is equally valuable for the human anatomist, neurophysiologist, neurosurgeon and neuroradiologist.

G. AMBACH

Genetic Mosaics and Cell Differentiation. Ed. by W. J. GEHRING. Results and Problems in Cell Differentiation. Volume 9. pp. 315. Springer Verlag, Berlin, Heidelberg, New York. 1978.

Price: DM 98.—

The volume deals with the methodological and theoretical aspects, results and perspectives of the works made on genetic mosaics produced by different techniques (e.g. X-ray inducation, recombination, etc.) provide a good opportunity for the study of cellular interaction and cell lineage during development. Their examination allows the investigator to determine the number and location of progenitor cells in the embryo and to construct fate maps on this basis, since the fate of a cell is decided largely by its location in a developing system.

The volume consists of the papers written by leading experts in the field, reviewing the results and problems of experimental works on genetic mosaics of Dorsophila and mouse. The chapters are well illustrated and supplied with lists of references covering practically all the papers published on the subject during the last years.

The volume is well worth reading for biologist of broad spectrum, and especially for those engaged in genetics, cytology and developmental biology.

J. KOVÁCS

Jürgen ROTH: *Experimentelle Pathologie.* Spl. 3. *The Lectins.* 186 pages, 3 tables, 26 figures. VEB Gustav Fischer 1978.

Price: M 59,—

Recent studies have clearly shown the importance of cell surface substances in regulating cellular functions in various stages of the cell cycle. It has been established that the glycoproteins of the cell surface play a central role in these processes. This excellent monograph deals with the significance of lectin. It contains 11 chapters concluded by a 12th summarizing one. References are listed comprehensively, electron micrographs, tables and schematic drawings serve properly their purpose.

The first chapter is a histotogical review, the second tells about function. The third chapter is concerned with the physico-chemistry of lectin, the fourth and fifth chapters with the purification of lectins and with the structure of so-called lectin-binding sites. The sixth chapter deals with the methods of demonstration of lectin used in cell biology and membrane research. A detailed account of cell agglutination isotope and microscopic techniques is given. Chapter seven compares the lectin component of normal and malignat cell-surfaces. Chapter eight consideres the effects of lectin on cell metabolism and function including that of lymphocytes, macrophages and blood platelets. Chapters nine and ten discuss the significance of lectin research and preparative and analytical biochemistry.

The book is recommended primarily for biochemists but morphologist may also benefit from it.

P. SÓTONYI

D. STARCK: *Vergleichende Anatomie der Wirbeltiere* auf evolutionsbiologischer Grundlage. Band I. Theoretische Grundlagen. Stammesgeschichte und Systematik unter Berücksichtigung der niederen Chordata. 274 pages with 100 figures. Springer Verlag, Heidelberg, Berlin, New York 1978.

Price: DM 88.—

Comparative anatomy has made a growing progress toward the synthesis of classical data with the modern functional results given by genetics, embryology, physiology, ethology, etc. Professor Starck's three volume work is a successful example of this tendency.

The first volume of the work is divided into three parts. The first part summarizes the history of comparative anatomy, the relation of onto- and phylogenetics, the basic laws of evolution and Darwin's theory.

In the second part there is an introductory chapter about lower vertebrates which is followed by a systematic description of Acrania, Tunicata, Haemichordata, Tentaculata and their phylogenetic connections.

The third part is a comprehensive classification of recent and fossie vertebrates on the basis of evolutionary relations including many anatomical, embryological and paleontological conclusions.

The traditional chapters of comparative anatomy such as the description of organ systems will appear in the future volumes *viz.* in the second volume the skeletal system and the types of locomotion; and in the third volume the skin and its derivatives, the muscular system, the coeloma, the immune system, and the organs of metabolism and reproduction.

The book is well illustrated, and contains a detailed subject index, a register of the animals and a list of the most important pertinent references.

All this makes the volume a very useful aid in understanding comparative anatomy for all those interested in research of teaching of the subject.

J. KOVÁCS

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Budapest, September 21—24, 1980

INTERNATIONAL ACADEMY OF PATHOLOGY

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Chinoin Pharmaceutical and Chemical Works Ltd. and Institute of Biology,
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EFFECT OF GLUTAURINE ON THE PINEAL GLAND OF THE RAT

L. Feuer, B. Madarász, F. Sudár and G. Csaba

(Received February 8, 1979)

Glutaurine (gamma-L-glutamyl-taurine), the recently discovered hormone of the parathyroid enhances the aggregation and subsequent degeneration of mitochondria in the pinealocyte processes of the rat pineal gland. It also stimulates autophagy, probably through its general lysosome activating effect.

Introduction

Glutaurine (gamma-L-glutamyl-taurine), the newly discovered hormone of the parathyroid [3] possesses vitamin A like effects [4, 5, 7] and acts on the function of different endocrine organs; it has been shown to antagonize the effects of cortisone and thyroxine in several systems [6, 11]. Since the putative precursor of glutaurine, the amino acid taurine, is known to affect the synthesis and/or release of melatonin by the pineal gland [1], it seemed worthwhile to investigate the morphological alterations caused in the gland by glutaurine.

Material and method

Sexually mature Wistar CB rats of our own breed, weighing 150—200 g, were used in the experiments in groups of five.

Rats of the first group were treated intraperitoneally with 10 μg glutaurine in 1 ml saline on a single occasion.

In the second group, 1 μg glutaurine in 1 ml saline was administered intraperitoneally to each rat on 7 consecutive days.

Rats of the third group were treated intraperitoneally with 1 ml saline, to serve as controls.

Treatment was always performed at 9 a.m., and the pineal gland was removed one hour after the last injection.

The pineal gland was removed surgically under ether anaesthesia. The abdominal aorta was ligated, and Karnowsky's solution was injected through the aorta for perfusion fixation. The rats then were decapitated, the pineal gland was removed, divided into two parts, and fixed in Karnowsky's solution for further 24 hr. The specimens were then post-fixed in osmium tetroxide for two hour, embedded in Araldite (Durcupan, Fluka AG., Buchs, Switzerland), sections were cut with an ultra-microtome (Tesla, Brno, Nat. Corp. CSSR), and examined with the electron microscope (Jeol 100 B, Japan).

Results and discussion

The pineal gland is composed of two cell types, pinealocytes and astrocytes (interstitial cells) [2, 9, 10, 12]. The astrocytes extend processes and contain few organelles. As their morphological appearance was similar in the experimental and control groups, further descriptions are centred on the pinealocytes.

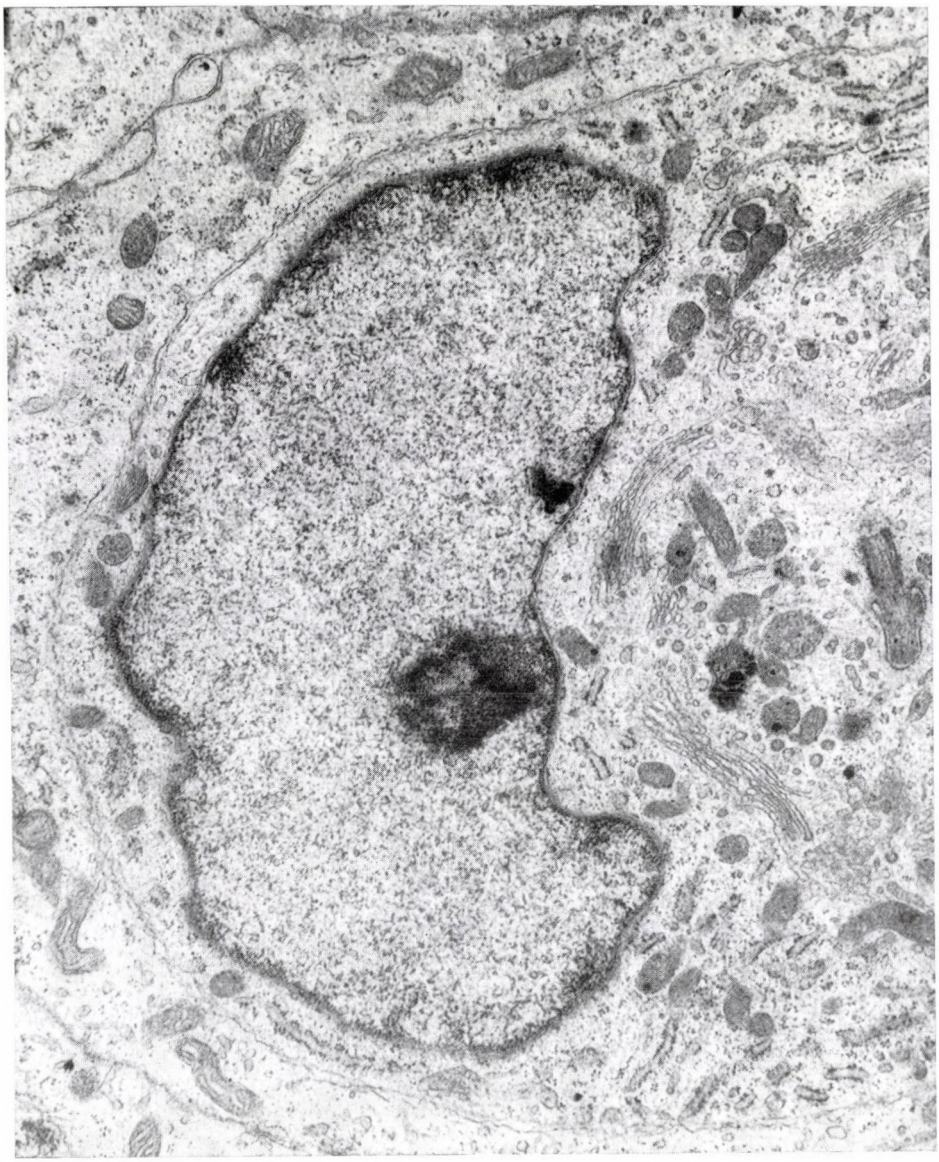

Fig. 1. Pineal gland of control rat. Euchromatic nucleus, prominent nucleolus, well-developed Golgi complex, centriole, diffusely localized mitochondria and few endoplasmatic reticula of pinealocytes. × 17 000

The pineal gland of the control rats showed a vigorous cellular activity corresponding to the hour of removal. The pinealocytes displayed euchromatic nuclei, prominent nucleoli, a well-developed Golgi apparatus (Figs 1 and 2), and enclosed many lipid droplets of various density and size. Some mitochondria clung to the lipid droplets and exhibited signs of transformation.

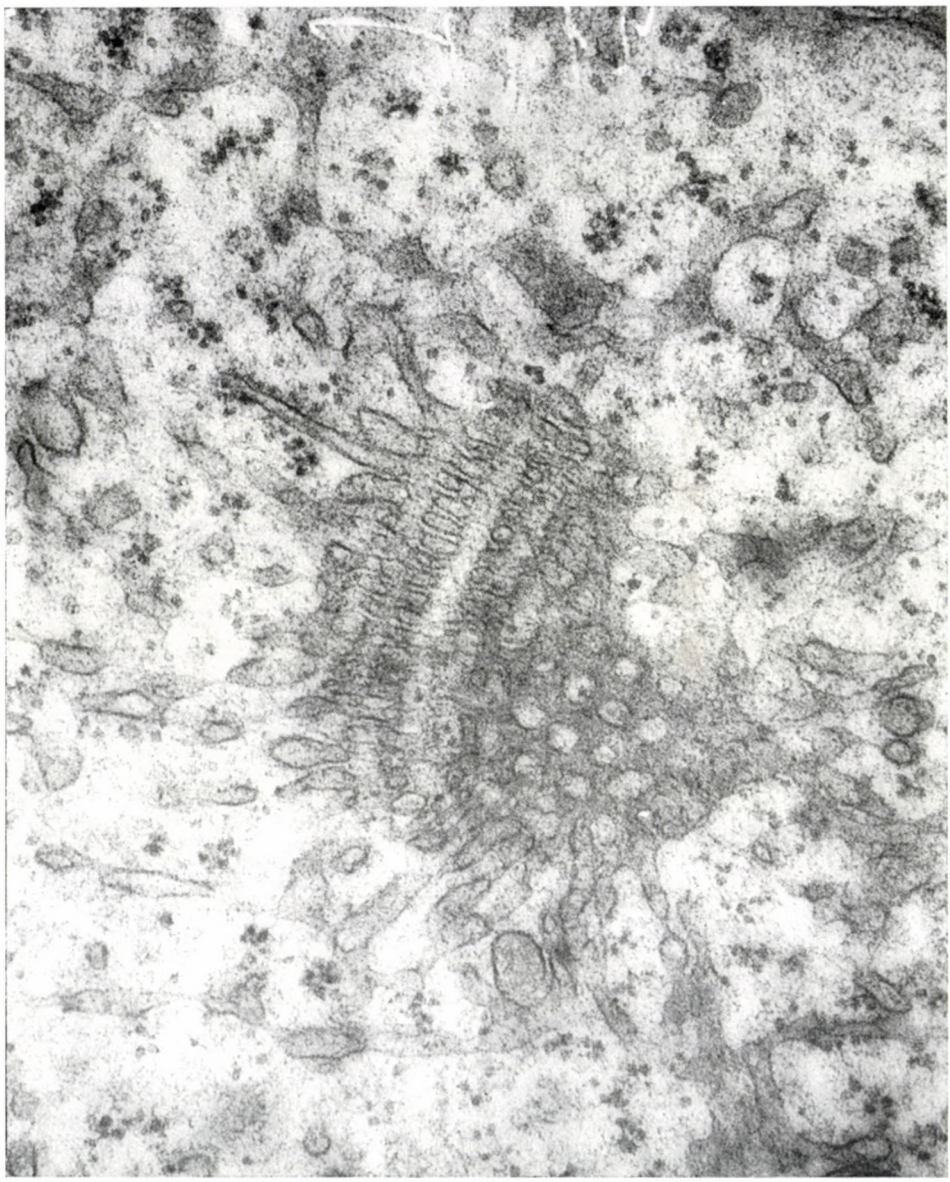

Fig. 2. Well-developed Golgi complex in a normal (control) pinealocyte, associated with the endoplasmic reticulum. × 71 300

Close connections between the Golgi apparatus and the rough-surface endoplasmic reticulum were visible in some places. The endoplasmic reticulum was usually fragmented, less often aggregated. The mitochondria communicated with the Golgi apparatus. In many cells, synaptic trabeculae carrying synaptic vesicles were seen.

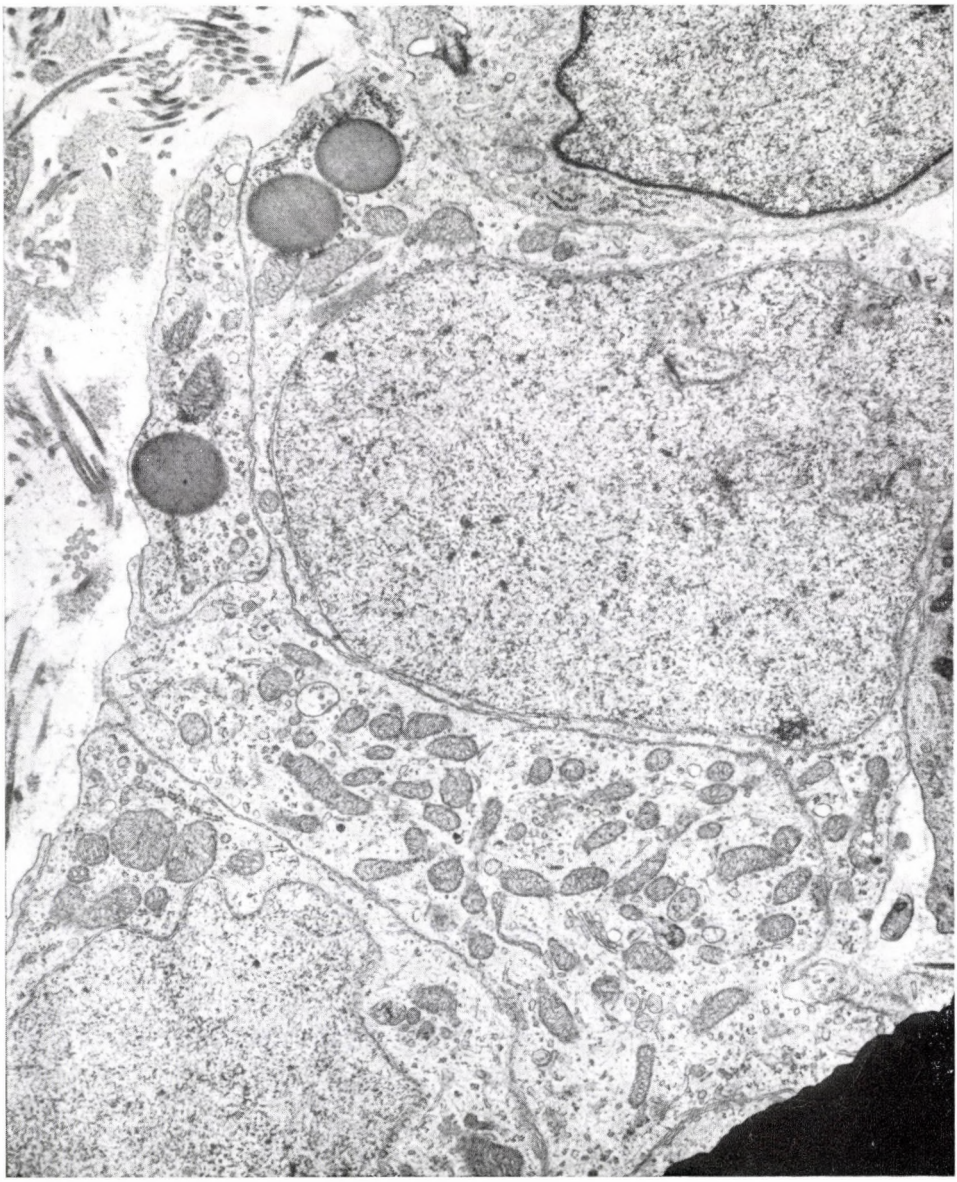

Fig. 3. Pinealocyte of rat treated with a single 10 μg dose of glutaurine. Euchromatic nucleus, cytoplasmic lipid droplets, and aggregation of mitochondria in the cell process. × 14 000

One hour after a single large dose of glutaurine the nucleus was still euchromatic and the nucleolus prominent, and neither the endoplasmic reticulum nor the Golgi complex was different from the control. Lipid was abundantly present in some cells. Aggregation of mitochondria in the processes of pinealocytes was a characteristic change (Figs 3 and 4); the organelles formed

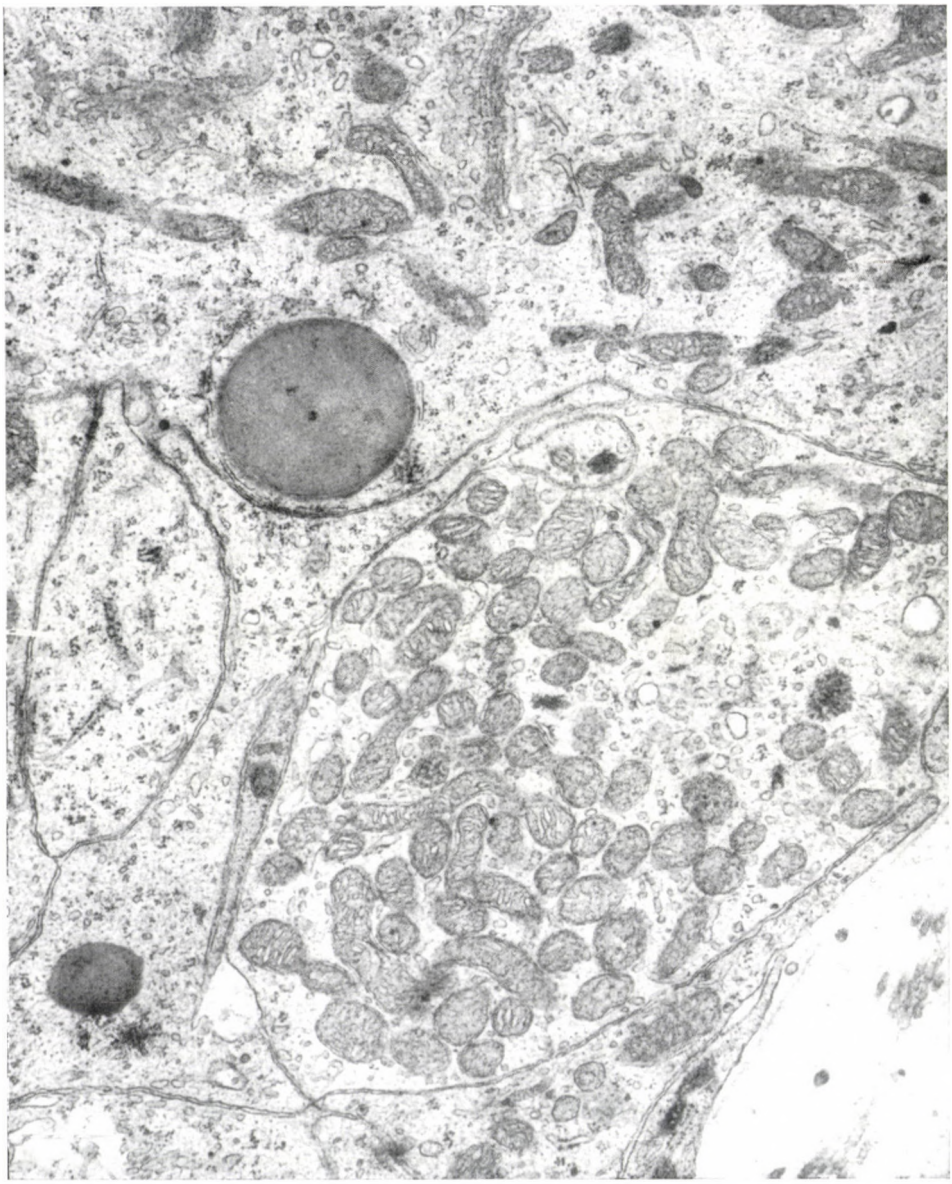

Fig. 4. Pinealocyte of rat treated with a single dose of glutaurine. Large groups of aggregated mitochondria in the pinealocytic process. × 22 500

densely arranged large groups. The autophagous vacuoles usually enclosed mitochondria.

The pinealocytes from rats treated with seven doses of glutaurine showed a slight nuclear heterochromasia, but did not notably differ from the controls. The nucleoli were prominent, mitochondria were abundantly present and

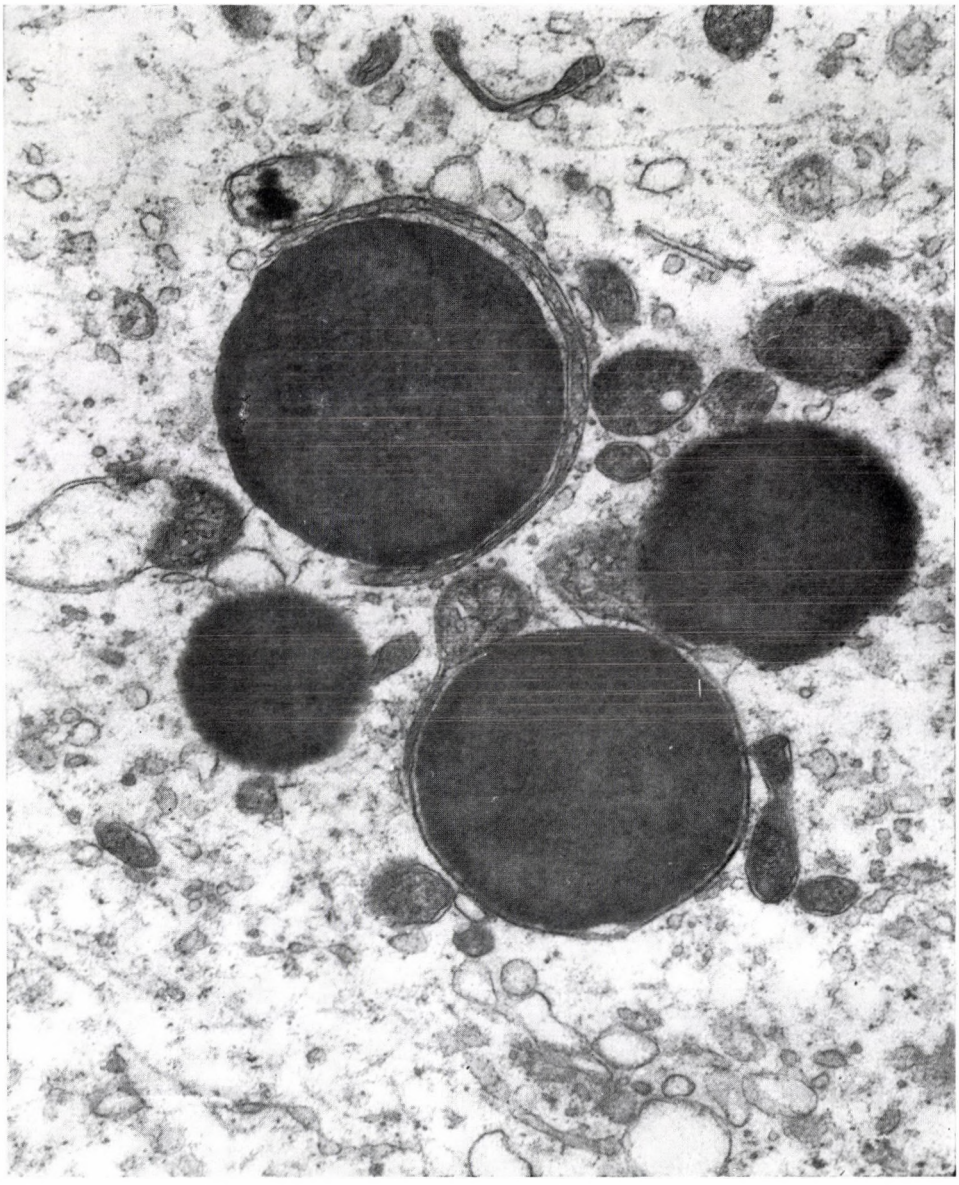

Fig. 5. Pinealocyte of rat treated intraperitoneally with 1 μg glutaurine daily for 7 days. Close association of large lipid droplets with mitochondria. × 24 200

showed the same characteristic aggregation pattern as after a large dose. Mitochondrium-lipid association or transformation was frequent (Fig. 5), with signs of delimitation or extrusion of the degenerating mitochondria (Fig. 6). Autophagous vacuoles were numerous in this group, and they contained both mitochondria and endoplasmic reticulum. The vacuoles varied in size, but

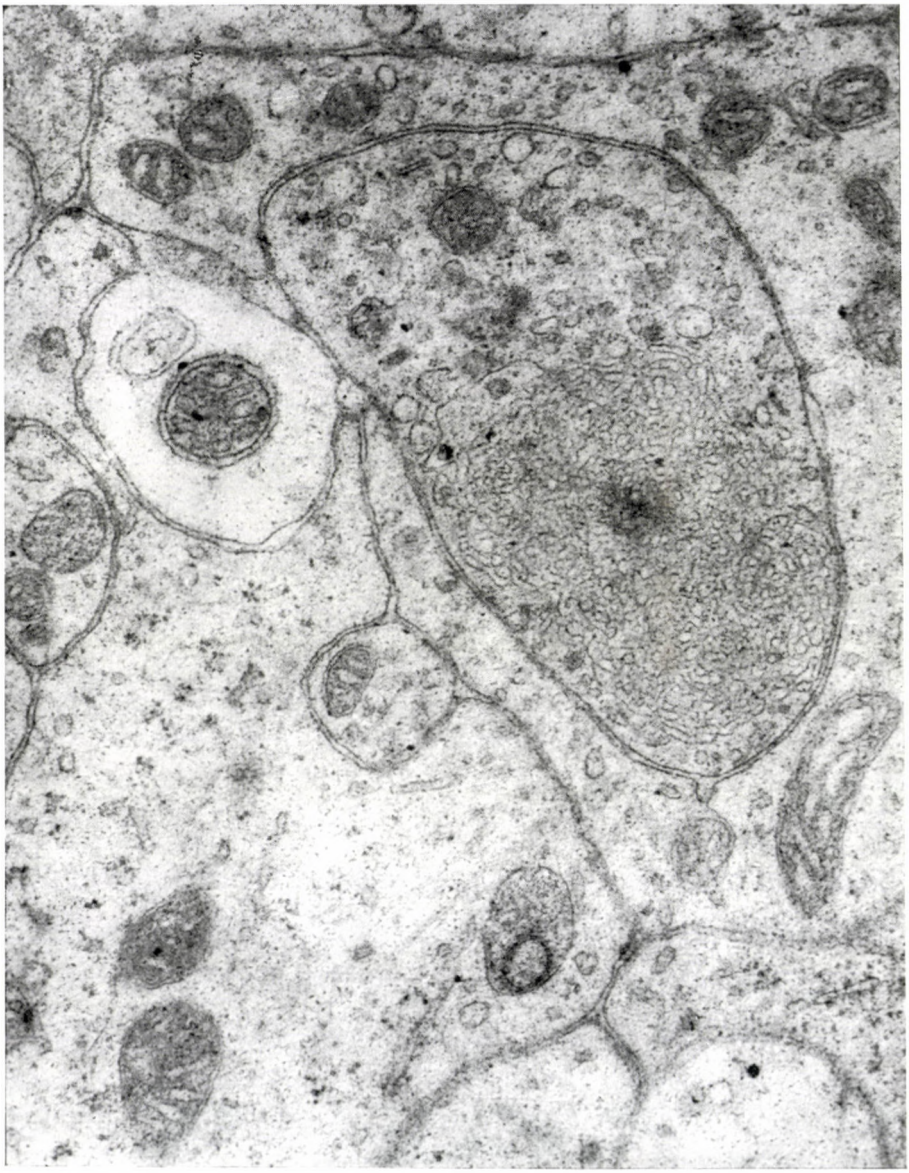

Fig. 6. Pineal gland of rat treated with 1 μg glutaurine daily for 7 days. Many autophagous vacuoles enclose intact or degenerated mitochondria. Separation of laden vacuoles from the cell. × 37 800

many of them were large. The mitochondria frequently showed bizarre, ramified or rectangular shapes, with transversal or longitudinal, or even mixed patterns of cristae (Fig. 7). Synaptic trabecules were found also in this group, in practically the same number as in the control specimens.

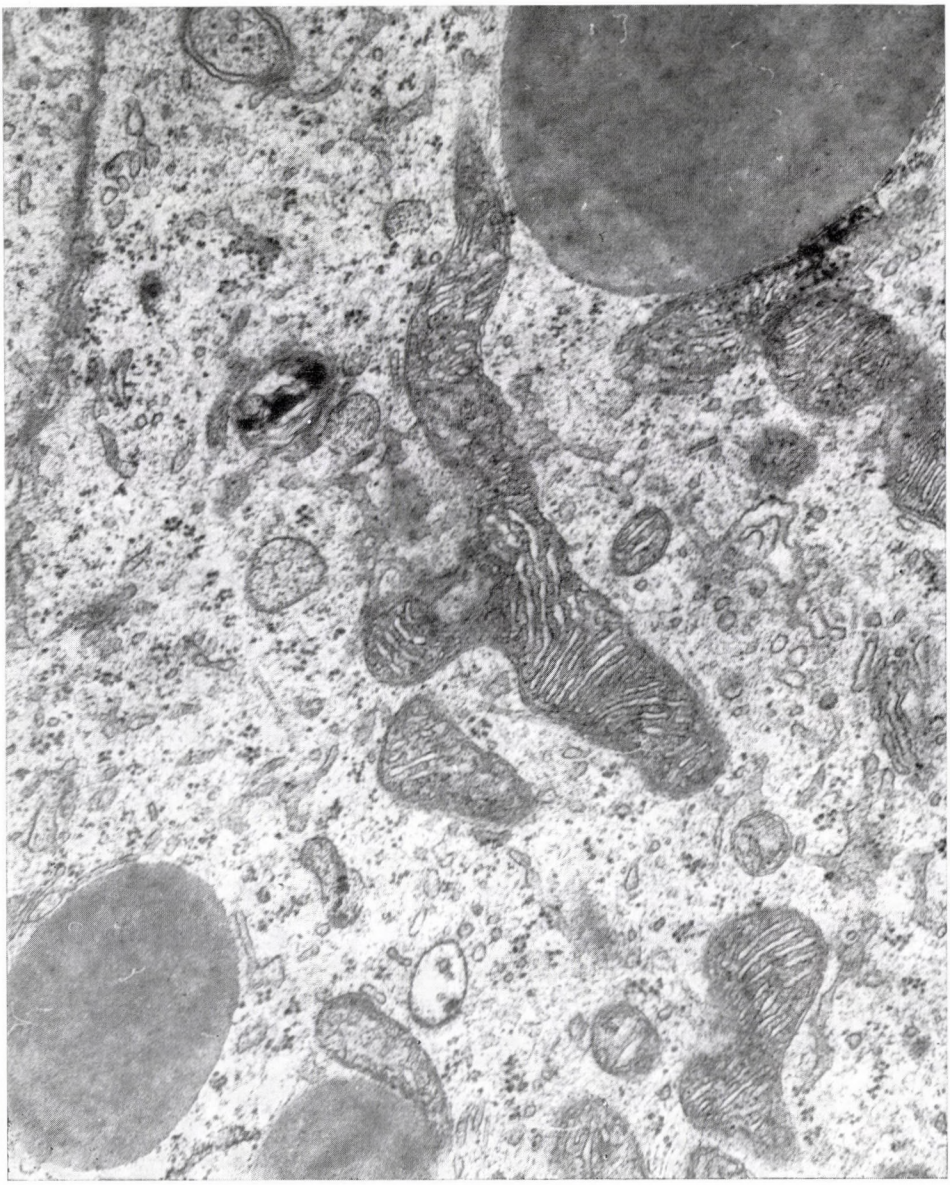

Fig. 7. Pineal gland of rat treated with 1 μg glutaurine daily for 7 days. Bizarre shape of mitochondria and their association with lipid droplets

The ultrastructural details of the pineal gland of control rats were in every respect similar as those described by other authors [2, 9, 10, 12]. The origin of the granules having the appearance of a "secretion" was not nearer pursued, as other authors, too, have failed to clarify their precise nature [12]. Exclusively those structures were studied in detail which permitted indirect but reliable conclusions as to a functional stimulation or inhibition by glutaurine.

The nuclei of the control pinealocytes were euchromatic, and the nucleoli were prominent, exactly as in the experimental groups, indicating that glutaurine treatment did not interfere with the melatonin synthesizing function of the rat pineal. The behaviour of mitochondria did, however, differ between the experimental and control groups. The pinealocytes of the glutaurine treated rats showed a numerical increase and aggregation of the mitochondria in the cell processes, and their degeneration, on prolonged treatment. At the same time, many mitochondria were seen inside autophagous vacuoles. It remains to be clarified whether the marked increase of mitochondrial autophagy was due to a degeneration of the mitochondria or to a direct effect of glutaurine, as the latter is known to stimulate lysosomal activity [4, 8]. If it is postulated that glutaurine, like its precursor taurine [1], acts by inhibiting release of melatonin the conclusion lies close at hand that this effect is exerted by the stimulation of autophagy.

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DIE WIRKUNG DES LITORALONS AUF DIE ZIRBELDRÜSE DER RATTE

L. FEUER, B. MADARÁSZ, F. SUDÁR und G. CSABA

Das vor kurzem entdeckte Hormon der Nebenschilddrüse, das Litoralon, bewirkt im Corpus pineale der Ratte die Aggregation und Degeneration der Mitochondrien in den Pinealozytenfortsätzen. Unter seiner Wirkung tritt starke Autophagie ein, was aller Wahrscheinlichkeit nach der allgemeinen Lysosomen-aktivierenden Wirkung des Litoralons zugeschrieben werden kann.

ДЕЙСТВИЕ ЛИТОРАЛОНА НА ШИШКОВИДНОЕ ТЕЛО КРЫСЫ

Л. ФЕЙЕР, Б. МАДАРАС, Ф. ШУДАР и Г. ЧАБА

Литоралон, недавно открытый гормон щитовидной железы, повышает в шишковидном теле крысы аггрегацию и вырождение митохондрий в отростках пинеалоцитов. Под его влиянием появляется автофагия значительного размера, что по свей вероятности можно приписать общему активирующему лизосомы действию Литоралона.

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Department of Animal Physiology, Teacher Training College, Krakow, Poland

DIURNAL RHYTHM OF HYPOPHYSEAL CELLS IN MICE. PART III. PARS NERVOSA*

H. Lach, S. Krawczyk, K. Dziubek and W. Szaroma

(Received April 21, 1979)

Karyometric analysis revealed a distinct diurnal rhythmicity of the nuclear volume of pars nervosa pituicytes in mice maintained on a 12 h light : dark schedule. Maximum nuclear volumes of pituicytes in both males and females were observed at 18^{00} o'clock while the minimum ones at 12^{00} o'clock. In addition, a distinct rhythm was observed in the amount of lipid vacuoles in the pituicytes of both sexes; they showed two peaks, a larger one at 18^{00} and a smaller one at 6^{00} o'clock, as well as two minima at 12^{00} and 24^{00} o'clock.

Introduction

The role of pituicytes in the activity of hypophyseal pars nervosa is not clear (Holmes and Ball [5]). The suggestion of Ranson et al. [20] that pituicytes could be the site of neurohormone synthesis was contradicted by many authors, although they continue to associate the function of these cells with the activity of the neurosecretory system. Romeis and Stahl [23], Kratzsch [6], Ortman [16], Hild [4], Brettschneider [1] and Legait [11] claim that pituicytes play an important role in releasing the neurosecretion from the posterior pituitary lobe. On the other hand, Rennels and Drager [21] suggest that the pituicytes take part in splitting biologically active hormones from their protein carriers, and in releasing these hormones into the blood.

An increase in neurosecretory activity is connected with the increase of the amount of lipid vacuoles within the pituicytes relationship observed by Kurosumi et al. [9]. Similar reactions were seen as a result of dehydration (Krsulovic et al. [8]) and of castration [25]. Deis [2] reported that the increase of neurosecretion in the posterior pituitary after castration coincided with the increase in number of lipid vacuoles within the pituicytes; this was then confirmed electron-microscopically by Zambrano [25]. Other authors [21, 13, 14, 15] reported that the activity of the neurosecretory system exhibits

* Supported by scientific program R-III-14 coordinated by Jagiellonian University, Krakow, Poland.

a diurnal rhythmicity in the neurosecretory neurocytes as well as in the amount of neurosecretory material in the pars nervosa of the hypophysis.

Since pituicytes are though to participate in hormone release and since fluctuations of the activity of the neurosecretory system seem to be connected with the pituicytes, a diurnal variation of pituicyte activity could be expected.

The present study was undertaken to determine the diurnal rhythmicity of pituicyte activity and of the amount of lipid vacuoles the pars nervosa pituicytes in male and female mice.

Material and method

Four months old white mice were used 25 females and 25 males, of 25 g mean weight. They were kept on a 12 h light-dark schedule. The animals were divided into 5 groups 5 of 5 males and 5 females each, and killed by decapitation at 12^{00}, 18^{00}, 24^{00}, 6^{00} and 12^{00} o'clock. The pituitaries were excised and fixed in Bouin-Holland fixative and then routinely processed for histological examination. 5 μm thick sections were stained according to HERLANT [3], using lead haematoxylin (PbH), periodic acid and Schiff reagent (PAS); and according to BARGMANN [19] using Gomori chrome haematoxylin and phloxin.

The measure of pituicyte activity was the volume of cell nuclei. According to PALKOVITS and FISHER [18] the nuclear volume is the morphometric index of functional changes of cell activity. Karyometric measurements were made using a micrometer and a 40 $\times$ objective. Two perpendicular nuclear diameters (longer L and shorter B) were measured. The nuclear volume was calculated by means of Palkovits's formula [17]: $V = \pi/6 \times L \times B^2$. Altogether 10 000 measurements were made. Besides, lipid vacuoles in the measured pituicytes were counted. Nuclear volumes and the average amount of lipid vacuoles in the pituicytes were then evaluated statistically using the Student-Gosset test.

Results

Data concerning diurnal changes of the mean nuclear volume of pars nervosa pituicytes are shown in Tables I, II and Fig. 1, while data concerning the amount of lipid vacuoles are seen in Tables III, IV and in Fig. 2. The largest mean volume of pituicyte nuclei was 135.178 μm^3 in males and 144.382 μm^3 in females; it was observed at 18^{00} o'clock. After that time the nuclear volume decreased to the minimum of 109.513 μm^3 in males, and 91.210 μm^3 in females, by 12^{00} o'clock. The results obtained at the investigative points of time showed in females statistically significant differences between all the intervals while in males the differences were statistically significant only between 12^{00} and 18^{00}, and 12^{00} and 24^{00} o'clock.

As to the amount of lipid vacuoles in the investigated pituicytes, two peaks were observed: the first at 18^{00} o'clock at of 37.2 in females and 27.6 in males, and the second at 6^{00} o'clock, of 16.0 vacuoles in females and 15.2 in males. Also two minimums of the mean amount of lipid vacuoles, one at 12^{00} and the other at 24^{00} o'clock, were observed in both males and females. The differences between the time intervals were significant statistically in both sexes.

Table I

Diurnal fluctuation of mean nuclear volume of pituicytes in pars nervosa in male and female mice

Hour	Sex	No. of animals	Mean nuclear volume (μm^3)	Standard deviation	Mean error	t (Student's test)
12^{00}	♂	5	91.210	10.607	4.744	7.386*
	♀	5	109.513	6.401	2.863	
18^{00}	♂	5	144.382	8.487	3.796	4.265
	♀	5	135.178	6.658	2.978	
24^{00}	♂	5	142.156	16.554	7.403	4.212
	♀	5	126.447	8.589	3.841	
6^{00}	♂	5	103.115	9.096	4.068	6.004*
	♀	5	124.885	15.681	7.013	
12^{00}	♂	5	91.210	10.607	4.744	7.386*
	♀	5	109.513	6.401	2.863	

* statistically significant at $P < 0.01$

Table II

Statistical comparison of mean nuclear volumes of pituicytes in pars nervosa of male and female mice

Hours	t (Student's test)	
	females	males
$12^{00}-18^{00}$	19.570*	13.896*
$12^{00}-24^{00}$	12.957*	7.902*
$12^{00}-6^{00}$	4.259	4.539
$18^{00}-24^{00}$	0.598	4.016
$18^{00}-6^{00}$	16.586*	3.021
$24^{00}-6^{00}$	10.334*	0.437

* statistically significant at $P < 0.01$

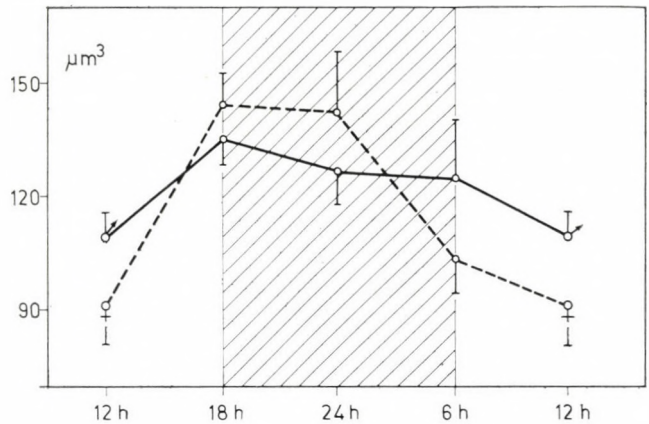
μm3
150
120
90
12 h
18 h
24 h
6 h
12 h

Fig. 1

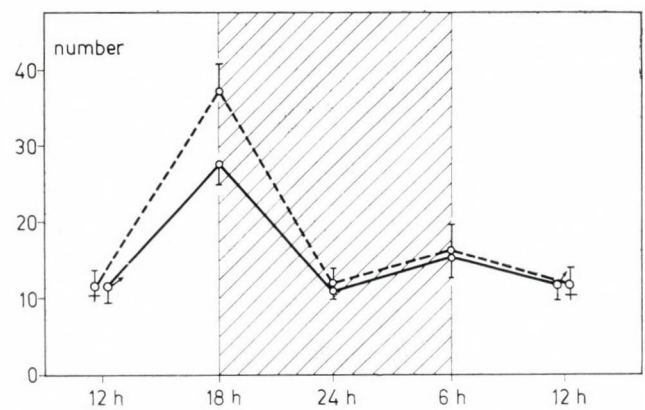
number
40
30
20
10
0
12 h
18 h
24 h
6 h
12 h

Fig. 2

Table III

Diurnal rhythm of the amount of lipid vacuoles in pituicytes of pars nervosa in female and male mice

Hour	Sex	No. of animals	Mean number of lipid vacuoles	Standard deviation	Mean error	t (Student's test)
12^{00}	♂	5	11.6	2.074	0.928	0.000
	♀	5	11.6	2.408	1.077	
18^{00}	♂	5	37.2	3.701	1.655	10.235*
	♀	5	27.6	2.881	1.288	
24^{00}	♂	5	11.6	1.949	0.872	1.370
	♀	5	11.0	1.000	0.447	
6^{00}	♂	5	16.0	3.536	1.581	0.925
	♀	5	15.2	2.490	1.114	
12^{00}	♂	5	11.6	2.074	0.928	0.000
	♀	5	11.6	2.408	1.077	

* statistically significant at $P < 0.01$

Table IV

Statistical comparison of mean amount of lipid vacuoles in pituicytes of the pars nervosa of male and female mice

Hours	t (Student's test)	
	females	males
12^{00}—18^{00}	30.153*	21.305*
12^{00}—24^{00}	0.000	1.149
12^{00}— 6^{00}	5.366*	5.195*
18^{00}—24^{00}	30.585*	27.213*
18^{00}— 6^{00}	20.703*	16.273*
24^{00}— 6^{00}	5.446*	7.821*

* statistically significant at $P < 0.01$

Discussion

From the results it is seen that the nuclear volume of pars nervosa pituicytes of male and female mice exhibits a distinct diurnal rhythm with the maximum at 18^{00} and minimum at 12^{00} o'clock.

The results presented agree with those of NIEBRÓJ [15], who formed the maximum nuclear volume of neurocytes in the supraoptico and paraventricular nuclei in mice at 18^{00} and the minimum at 3^{00} o'clock. At the same time an increased diuresis was observed while at night it was markedly decreased. Similar results were reported by MÖDLINGER-ODORFER [14] according to whom a large amount of neurosecretory material in the neurocytes of hypothalamic nuclei was observed at 18^{00}, and a minimum at 6^{00} and 24^{00} o'clock. RINNE and SONNINEN [22] too showed the highest amount of neurosecretory material during the day while the least amount between 24^{00} and 4^{00} o'clock. When comparing the diurnal rhythm of the neurosecretory system with that of the nuclear volume of pituicytes, the coincidence in the reactivity of the neurosecretory system and the pituicytes at various time intervals is clearly seen. The results suggest that the pituicytes are functionally connected with hormone release into blood.

In addition, the amount of lipid vacuoles in the pituicytes of male and female mice exhibits a distinct diurnal rhythm simultaneously with the diurnal rhythm of the nuclear volume, in agreement with the results by SUROWIAK [24] who showed that acid phosphatase activity in the hypothalamus exhibited a diurnal rhythm with the maximum at 18^{00} and a minimum at 24^{00} o'clock. At the same time is has been shown that an increase in neurosecretory activity is connected with an increase in the amount of lipid vacuoles and that the changes follow the diurnal rhythm of the whole neurosecretory system. In the light of the finding [7, 8, 9] that the amount of lipid vacuoles in the pituicytes is an additional index of the release of neurosecretory substances from the posterior pituitary lobe, it is easy to understand the diurnal rhythm of the vacuoles.

Acknowledgements

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DER CIRCADIANE RHYTHMUS DER HYPOPHYSENZELLEN DER MAUS III. PARS NERVOSA

H. LACH, S. KRAWCZYK, K. DZIUBEK und W. SZAROMA

An Mäusen, die bei je 12stündigen dunklen und hellen Zyklen gehalten wurden, lassen sich durch karyometrische Analyse die im circadianen Rhythmus erfolgenden Kernvolumenveränderungen in Pars nervosa nachweisen. Die größten Kernvolumina wurden sowohl bei männlichen als auch bei weiblichen Mäusen um 18 Uhr ermittelt, entgegen dem Minimum um 12 Uhr. Darüber hinaus ließ sich bei beiden Geschlechtern eine ausgeprägte Rhythmizität in der Größe der Lipidvakuolen beobachten. Zwei Maxima, eine größere um 18 Uhr und eine kleinere um 6 Uhr, ferner zwei Minima um 12 und um 24 Uhr wurden festgestellt.

СУТОЧНЫЙ РИТМ ГИПОФИЗАРНЫХ КЛЕТОК У МЫШИ III. PARS NERVOSA

Х. ЛАХ, Ш. КРАВЧИК, К. ДЗИУБЕК и В. ШАРОМА

У мышей, содержанных в двенадцатичасовых циклах на свете и в темноте, при помощи кариометрического анализа можно выявить в суточном ритме изменения ядерного объема Pars nervosa. Как у мужских, так и у женских мышей наибольший ядерный объем был выявлен в 18 часов, а наименьший в 12 часов. Наряду с этим у обоих полов наблюдалась выраженная ритмичность в изменениях размера липоидных пузырьков. Авторы определяли два максимума, более высокий в 18 часов и меньший в 6 часов, а также два минимума в 12 и 24 часов.

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DISTRIBUTION AND FINE STRUCTURE OF THE INTERSTITIAL CELLS OF CAJAL

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Distribution and fine structure of the interstitial cells of Cajal were studied in the cat's small intestine. They were only found in the circular muscle layer. They had a slender structure with numerous mitochondria, rough endoplasmic reticulum and granular or secretory vesicles 100 to 300 nm in diameter. The closest distance between the membrane of smooth muscle cells and interstitial cells was 40 to 60 nm. Some nerve bundles were found in close relation to their processes, but neither pre- nor postsynaptic differentiation could be observed. After nialamide treatment the quantity of secretory vesicles increased and therefore it is suggested that these cells have a secretory function producing some kind of peptide and so they may act directly on the smooth muscle cells.

Introduction

The study of the interstitial cells in the several organs has been confined for many years to light microscopy using different kinds of impregnation and methylene blue staining [1—5, 10, 11, 14, 15, 20, 21]. Some of these authors considered the interstitial cells to be small nerve cells; according to others, these cells were connective tissue cells; and a third group of investigators thought these cells to be special Schwann cells.

TAXI [22, 23] and RICHARDSON [18, 19] using electron microscopy, could differentiate the interstitial cells from Schwann cells. COUPLAND and HOLMES [7] and LEAMING and CAUNA [16] found that they gave a negative cholinesterase reaction, while, according to GUNN [9], this reaction was non-specific.

We have studied the distribution and the structure of the interstitial cells of Cajal at the electron microscopic level, to determine their possible target of action.

Materials and methods

Adult cats of both sexes were used. The materials were stained by the zinc-iodine-osmium technique for light microscopy and for electron microscopy the animals were perfused with Karnovsky's fixative [13]. Some of the animals were treated with 200 mg/kg nialamide for 2 h before the perfusion. Small pieces of ileum were excised and postfixed in 1% osmic acid for 2 h and embedded into Epon. Ultrathin sections were stained with uranyl acetate and lead citrate. Electronmicrographs were taken with a Tesla BS 500 electron microscope.

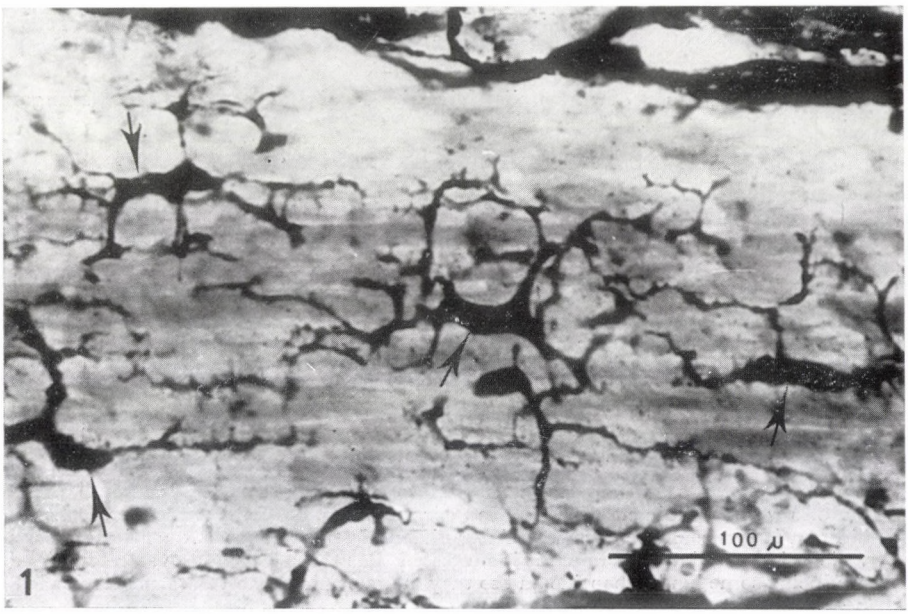

Fig. 1. Part of the circular muscle layer. Arrows point at star-shaped interstitial cell

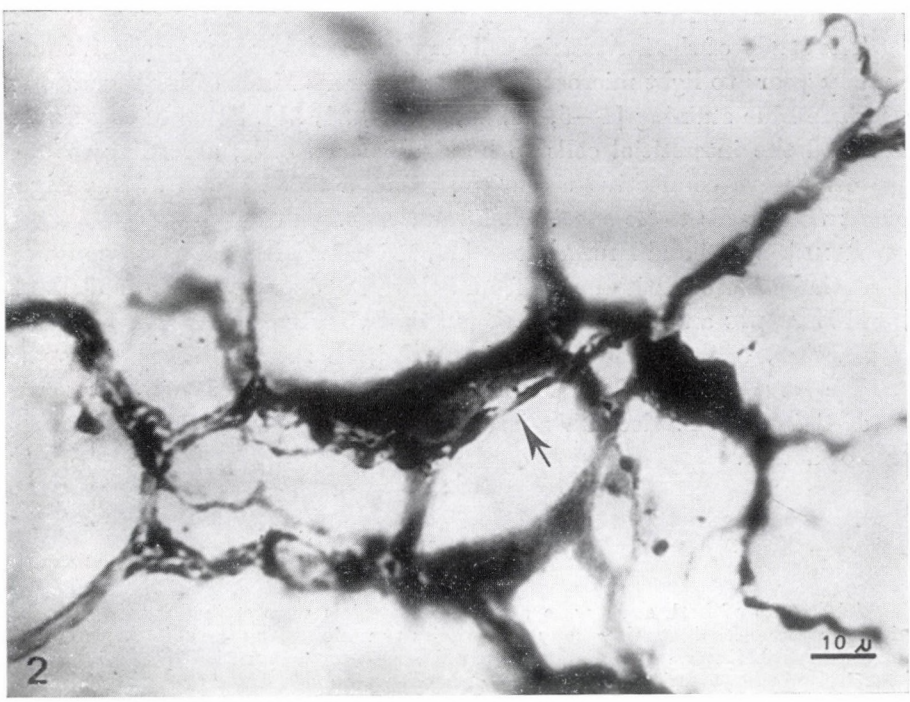

Fig. 2. Interstitial cell of Cajal with its processes. Arrow points to a close nerve bundle

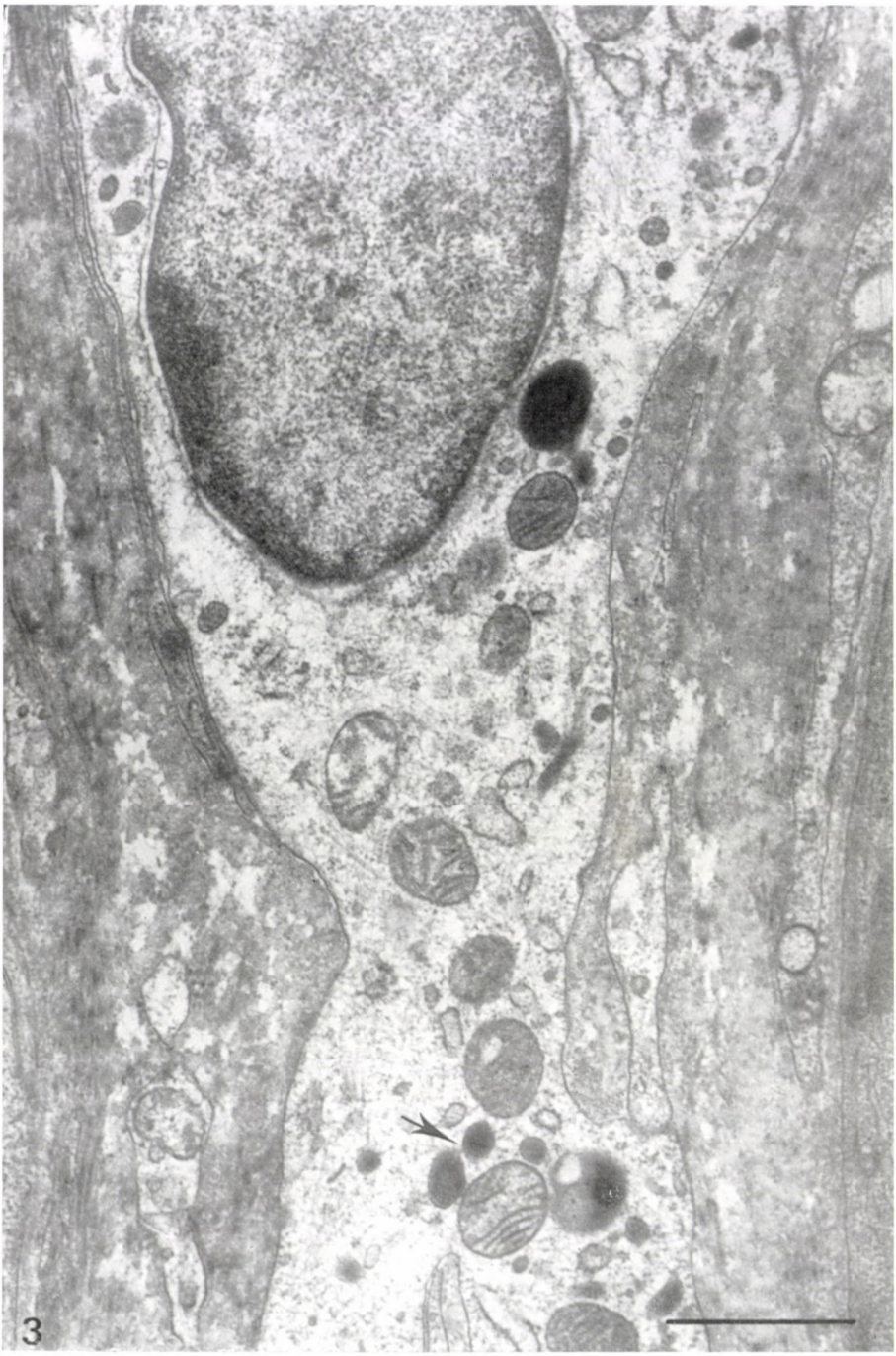

Fig. 3. Electronmicrograph of an interstitial cell of Cajal. Arrow points to secretory vesicles in the cytoplasm

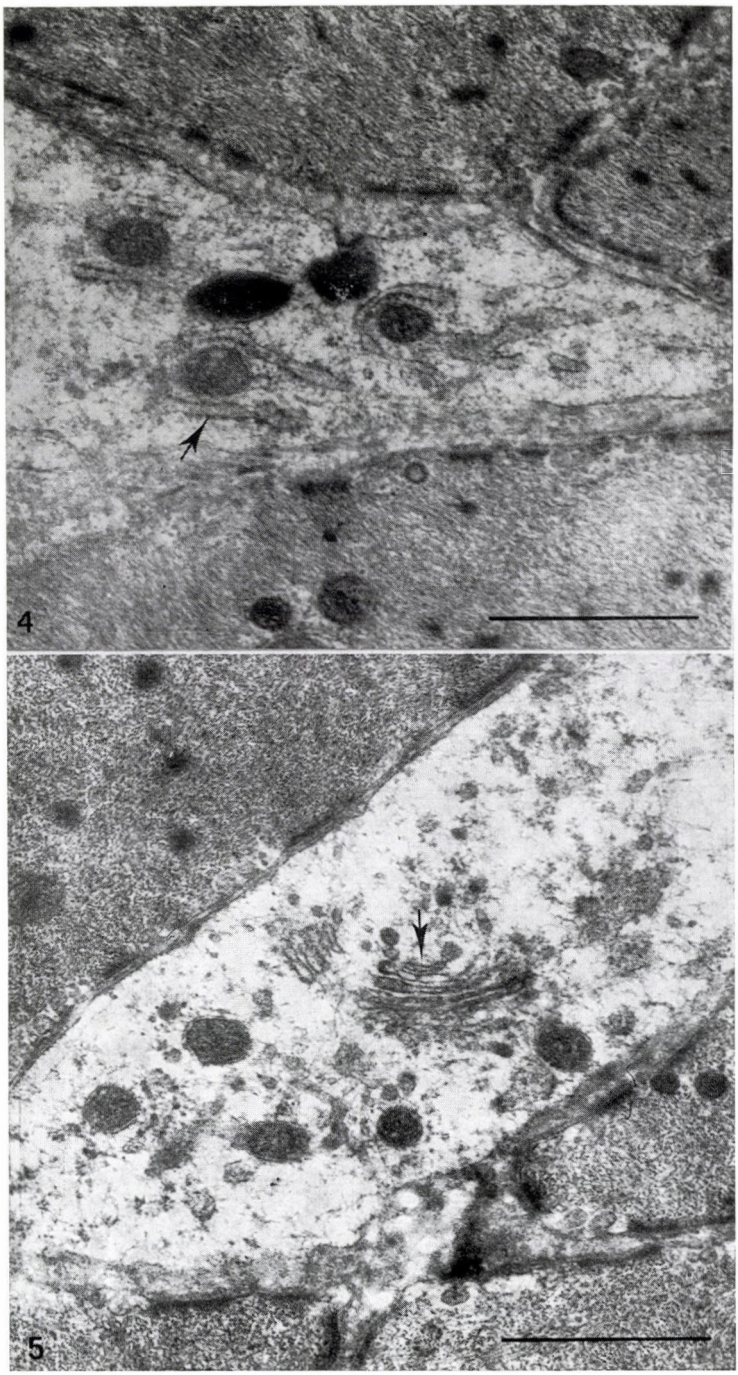

Fig. 4. Process of an interstitial cell of Cajal. Arrow indicates rough surface endoplasmic reticulum

Fig. 5. Process of an interstitial cell in close relation to smooth muscle cells. Arrow points to Golgi area

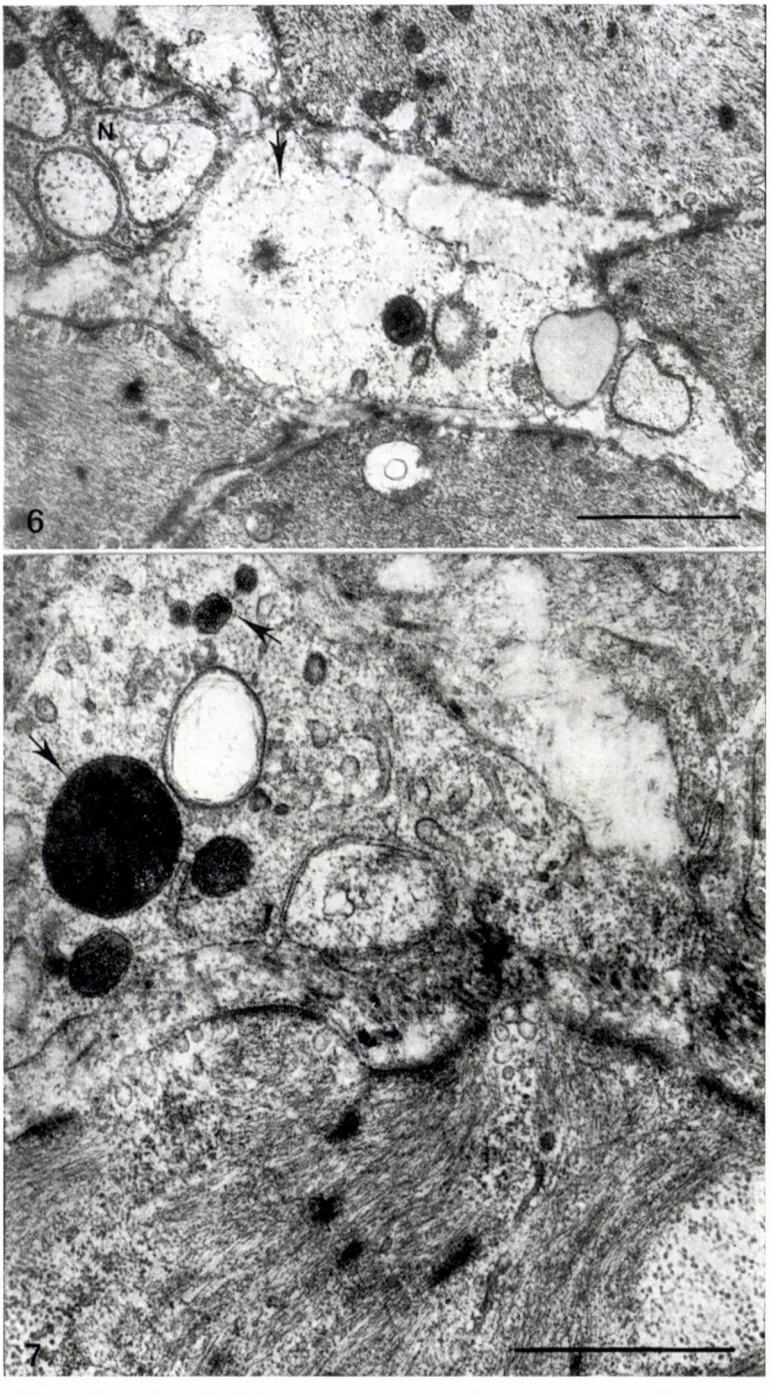

Fig. 6. Nerve bundle (N) close to a process of an interstitial cell (arrow)

Fig. 7. After nialamide treatment the quantity of secretory vesicles (arrows) increased in the interstitial cell

Results

The interstitial cells are star shaped cells with anastomosing cytoplasmic processes. They lie in the connective tissue among the smooth muscle cells of the circular muscle layer (Fig. 1). Their processes are coarse at their origin, but, due to repeated ramifications, they gradually become finer. Some small nerve bundles could also be observed in close relation to the interstitial cells (Fig. 2).

Under the electron microscope the nuclei of these cells were found to be round or oval. The cytoplasm was slender and extended into attenuated processes (Fig. 3). The interstitial cells are covered by amorphous intercellular substance. Numerous large elongated mitochondria, rough endoplasmic reticulum, individual ribosomes and medium developed Golgi areas were observed. They contain a large number of granular or secretory vesicles 100 to 300 nm in diameter, having a dense finely granulated material in the central zone (Figs 3, 4). There is no basement membrane bordering the cells; their irregular cytoplasmic processes penetrate into the circular smooth muscle cells. The distance between the opposing plasma membranes of the two cells at the region of close contacts is 40 to 60 nm. Sometimes a slightly dense material could be observed on the membrane of the smooth muscle cells (Fig. 5).

At certain points the processes of the interstitial cells may be in immediate contacts with the nerve bundles (Fig. 6), but even in these cases the nerve fibres are embedded into Schwann cell cytoplasm. Sometimes the closest gap between the nerve fibre and the membrane of interstitial cells is 30 to 40 nm wide. Neither pre- nor post-synaptic differentiation could be observed.

After nialamide treatment the quantity of granular vesicles (secretory vesicles) increased in the processes of interstitial cells (Fig. 7).

Discussion

Electron microscopy revealed that the interstitial cells of Cajal are specialized ones [6] and that they are in close contact with the smooth muscle cells [12, 24]. The ultrastructure and the large number of dense core secretory vesicles suggests a secretory function. After monoamino oxidase inhibitor (nialamide) treatment the quantity of the vesicles increased, therefore they may belong to the APUD series [17] and their primary function would be the synthesis and secretion of some kind of polypeptide which acts on the neighbouring smooth muscle cells. LAWRENTJEW [15] and van ESVELD [8] supposed that the autonomic interstitial cells were intercalary cells functionally and morphologically interposed between the postganglionic axons and the effector cells. According to GUNN [9] the interstitial cell is an inbuilt end-apparatus communicating with the true nerve fibres. In view of the close

association of several nerve fibres with the interstitial cells, it is reasonable to suppose that the diffuse release of neurotransmitter substance along the length of the nerve processes would activate the interstitial cells. This morphological feature may mean that these cells are, in fact, establishing functional contacts between the nerve fibre and the smooth muscle cell.

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UNTERSUCHUNG DER VERTEILUNG UND DER FEINSTRUKTUR DER CAJALSCHEN ZELLEN

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Im Dünndarm von Katzen wurde die Verteilung und die Feinstruktur der Cajalschen interstitialen Zellen untersucht. Diese Zellen finden sich nur in der inneren zirkulären Muskelschicht, haben ein helles Plasma und enthalten zahlreiche Mitochondrien, ein endoplasmatisches Retikulum mit grober Oberfläche sowie körnige oder sekreterische Bläschen mit 100–300 nm. Durchmesser. Die kleinste Spalte zwischen der Membrane der glatten Muskelzelle und der Membrane der interstitialen Zellen beträgt 40–60 nm. Neben den Ausläufern der interstitialen Zellen finden sich häufig auch Nervenfasernbündel. Nach Nialamid-Behandlung hatte sich die Anzahl der körnigen Vesikel erhöht; somit ist anzunehmen, daß die Zellen irgendein Peptid produzieren, das auf die glatten Muskelzellen eine direkte Wirkung ausübt.

РАСПРЕДЕЛЕНИЕ И ТОНКАЯ СТРУКТУРА ИНТЕРСТИЦИАЛЬНЫХ КЛЕТОК КАХАЛЯ

Й. ВАЙДА и Эржебет ФЕХЕР

Авторы изучали распределение и тонкую структуру интерстициальных клеток Кахаля в тонкой кишке кошки. Эти клетки наблюдаются только во внутреннем циркулярном мышечном слое, они имеют светлую плазму, содержат много митохондрий, эндоплазматическую сетчатку с грубой поверхностью и зернистые или секреторные пузырьки с диаметром в 100–300 nm. Наименьшая щель между мембранами гладкомышечных и интерстициональных клеток составляет 40–60 nm. Возле отростков интерстициональных клеток часто наблюдаются и пучки нервных волокон. После применения ниаламида количество зернистых пузырьков повышается, следовательно можно полагать, что клетки вырабатывают какой-то пептид, непосредственно действующий на клетки гладких мышц.

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TERATOGENIC EFFECT OF 2'-THIOUREA IN THE RAT

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(Received September 28, 1979)

The compound 2'-thiourea was studied for teratogenic effect in fetal rats. A 0.2% aqueous solution of 2'-thiourea (Merck) was administered ad libitum to pregnant rats from the first to the 14th day of pregnancy. The pregnancy was counted from the day when sperm was found in the vaginal smear. In the treated rat fetuses hypoplasia of the brain and spinal cord, internal hydrocephalus, hypoplasia of the spinal ganglia, kypholordosis, micromelia, micrognathia, cleft palate, retarded tooth development, exophthalmus, coloboma, and cataract were observed. In addition, generalized haemorrhages were found all over the body. The changes were similar to those produced by ethylene thiourea treatment in rat fetuses.

Introduction

The 2'-thiourea and its derivatives are natural components of plants. Lately, their quantity has increased in the environment as they are widely applied as a fungicide in agriculture and in the dye, paper, photographic, plastic, pharmaceutic and rubber industries. This is especially the case with ethylene thiourea which, as a metabolite of the fungicide ethylene-bisthiocarbonate, is frequently found in agriculture [1, 8].

The effect of thiourea derivatives on the thyroid is well known [3, 12], and their tumourigenic effect has been demonstrated in animals [2, 5, 9, 10, etc.]. In addition, ethylene thiourea has been show to exert a teratogenic effect on several organ systems [7].

There are no data on the teratogenic effect of 2'-thiourea, though it is known to pass through the placenta and to affect the iodine metabolism of the offspring [6, 11]. We have therefore studied the teratogenicity of the compound and the nature of these changes, and is compared them to those produced by ethylene thiourea.

Material and method

LATI: CFY rats were used in the experiments. The animals were divided in groups, each consisting of a male and two females placed in a polyethylene cage. Vaginal smears were taken every morning until the appearance of the sperm plug. From this day on, which was considered the first day of pregnancy, the pregnant rats were isolated and divided into two groups. Rats in the experimental group were given tap water containing 0.2% of 2'-thiourea (Merck) from the first to the 14th day of pregnancy. Rats in the control group received com-

mon tap water. Animals in both groups were fed LATI rat food. The fetuses were removed transabdominally on the 20th day of pregnancy.

Part of the fetuses of each group was processed according to HOOD—NEILL [4] to make transparent preparations stained with alizarin red, for the demonstration of the degree of bone calcification. The rest of the fetuses was used for histological studies. Specimens were fixed in a mixture of alcohol and formol (4 : 1) for 24 hours, embedded in paraffin and sectioned serially at 7 μm. Histological examinations were carried out on sections stained with haematoxylin-chromotrope. For the demonstration of mineralization the Kossa reaction was used. The head, spine and hind limb buds of the fetuses were examined.

Results

In 2'-thiourea treated rat fetuses retarded growth, malformations (Fig. 1) and abnormalities were noted in nervous and skeletal development, in the eye and vessels. Some malformations appeared as morphological changes, such as micromelia, hydrocephalus, exophthalmus, micrognathia, gnatoschisis, kypholordosis and shortening of the tail. In the transparent preparations, hypoplasia of the skeletal system was apparent (Fig. 1). The ossification centres were smaller in the treated animals than in the controls. Besides, ossification centres were absent in the vertebrae, in the extremital bones and in the occipital squama. In the cranium, retardation of ossification was limited to the squama of the parietal and temporal bones. The palate was cleft, the mandible smaller than normal and cleft.

Histology

Nervous system. In the treated rats the spinal medulla is thin and hypoplastic (Fig. 2) and the central canal narrow and displaced ventrally. The ependymal cells from the central canal proliferate into the anterior median

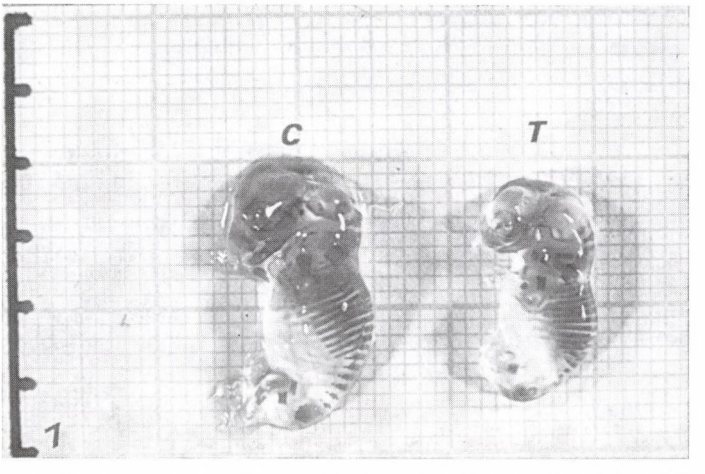


Fig. 1. Transparent preparations stained with alizarin red showing the ossification centres in control (*C*) and in 2'-thiourea treated (T) rat fetuses. Note smaller body size and hypoplastic skeletal system of the treated fetus

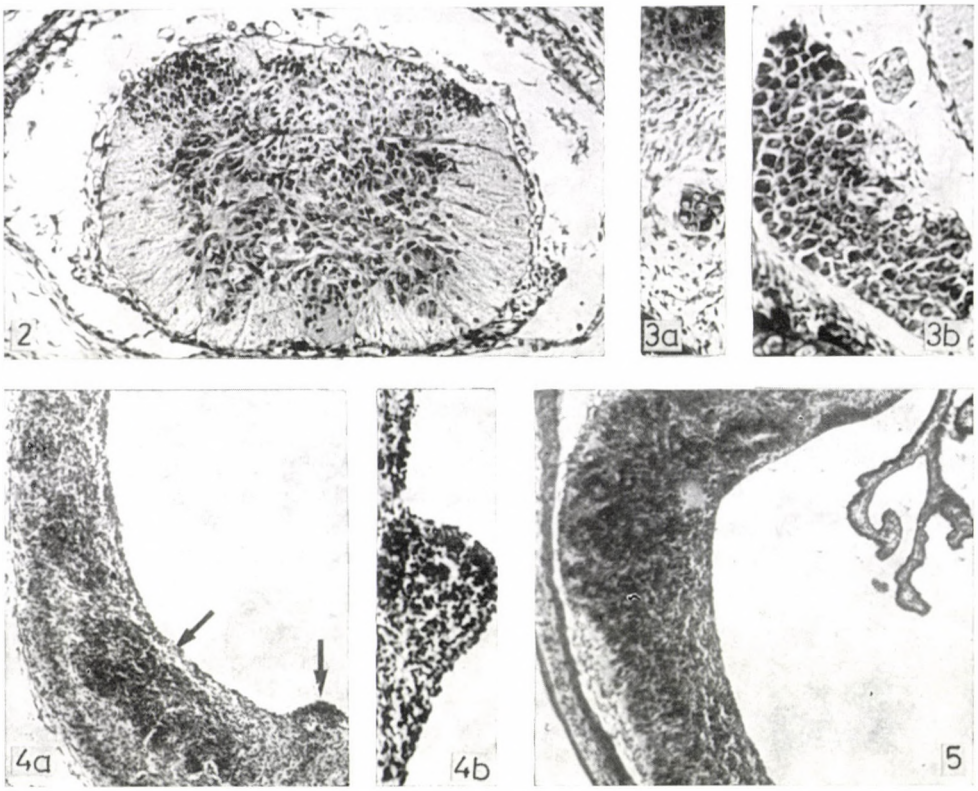


Fig. 2. Cross section of the spinal cord of a rat treated with 2'-thiourea showing the hypoplastic spinal medulla and undifferentiated cells in the dorsal horn region. Haematoxylin-chromotrope. $10 \times 6.3 \times 0.5$

Fig. 3. Spinal ganglia from 20-day old rat fetuses. *a:* Very small spinal ganglion from a fetus treated with 2'-thiourea. *b:* A spinal ganglion from a control fetus. Haematoxylin-chromotrope. $10 \times 6.3 \times 0.5$

Fig. 4 a. Telencephalon of a rat fetus treated with 2'-thiourea; undifferentiated rosette-like cell-groups and metaplastic nodes (arrows). $4 \times 6.3 \times 0.5$. *b:* Metaplastic node, containing proliferating cells. Haematoxylin-chromotrope. $10 \times 6.3 \times 0.5$

Fig. 5. Choroid plexus in the widened cavity of the forebrain vesicle in a treated rat fetus. Haematoxylin-chromotrope. $4 \times 6.3 \times 0.5$

fissure. The metaplastic node is well circumscribed. The dorsal parts, which develop from the alar plates, show retarded differentiation. In the dorsal part of the grey matter karyorrhexis and cell decay are observed. The spinal ganglia (Fig. 3a, b) are small, consisting of few cells. This may account for the lack of differentiation and cell death in the dorsal horn.

The five brain vesicles are discernible, but their wide ventricles are surrounded by thin undifferentiated nervous tissue (Fig. 4a). In the reduced brain substance of the telencephalon, undifferentiated rosette-like cell groups

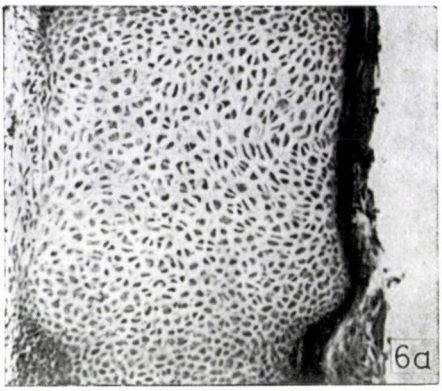
are seen. In the centre of these cell groups lumine lined with ependymal cells are visible. The hypoplastic cortical substance contain a number of metaplastic nodes. They are produced by proliferating undifferentiated cells and covered by flattened ependyma (Fig. 4b). The nodes protrude into the widened cavities of the brain vesicles where the choroid plexus can be recognized (Fig. 5). Circulation of the cerebrospinal fluid is presumably inhibited by the hypoplastic spinal medulla and this may account for the hydrocephalus.

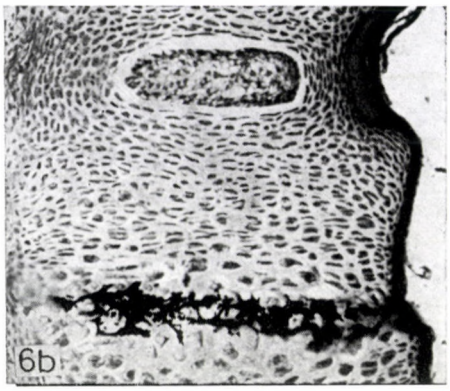
Skeletal system and arteries

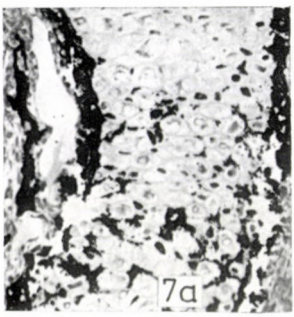
In conformity with the hypoplasia of the spinal cord, the vertebral foramina are also narrowed. Vascular ingrowth cannot be observed in the secondary vertebrae (Fig. 6a). The chorda dorsalis is fully developed and surrounded by the cartilagineous primordia of secondary vertebrae. The nucleus pulposus is not discernible, the intervertebral fissures are already present. Ossification has not begun. Vertebral hypoplasia causes kypholordosis which, in some cases, leads to the formation of humps. In control animals of the same age (Fig. 6b) enchondral ossification can be seen in the vertebral bodies. Among swollen chondrocytes, remnants of the ground substance contain calcium deposits. The nucleus pulposus is fully developed, and there are no chordal remnants in the vertebral bodies.

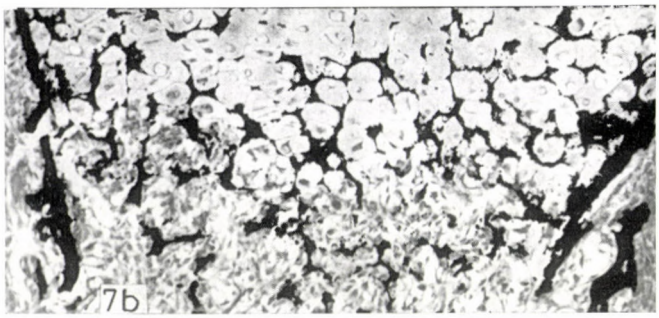
There is a retardation of growth in length and thickness of long bones. The cartilage islets are surrounded by periostal bone rings in some long bones. In the centre of the cartilage primordium of limbs, hypertrophic chondrocytes and calcium deposits are observable indicating the beginning of enchondral ossification (Fig. 7a). In the long bones of the control animals (Fig. 7b) the metaphysis is separated from the epiphysis. The metaphysis consists of compact and spongious bone substance surrounding the medullar cavity which is filled with haemopoietic elements. Enchondral ossification occurs still at the epiphyseal—metaphyseal border; an ossification centre, however, cannot be seen in the epiphysis.

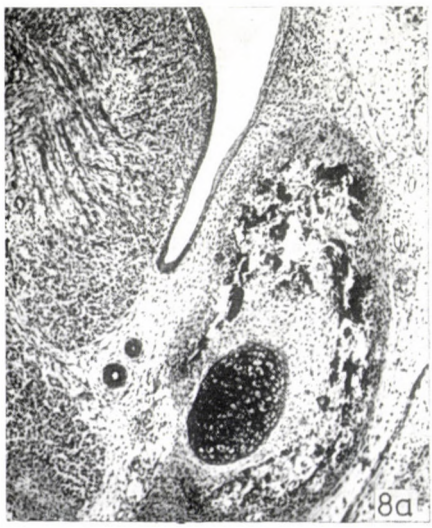
In the cranial bones, changes are seen in the facial bones and the skull which ossify intramembranously. The mandible is hypoplastic (Fig. 8a). The mandibular process of Meckel's cartilage surrounded by desmogenous bone, is smaller than in the controls (Fig. 8b). Numerous typical mesenchymal cells and intense osteoblastic activity can be observed. In the treated animals no tooth development is seen while tooth primordia are well visible in the controls. Desmogenous bone primordia in the maxillar process are well discernible, but they are smaller than in the control rats. The most striking changes occur in the palatine process (Fig. 9); it is smaller, its bony axis is thinner and smaller than in the control animals. The right and left processes are far apart, the nasal and oral cavities communicate with one another. Tooth primordia can-











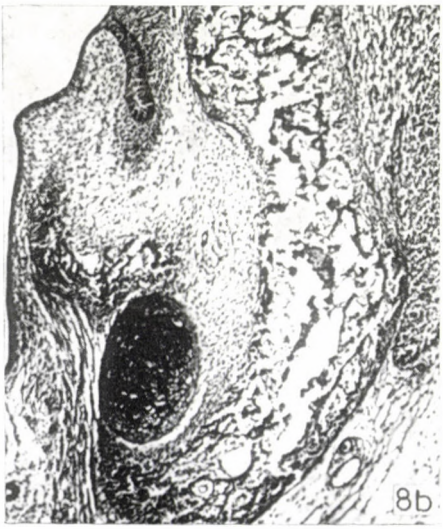
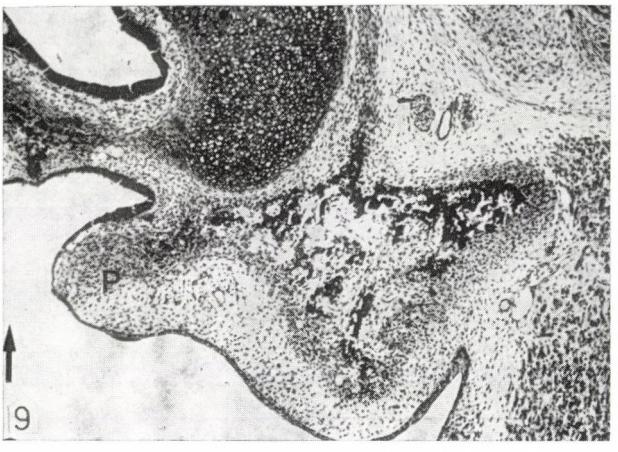
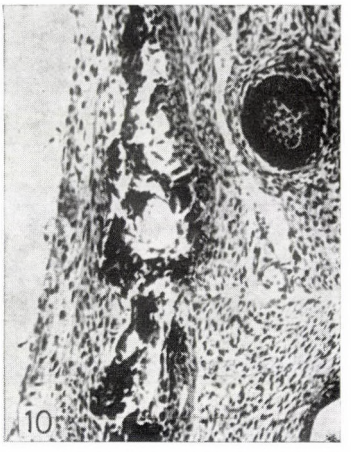


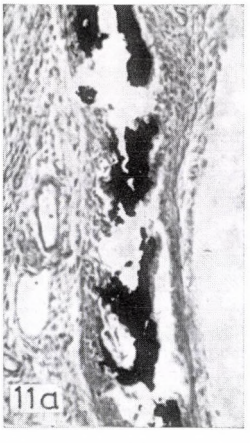
Fig. 6. Thoracic vertebrae of 20-day old rat fetuses. a: Following 2'-thiourea treatment, well differentiated cartilage is seen without ossification. b: Control cartilage chondrocytes with beginning ossification. Kossa reaction. $40 \times 6.3 \times 0.5$

Fig. 7. Tibiae of 20-day old rat fetuses. a: After 2'-thiourea treatment mature, hypertrophized cells and calcified ground substance are seen. b: In the control intensive ossification of the cartilage is prominent. Kossa reaction. $10 \times 6.3 \times 0.5$

Fig. 8. Mandibles of 20-day old rat fetuses. a: 2'-thiourea treatment causes hypoplastic mandible and a small Meckel cartilage. b: In the control, intramembranous ossification and a large Meckel cartilage are seen. Haematoxylin-chromotrope. $4 \times 6.3 \times 0.5$







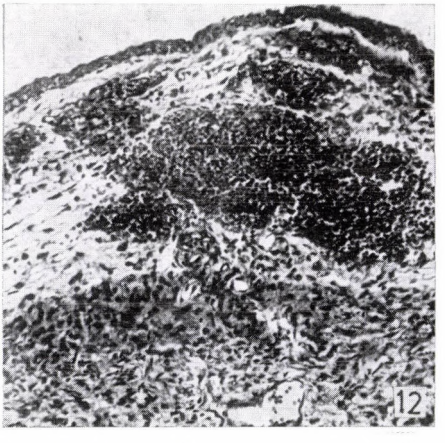


Fig. 9. Maxillar primordium of a rat fetus treated with 2'-thiourea. The process (P) is smaller than in the control, resulting in a wide palatal fissure (arrow). Haematoxylin-chromotrope. $4 \times 6.3 \times 0.5$

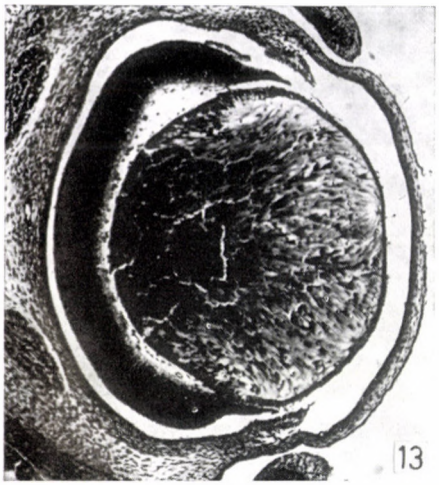
Fig. 10. Frontal bone of rat fetus treated with 2'-thiourea. Thin bone trabecules and narrow medullar cavities. Haematoxylin-chromotrope. $10 \times 6.3 \times 0.5$

Fig. 11. Frontal bone of 20-day old treated (a) and control (b) rat fetus. Sparse calcification and hypoplastic bone in the treated fetus. Kossa reaction. $10 \times 6.3 \times 0.5$

Fig. 12. Galea of a rat fetus treated with 2'-thiourea. Extensive haemorrhages in the loosened subcutis. Haematoxylin-chromotrope. $4 \times 6.3 \times 0.5$

not be found in the maxilla, whereas in the control animals the dental laminae are already present.

In the frontal, parietal, temporal, and occipital bones, the squama is hypoplastic and reduced in thickness. The bone trabecules are thin, the medullary cavities narrow and there are signs of enhanced osteoblastic activity (Fig. 10). Some calcium incorporation is characteristic of all hypoplastic bones of the skull (Fig. 11a, b). Hypoplasia is most marked in the occipital and



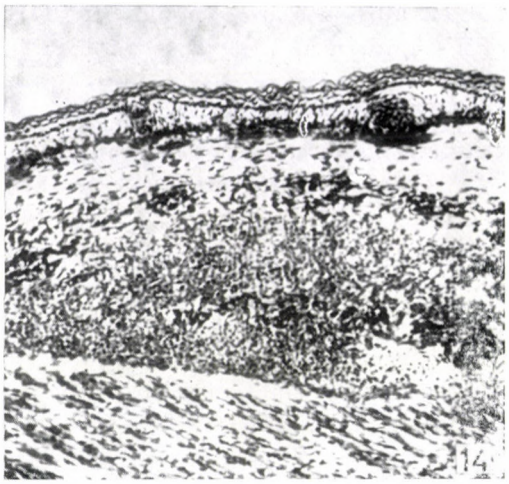


Fig. 13. The eye of a rat fetus treated with 2'-thiourea. The lens is enlarged, the cornea protruding, the vitreous is decreased in mass. Haematoxylin-chromotrope. $4 \times 6.3 \times 0.5$
Fig. 14. Skin of a rat fetus treated with 2'-thiourea, showing subdermal haemorrhages. Haematoxylin-chromotrope. $10 \times 6.3 \times 0.5$

parietal bones. Calcium incorporation is strongly reduced in the former. The connective tissue layer of the calvaria is thickened in the middle, containing numerous loosely arranged fibres (Fig. 12). Above the thick connective tissue layer, the subcutaneous tissue is loose and contains many mesenchymal cells. In the loosened subcutis, extensive haemorrhages make the calvaria even more protuberant. There are haemorrhages in the subcutis all over the body (Fig. 14). Presumably, a general developmental disturbance of vascular walls leads to the formation of these haemorrhagic areas.

Eye

In treated fetuses the eyelids are not fused. The lens is unusually large with swollen fibres that do not stain uniformly. The enlarged lens makes the cornea protrude beyond the line of the eyelids. Extending posteriorly, the lens occupies the place of the vitreous which is strongly reduced in mass (Fig. 13).

Discussion

The results indicate that 2'-thiourea, similarly as ethylene thiourea, exerts a teratogenic effect in the rat. Changes in the nervous system occurring under the effect of 2'-thiourea are similar to those induced by ethylene thiourea [7] with the difference that in our material subdural oedema was absent. Changes in the skeletal system are conspicuous after 2'-thiourea treatment;

almost all bones are hypoplastic. There is a marked difference in the effect of 2'-thiourea and ethylene thiourea in that the former compound disturbs the development of the cerebro- and viscerocranium with consequent cleft palate and micrognathia. Such changes were not noted by KHERA [7] after ethylene thiourea administration.

Our histological studies have shown that endochondral and intramembraneous ossification is equally retarded. Changes in the nervous and skeletal system are due to disturbed differentiation. Everywhere we find younger, undifferentiated cells among which there are many degenerating and decaying cells. We cannot explain the phenomenon of metaplasia in the nervous system.

Thus, 2'-thiourea, which is a carcinogenic substance, is capable of inducing metaplastic and hypoplastic changes in rat fetuses. The significance of this finding is underlined by the fact that more and more carcinogenic agents are proved to be teratogenic as well.

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DIE TERATOGENE WIRKUNG VON 2'-THIOUREA BEI RATTEN

M. KERN, ZS. TATÁR-KISS, P. KERTAI und I. FÖLDES

Die teratogene Wirkung von 2'-Thiourea auf Rattenembryonen wurde untersucht. Die 0,2%ige wäßrige Lösung von 2'-Thiourea (Merck) wurde trächtigen Ratten vom 1. bis zum 14. Tag der Schwangerschaft statt Trinkwasser ad libitum gereicht. Den Tag, an dem

spermapositiver Vaginalabstrich erhalten wurde, betrachtete man als 1. Tag der Schwangerschaft. Folgende Veränderungen wurden registriert: Gehirn- und Rückenmarkshypoplasie, Hydrocephalus internus, Hypoplasie des Ganglion spinale, Hypoplasie und Mißbildungen am Knochengerüst: Kypholordose, Mikromelie, Mikrognathie, Gnathopalatoschisis, retardierte Zahnbildung, Anomalien der Augenentwicklung: Exophthalmus, Kolobom, Katarakt, generalisierte Hämorrhagien am ganzen Körper.

Es wurde festgestellt, daß die Entwicklungsanomalien den durch Äthylenthiourea bewirkten teratogenen Veränderungen ähnlich sind.

ТЕРАТОГЕННОЕ ДЕЙСТВИЕ 2'ТИОУРЕИ У КРЫСЫ

М. КЕРН, Ж. ТАТАР-КИШШ, П. КАРТАИ и И. ФЁЛДЕШ

Тератогенное действие 2'тиоурей было изучено на зародышах крыс. Беременным крысам давали вместо питьевой воды 0,2%-ый раствор 2'тиоуреи (Мерк) от 1-го до 14-го дня беременности. Как первый день беременности авторы рассматривали день получения спермаположительного мазка из влагалища. Наблюдали следующие изменения: гипоплазию головного и спинного мозга, внутреннюю гидрокефалию, гипоплазию спинального узла, гипоплазию и уродство костного скелета: кифолордоз, микромелию, микрогнатию, гнатопалатошиз, замедленное развитие зуб, аномалии развития глаз: пучеглазию, колобому, катаракту, генерализованные геморрагии по всему телу. Было установлено, что наблюдаемые аномалии развития подобны тератогенным изменениям, вызванным дачей этилентиоуреи.

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ULTRASTRUCTURE AND LOCALIZATION OF CALCIUM IN UTERINE SMOOTH MUSCLE

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General ultrastructure and subcellular calcium localization was studied in the longitudinal muscle layer of the rabbit's post partum myometrium by means of the lead acetate and potassium pyroantimonate cytochemical techniques. The finding of abundant surface vesicles (SV) and intracellular tubules of smooth endoplasmic reticulum (SER), their close contacts in some parts of the fibre, and the isolated compartment between plasma membrane (PM) and basal membrane (BM) may be important in the understanding of characteristic features of excitation-contraction coupling in this tissue. In the resting fibre (Mn^{2+}-induced relaxation) the lead acetate and pyroantimonate reaction product was localized in the PM, SV, SER and mitochondria (M), indicating the possible in situ Ca-sequestering properties of the above structures. Tissues fixed at the peak of K^+-contraction revealed abundant precipitates in the cytoplasm, while little reaction product was seen at the periphery of the fibre.

The specificity of the cytochemical reactions was verified by Ca-extraction EGTA in situ (lead acetate) and by electronprobe X-ray microanalysis (pyroantimonate). These findings indicate that redistribution of the precipitate during the contraction-relaxation cycle is the result of intracellular calcium translocation in the rabbit myometrium.

Introduction

The determinant of uterine smooth muscle contractile state is intracellular Ca^{2+} activity [5, 6, 8, 14, 18]. This activity depends on the rates of Ca^{2+}-influx and efflux across the plasma membrane and rates of Ca^{2+} release and uptake into intracellular pools [8, 14]. There is physiological evidence of the existence of two major Ca-pools in the myometrium: a loosely bound superficial fraction localized in the extracellular space and plasma membrane, and a tightly bound intracellular fraction [6, 8, 18—20]. Studies of calcium uptake, binding and accumulation by subcellular fractions from the myometrium of different species indicate that intracellular Ca-sequestring sites are located in the plasma membrane [8, 14], in the smooth endoplasmic reticulum (SER) [4] and in the mitochondria [1]. In contrast to vascular [10, 17, 23, 24] and intestinal [11, 12, 13, 25] smooth muscle, in the myometrium no one has applied selective Ca-stains to determine which of these possible sites contain Ca under physiological conditions, and to study the effect of procedures associated with changes in intracellular Ca.

The present study was designed to investigate the general ultrastructure and subcellular Ca-localization in the rabbit's post partum myometrium by the lead acetate [3] and potassium pyroantimonate [25] methods. A further aim of the study was to determine intracellular Ca-translocation during the contraction-relaxation cycle. Chemical and physical analyses were applied to determine the specificity of the methods employed.

Materials and methods

A total of 55 longitudinal uterine strips (10 mm long and 5 mm wide) excised from 31 white female New Zeland rabbits 8—12 h after normal parturition were mounted in an experimental chamber (pyrex tube, 10 ml) filled with oxygenated Krebs—Ringer bicarbonate solution (KRB) at 37 °C. One end of the strip was connected to a strain gauge (Grass FTO3C) to record isometric tension, and the other end was clamped.

Stained tissue preparations

After 15 min incubation in KRB the preparations (n=10) were fixed in 3% glutaraldehyde (GTA) buffered with cacodylate (pH 7.4), for 30 min. The external longitudinal muscle layer was separated from other tissues (circumferential muscle, endometrium) and cut into small pieces. Subsequently the muscle fragments were immersed in 1% osmium tetroxide (OsO_4) buffered with 0.01 M acetic acid (pH 7.4). The specimens were dehydrated by cold ethanol, embedded in Durcupan (ACM Fluka) and ultrathin sections were cut by a Reichert OH U2 ultramicrotome. The sections were stained by uranyl acetate and lead citrate and examined in a JEM 100B electron microscope at 80 kV acceleration voltage.

Lanthanum method

Five uterine strips were fixed by GTA containing 2.5% lanthanum hydroxide. Electron microscopic localization of the extracellular marker lanthanum was carried out by the method of KARNOVSKY et al. [15].

Subcellular calcium localization

Experimental procedure. Calcium localization was examined in two uterine strips excised from the same uterine horn. One strip was immersed in KRB containing 4 mM $MnCl_2$ for 10 min, and was fixed by GTA or potassium pyroantimonate (see below) with no detectable resting tension or active tension development. The other strip was incubated in isotonic (127 mM) solution and fixation was done at the peak of the resulting contraction. The fall of tension at the completion of fixation was 5—10% of the maximum.

Lead acetate method. Following 15 min incubation in KRB, the relaxed (n=8) and contracting (n=8) uterine strips (see Experimental procedure) were placed in 3% GTA for 30 min. The external longitudinal muscle layer was separated from other tissues and cut into small fragments. Following prefixation the muscle pieces were rinsed three times in distilled water and then incubated in 4% lead acetate solution for 15 min at 37 °C. Subsequently the tissue was washed again in distilled water and postfixed in 1% OsO_4. The samples were then dehydrated, embedded and ultrathin sections were cut (see Stained tissue preparations). The sections were examined in a JEM 100B electron microscope at 80 kV acceleration voltage.

In control samples (n=4) the tissue was soaked in 10 mM EGTA for 20 min prior to lead acetate staining.

Potassium pyroantimonate method. After 15 min incubation in KRB the strips [relaxed (n=8) and contracting (n=8)] were immersed in isotonic KCl solution containing 2.5 mM potassium pyroantimonate [$K(Sb/OH_6)$] for 10 min to introduce the pyroantimonate into the fibres during membrane depolarization [25]. The preparations were then returned to KRB. After each experiment the tissue was fixed by replacing the experimental solution (see Experimental procedure) with 1% OsO_4 containing 2.5% potassium pyroantimonate. The external

muscle layer was separated from other tissues and cut into small pieces. The specimens were dehydrated, embedded, cut and examined without further staining as described above (see Stained tissue preparations).

Electron probe X-ray microanalysis

For chemical identification of the electron opaque precipitates, the sections were placed on copper grids and analyzed with an energy dispersive X-ray microanalyzer (ORTEC) attached to a JEOL 100B electron microscope provided by a JEOL scanning unit. Acceleration voltage was 40—80 kV, counting time 250 sec, and the procedure was carried out in scanning operation mode.

Results

General ultrastructure

Ultrastructural features of the longitudinal muscle layer of the post partum rabbit myometrium are demonstrated in Fig. 1. In the longitudinal section of two adjacent smooth muscle cells the following structures are visible (Fig. 1A): intercellular space (IS), plasma membrane (PM), surface vesicles

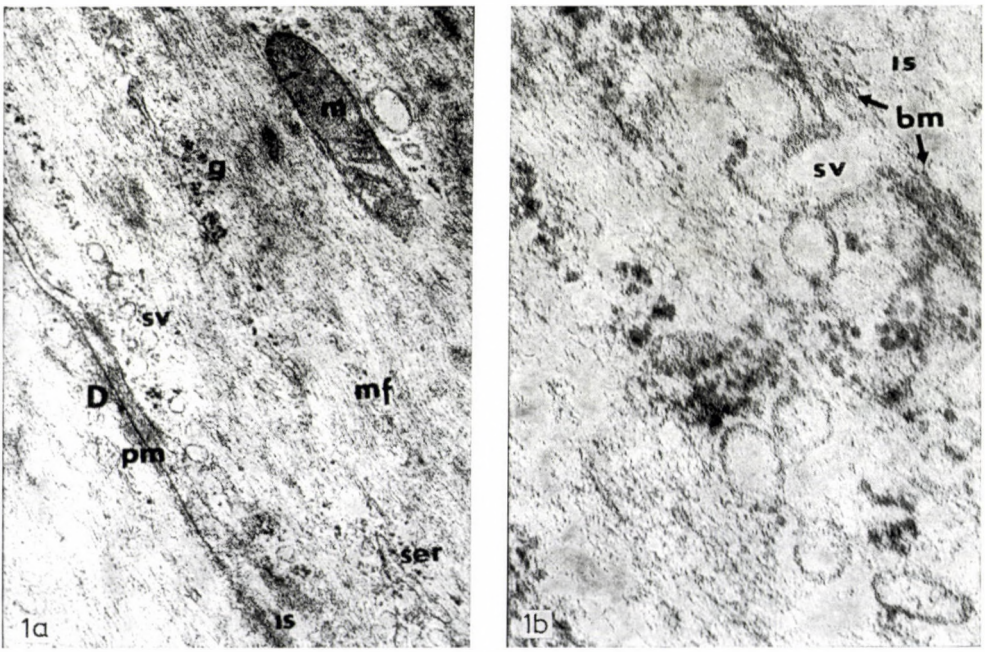

Fig. 1. a: Cross section of two adjacent myometrial smooth muscle cells (post partum rabbit uterus, external longitudinal muscle layer). Fixation by 3% GTA-Uranyl acetate + lead citrate staining. Magnification × 24 000. IS = intracellular space; PM = plasma membrane; SV = surface vesicles; D = desmosome-like structure; m = mitochondrium; ser = smooth endoplasmic reticulum; mf = myofilaments; g = glycogen. b: Enlarged picture of the region near to the plasma membrane. Surface vesicle is in direct connection with intercellular space. Well developed basal membrane (bm) is present; it does not penetrate the surface vesicle. Magnification: × 85 000

(SV), desmosome-like structures (D) in the region of tight connection between the two cells, vesicles and tubules of smooth endoplasmic reticulum (SER), mitochondrium (M), myofilaments, dense-bodies (DB), and glycogen particles (C). At higher magnification the SV was seen to communicate directly with the IS, and that the basal membrane (BM) does not penetrate into the SV (Fig. 1B).

Lanthanum is localized in the extracellular space and penetrates into the surface vesicles (Fig. 2). Since lanthanum remains in the IS, the SV can easily be separated from SER. In some parts of the cell, peripheral tubules of SER are in close contact with SV.

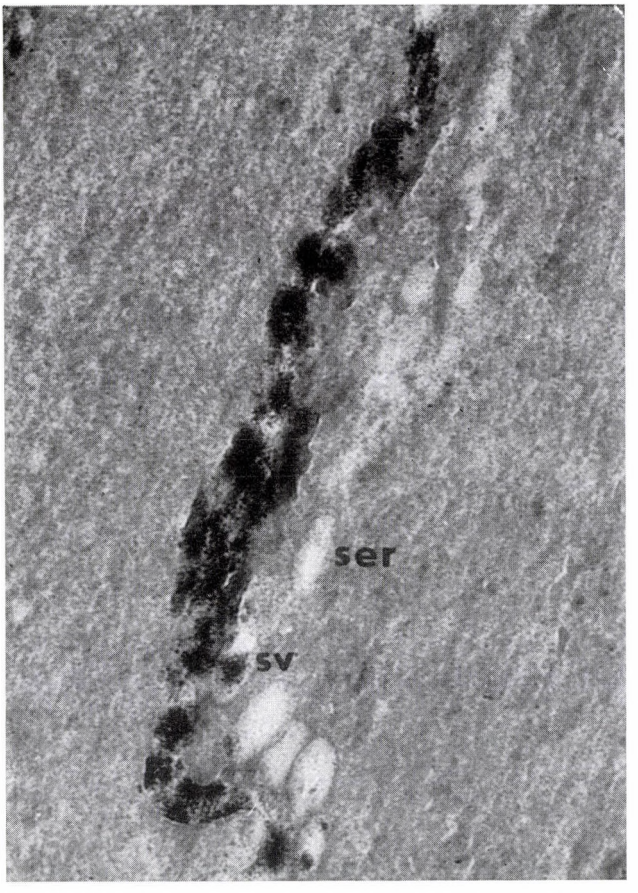

Fig. 2. Cross section of two adjacent myometrial smooth muscle cells. The extracellular marker lanthanium hydroxide is localized in the intercellular space. Surface vesicles filled with lanthanium are well separated from the "empty" intracellular vesicles of SER. Magnification: × 32 800

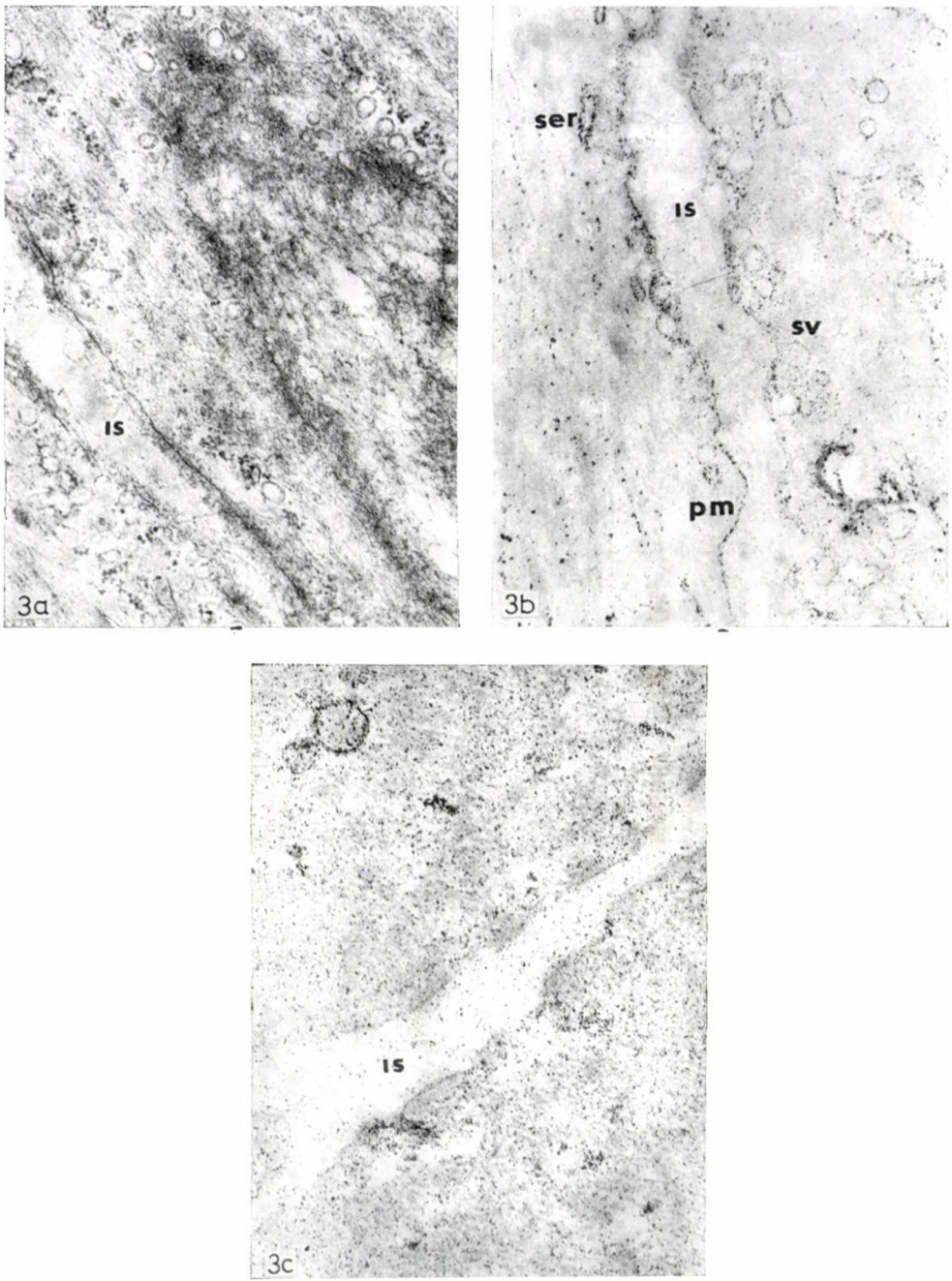

Fig. 3. a: Cross section of two adjacent myometrial smooth muscle cells, lead acetate staining. Control sample. The tissue was soaked in EGTA (10 mM) for 15 min prior to lead acetate staining. Note the absence of electron-opaque reaction product. Magnification: × 28 000. b: Lead acetate staining. The tissue was incubated in Krebs-solution containing 4 mM Mn^{2+} (totally relaxed sample) prior to GTA fixation. Intensive reaction product in pm, sv, ser and m. Magnification: × 32 800. c: Lead acetate staining. The muscle strip was fixed by GTA at the peak of K^+-contraction (127 mM KCl). Translocation of reaction product from the periphery of the fibre to the cytoplasm. Magnification: × 28 800

Calcium localization and translocation during the contraction-relaxation cycle

Characteristic, reproducible subcellular electron opaque precipitate distribution was revealed by the lead acetate method in the relaxed (Mn^{2+}) myometrial cells (Fig. 3b). An intensive lead phosphate reaction was present in PM, SV, SER and M. Precipitates were hardly recognized in the IS and cytoplasm. When calcium was removed by the specific chelator EGTA prior to soaking the tissue in lead acetate (control samples), no reaction product was observed at any site (Fig. 3a). The precipitates in M were restricted to the space between the outer and inner membrane and within the cristae (Fig. 4). Vesicles and tubules of SER contained reaction product in all parts of the cell, which was attached to the inner surface of the SER membrane (Fig. 4). At the peak of K^+-contraction the precipitate was diffusely distributed in the cytoplasm in the form of small particles, while little precipitate was seen at the periphery of the cell (Fig. 3c).

In the resting fibres relaxed by Mn^{2+}, the pyroantimonate precipitate, forming large particles was localized along the PM and at the periphery of the cytoplasm (Fig. 5a). The intracellular structures could not be recognized since no staining was used in the cytochemical procedure (see methods). In smooth muscle fibres fixed at peak of K^+ contraction, the precipitate was diffusely distributed in the cytoplasm in the form of small particles (Fig. 5b) similar to those revealed by the lead acetate method.

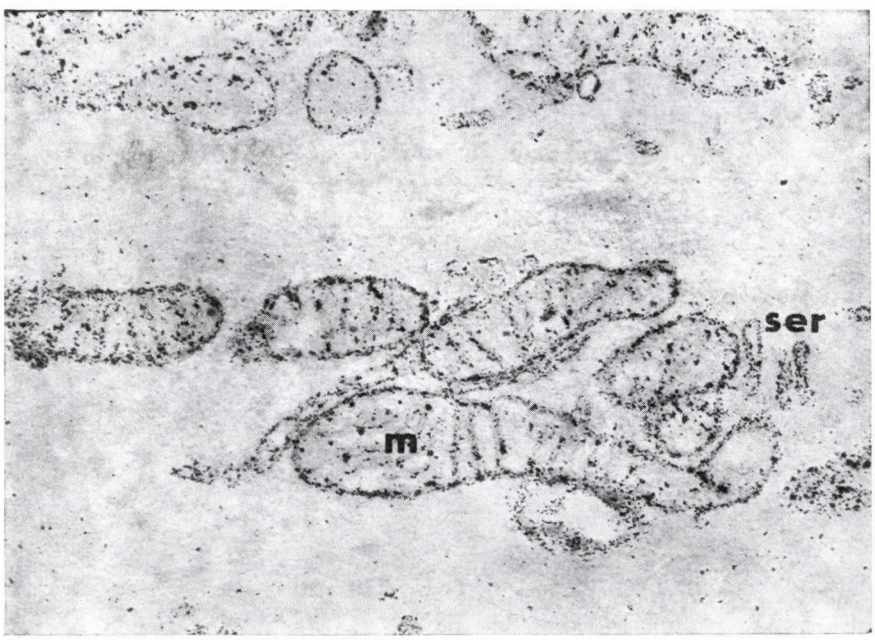

Fig. 4. Lead acetate staining. Mitochondrium (m) and vesicles of SER are packed with electron opaque precipitate (the muscle was relaxed by Mn^{2+} prior to fixation). Magnification: × 86 400

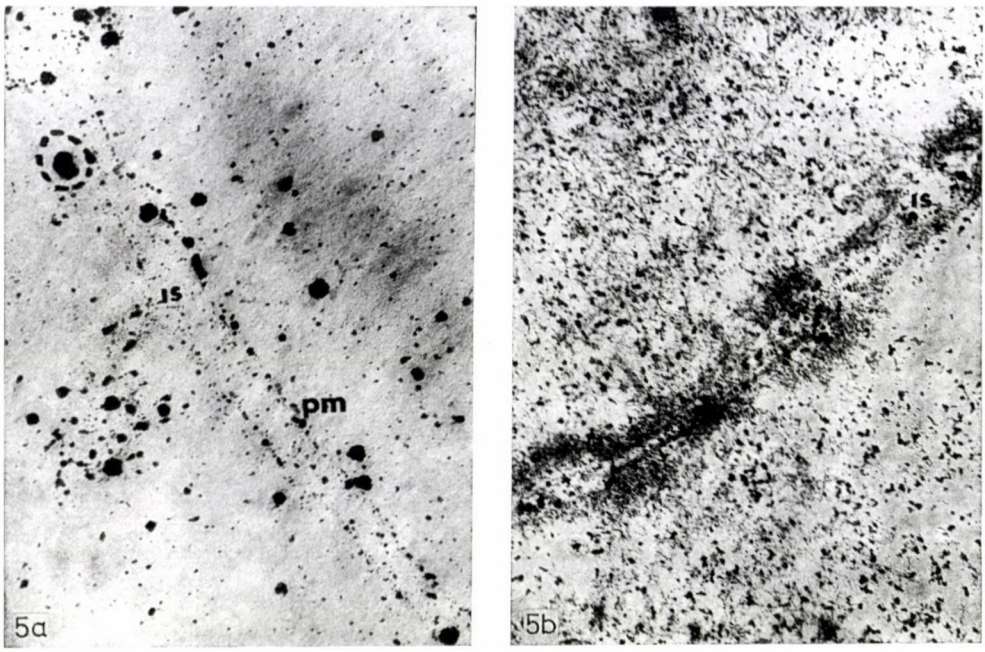

Fig. 5. Potassium pyroantimonate staining. a: Relaxed tissue (Mn^{2+}): the precipitate is localized along the PM, and in the cytoplasm in the form of large, dense particle. Magnification: × 30 000. b: Contracted tissue (K^+). Diffusely distributed reaction product in the cytoplasm. Magnification: × 30 400

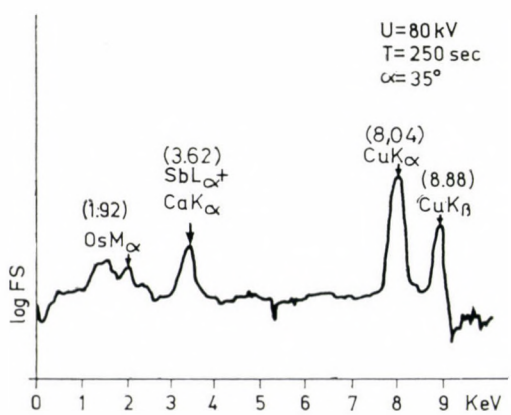

Fig. 6. Electronprobe X-ray spectrum of the pyroantimonate precipitate encircled in Fig. 5a. The ordinate represents total activity measured during the 250 sec observation period on a logarithmic scale (log FS). Beside Os M_α and Cu K_α and K_β lines, a characteristic peak can be observed at 3.62 keV. This peak is the result of combined spectrum of Sb L line with Ca K_α lines (see text)

X-ray microanalysis

In the lead phosphate precipitate particles or in areas of the cytoplasm not occupied by the reaction product, only X-ray peaks due to silicium, osmium and copper could be identified. All these elements were present as a result of the technique used in preparing and mounting the tissue for electron microscopic examination.

The X-ray spectrum of the pyroantimonate reaction product obtained from the encircled region of Fig. 5a, is demonstrated in Fig. 6.

A distinct peak at 3.62 KeV could be identified; the peak was the result of the combination of Sb L_α-line with Ca K_α-line (see Discussion).

Discussion

Ultrastructure

The ultrastructural features of the longitudinal muscle layer of the post partum rabbit myometrium are similar to those observed in the oestradiol-treated rat myometrium [7]. Surface vesicles (SV) are abundant and they could easily be separated from the intracellular vesicles and tubules of SER by the extracellular marker colloid lanthanum. In several regions of the smooth muscle fibre close contacts between SER and SV were observed. Similar close contacts were described in vascular [24], intestinal [11] and rat uterine smooth muscle [7] as well. It was supposed that in these junctional areas the action potential may activate Ca^{2+}-release from the SER [2, 23, 24], and they may play a role in specific ion exchange [2]. A well-developed basal membrane (BM) was observed close to the external surface of the plasma membrane (PM). It was found that BM does not penetrate into the SV, producing thereby closed compartments in the intercellular space. These areas, forming compartments with restricted ion diffusion [16], may play an important role in the determination of extracellular ion distribution [2, 8, 23].

Subcellular Ca-localization

The present study was the first attempt to localize calcium binding sites by cytochemical techniques in uterine smooth muscle fibres. Reproducible electron opaque precipitate distribution was present in the longitudinal muscle layer of the post partum rabbit myometrium, the most intensive reaction product being localized in the PM, SV, SER and M with both the lead acetate and the pyroantimonate methods.

In the resting fibre (Mn^{2+}-induced relaxation) the reaction product was localized at the periphery of the fibre (PM, SV) and in some intracellular structures (M, SER). This finding indicates that as in other types of smooth

muscle [10—13, 17, 24, 25], in the rabbit myometrium too, the plasma membrane, mitochondria and smooth endoplasmic reticulum are the most important Ca-storing and sequestring sites. This may play a leading role in the induction of relaxation by reducing the free cytoplasmic Ca^{2+}-concentration.

In the fibres fixed at the maximum of K^+-contraction, abundant lead phosphate and pyroantimonate reaction product was present in the cytoplasm, and the intensity of the cytochemical reaction decreased at the periphery (PM) of the fibre. This translocation of the precipitates from the periphery to the cytoplasm was similar to that found in the guinea pig taenia coli during acetylcholine contraction [25].

These findings are in good agreement with physiological data showing that superficial activator calcium is localized in or near to the plasma membrane, and it is mobilized during different types of activation [18, 22]. Demonstration of the reaction product in the PM of the relaxed fibre emphasizes the importance of Ca^{2+} in the regulation of membrane ion permeability, i.e. in the relaxed (polarized state firm binding of Ca^{2+} to the PM inhibits cation permeability, and its mobilization during membrane depolarization enhances the influx of external activator Ca^{2+} [18, 20]. Further studies are needed to elucidate the effect of hormonal influences on PM Ca-binding since recent data indicate that hormonal regulation of uterine contractile activity is linked to the control of superficial calcium binding in the rabbit myometrium [18—22].

Specificity of the cytochemical methods

To control the specificity of the lead acetate method a procedure of calcium extraction *in situ* was employed. The small and not very dense granules disclosed by this techniques make the reaction product unsuitable for specific X-ray microanalysis [9, 24].

Control samples obtained after washing the tissue in EGTA were always negative (electron microscopy revealed no end-product deposits). The specificity of the lead acetate reaction is indicated by the fact that results of the lead acetate reaction are in good agreement with those yielded by the pyroantimonate method.

The large and dense granules obtained by the pyroantimonate method were suitable for electronprobe X-ray microanalysis of their chemical composition. The X-ray spectrum of the pyroantimonate precipitate showed a distinct peak at 3.62 keV (Fig. 6). Model tests with calcium and antimonate showed that this spectrum resulted from the combination of Sb L_α-line (3.6 keV) with Ca K_α-line (3.69 keV) [25].

These findings indicate that a redistribution of the reaction product during the contraction-relaxation cycle in the post partum rabbit myometrium is the result of intracellular calcium translocation.

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KALZIUMKOALISATION UND ULTRASTRUKTUR IN DER GLATTEN MUSKULATUR DER GEBÄRMUTTER

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Die allgemeinen ultrastrukturellen Charakteristika sowie die subzelluläre Lokalisation des Kalziums im Myometrium wurden an aus Kaninchen post partum isolierten länglichen Uterusstreifen untersucht. Außer Einschnürungen der Zellmembran (PM) (oberflächliche Bläschen, FV) wurden Baselmembranen (BM), intrazelluläres endoplasmatisches Retikulum mit glatter Oberfläche (SER) sowie Mitochondrien (M) beobachtet. Zwischen den obigen Strukturen waren an vielen Stellen enge Kontakte zu erkennen, denen in der Entstehung der Exzitation—Kontraktion—Beziehung des Myometriums eine Bedeutung zukommen dürfte. In dem in Anwesenheit von 4 mM Mn^{2+} vollkommen erschlafften Muskel war das Reaktionsprodukt des für den Kalziumnachweis gebrauchten Bleiazetats und Pyroantimonats in erster Linie entlang der Zellmembran (PM), des SV, des endoplasmatischen Retikulums (SER) sowie der Mitochondrium (M) lokalisiert. In dem beim Maximum der K*-Kontraktion fixierten Muskel war das Reaktionsprodukt aus dem Bereich der Zellmembran (PM) und der oberflächlichen Bläschen (FV) verschwunden, während im Zytoplasma zahlreiche Präzipitate zu beobachten waren. Die Spezifität der zytochemischen Reaktionen wurde teils mit in situ Ca-Extraktion (EGTA), teils mit Hilfe von Mikrosonden mittels Röntgenmikroanalyse (Pyroantimonat) gesichert.

ЛОКАЛИЗАЦИЯ КАЛЬЦИЯ И УЛЬТРАСТРУКТУРА В ГЛАДКОЙ МЫШЦЕ МАТКИ

Г. РУБАНЬИ, И. БАЛОГ, А. Г. Б. КОВАЧ, Е. ШОМОДЬИ и П. ШОТОНЬИ

Общие ультраструктурные характеристики миометрия и подклеточная локализация кальция были изучены на подольных полосах матки, изолированных из кроликов после родов. Кроме зашнуровываний клеточной мембраны (PM), (поверхностные везикулы, FV), наблюдались базальные мембраны (BM), внутриклеточно расположенная эндоплазматическая сетчатка с гладкой поверхностью (SER), а также митохондрии (M). Между вышеуказанными структурами на многих местах наблюдался тесный контакт, что предположительно имеет значение в образовании эксцитационно-контракционных связей в миометрии. В мышце, совершенно расслабленной в присутствии 4 мМ Mn^{2+}, продукт реакции уксуснокислого свинца и пироантимоната, примененных для выявления кальция, располагается прежде всего вдоль PM, SV, SER, M. В миометрии, фиксированном при максимуме K^+-сокращения, продукт реакции исчез из области PM и FV, причем, однако, в цитоплазме наблюдались многочисленные осадки. Специфичность цитохимических реакций была подтверждена с одной стороны с экстракцией кальция (EGTA) (уксуснокислый свинец), а с другой, рентгеновским микроанализом посредством микрозонда (пироантимонат).

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THE STUDY OF BONE TISSUE WITH NEW ARGYROPHILIC TECHNIQUES

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Fractured and necrotic bones were examined by new silver impregnation techniques based on the catalytic activity of the bone. The new methods proved to be useful in differentiating between bone tissues of various ages and maturity and between living and necrotic bones.

Introduction

Routine histological stainings (haematoxylin-eosin, Schmorl) give little if any help in establishing the age, maturity and early necrosis of bone. We have employed new silver impregnation techniques [4, 5] for studying experimental bone necrosis. The methods can readily be adopted to formaldehyde-fixed paraffin-embedded tissue, thus they provide a reliable means for everyday diagnostics.

Material and methods

In 32 chinchilla rabbits the neck of the right femur was surgically exposed and broken, while on the left side the vessels supplying the head of the femur were electrocoagulated. Development of the traumatic-fractural and electrocoagulatic-vascular aseptic necrosis was studied at 1—2—3—4—5—6 days, 1—2—3—4 weeks and 1—2—3—4—5—6 months after operation [1].

The tissue specimens were fixed in 5% formaldehyde at least for 24 h. Decalcination was performed at room temperature for 18—48 h depending on the hardness of the bone, in a medium containing 24 ml 85% formic acid and 50 ml 35% hydrochloric acid in 126 ml distilled water. After washing and dehydration the specimens were embedded in paraffin; 5 μm thick sections were cut, deparaffinated and stored in absolute alcohol prior to silver impregnation.

Impregnation was performed by the following procedure:

1. Esterification in one of the solutions listed:

a) 100 ml 100% methyl alcohol + 0.5 ml concentrated sulphuric acid, 56 °C, 20 h.

b) 100 ml 100% propyl alcohol + 0.5 ml concentrated sulphuric acid, 56 °C, 20 h.

c) 100 ml 100% octyl alcohol + 0.5 ml concentrated sulphuric acid, 56 °C, 20 h.

2. Washing in descending alcohol series (100—96—50—20%) and then in distilled water.

3. Physical development until the required colour is obtained (approx. 5 min).

The physical developer has to be prepared before use from two stable stock solutions:

A. 5 g anhydric sodium carbonate in 1000 ml distilled water.

B. 2 g ammonium nitrate, 2 g silver nitrate and 10 g silicotungstic acid dissolved in 1000 ml distilled water, then 3 ml 40% formaldehyde is added in drops.

To 50 ml A 50 ml B is added gradually, under continuous stirring. In the first minute slides immersed into the developer should be agitated. Optimum development-time can be assessed by the colour of sections. Until sufficient skill and experience are gained, microscopic control during the procedure is recommended. For this purpose the slides are rinsed in 1% acetic acid and checked wet under a microscope. If further impregnation is needed, the sections are re-immersed into the developer.

4. Washing in 1% acetic acid for 10 min, dehydration and mounting in balsam.

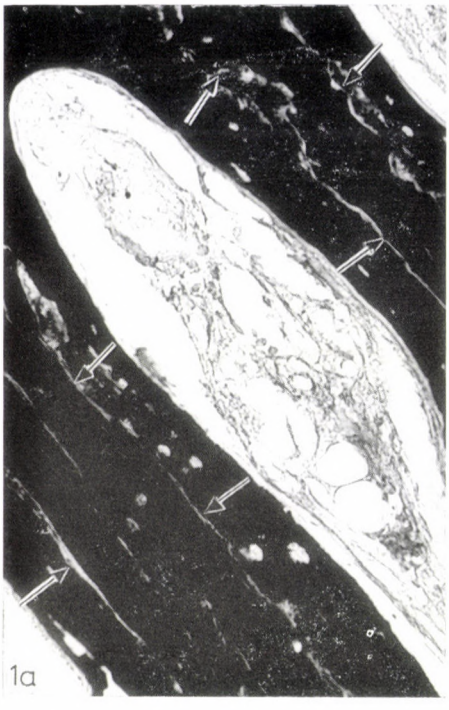
Results

1. As a result of methanol esterification followed by physical development, mature lamellar bone stains black. From this background cement lines of the bone tissue are apparent (Fig. 1a).

Immature bone stains faintly, cartilage and osteocyte nuclei are unstained (Fig. 1b). Chondrocyte nuclei, on the other hand, are best seen after this pretreatment when comparing the three esterification procedures.

2. If esterification is carried out by propanol, differentiated lamellar bone is stained tobacco-brown; immature bone is yellow. Cement lines are not conspicuous (Fig. 1c). The basic substance of cartilage is not stained. Nuclei of osteocytes are prominent. Necrotic cells are not stained. Around empty lacunae a tobacco-brown rim is formed (Fig. 1d). As compared to haematoxylin-eosin preparations, the number of empty lacunae is increased as early necrobiotic cells ("ghost-cell") still showing some staining with haematoxylin-eosin, are not impregnated. A very early stage of necrobiosis is indicated by the decreased impregnation of the cell and by the brown rim at the edge of its lacuna. Hence there is no transition in staining between intact and necrotic cells according to the "all or none" nature of silver impregnation techniques. In contrast, haematoxylin-eosin preparations show a gradual decrease of staining intensity with the progress of necrosis. Due to the presence of transitional forms, the assessment of necrosis is more difficult in haematoxylin-eosin preparations than in impregnated ones.

Fig. 1 a. Differentiated lamellar bone, 5-month necrosis. Physical development after methanol treatment. 25 × 6.3. The bone tissue is permeated by parallel cement-lines. The middle segment of the trabecule (arrows) corresponds to the original bone structure, the two outer layers are sheaths formed during reparation. Osteocytes are not stained, the marrow is poor in details. b. Epiphyseal cartilage and its surroundings. One-week necrosis. Physical developing after methanol pretreatment. 12.5 × 6.3. Cartilage ground substance is not impregnated, chondrocytes arranged in rows are conspicuous. Differentiated lamellar bone (upper right) is black, immature tissues (lower left) are stain faintly. c. Cortical portion, 2-week necrosis. Physical development after propanol pretreatment. 12.5 × 6.3. Mature bone is tobacco-brown, cement lines are wide and vaguely visible (arrows). Marrow elements stain well. d. Partially necrotic subchondral cortical area, 2-week necrosis. Physical development after propanol pretreatment. 12.5 × 6.3. Living osteocytes (arrow) are well impregnated. At the edges of their capsule dark rim is visible (double arrow). These cells correspond to early necrobiotic forms. Empty lacunae (marked by X) have a dark rim indicative of a more advanced stage of necrobiosis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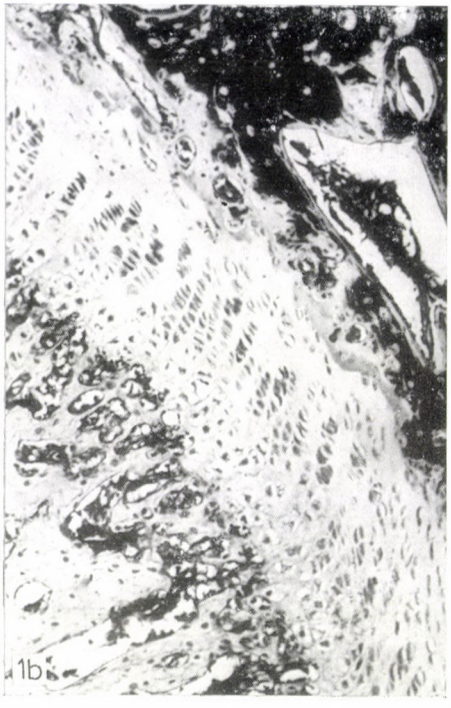
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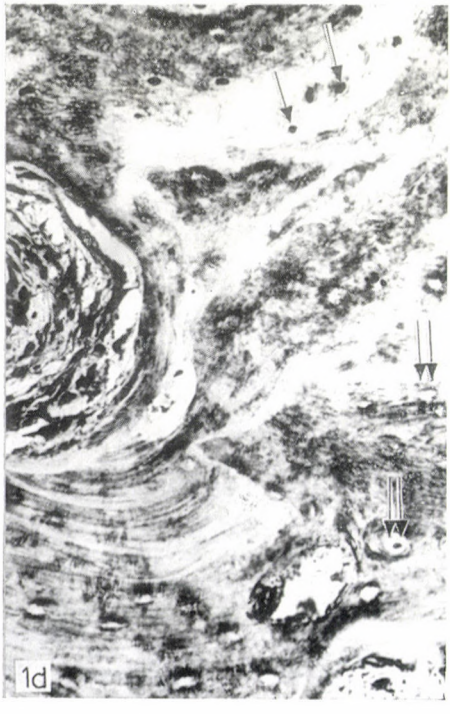
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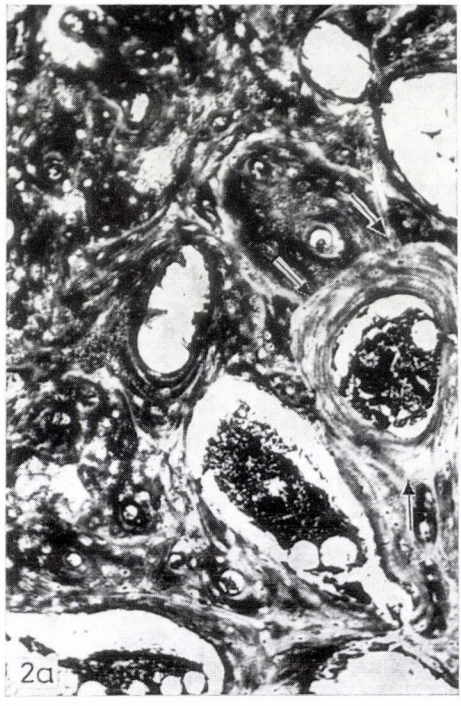
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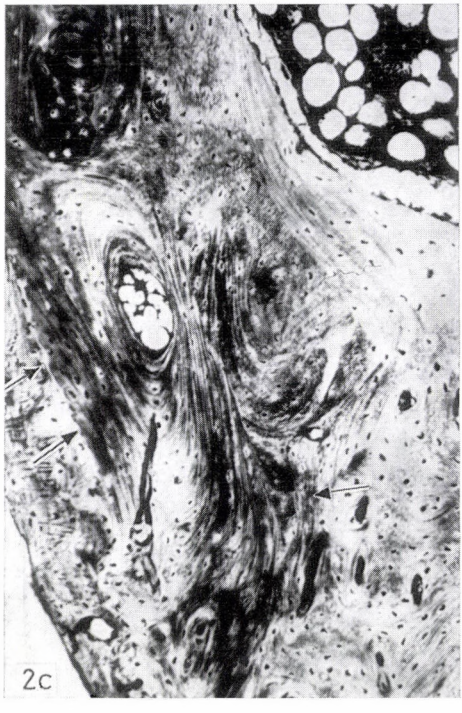
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3. To distinguish between bone tissues different in age, octyl alcoholic esterification proved to be the best. The intensity of the brown colour is indicative of the stage of differentiation (Fig. 2a, b, c) and the approximate age of undifferentiated bone (Figs 3a, b). Osteocyte nuclei are stained like after propanol but no brown rims of the lacunae are formed (Figs 3c, d).

For the study of bone marrow, methanol pretreatment is not suitable. Propanol, but especially octyl alcohol, give better results. The fibre network of the marrow and fibres and muscle cell nuclei of its vessels are particularly well demonstrated.

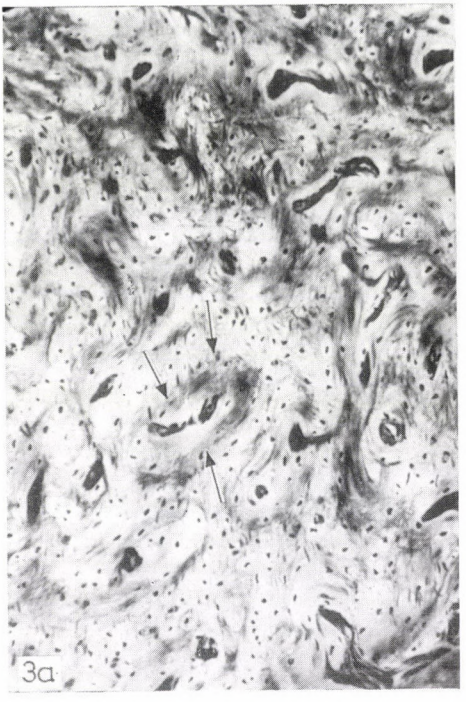
After octyl alcohol a linear system is revealed which is independent of the cement lines and does not respect inner territorial boundaries. Parallel lines display a fishbone-like pattern by running obliquely from the periosteum in a 30—45° angle to the cortex. They are particularly conspicuous in the large tubercles. In more distal parts of the bone they fade and appear as sporadic thin lines. These latter may correspond to bone canalicules (Fig. 2d).

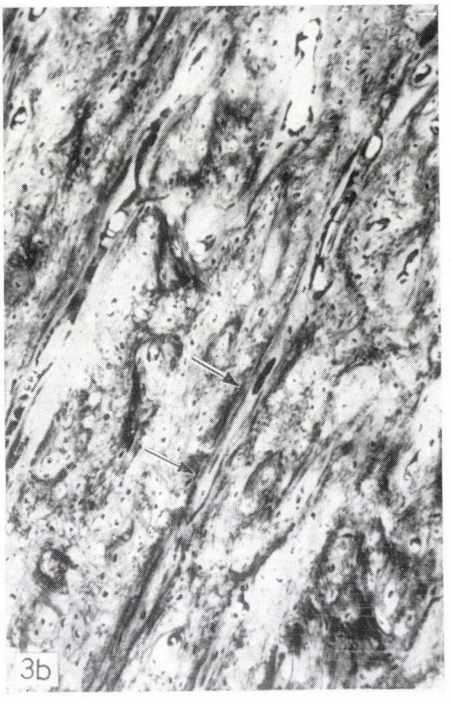
Rough lines not respecting inner boundaries are thought to result from silver deposition into transected bone channels. This is apparent in preparations developed for a shorter period.

Discussion

Argyrophilic procedures, i.e. those requiring photographic development, comprise two basic steps: formation and growth of foci [6, 8, 9]. While the latter has a mechanism similar in all procedures, the former may occur in three different ways [4]. 1. The classical argyrophilic reaction: reducing groups of the tissue, when exposed to the medium containing silver ions form of foci colloidal silver; 2. Onto certain tissue elements colloidal silver-salts are precipitated by means of a chemical reaction (silver-salt foci); 3. Certain tissue elements catalyze an interaction of silver ions and dissolved reducing molecules as a result of which colloidal metal silver grains are formed. In this reaction reducing tissue elements are not involved.

Fig. 2 a. Almost totally necrotic subchondral cortical area, 5-month necrosis. Physical development after octyl alcohol pretreatment. 12.5×6.3. The younger differentiated necrotic lamellar bone stains faintly as compared to the older bone (arrows point to the border of the younger bone tissue). b. Partially necrotic cortical bone, 4-month necrosis. Octyl alcohol pretreatment. 12.5×6.3. Necrotic differentiated lamellar bone stains dark, newly formed undifferentiated bone stains lighter (arrows). The youngest bone tissue (labelled by X) is almost unstained. c. Portion of a callus, 3-month necrosis. Physical development after octyl alcohol pretreatment. 12.5×6.3. The dark mature, differentiated lamellar bone (arrows) and the light immature, undifferentiated bone can well be distinguished. d. Portion of cortical bone. Physical development after octyl alcohol pretreatment. 12.5×6.3. The parallel lines correspond to the canaliculo-lacunar network system


3a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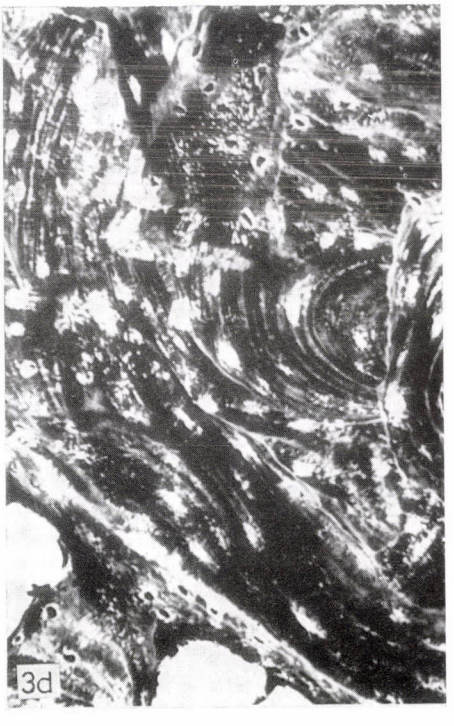
3c


3d

Focus formation of the 3rd type may occur only under stabilized conditions in solutions where silver ions do not react or react very slowly with dissolved molecules. Such solutions are called physical developers. To slow down the spontaneous reaction, these solutions contain a protective colloid. In these physical developers the size of both colloidal metal silver grains and silver-salt grains is rapidly increased [7] by autocatalysis.

The histotechnical application of physical developers was rendered possible by recognizing that esterification enhances the activity of tissue catalytic points. Tissue elements containing catalytic points are impregnated faster than those lacking such points. The number of carbon atoms in the alcohol used for esterification influences the activity of catalytic points. Accordingly, the chemical basis of the procedure is provided by the different activity of catalytic tissue-points at different stages of bone maturation, differentiation, aging and necrosis.

For studying bone-lines, esterification with methanol appears to be the method of choice. By demonstrating inner territorial boundaries it yields information concerning the dynamics of bone formation and destruction. Regular and irregular lines are indicative of older and newer formation and destruction processes, respectively.

After esterification with propyl, and octyl alcohol a fairly good assessment of bone age is possible: the older the bone, the darker its staining. With these methods intact and necrotic bone cells can be differentiated, whereas routine histological stains often show a wide range of transitional forms.

There seems to be a parallelism between collagen-content and staining intensity. In the older, differentiated lamellar bone more collagen is found than in the immature one (Fig. 2a). This is reflected by the intensity of the impregnation (Figs 2b, c).

A landmark of bone tissue necrosis is the disappearance of osteocytes with accompanying changes in collagenous ground substance. The fainter staining of the necrotic bone can be explained by the destruction of its collagen apparatus (Figs 3c, d). This does not mean that the collagen is impregnated. The changes in proteoglycan content of the ground substance are probably responsible for the phenomenon.

Fig. 3 a. Newly formed bone tissue, 3-month after traumatic fracture. Physical development following octyl alcohol pretreatment. 12.5×6.3. Fibres accumulated around blood vessels correspond to the primary osteon (arrows). The organized bone structure stains darker. b. Newly formed bone tissue, 4-month after traumatic fracture. Physical development following octyl alcohol pretreatment. 12.5×6.3. As compared to Fig. 3a, the bone tissue is more mature and the fibre ring of primary osteons stains darker (arrows). c. Intact subchondral bone tissue. Physical development, following octyl alcohol pretreatment. 25×6.3. Differentiated lamellar bone is impregnated dark-brown. Cement-lines are well visible. d. Almost totally necrotic are of subchondrial cortical bone, 6-month necrosis. Physical development following octyl alcohol pretreatment. 25×6.3. Empty lacunae are enlarged, their edges are fringed. Fibre bundles are less compact and stains fainter than the intact bone

After using propanol, an intensely stained rim appears at the edge of the osteocyte capsule. This is an early necrobiotic sign of osteocytes. Lysosomal and other enzymes liberated from necrobiotic osteocytes can destroy the proteoglycan ground substance of the capsule. The disappearance of cells can be verified by the ABT-reaction of ROMHÁNYI [2, 10].

The more resistant collagenous skeleton survives and silver is deposited at the site of the disappeared ground substance. This assumption is supported by the fact that cartilage containing a large amount of ground substance does not take the stain, nor can immature tissues be impregnated. Mature tissues in which the amount of ground substance is low in comparison to the amount of collagenous fibres, stain well.

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DIE UNTERSUCHUNG DES KNOCHENGEWEBES MIT NEUARTIGEN ARGYROPHILEN VERFAHREN

M. BÉLY und F. GALLYAS

An traumatisch frakturiertem sowie vaskulär bedingtem nekrotischem Knochengewebe wurden Untersuchungen mit neuartigen Versilberungsverfahren durchgeführt. die — entgegen den bekannten argyrophilen und argentaffinen Versilberungsverfahren — nicht auf der Reduktionsfähigkeit sondern auf der katalytischen Aktivität der Gewebe beruhen. Die Verfahren waren zur Unterscheidung von Geweben verschiedener Altersstufen und verschieden Reifegrades, ferner des lebenden und nekrotischen Knochengewebes geeignet.

ИЗУЧЕНИЕ КОСТНОЙ ТКАНИ ПРИ ПОМОЩИ АРГИРОФИЛЬНЫХ МЕТОДОВ НОВОГО ТИПА

М. БЕЛЬ и Ф. ГАЙЯШ

Авторамы была изучена травматически переложанная костная ткань, а также костная ткань с некрозом васкулярного происхождения методами серебрения нового типа, основывающимися — в противоположность известным аргирофильным и аргентаффинным методам — не на восстанавливающей способности тканей, а на их каталитическую активность. Новые методы исследования оказались пригодными для различения костных тканей различного возраста и различных степеней зрелости, а также для различения живой и мертвой ткани.

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DIAGNOSIS OF SKULL FRACTURES BY AUTOPSY AND RADIOLOGY

E. Ehler[1], D. Ivánkievicz[2] and G. H. Schumacher[1]

(Received 29 May, 1978)

Fifteen human cadaver heads after fixation in formalin were studied. Half of them were exposed to force applied fronto-occipitally, in the remainder the force was applied roughly vertically from the parietal bone towards a line connecting the two internal acoustic pores. The loads were increased until a fracture had occurred. The analysis was based on both halves of each head. Total number of fractures detected by autopsy and radiology.

Correct diagnosis	Skull roof	Skull base	Front of skull
a) autopsy	22	19	19
b) X-rays	10	0	0
b) in percent of *a)*	45.45	0	0

Those who remember that the conditions of X-rays of live subjects are substantially more complicated than those of a severed cadaver head will certainly be very cautious when examining X-ray material for signs of a fracture of the skull basis under clinical conditions.

Our data are based on fifteen skulls. It must nevertheless be assumed that results obtained from a larger number of samples would not differ appreciably.

Introduction

The numerous statistics published on accidents during the last 30 years [5, 6, 7, 10] show widely diverging figures. This is due in part to differences in the diagnosis, for instance, clinicians repeatedly find fractures which subsequently are not confirmed by radiological examination. We have studied this problem with test specimens, to ascertain whether identical forms of fracture could be obtained by applying forces of defined magnitude and direction. The question was answered in the negative [1]. This led to the question of agreement between radiological and autopsy findings, a problem which has scarcely been studied [3, 9].

Material and method

Fifteen human cadaver skulls (average age, 72 years) with undisturbed viscera were obtained from the Institute of Anatomy in Rostock. They were fixed in formalin in the usual manner. The two first vertebrae of the neck were left attached to the skull. The experiments

were performed in the Material Testing Laboratory of the Shipbuilding Faculty of Wilhelm Pieck University, Rostock, where each skull was subjected to static loading until it had fractured. The actual fracture was indicated in all cases by definite phenomena (sound of fracturing, breakdown of load absorption properties).

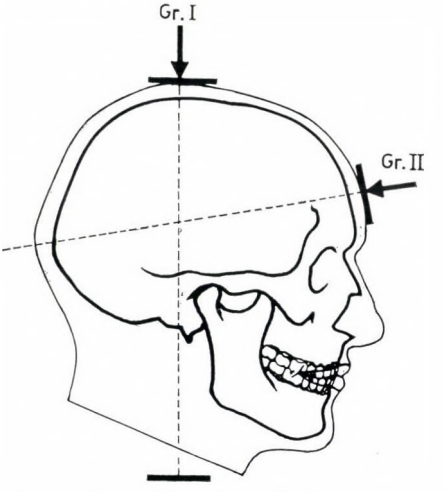

Fig. 1. The forces applied to the cranium. I Force applied to parietal bone towards the external acoustic pore. II Force applied to frontal tuber towards the external occipital protuberantia

The experiments were performed in two groups, the direction of force application being different in each group. Group I[4] compared heads 21 and 23 to 28. In this group the force was applied to the parietal bone and directed in the median sagittal towards the middle of the line connecting the left and right porus acusticus externus [4]. In cases 10 to 17 of Group 2, the load was applied in the median sagittal level of the frontal tubers and directed towards the occipital protuberantia externa [2] (Fig. 1).

The force was applied in all cases through a steel rod measuring $1 \times 1 \times 10$ cm. The longitudinal axis was positioned frontally in half of the cases in each group and sagittally in the remaining cases. The skulls were examined radiologically before and after each loading experiment.

Results

Comparison of fractures determined radiologically and by autopsy, Load application I (cf. Fig. 1).

Load transfer by rod positioned frontally

Skull No.	Roof of skull		Skull base		Front of skull	
	le	ri	le	ri	le	ri
25	+	+	−	−	−	−
27	−	−	+	+	−	+
28	+	+	+	+	+	+

Load transfer by rod positioned sagittally

Skull No.	Roof of skull		Skull base		Front of skull	
	le	ri	le	ri	le	ri
21	−	+	+	+	+	+
23	+	+	−	−	+	+
24	+	+	+	+	+	+
26	+	+	+	−	+	+

Load application II (cf. Fig. 1).

Load transfer by rod positioned frontally

10	+	+	−	−	−	−
11	+	+	−	−	−	−
12	−	+?	+	+	+	+
13	+	+	+	+	+	+

Load transfer by rod positioned sagittally

14	−	−	+	+	−	+
15	−	−	+	+	−	−
16	+	+	−	−	+	+
17	+	−	+	+	+	−

Key to symbols:

+ = fracture − = no fracture

le = left side of head ri = right side of head

Only the crosses in circles denote radiologically determined fractures. The question mark beside the cross for head 12, roof of skull, right, indicates that the fracture was suspected on the basis of the X-ray examination but could not be diagnosed with certainty.

Discussion

Comparison of the diagnoses obtained by radiological examination and autopsy showed that the latter is substantially more accurate.

Fractures situated at the roof of the skull were often detected radiologically whereas none of the fractures at the base or front of the skull were

diagnosed although the cadaver heads posed considerably less difficulty regarding X-ray exposure than live patients as all kinds of positioning could be used.

The fixative scarcely affected radiological transparency. In this respect the experimental conditions were identical to practical conditions.

Nevertheless, some postmortem conditions definitely affected the accuracy of diagnosis. For example, fractures in living subjects cause haemorrhages which are absent in cadavers. The changes of radiological detection increase if the diastasis is enlarged by a haematoma. The accuracy of diagnosis may also depend upon the distance between the edges of a fracture; a diastasis of 1 mm will probably be more easy to detect than a smaller one.

The position of the fracture also effects diagnostic accuracy. As our experiments clearly showed, fractures of the skull roof are considerably easier to detect radiologically than fractures of the front or base of the skull.

Superficial injuries are easier to detect than deeper ones. In our cases, however, radiological conditions at the skull base hardly differed from those at the skull roof with regard to positioning of the skull; the skulls could be placed without restriction for exposure; adjacent parts (trunk) imposed no constraint.

The differences between the accuracy of findings obtained by X-rays and by autopsy were obviously due to morphological criteria, owing mostly to the fact that the three layers of the skull roof together are in most places thicker than at the skull base or front. Pneumatisation may have effected the fragility of skulls 10 and 11, but it could not have played a major role in the results.

If the distance between the fracture edges affects diagnostic accuracy, in our experiments the skull roof fractures produced were wider than the fractures of the skull front and base. Since our samples, with their intact viscera, conformed broadly with practical conditions involving live subjects, it may be assumed, that a sort of servo mechanism is involved which is intended to protect the blood supply to the brain through the arteries at the skull base and to reduce the chances of injury of the brain nerves emerging through the skull base. The broad openings of fractures in the skull roof may be due to tension from the aponeurotic galea (mimicry), but strains in the cranium might be involved to some extent. The latter conjecture has, however, to be checked by further studies on skulls following removal of the viscera. That this conjecture cannot be simply ignored is shown by the fact that broad cracks occurred in our samples possibly because no mimicry could take place. The fact that no fractures were detected radiologically in the front or base of the skull, whereas in the skull roof they were often revealed by X-ray, shows that the fractures in the skull roof were broader.

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BEITRÄGE ZU DER AUTOPTISCHEN UND RÖNTGENOLOGISCHEN DIAGNOSTIK DER SCHÄDELBRÜCHE

E. EHLER, D. IVANKIEVICZ und G. H. SCHUMACHER

15 formalinfixierte Köpfe menschlicher Leichen wurden in sagittaler und transversaler Richtung einer Druckbelastung bis zum Bruch der Schädelknochen unterworfen. Die Ergebnisse der Experimente wurden an beiden Schädelhälften bewertet und so insgesamt die Angaben von 30 Schädelhälften registriert. Die mittels Autopsie bzw. Röntgenaufnahmen festgestellten Frakturen zeigt folgende Zusammenstellung:

Diagnose	Schädeldach	Schädelbasis	Gesichtsschädel
a) mittels Autopsie	22	19	19
b) röntgenologische	10	0	0
c) in % von *a)*	45,5	0	0

Am lebenden Menschen sind die röntgenologischen Bedingungen bedeutend ungünstiger als an Leichenköpfen. Aufgrund der Untersuchungsergebnisse der 15 Leichenköpfe sind die Verfasser der Ansicht, daß die obigen Relationen auch im Falle eines großen Untersuchungsmaterials keine wesentliche Abweichung aufweisen würden.

ДАННЫЕ К АУТОПТИЧЕСКОЙ И РЕНТГЕНОЛОГИЧЕСКОЙ ДИАГНОСТИКЕ ПЕРЕЛОМОВ ЧЕРЕПА

Э. ЭЛЕР, Д. ИВАНКИЕВИЧ и Г. Х. ШУМАХЕР

На 15 черепах человеческих трупов, фиксированных формалином, авторы проводили нагрузку давлением в сагиттальном и трансверсальном направлениях, вплоть до перелома черепов. Результаты были исследованы на обеих половинах черепов и таким образом удалось зарегистрировать данные всего 30 половин черепов. При вскрытии и при рентгеновском исследовании черепов были обнаружены следующие виды переломов:

Диагноз	Черепной свод	Основание черепа	Лицевой череп
а) При вскрытии	22	19	19
б) При рентгеновском исследовании	10	0	0
в) В процентах «а»	45,5	0	0

При жизни условия рентгенографии значительно более трудные чем на удаленных черепах трупов. На основе данных 15 черепов трупов авторы придерживаются того мнения, что при исследовании более большого материала вышеприведенное соотношение не изменилось бы в значительной мере.

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NEEDLE-BIOPSY CYTOLOGY IN THE PREOPERATIVE DIAGNOSTICS OF BREAST DISEASES

I. DEGRELL

(Received July 14, 1979)

The author has performed 1000 breast needle-biopsies during a period of 20 years. Based on his own experiences and on the data of the literature the method is evaluated as a useful, informative and complementary tool in the preoperative diagnosis of breast diseases.

The method is reliable, quick, painless and performable on out-patients, it causes no dissipation of the tumour, therefore, it is recommended as a routine diagnostic method.

Introduction

In the preoperative diagnostics of breast diseases conventional procedures such as mammography, galactography, pneumocystography, thermography and ultrasonic examination have now been complemented with aspiration needle-biopsy. In this field four institutions have earned an international reputation, the New York Memorial Hospital, the Herzen Oncology Institute in Moscow, the Curie Foundation in Paris and the Radiumhemmet in Stockholm. The procedure is rapidly gaining routine application in several other institutions. The works of several researchers [1, 2, 3, 11—19, 22—24], were pioneering studies in the field. An important recent development was the introduction of stereotaxic needle-biopsy by NORDERSTRÖM and ZAJICEK [2] which proved a valuable tool in the diagnostic of non-palpable changes. Experience with ANB has been reported also by BODÓ et al. [4, 5] and DEGRELL [6—10].

Method

A Cameco punction device was used, equipped with a disposable plastic syringe and a 0.6 mm needle. After fixing the punction area by hand, aspiration biopsy is done. The sample is spread on a slide glass, fixed for 30 min in ether-alcohol and stained according to Papanicolaou or with haematoxylin-eosin. Some samples were dried and stained unfixed with May-Grünwald-Giemsa (air-dried smears).

Results

An indispensable guide for cytological evaluation is the WHO proposal for classification of proliferative processes and tumours of the breast [21]:

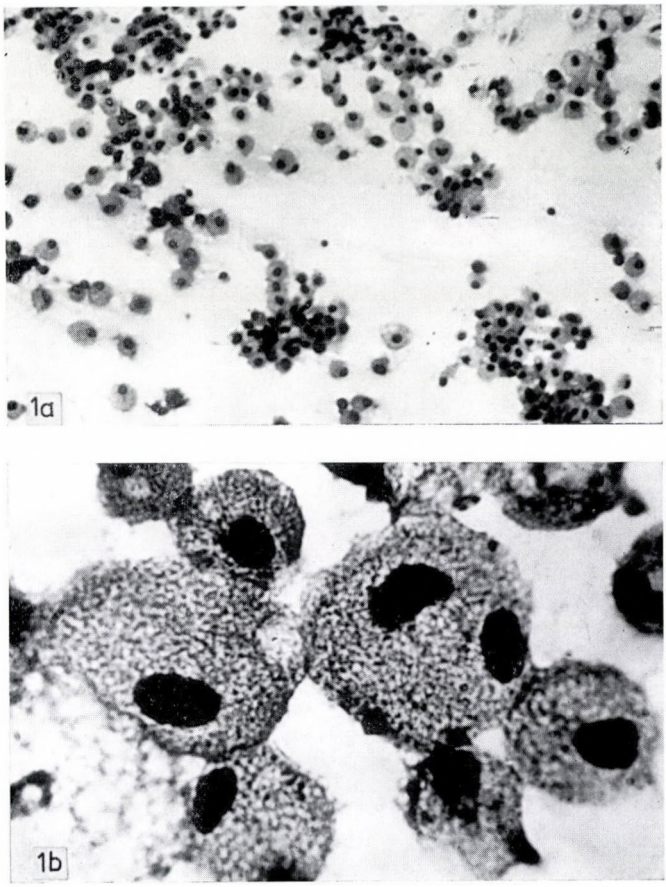

Fig. 1. a. Foam-cells, × 280; b. Foam-cells, × 320

A. Dysplasia (I. cyst — simple or papillary, II. adenosis, III. epithelial proliferation in ducts and lobules, IV. dilatation of ducts, V. fibrosclerosis, VI. gynaecomastia, VII. other non-tumorous proliferative changes.

B. *Non-malignant tumors.* I. adenoma, II. mammillary adenoma, III. ductal papilloma, IV. fibroadenoma, cystosarcoma phylloides (giant fibroadenoma of Brodie).

C. *Cancer.* I. Intraductal and intralobular non-infiltrative cancer, II. infiltrative cancer, III. cancer with typical histology: medullary, cribriform, gelatinous, lobular, planocellular, mammillary Paget disease, intracanalicular fibroadenomatous cancer.

D. *Sarcoma*

E. *Carcino-sarcoma*

F. *Tumours difficult to classify*

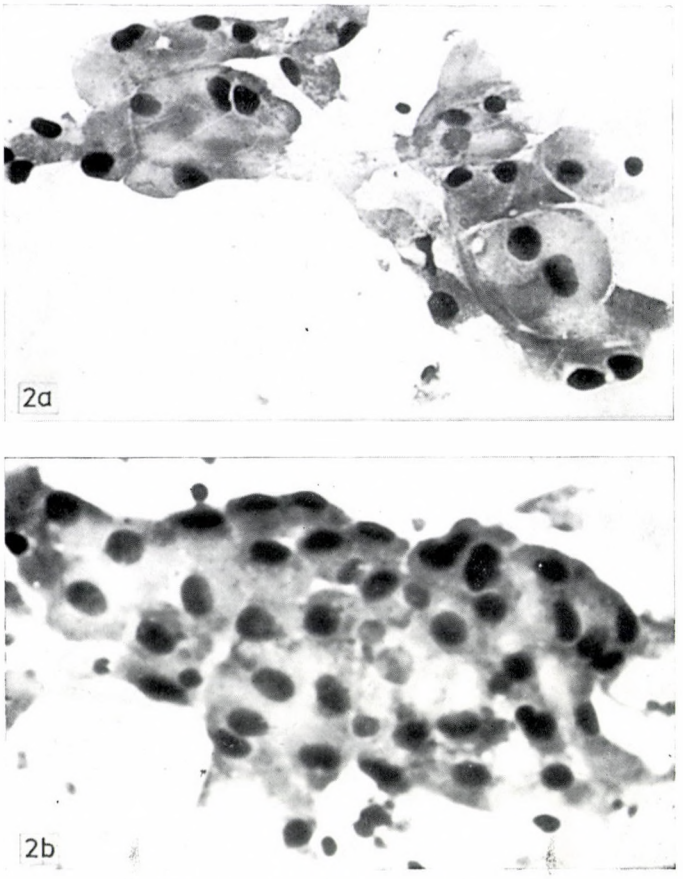

Fig. 2. a, b. Oncocytic apocrine cells, × 320

In the following, cell types found in non-malignant and malignant breast changes will be dealt with.

A. *Cells occurring in dysplasia*

1. *Foam cells:* they occur either in clusters or diffusely. They vary in size, possess a finely vacuolated cytoplasm, and not infrequently double nuclei. The nucleus is excentrically located with finely granular chromatin substance (Fig. 1a, b). Foam cells occur in addition in chronic mastitis and some other tumours.

2. *Apocrine epithelial metaplasia.* Apocrine oncocytes are found in clusters with eosinophilic cytoplasm around the centrally located nucleus. The chromatin is dense (Fig. 2a, b).

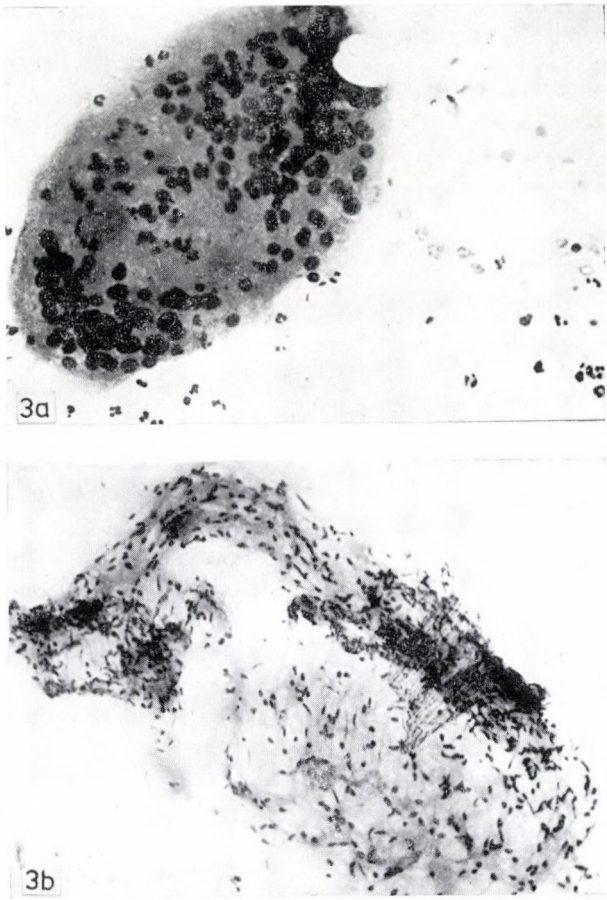

Fig. 3. a. Giant cell, × 25; b. Cluster of fat cells and fibrocytes with some epithelial cells, × 25

3. *Giant cells.* Large, round or ovoid, multinuclear cells occurring in groups. They can also be found in chronic mastitis, granulation tissue and tumours (Fig. 3a).

4. *Clusters of fat-cells and fibrocytes.* They are consistently present in the smears. The clusters often contain epithelial cells (Fig. 3b).

5. Usual constituents of the smears are erythrocytes, granulocytes, lymphocytes, monocytes, histiocytes, amorphous cell debris.

6. Normal lobular or ductal epithelial cell.

B. *Cytology of non-malignant tumours*

1. *In fibroadenoma,* the cell nuclei are normal, round or oval with finely granulated chromatin. Cells occur in groups (Fig. 4a). The cytoplasm is a nar-

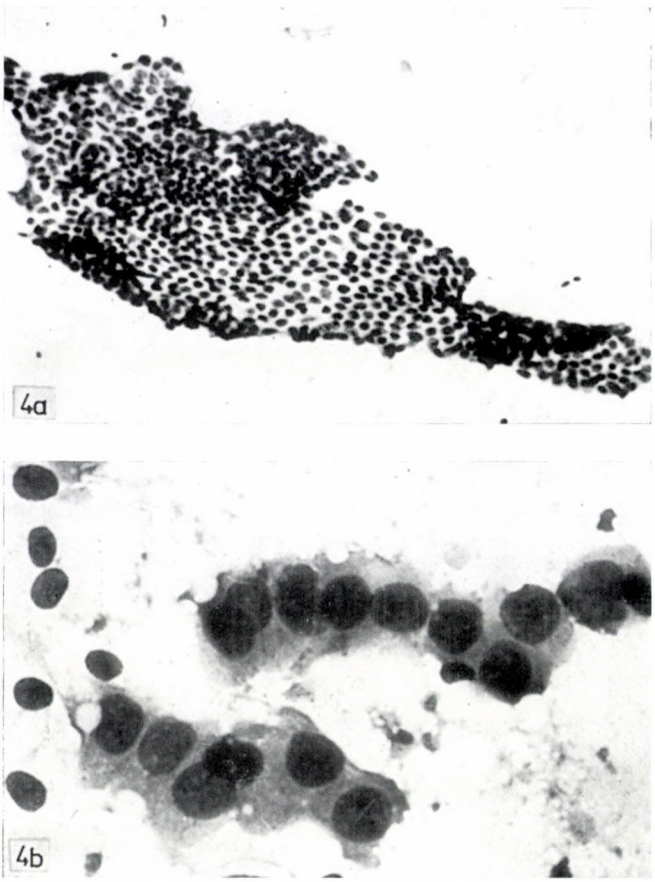

Fig. 4. a. A group of round and ovoid cell nuclei in a fibroadenoma. b. Two non-malignant cell bundles in a fibroadenoma. On the left, scattered naked nuclei. × 400

row rim around the nucleus. The "naked nuclei" of myothelial origin may also be present; they are round or ovoid and, as seen in Fig. 4, located outside the groups of epithelial cells.

2. *Papilloma.* The cytology is similar to that of fibroadenoma, but the nuclei are frequently elongated (Fig. 5a) and the cells show some degenerative signs: pyknosis of nuclei, cytoplasmic vacuoles, ring formations (Figs 5b, c).

C. *Cytology of malignant tumours*

Malignant cells found in these cases vary in nature according to the type of the tumour and can be classified as outlined above; the most widely accepted classification is that of ZAJICEK et al. [25].

1. *Duct-cell type cancer.* This is the most common type of cancer cell (80%). It may occur in clusters or diffusely, it is monomorphic and small,

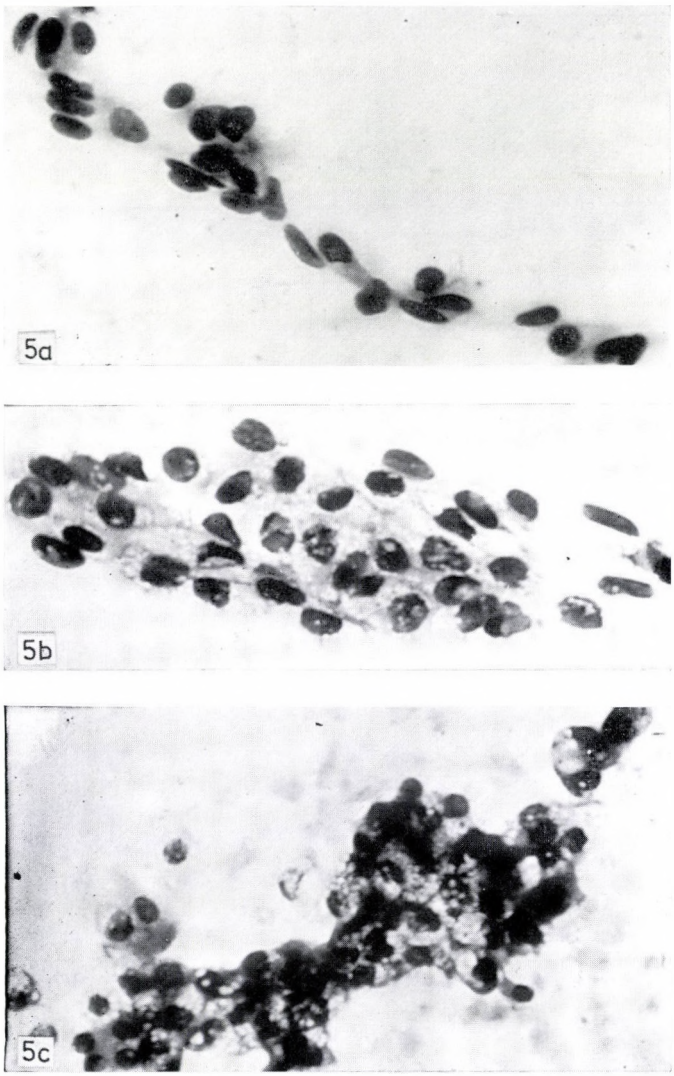

Fig. 5. a. Papilloma. Chain-like round or ovoid nuclei, × 320. b. Papilloma. Pyknotic nuclei in clusters, × 320. c. Papilloma. A group of pyknotic, vacuolated epithelial cell, × 320

medium and large in size. Few mitoses are found (Fig. 6a). Histologically, they may originate from tubular, ductal and lobular cancers.

2. *Acinic-cell cancer.* This form is less frequent (9%). The cytoplasm is finely granular, the nuclei contain several nucleoli (Fig. 6b). Isolated nuclei also occur. Histologically these cells represent medullary, infiltrative ductal cancer.

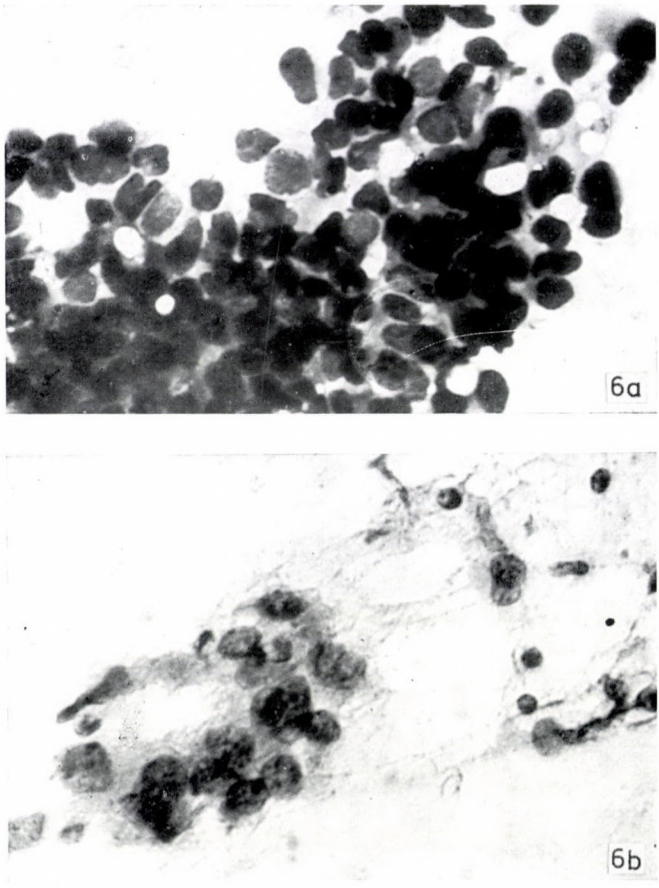

Fig. 6. a. Duct cell type carcinoma, × 320. b. Acinic cell carcinoma, × 320

3. *Apocrine-cell cancer* (6%). According to their stage of differentiation, these cells are either poly- or monomorphic with granular cytoplasm. Their nuclei contain several nucleoli (Fig. 7a). Histologically they may be comedo or ductal carcinoma.

4. *Mucous carcinoma* (colloid, gelatinous, 3%). These cells form clusters surrounded by a mucous substance (Fig. 7b). A rarely encountered form is the mucous-cell carcinoma (signet ring carcinoma) characterized by signet-ring cells (Fig. 7c).

The rest is constituted by *papillary carcinoma* cells (Fig. 8a) with elongated nuclei arranged in palisade form, and by *Paget-cancer* cells of the mammilla (Fig. 8b).

It often happens that needle-biopsy is carried out for other purposes and a sarcoma or mesenchymal malignoma is diagnosed by chance. With other

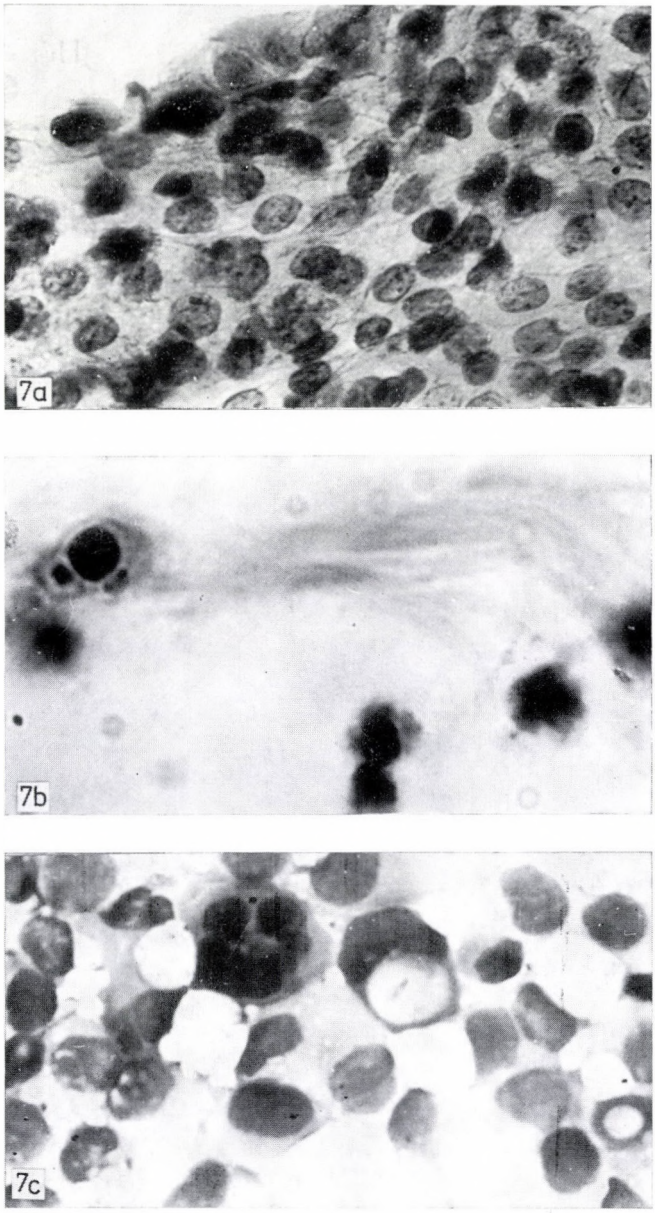

Fig. 7. a. Apocrine cell carcinoma, × 320. b. Mucous, colloid carcinoma, × 400. c. Signet-ring cell carcinoma, × 400

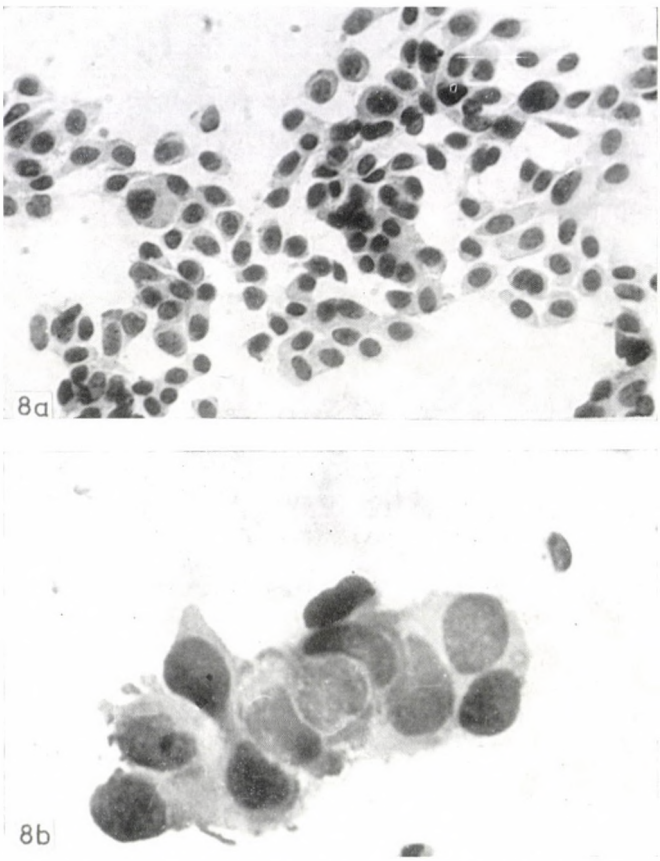

Fig. 8. a. Papillary carcinoma, × 320. b. Paget cells

diagnostic techniques these tumours are easily mistaken for fibroadenomas. In the smears polymorphic, ovoid or spindle-shaped malignant cells occur with densely chromatinous nuclei (Fig. 9a) which can readily be distinguished from carcinoma-cells. One of our cases proved to be a curiosity: in a tumour which appeared to be a fibroadenoma, malignant cells containing melanin-like pigment were found (Fig. 9b). Histology verified a primary melanoblastoma.

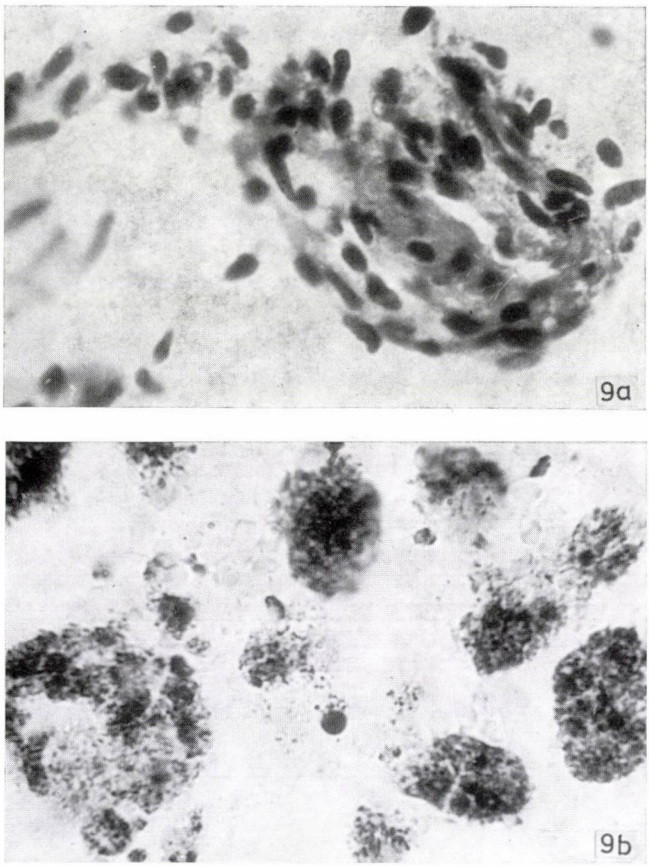

Fig. 9. a. Fibrosarcoma: polymorphic, spindle shaped mesenchymal cells, × 320. b. Melanoblastoma. Malignant cells vary in size and shape and are filled with melanin pigment granules (oil-immersion)

Conclusions

In the past 20 years we have performed needle-biopsy in 1000 cases. These were:

Non-malignant tumours	360
Dysplasia	250
Chronic mastitis, lipogranuloma, galactokele and other non-malignant processes	143
Carcinoma	220
Mesenchymal malignoma	27

The accuracy of diagnosis ranged between 90—95%.

The highest accuracy (96%) was achieved in carcinomas, and slightly lower one in mesenchymal malignomas. The cytological diagnosis was routinely completed with histology.

Correlating the diagnostic value of cytology with radiological and clinical diagnoses [3] it appears that while clinical and radiological diagnoses are of 60 and 90% accuracy, respectively, cytology has always been reliable.

ANB of the breast is a useful tool in preoperative diagnostics especially when employed in combination with other diagnostic techniques. It is reliable, painless and can be performed in out-patients as well. The danger of tumour dissemination is non existent. In conclusion, ANB is recommended as a routine diagnostic method.

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DIE ZYTOLOGISCHE BEDEUTUNG DER FEINNADELBIOPSIE IN DER PREOPERATIVEN DIAGNOSTIK DER MAMMAERKRANKUNGEN

I. DEGRELL

Im Verlauf von 20 Jahren hat der Autor nahezu 1000 Feinnadelbiopsien der Mamma durchgeführt. Aufgrund seiner Erfahrungen und der Literaturangaben stellt er fest, daß dieses Verfahren eine nützliche, aufschlußreiche, ergänzende Methode für die präoperative Diagnostik der Mammaerkrankungen darstellt und mit den übrigen Untersuchungsverfahren (Mammographie, klinische Untersuchungen) eine funktionale Einheit bildet. Die Punktionszytologie ist rasch, schmerzlos, ambulanter durchführbar und ist im Hinblick auf die Tumorstreuung ungefährlich. Ihre routinemäßige Anwendung empfiehlt sich in sämtlichen Anstalten, die über die nötigen personellen und instrumentellen Voraussetzungen verfügen.

ЗНАЧЕНИЕ ПУНКЦИОННОЙ ЦИТОЛОГИИ В ОБЛАСТИ ПРЕДОПЕРАЦИОННОГО ДИАГНОЗА ЗАБОЛЕВАНИЙ ГРУДНОЙ ЖЕЛЕЗЫ

И. ДЕГРЕЛЛ

В течение 20 лет автором было проведено почти 1000 пункционной цитологии грудной железы. На основе своих наблюдений из литературных данных он устанавливает, что это полезный и информационный дополнительный метод для предоперационного диагноза заболеваний грудной железы, который, вместе с другими исследованиями (маммография, клинические иссследования), образует функциональную единицу. Это надежный, быстрый безболезненно проводимый в амбулатории метод, являющийся с точки зрения разноса опухолей безопасным. Повседневное применение пункционной цитологии рекомендуется во всех учреждениях, в которых обеспечены персональные и инструментальные требования.

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AN AIR-DRYING METHOD FOR PREPARING METAPHASE FROM SPERMATOGONIAL CELLS OF CHINESE HAMSTER (CRICETULUS GRISEUS)

SHORT COMMUNICATION

S. PÉTER

(Received May 2, 1979)

Introduction

An analysis of chromosomes of germ cells provides important data for judgement of the mutagenic activity of chemicals. In recent years a variety of techniques have been published for the study of meiotic chromosomes [11, 3, 4, 9, 7], but few papers describe methods for the preparation of spermatogonial metaphases [2, 5]. These methods have several disadvantages. The squash-technique employed for the preparation of spermatogonial chromosomes [2, 11] does not yield enough metaphases for examination. The phase-contrast method employed for analysis by BROKS et al. [2], also has disadvantages regarding the presence of spermatogonic-wave in the semiferous tubules [5]. Evans' air-drying method [4] produces a large number of analyzable meiotic cell chromosomes, eliminating the disadvantages of the mentioned technique; nevertheless the method is only suited for the analysis of diplotene, diakinesis, metaphase I—II, and only few mitoses of spermatogonia can be found. More recently, MEREDITH [7], HOO and BOWLES [5] published an air-drying method for preparing meiotic and mitotic metaphases using 60% acetic acid and a hot plate. We have achieved little success with this and other air-drying methods preparing Chinese hamster spermatogonium chromosomes. We have therefore worked out a simple and quick method which provides a sufficient amount of well-spread metaphase chromosomes of spermatogonium cells with good morphology, a method free from the shortcomings of those mentioned above.

Method

Metaphase chromosomes from the spermatogonial cell of sexually mature, 8-week-old Chinese hamster can be obtained in the following manner.

1. Animals are sacrified 5 hours after the administration of 8 mg/kg of colchicine by the intraperitoneal route.

2. One testicle is carefully prepared and placed in a glass at 37 °C temperature.
3. The tunica albuginea is incised and removed. The tubules are minced by scissors.
4. 1 ml 0.8% sodium citrate solution at 37 °C is added to the minced tubules.
5. The material is than transferred into a 10 ml tube and 9 ml 0.8% sodium citrate solution at 37 °C is added.
6. After 5 min sedimentation the supernatant is carefully removed and the sediment is resuspended in 10 ml fresh 0.8% sodium citrate at 37 °C.
7. After 4.5 min the tube is centrifuged at 1000 r.p.m. for 0.5 min and the supernatant is removed and discarded.
8. The cells are resuspended and fresh fixative, 3 parts of ethyl alcohol or methyl alcohol to 1 part of glacial acetic acid is added drop by drop flicking the tube after each drop. The total volume of fixative is approximately 10 ml.
9. The tube is stored for 1 h at 4 °C, then centrifuged at 1000 r.p.m. the supernatant carefully removed and the sediment resuspended and fixed again in 10 ml fixative.
10. The tube is stored overnight at 4 °C. On the following day it is centrifuged, the supernatant is removed, and the cells are resuspended by flicking the tube with the forefinger so that tubule segments adhere in 3—5 cm height to the wall of the tube.
11. 0.5 ml of suspension is taken up into a small pipette and from a height of 0.5—1 m it is dropped on grease-free, wet ice cold slides. The slides are dried, stained with 5% Giemsa solution pH 7.0—7.2 for 10 min, and subsequently washed in distilled water dried and covered using Eukitt or DPX and a coverslip.

Discussion

Examination of the slides showed a great number of spermatogonial metaphases. The tubules and the spermatogonia sediment in the hypotonic solution, while the spermatocytes and spermatides remain in the supernatant which can thus be removed. One metaphase of spermatogonium and its quality is illustrated in Fig. 1. In recent years several papers have pointed out the importance and indispensability of analyzing spermatogonial metaphases, underlining their sensitivity [6, 8] and demonstrating the positive mutagenic effect detectable in them as opposed to the negative results obtained in sperma-

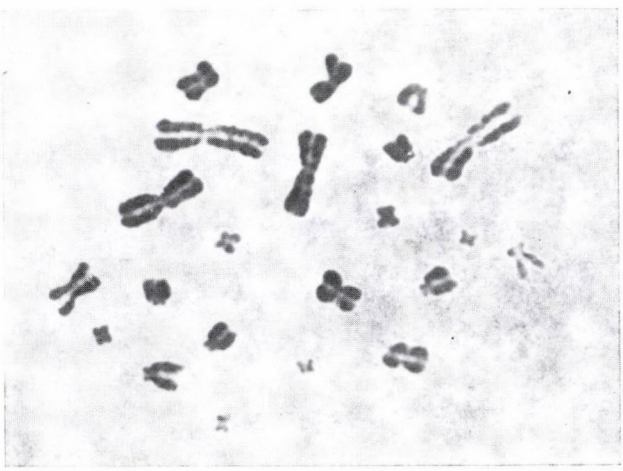

Fig. 1. 2n = 22XY normal chromosomes of Chinese hamster (Cricetulus griseus) spermatogonial cell

tocytes [1, 10]. The great advantage of the described method is that it allows to analyze a large number of spermatogonial metaphases. The method therefore seems to be reliable for the qualitative and quantitative evaluation of the genetic risk involved by the use of chemical mutagens.

Acknowledgement

I am indebted to Prof. Dr. G. Röhrborn (Düsseldorf, W-Germany) for a critical reading of the manuscript.

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RECENSIONES

I. Gy. Fazekas and F. Kósa: *Forensic Fetal Osteology*. Akadémiai Kiadó, Budapest 1970. 414 pages with 62 figures, 207 tables and 89 diagrams

The aim of forensic examination of fetal bones is to determine its human origin, the time of death and, in particular, the age of the fetus. In selected cases the forensic expert is also expected to give an indication of the possible reason of abortion, i.e. the death of the fetus. The authors attempted to answer these questions by studying 138 fetal skeletons from the museum of the Forensic Institute of Szeged Medical School. Their book is the most detailed account of the problem found in the international literature. It summarizes current knowledge and offers new information.

After listing the questions asked in such cases by the authorities from the forensic expert, the material, the methods and the measures of the skeletons are described. These data are valuable in forensic practice and the bone sizes are shown by photographs and drawings, the measuring points are indicated. The methods of biometric analysis are dealt with and a series of standard skeletal parameters are defined. Skeletons from the 3rd months of pregnancy are shown in 1/2-monthly intervals. Unfortunately, no exact age determination was possible and therefore a ± 14 days inaccuracy has to be reckoned with. Chapter 7 deals with the development of individual bones, giving exact sizes for each age group. Regression curves enable to determine the age and length from one single bone. The value of the data presented for assessing the age of the fetus is without any doubt higher than that of any previous method. The authors also discuss the possibility of blood-group determination from fetal bones and the problem of studying combusted bones. Finally, sex determination is described based on the study of the fetal pelvis. The list of references may satisfy any special demand. The illustrations and diagrams are of good quality. The book is recommended for forensic experts as well as for anatomists and embryologists.

L. Harsányi

British Medical Bulletin. The British Council, London. Volume 15. No. 2. The issue contains 16 reports on reproduction of the fields of developmental biology and experimental embryology.

The studies are focused on two main problems. The first concerns the results and further prospects of the experiments in Britain with human "flask-babies". In 1978 a human ovocyte was to fertilized *in vitro* and reimplanted into the mother. After an undisturbed pregnancy a healthy baby was born. The other main subject of studies is the overpopulation of some areas of the world against which in various countries birth control measures have been introduced since 1959. Several problems related to birth control are still unresolved.

Although all the papers deal with birth problems, in the first seven papers the first, in the following nine, the second topic is considered more emphatically. The paper of D. G. Wittingham "*In vitro fertilization embryo transfer and storage*" describes the pretreatment of germinal cells and the process of *in vitro* fertilization. It deals with the alternative possibilities of embryo transfer to the tuba or uterus of the mother or of its storage. To this question the paper of Moor and Warnes "*Regulation of meiosis in mammalian oocytes*" provides data on the energy sources of the maturing oocyte, its metabolic processes and membrane alterations. It summarizes factors influencing the maturation and intrafollicular inhibitory effects of granulosa cells. The maturation of a human oocyte and follicle and the role of steroids was

studied six hours after LH release, oocytes removed from preovulatory follicles can be transferred to the tuba of a recipient where they develop normally. The work of ADAMSON and GERDNER, "*Control of early development*", deals with the maternal genome effect on early development after the two-cell stage through mRNS. The most significant change in protein synthesis occurs in the one- and two-cell stages. Functioning mRNS template transcribed by the genome of the embryo appears at the 6—8-cell stage. In the 8-cell stage, surface antigens are determined by the paternal genome. These observations were made in mice and rabbits.

R. V. SHORT considers the role of the H-Y antigen in differentiation and sex determination, with special reference to intersex development in animals and in man. The author is rather sceptical about the success of attempts to influence primary sex but he sees some advance in the research of neural control of sex determination.

The embryo is emitting pregnancy signals as early as the blastocysts stage. This seems to be mediated by the corpus luteum although the duration of progesterone release varies in species. It is still argued whether the signal is evoked by the changes of the endometrium due to implantation. As the endometrial environment affects embryonic growth, description of the biochemical changes accompanying pregnancy is of interest (HEAP et al.: *Role of embryonic signals in the establishment of pregnancy)*. Proteins of the uterine environment influence the growth of the cells of the embryo. Most pregnancy abnormalities develop during implantation and they are mostly due to genetic defects of the male. In domestic animals the maternal factor seems to be more important as it prevents embryonic signals in the preimplantation period.

Research of the past 20 years has corroborated that abnormalities of the fetus result from disturbed intrauterine growth (ROBINSON: *Growth of the fetus)* caused by undernutrition of the mother, hypertension, preeclampsia, smoking, alcoholism, drug addition, etc. Usefulness of the ultrasonic determination of the embryos size is emphasized. As shown by experiments, retardation in fetal growth can be caused by changes in oxygen *tension, nutrition and hormones.* Further plasma tests are still required to the exact assessment of the degree of retardation; a prerequisite to induce normal growth. The author warns of hazardous growth-inducing manipulations (intrauterine feeding, maternal infusion, addition of supposedly deficient substances).

In most mammals the fetal adrenals play a role in starting delivery and the chorion too is thought to have a similar effect. In human pregnancy the fetal adrenals seem to be less important (LIGGINS: *Initiation of parturition)*. Parturition is probably induced by prostaglandin secreted by uterine epithelial cells. Oestrogen stimulates the synthesis of prostaglandin F20. Changes in progesterone levels must be expected at a cellular level.

Some interesting papers deal with gonadotropin secretion. One of them (FINK: *Neuroendocrine control of gonadotropin secretion)* considers the role of hypothalamic releasing hormones and a supposed feed-back mechanism between monoaminergic neurons, releasing hormones and inhibin, a non-steroid substance. The centre of this control system is a cellular mechanism in which steroid effects are manifested on genomes, neurons and on the synthesis and secretion of gonadotropins. The dissociation of LH and FSH is also dealt with. The author is committed to the single releasing-hormone theory and describes how steroids affect the quality of gonadotropin synthesis by the anterior pituitary. Remarkable findings are presented by HENDERSON *(Gonadotrophic regulation of ovarian activity)* concerning a problem difficult to study in humans.

According to LINCOLN *(Pituitary control of testicular activity)* LH and FSH are the primary factors in testicular control, prolactin is only secondary. The two gonadotropins from a complex (LH-FSH) produced by the same anterior pituitary cells under the control of gonadotropin releasing hormone. This latter is produced by neurons of the median eminence. Gonadotropin acts directly on the somatic cells of the testicle while LH stimulates the Leydig-cells, FSH the Sertoli-cells. This is the environment for germinal cell maturation. Testosterone is a product of the Leydig-cells and has an important role in the formation of testicular parenchyma. Testosterone and other testicular substances are parts of a feed-back system involving the hypothalamus, hypophysis and LH-FSH.

Theoretically the contraceptive effect of prostaglandins can be explained in various ways. The problem is dealt with in KARIN's paper: *Prostaglandin in the control of animal and human reproduction.* The transport of spermia, alterations in the motility of the tuba and uterus, altered penetration of spermia through the cervical canal, interference with steroid hormone synthesis and luteolytic effect are the possible alternatives. The luteolytic effect has not been confirmed in man. In his paper, "*Effects of lactation on fertility*", MCNEILLY searches for the causes of the absence of menstruation and fertilization during the period of lactation; and it is suggested that lactation has a natural contraceptive role.

"*The chromosomal basis of human fertility*" by A. C. CHANDLE surveys abnormalities of gametogenesis (translocation, meiosis mutation) as the causes of infertility. It is concluded that a wide range of chromosomal abnormalities are found in animals and in man. Sex-chromosomal aneuploidy and gametogenic defect which occur jointly in males may cause secondary sterility, i.e. the genetically deficient fetus is aborted.

Two papers are dealing with the hormonal background of male sterility (LINCOLN: *Pituitary control of testicular activity*, and DE KRETSER: *Endocrinology of male infertility):* they concentrate on the interaction of hormones affecting Leydig- and Sertoli-cell functions. Leydig-cells contain oestradiol-sensitive receptors whereas the Sertoli-cells transform testosterone to oestradiol. It is assumed that the damage of spermatogenesis by the inhibin produced by the Sertoli-cells causes a rise in FSH. This stimulates the transformation of testosterone to oestradiol by Sertoli-cells which, in turn, decreases testosterone-production by Leydig-cells. Thus, the testicle should be regarded as a functional unit responding sensitively to any impairment of spermatogenesis.

Causes of female sterility are surveyed in "*Endocrinology of female infertility*" by D. T. BAERD. It is pointed out that female infertility can be treated successfully. Hypothalamic, hypophyseal and ovarian tests provide a basis of classification of menstruation disturbances. Thus, adequate therapy can readily be chosen and pregnancy can be achieved without any hazard.

R. J. AITKEN writes about experiments with contraceptives (*"Contraceptive research and development"*). As to future prospects, he stresses the need of continued financial support of contraception-research as only long-term experiments can make contraception socially acceptable and help to find the contraceptive most tolerable to women.

I. TÖRŐ

INDEX

Morphologica Normalis et Experimentalis

Pathologia

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POST-CONGRESS MEETING

Budapest, September 21—24, 1980

INTERNATIONAL ACADEMY OF PATHOLOGY

GENERAL INFORMATION

The Post-Congress Meeting will be held in Budapest (Hungary), 21—24 September, 1980, as an extension meeting to the XIII. IAP Congress, Paris.

Location of the Meeting: Cultural Center (Budapest, V., Zrínyi u. 5.)

Social programme: Starts with a sightseeing tour on the 21st September (Sunday) and a Reception will be offered on the eve of 21st September. Detailed information will be given in the Second Announcement.

Language: English will be the working language of the Meeting.

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Exhibition: There will be a display of pharmaceutical products, medical instruments and of scientific books at the site of the Meeting. Advertisements can be inserted in the Programme. For information please contact the MH-Advertising Service (H-1093 Budapest, Közraktár u. 6—8.) or the MOTESZ Congress Bureau (H-1361 Budapest, POB 32).

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A MAGYAR TUDOMÁNYOS AKADÉMIA ORVOSTUDOMÁNYI KÖZLEMÉNYEI

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Professor Imre Törő

It is a special privilege for the Editorial Board to greet Imre Törő, member of the Hungarian Academy of Sciences, emeritus professor, Kossuth Award laureate on occasion of his 80th birthday.

Professor Törő is one of the founders of Acta Morphologica Acad. Sci. Hung. and served for many years as its Editor-in-Chief with undiminishing enthusiasm, deep wisdom and executive skill. He is still an active member (and Chairman) of the Editorial Board. One of the highly respected members of the international scientific community of cell and tissue research Professor Törő recognized the significance of the thymo-lymphatic system decades before the full impact of this field upon biomedical sciences was understood, this made him one of the early pioneers of this important territory. — Professor Törő had an exceptionally clear foresight for the future significance of new research techniques, so that it was the Department under his guidance that played a pioneering role in the introduction of electron microscopy, tissue culturing, histochemistry, autoradiography in Hungary. Generations of medical students have studied histology and embryology from his textbooks. It was due to his influence that medical biology and cytology was established as a discipline in its own right in the medical curriculum.

The members of the Editorial Board greet and congratulate Professor Törő on this occasion, wishing him further success and good health for many years to come.

Acta Morphologica Aacd. Sci. Hung. 28 (4), pp. 317—335, 1980

Department of Biology, Semmeilweis University of Medicine, Budapest

EFFECT OF GONADOTROPINS AND THYROTROPIN ON THE TESTES AND OVARIES OF THE NEWLY HATCHED CHICKEN

M. A. Shahin, G. Csaba and O. Dobozy

(Received Septembre 3, 1979)

In newly hatched male and female chickens thyrotropin (TSH) and gonadotropins (FSH, LH) were found to increase the number of spermatogonia or Sertoli cells in the seminiferous cord, and the number of granulosa cells in the ovarian follicle. The effect of gonadotropins was more pronounced than that of TSH. Regarding the overlapping effect of thyrotropin and gonadotropins, in both male and female neonatal gonads the hormones similar in structure seem to be able to substitute each other. They do not however, change each other's effect on the parenchymatous tissue (number of spermatogonia and Sertoli cells in the male, and number of follicles in the female) if they are administered one after the other.

Introduction

It has been shown in mammals that gonadotropins, as well as other peptide hormones exert their action by binding to specific receptors on the membrane of the target cells in the gonads [7, 25, 26].

Membrane receptors undergo a maturation process, thus the hormone receptors have a low binding capacity in embryonic and neonatal age, reaching their total maturity in several weeks. The receptors appear to be influenced at the time of their maturation, and their sensitivity can be reduced or increased permanently [4, 37].

Gonadotropins and TSH molecules have the same α-subunit, in their chemical structure, but differ in their β-subunit, on which their specificity depends [8, 33, 41]. In previous experiments it was found that the difference in β-subunits is not so distinct that in the neonatal age the receptors could distinguish between the two hormones, and their distinguishing capacity is not yet developed [9, 10, 11]. Both hormones have a clear effect on the weight of the testes as well as on the diameter and structure of the seminiferous tubule of newly hatched chicks [12, 13, 16]. In a previous study we found that TSH and gonadotropins affected the gonads (testis and ovary) of newly hatched chickens. Gonadotropins act mainly on the parenchymatous tissue, and thyrotropin acts on the interstitium, but the effect of the latter on the parenchymatous tissue cannot be neglected either. Moreover, it was postulated that gonadotropins increased the effect of thyrotropin on the interstitium [14].

The present study was undertaken to investigate [1] the effect of gonadotropins and thyrotropin on the histological structure of the testes and ovaries of newly hatched chicks; [2] the overlapping effect of TSH and gonadotropins when administered one after the other, on the testes and ovaries of the newly hatched chicks.

Materials and methods

Newly hatched chickens of both sexes, of the Hubbard-broyler strain were fed a commercial chick food and water.

The hormones used were, human gonadotropins; 50% FSH and 50% LH (Pergonal, Budapest, lic. Sereno-Roma) and thyrotropin (Ambinon, NV. Organon, Oss, Holland). The hormone fractions were dissolved in saline and injected subcutaneously. Each experimental chick received 0.2 ml of the solution (40 or 20 μg) at one time and each control chick received hormone free saline. The first injection was performed at 6:00 p.m. on the day after hatch (two days old chicks). Subsequent injections were performed at 12-hr intervals. Details of the experimental groups were as follows.

Group I. control chickens received subcutaneous injections of saline two times daily for 4 days. Group II chickens received subcutaneous injections of thyrotropin (TSH) two times daily for 4 days (40 μg on the first day and 20 μg on the other 3 days). Group III chickens received subcutanous injections of gonadotropins (FSH+LH) two times daily for 4 days (40 μg on the first day and 20 μg on the other 3 days). Group IV chickens received subcutaneous injections of 40 μg thyrotropin (TSH) twice on the first day, and 20 μg of gonadotropins (FSH+LH) twice daily on the next 3 days. Group V chickens received subcutaneous injec-

Table I

Mean number of spermatogonia and Sertoli cells in the seminiferous cords of newly

Group	Treatment	Dose μg	No. of chickens	No. of identified cells in all chicks (in ten cords)
I	Control	—	11	1881
II	TSH	2×40 6×20	12	2624
III	Gonadotropins	2×40 6×20	11	4387
IV	TSH + Gonadotropins	2×40 6×20	11	2821
V	Gonadotropins + TSH	2×40 6×20	12	3355

(All results compared with control are significant ($p < 0.001$). All results in the experimen-

*No. of Spermatogonia/No. of Sertoli cells

tions of 40 μg gonadotropins (FSH+LH) twice on the first day, and 20 μg thyrotropin TSH twice daily on the other 3 days.

Histomorphometry

Chicks were sacrificed 12-hr. after the last injection (6 day-old chickens). The left testes and ovaries were processed histologically by Bouin fixation, paraffin embedding (section thickness 6 μm) and haematoxylin-eosin staining.

The selected parameter in the testis was the number of Sertoli cells and spermatogonia. The number of Sertoli cells and spermatogonia were counted in 10 cords in every chick, and the mean number of both cells in all chickens in every group was calculated. Only cords absolutely round in cross-section are suitable for such counting. A median section was chosen after counting serial sections of the left testis of each individual [23]. The number of each cell type, compared to the whole number of cells, the percentage of each type, the mean total cell number in each group, the mean number of whole cells and the mean percentage of various cell types, were calculated.

In the ovaries, the average number of granulosa cells was counted in 10 follicles of the 10th section in every specimen and the mean number in all animals was calculated. Analysis of variance (Student's t test) was used to evaluate the difference in the different cell types in the testis, and the granulosa cells in the ovary.

Results

Male chickens

Histological observations. Examination of the testis in the control (Fig. 1) and the experimental groups (Figs 2—6), showed it to consist of seminiferous

hatched cockerels treated with TSH and gonadotropins (FSH + LH) (Mean ± SD)

Number and per cent of cells in ten cords						SPG/SC*
Spermatogonia + Sertoli cells		Spermatogonia		Sertoli cells		
average No. of cells in ten cords	per cent	average No. of cells in ten cords	per cent	average No. of cells in ten cords	per cent	
171 ±2.32	100	49.18 ±1.33	28.76	121.81 ±2.08	71.24	0.40
218.6 ±1.49	100	52.08 ±0.79	23.81	166.58 ±1.08	76.18	0.31
398.8 ±1.25	100	68.82 ±0.75	17.25	330	82.74	0.21
256.5 ±1.21	100	55.18 ±1.07	21.52	201.27 ±0.65	78.48	0.27
279.6 ±2.15	100	58.25 ±1.5	20.83	221.33 ±0.98	79.17	0.26

tal groups compared to each other are significant ($p < 0.001$ or $0.02 > p > 0.01$).)

Table II

Mean number of granulosa cells in one follicle of newly hatched female chickens treated with TSH and gonadotropins

(Mean ± SD)

Group	No. of female chickens	Treatment	Dose μg/chick	Total dose μg/chick	Mean No. of granulosa cells in one follicle
I	10	Control	—	—	11.9 ±0.77
II	10	TSH	2×40 6×20	200	14.2** ±0.63
III	10	Gonadotropins	2×40 6×20	200	21.3* ±0.72
IV	10	TSH + Gonadotropins	2×40 6×20	80 TSH 120	14.2 ±0.99
V	10	Gonadotropins + TSH	2×40 6×20	80 120 TSH	14.9** ±0.48

* Significant as compared to all groups ($p < 0.001$)

** Significant as compared to control ($0.05 > p > 0.02$)

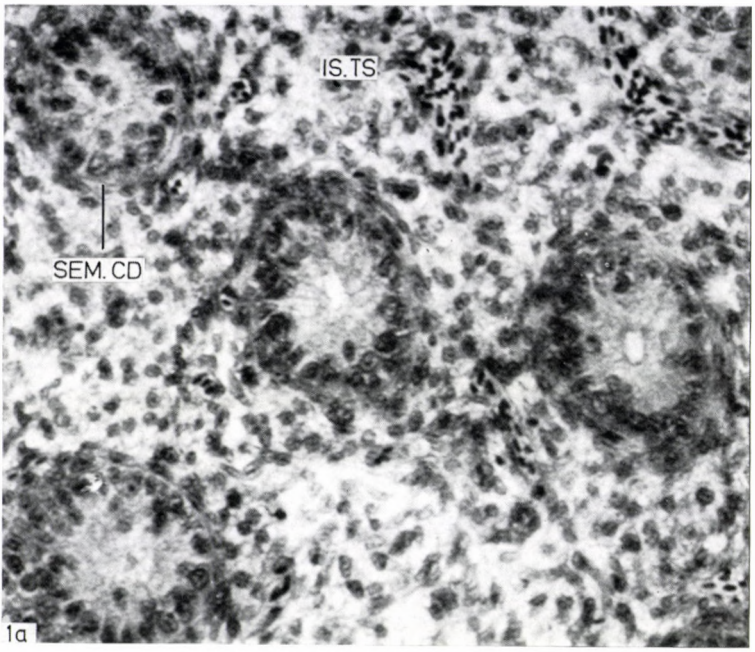
cords and interstitial tissue. The seminiferous cords consist of two cellular components, the Sertoli cells and the spermatogonia. The Sertoli cells are more numerous and appear as epithelial cells possessing round or oval, darkly stained nuclei, which lie near the basement membrane and their cytoplasmic strands extend to the centre of the cord. They appear syncitial in nature, since their cell boundaries are indistinct. The spermatogonia are less numerous. They are of moderate size and have large round eccenteric nuclei with darkly stained nucleoli. They are located more internally in the cord.

In group II was noticed the largest testicle and the largest amount of interstitial tissue. This is followed by the group V, III and IV.

The largest seminiferous cord size was in group III than in the groups V, IV and II (Figs 2 and 3).

In groups III and IV the Sertoli cells appear as high columnar epithelial cells with ramified apical ends. These changes were most marked in group III (Fig. 4). In groups II and V no structural changes were noticed in the Sertoli cells (Fig. 5). Mitotic figures were frequently observed in the seminiferous cords of groups III and IV, while they were infrequent in the control group or in the groups II and V.

The spermatogonia show more proliferations in group III than in the others especially in group IV.



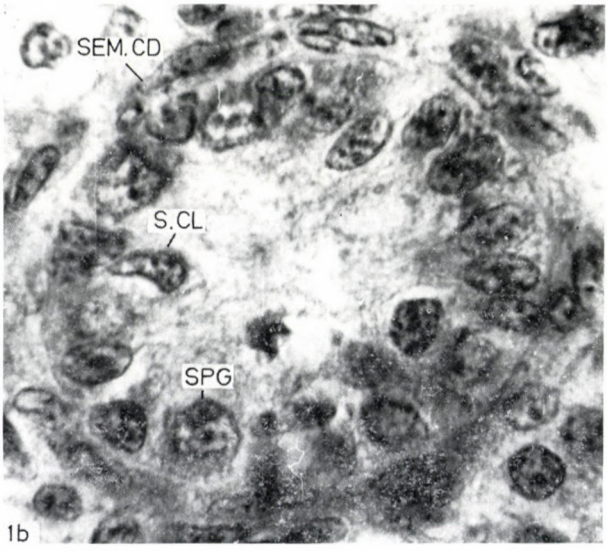
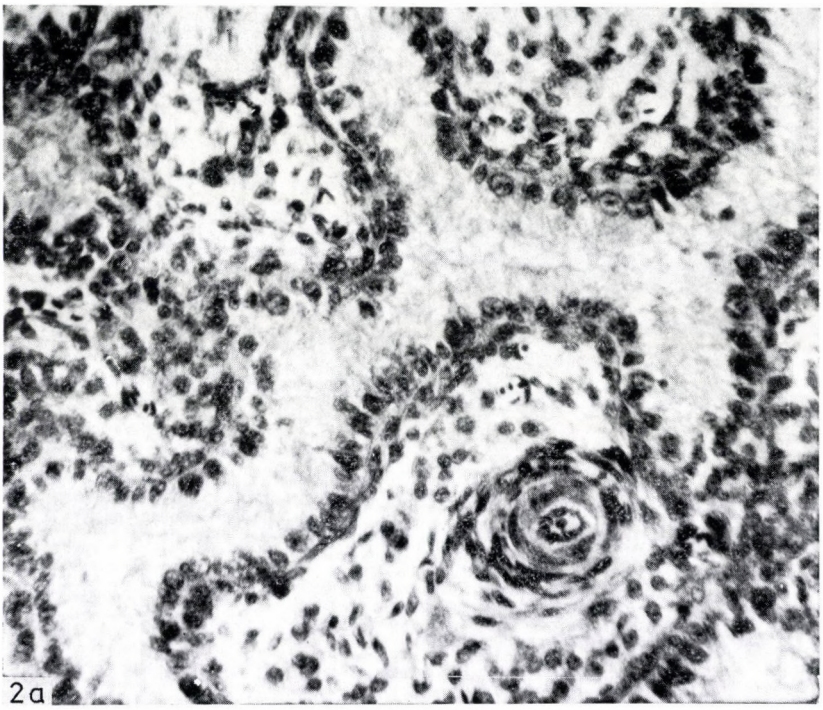


Fig. 1a. Control testis of newly hatched chick. Seminiferous cords and interstitial tissue.
b. Enlarged seminiferous cord.
a ×700, *b* ×1500



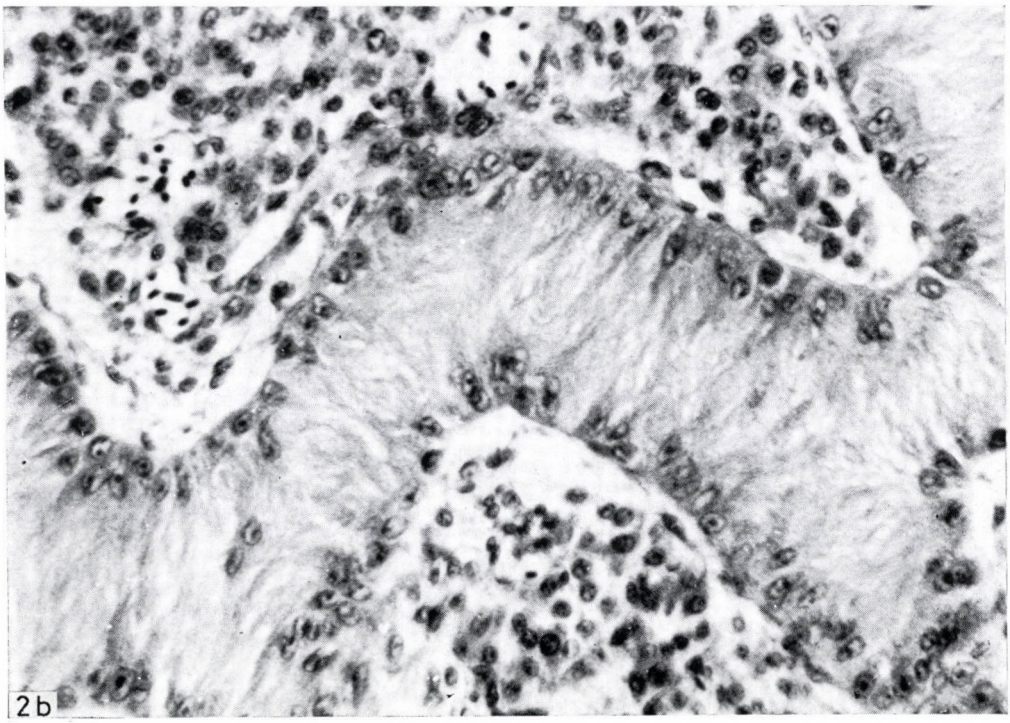
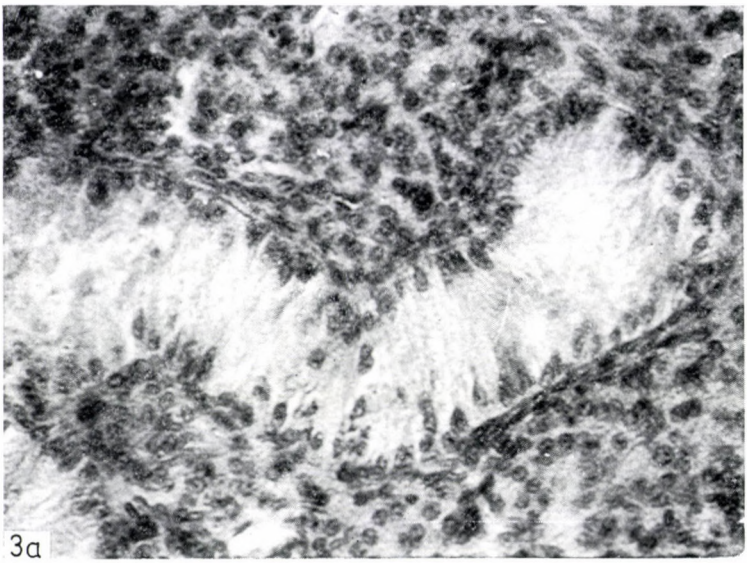


Fig. 2. Testes of newly hatched chickens treated with TSH (*a*) and gonadotropins (*b*). The size of the seminiferous cord is larger in *a* than in *b* or in the control groups. ×700



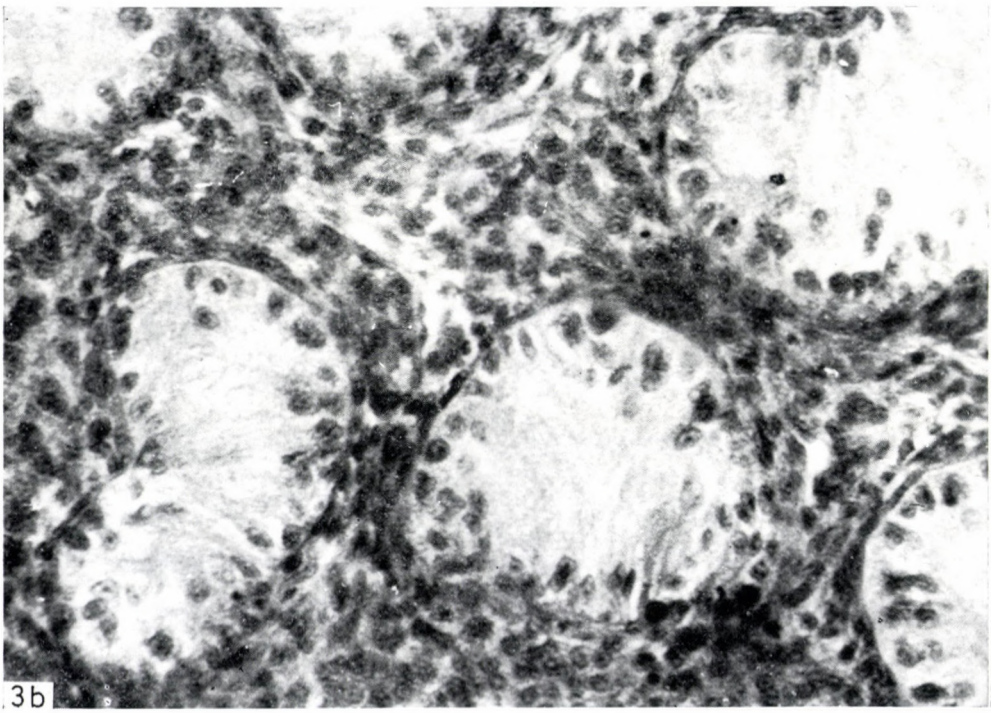


Fig. 3. Testes of newly hatched chickens injected with TSH and gonadotropins (*a*) and gonadotropins and TSH (*b*). The seminiferous cords are smaller than in gonadotropis treated chickens (Fig. 2*b*)
×700

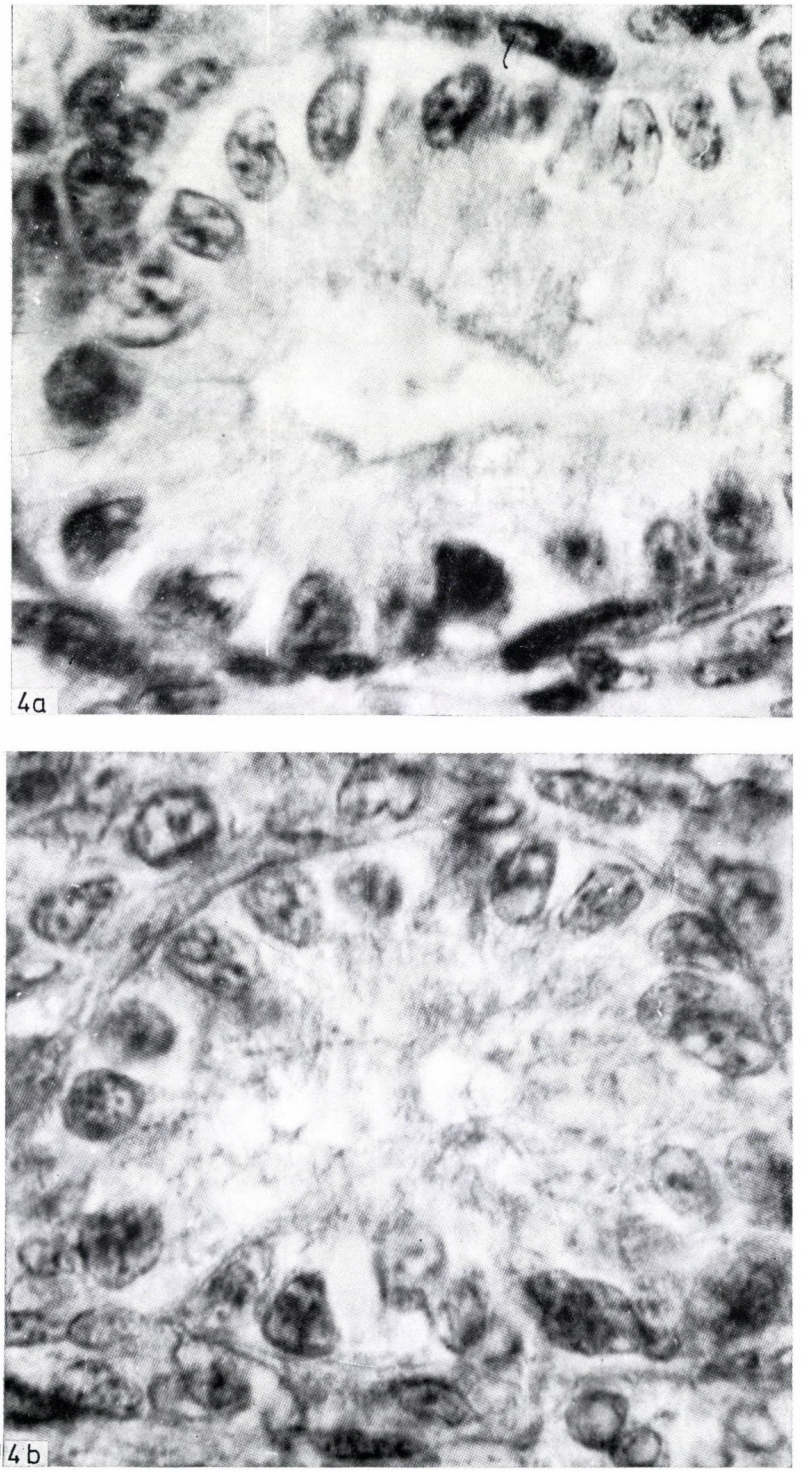
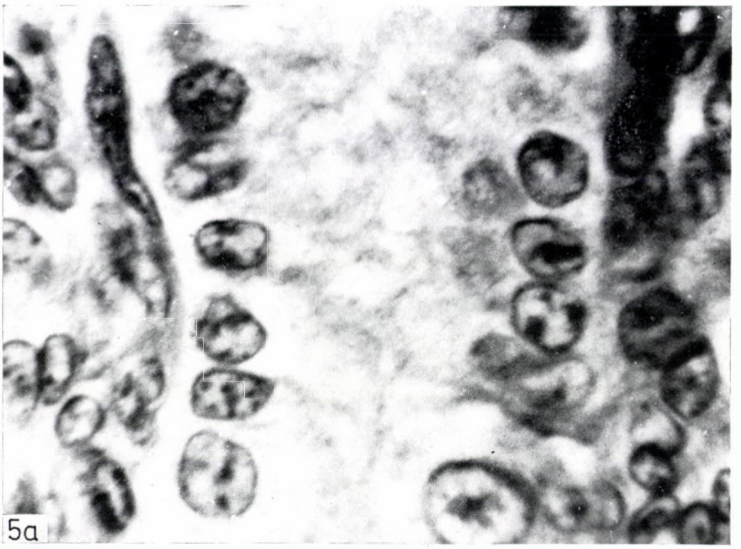


Fig. 4. Enlarged seminiferous cords of newly hatched chickens treated with gonadotropins (*a*) and TSH and gonadotropins (*b*). The Steroli cells appear as high columnar epithelial cells. Vacuolization, is most marked in (*a*)
×1500



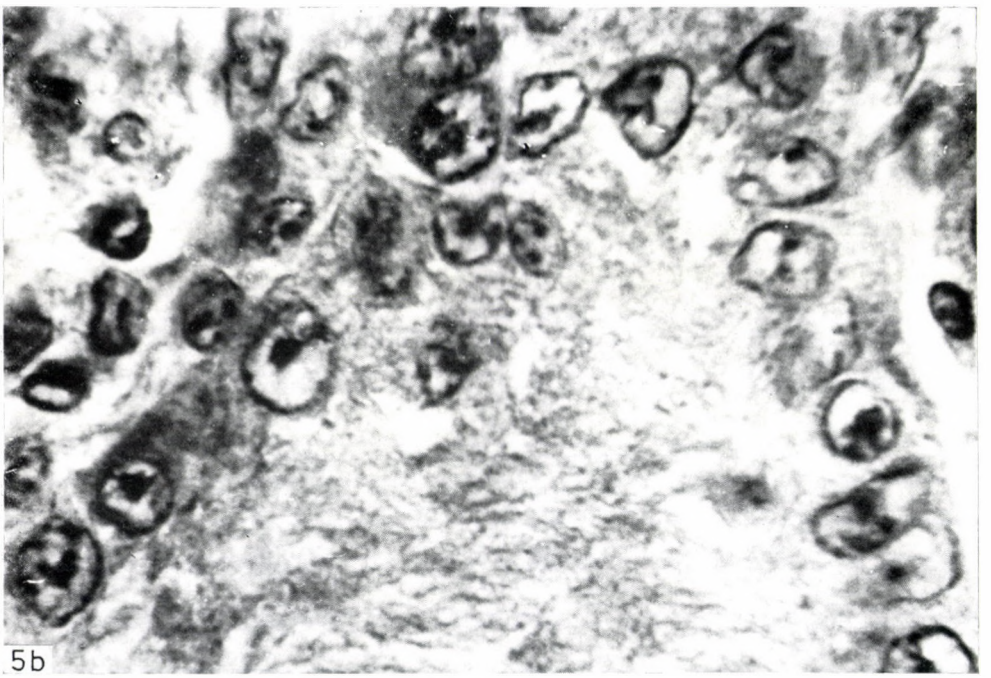


Fig. 5. Enlarged seminiferous cords of newly hatched chickens treated with TSH (*a*) gonadotropins and TSH (*b*). The sertoli cells display no structural changes as compared with the control (Fig. *1b*). Seminiferous cord cavities are absent as compared with Figs *4a* and *b* ×1500

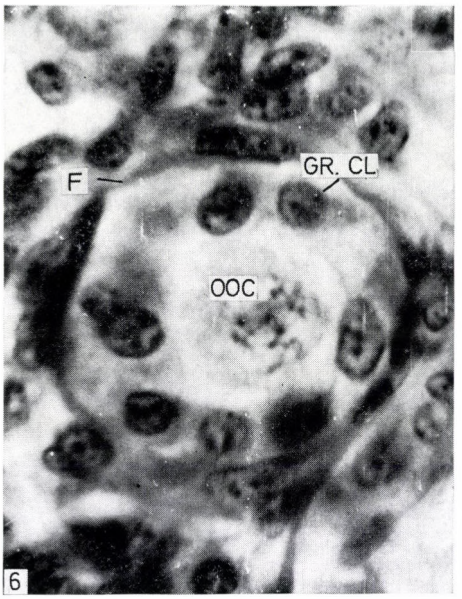


Fig. 6. Enlarged follicle in the left ovary of newly hatched control chickens. The follicle consists of an oocyte surrounded by a single layer of granulosa cells ×1500

The process of vacuolation (cavity formation) was most marked in groups III and IV (Fig. 4) while in groups II and V there was a slight increase in such process compared with control. The vacuolation, however, formed no true cavities, thus the cords did not develop into tubules.

Quantititative analysis. In the control group the percentage of Sertoli cells was about 71% while that of the spermatogonia, 29%.

All the experimental groups showed a marked increase in the whole number of cells as well as in the number of spermatogonia or Sertoli cells in the seminiferous cord (Table I).

In *group II* (TSH treated), although the difference in the whole number of cells (spermatogonia + Sertoli cells) as compared with the control was significant, it was not large as compared to groups III, IV and V, and showed the lowest increase in the number of spermatogonia and Sertoli cells (alone or together).

In *group III* (gonadotropins treated) chickens appears clearly the large number of cells with the highest increase in the number of spermatogonia and Sertoli cells. The increase was in the number of Sertoli cells rather than in the number of spermatogonia which was about three times the number in the control group.

In *group IV* (TSH + gonadotropin treated) chickens although the difference in the number of Sertoli cells and spermatogonia (together or alone)

was significant as compared to the controls and group II, the rate of the increase was higher in group II (Table I), especially in the number of spermatogonia.

In group V gonadotropin + TSH treated chickens the number of spermatogonia and Sertoli cells showed after group III the second highest increase. The whole number of cells was double of that in the control group.

It was characteristic of all the groups that as compared with the control the increase in the number of Sertoli cells was higher than that in the spermatogonia.

Female chickens

The ovaries and oviducts were present in all groups, but the right ovary and right oviduct were undeveloped.

The largest left ovary was in group V, this was followed by groups II and III, in the latter, the ovary was approximately of the control size.

No morphological differences of the reproductive system were observed in any of the groups.

Histological observations. Examination of serial transverse sections of the left ovary showed a normal structure in all groups.

The left ovary consisted of an outer cortex and inner medulla. The cortex was composed of cortical cysts, containing groups of oocytes and scattered follicle cells.

The developed oocytes were surrounded by large squamous granulosa cells (follicular cells) forming the follicle. The follicle consists of an oocyte surrounded by a single layer of flattened granulosa cells. The oocyte fills the developing follicle (Fig. 6). The granulosa cells change in shape from a simple squamous to one of either cuboidal or short columnar cells.

The medulla is composed of medullary cords consisting of connective tissue, clumps of variably shaped cells which fill the spaces in the medulla; and some of them singly fill the space between the developing follicles. Such structures are referred to as the ovarian stroma. The interstitial tissue is scattered in the stroma.

No clear differences in the number of oocytes, of cortical cords or oocyte structures appeared in any group.

In groups II and V, the ovarian stroma increased greatly in size and filled most of the ovary. The interstitial tissue islets increased in amount.

Such phenomena were absent from the control as well as the III and IV groups.

Quantitative analysis. The number of granulosa cells in the follicles, is considered parameter of ovarian development.

Comparing the mean number of granulosa cells in the experimental groups and the control group, all experimental groups had an increased number

of granulosa cells, but the increase was not significant between all groups (Table II, Figs 6 and 7).

The highest number of granulosa cells, and the highest increase was found in group III (Fig. 7b). The difference in the number of granulosa cells between group III and the other groups (II, IV, V and control)was significant ($p < 0.001$).

The difference in the number of granulosa cells between groups II and V and the control was also significant ($0.05 > p > 0.02$).

The difference between group IV and the control group was not significant. The difference in the number of granulosa cells between the groups II, IV and V was not significant, either.

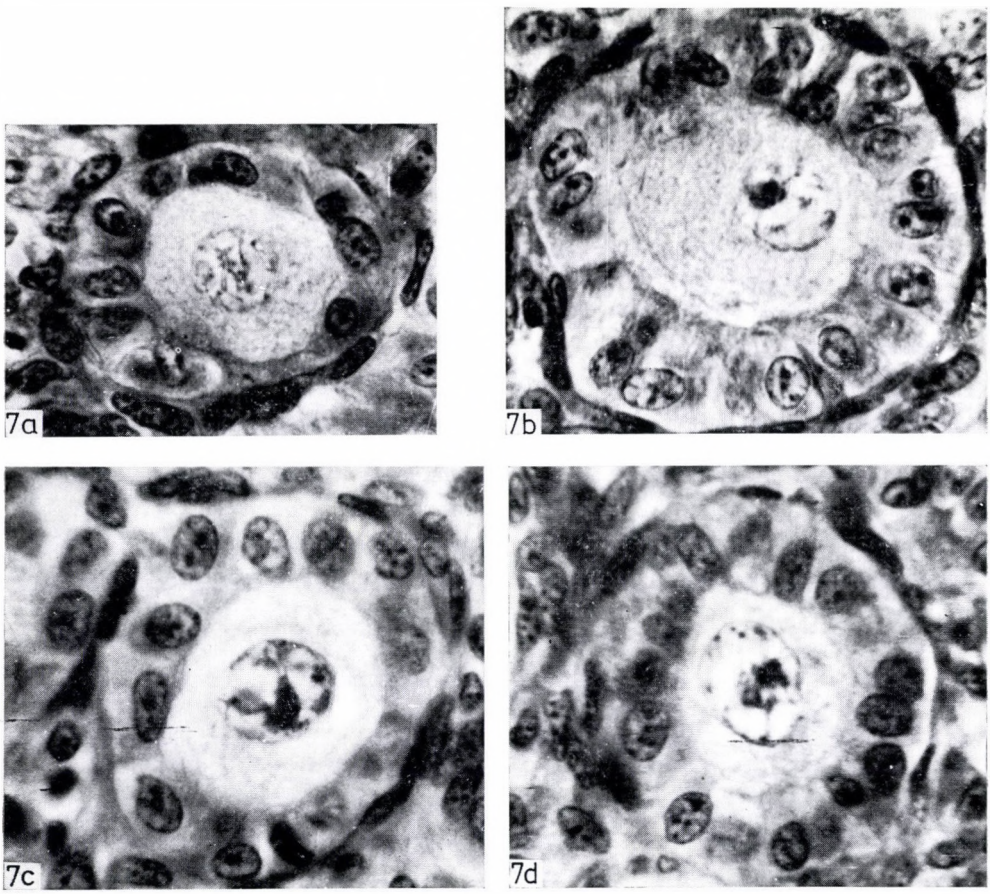


Fig. 7. Enlarged follicles in the left ovary of newly hatched chickens injected with TSH (*a*), gonadotropins (*b*) TSH and gonadotropins (*c*) and gonadotropins and TSH (*d*). Notice the highest number of granulosa cells in the gonadotropins treated chickens (*b*) ×1500

Discussion

During embryogenesis and the first few postnatal days, the gonads are very sensitive to the influence of various exogenous factors, particularly to hormones [32]. In mammals, the gonadotropins and the other peptide hormones exert their action by binding to specific receptors on the plasma membrane of the target cells of the gonads [7, 25, 26].

The mechanism regulating growth and development of the testis is not clear. At present, quantitation of germ cells is the predominant method of assessing the extent of maturation within the seminiferous tubule [20].

NISHIDA and YASUDA (1957) demonstrated that spermatogonia were less numerous than Sertoli cells in the seminiferous tubule of newly hatched cockerels [29]. This finding agrees with our results in the control group, where the number of spermatogonia amounted to 40% of Sertoli cells (Table I).

Chronic FSH treatment of immature rats results in an increase in the size of the gonads. This size change is attributable in part to increase in the wet and dry weight of the testis as well as to the marked increase in the diameter of the seminiferous tubules [22]. In birds, purified ovine FSH stimulates specifically the increase in diameter of the seminiferous tubules of the newly hatched cockerel [38]. Pure FSH causes an increase in weight and tubule diameter in the testes of newly hatched chickens [13].

In 1942 GREEP et al. [19] reported an increase in the number of spermatocytes per tubular cross section when immature male rats were given injections of FSH. This was confirmed by demonstrating an increase in the number of primary spermatocytes per tubule when FSH was administered for 5 days to immature male rats from the 15th day of age. This increase in primary spermatocytes suggested that FSH might cause a stimulation of mitotic activity [27]. FSH induced a significant increase in the number of mitoses per tubule within 9 hr following a single injection [27].

In the present work we found that gonadotropins (FSH, LH) cause a marked increase in the number of spermatogonia and Sertoli cells as compared with the control. This effect was attributed to the stimulatory effect of the hormone on mitotic activity.

These results agree with our previous findings, where FSH caused an increase in number and mitotic activity of the germ cells and Sertoli cells [16]. More recently we have found that gonadotropins cause a significant increase in seminiferous cord diameter in the newly hatched cockerels [14].

We attributed the increase mainly to the increase in the number of spermatogonia and Sertoli cells, and to a less extent to the hypertrophy of Sertoli cells. These findings contradict the findings of other authors, who attributed the increase in tubule diameter to the Sertoli cell hypertrophy.

MURPHY [1965] stated that the increase in seminiferous tubule diameter after FSH in the rat did not depend upon cell division but rather upon Sertoli cell hypertrophy and secretion resulting in an accumulation of fluid [28]. Although TSUTSUI (1978) found an increase in the total number of Sertoli cells in the testis of the Japanese quail treated with FSH and testosterone as well as an increase in the total number of gonocytes, he attributed the increase tubule diameter to the hypertrophy of Sertoli cells and not to the increase in the number of cells [40].

On the other hand, our results agree with those of HUCKINS [21] according to whom FSH had a direct effect on the proliferating spermatogonial population in the rat testis. Although gonadotropin caused an increase in the number of spermatogonia and Sertoli cells, the increase involved especially the Sertoli cells.

The density of FSH receptors on the Sertoli cells and spermatogonia is practically similar [31]. The presence of FSH receptors on these cells supports the suggestion that FSH may have a direct effect on spermatogonia [31]. Autoradiography revealed specific binding sites for human FSH on Sertoli plasma membranes in the basal compartment of the rat seminiferous tubules. FSH-binding sites occur on the surface of spermatogonia in concentrations similar to those of Sertoli cells [30]. A comparison of the density of FSH receptors on Sertoli cells with that on the spermatogonia yielded a ratio (Sertoli to spermatogonia) of 1.68 [30]. This agrees with our results where the number of spermatogonia increases to 1.4 fold the number of control and the number of Sertoli cells increases about 3 fold as compared with the control (Table I).

In thyrotropin treated cockerels, the effect was similar as that of gonadotropins only somewhat slighter. Thyrotropin increased the number of Sertoli cells to 1.4 fold the control number and caused a slight increase in the number of spermatogonia. This effect was much weaker than that of gonadotropins. In the gonadotropins group the number of Sertoli cells and spermatogonia was about 2 fold and 1.3 fold the number in the thyrotropin group. These findings agreed with our previous results in that TSH was found to have an effect on both Sertoli and germ cells [16]. bTSH binds reversibly and with a high affinity to receptors in the rat testis that are either the same as receptors for hCG and LH or that interact therewith [2].

The apparent affinity of bTSH receptors in the rat testis is several orders of magnitude lower than that of hCG [2]. As observed earlier in perinatal age TSH can bind to the receptors in the cockerel testis, and has approximately the same effect as that of gonadotropins [16]. Interstitial tissue appears to be more sensitive to thyrotropin than to gonadotropins. This agrees with the findings of AMIR (1978) that the binding of TSH to testicular homogenate was localized to the interstitial tissue [2].

Although the half amount of the gonadotropins used consisted of LH,

the effect of the latter on the intersitial tissue appears to be much weaker than that of thyrotropin. The interstitial tissue, which contains undifferentiated interstitial cells, hardly responds to exogenous LH [23].

Recently [14], we have observed an intense increase in the weight of the testes of thyrotropin treated chickens. On the basis of the present histological observations we attribute the increase in weight mainly to the increase in the amount of interstitial tissue.

In chickens treated on the first day with thyrotropin (80 μg), and for the next three days, with gonadotropins (120 μg), the number of spermatogonia and Sertoli cells was increased, as compared with the controls. The increase was, however, slighter than after gonadotropins treatment (200 μg) for four days, and somewhat larger than after thyrotropin treatment (200 μg) for four days.

In group V the number of spermatogonia and Sertoli cells was increased as compared with the controls or with chickens in group IV. Although the amount of gonadotropins in group V was 80 μg compared to 120 μg in group IV, the number of spermatogonia and Sertoli cells increased more in group V. This was due to that the effect of 80 μg gonadotropin on its target cells on the first day was much stronger than the effect of the 1.5 fold dose (120 μg) since the receptors on the target cell have been occupied by another hormone (TSH) of approximately the same structure. Moreover, this indicates that the increase in the number of cells and of their mitotic activity is due mainly to gonadotropins, but the role of TSH in those processes must not be neglected either.

The apparent affinity of bTSH to receptors in the rat testicle is several orders of magnitude less than that of hCG [2].

Our findings partly [12, 14, 16] disagree and partly agree with the above statement. They disagree as in birds TSH was found to increase the weight of the testicles and the increase was more marked than that caused by gonadotropins [14] or FSH [12, 16].

On the other hand they agree in that the effect of TSH on the seminiferous tubule diameter as well as on the number of germ and Sertoli cells was much weaker than that of gonadotropins [14].

It is therefore concluded that the gonadotropins play an important role in the initiation and possibly regulation of the process of spermatogenesis in avian testes. At the same time they exert an effect on the process of mitosis in the germinal epithelium. In addition, gonadotropins, cause a marked increase in the number of Sertoli cells. Thyrotropin (TSH) had a similar but slighter effect. Regarding the overlapping effect of thyrotropin and gonadotropins on the testicle, these hormones which are similar in structure did not change or increase other's each effect when they were administered one after the other in the perinatal period.

In general, the ovary of the immature bird responds poorly to gonadotropin [3, 5, 6, 17]. Chicks at the age of 180 days responded to mammalian preparations of fairly pure FSH and LH with pronounced follicular growth and premature ovulation [3]. TABER (1948) attempted to distinguish between FSH and LH effects but found, as did also KORNFELD and NALBANDOV (1959), that mammalian preparations did not simulate exactly the action of endogenous gonadotropins [24, 39].

In mammals, a combination of FSH and LH given to the hypophysectomized rat elicits a twofold increase in ovarian weight follicle maturation and stimulation of intersitial tissue [19, 18]. Still, in a previous work we found that gonadotropins did not increase the weight of the left ovary of newly hatched chickens [14].

Antigonadotropic studies designed to localize the specific intraovarian binding sites for FSH and LH in the immature rat ovary showed that ^{125}I—FSH was bound to follicles in many stages of development including those in the early stage [35]. This strongly suggests that early follicles of the rat already possess the equipment necessary to react with FSH. In the immature rat, ovine FSH increased greatly LH receptors in the granulosa cells of the developing follicles [42]. In the present study, the number of granulosa cells showed a marked increase as compared with the controls. Consequently, the gonadotropins, affected the mitotic proliferation of granulosa cells. This is supported by the finding of GREEP et al. (1942) who stated that the mitotic proliferation of granulosa cells is dependent on FSH [19].

In thyrotropin treated chickens we noticed a slight increase in the number of granulosa cells and the same was noted in groups IV and V. This was attributed to that the effect of any given hormone appears to be related to the type of the cell and the stage of its differentiation. Thus, although the intraovarian distribution of FSH binding suggests that FSH can bind to granulosa cells of the follicles in many if not every stage of growth, there are data that the effect of FSH is dramatically modified by the presence of oestradiol [34]. Thus, hormone regulation of hormone receptors appears to be an important aspect of follicular maturation [36].

In conclusion, we can say that the left ovaries of newly hatched chickens respond to gonadotropins and thyrotropin. The response to the former is much greater than that to the latter, and it manifests itself with an increase in the number of granulosa cells i.e. an increase in the mitotic proliferation of granulosa cells.

In the case of the testis, gonadotropins and TSH did not increase essentially each other's effect when they were administered to newly hatched chickens one after the other within a short time, however the effect of previously administered gonadotropins, to TSH was stronger (and perhaps amplifying) than TSH to gonadotropins.

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List of abbreviations

F	Follicle
GR . CL	Granulosa cell
IS . TS	Interstitial tissue
OOC	Oocyte
S . CL	Sertoli cell
SEM . CD	Seminiferous cord
SPG	Spermatogonium

DIE WIRKUNG DER GONADOTROPINE UND DES THYREOTROPINS AUF DIE HODEN UND DAS NEUGESCHLÜPFTER KÜKEN

In neugeschlüpften männlichen und weiblichen Küken erhöhen das TSH und die Gonadotropine (FSH, LH) die Zahl der Spermatogonien und der Sertoli-Zellen in den gewundenen Hodenkanälchen sowie eine Menge der granulösen Zellen in den Follikeln des Ovars. Die Wirkung der Gonadotropine war ausgesprägter als die des TSH. Aufgrund der Versuchsergebnisse hat es den Anscheien, daß im Falle der männlichen und weiblichen Gonaden neugeschlüpfter Küken das TSH und die Gonadotropine, deren chemische Struktur ähnlich ist, ihre Wirkung gegenseitig verdecken bzw. einander ersetzen können. Nacheinander angewandt erhöhen sie aber nicht gegenseitig ihre auf das parenchymatöse Gewebe (auf die Anzahl der Spermatogonien und Sertoli-Zellen in den männlichen Geschlechtsdrüsen sowie auf die Follikelzellen in den weiblichen Gonaden) ausgeübte Wirkung.

ДЕЙСТВИЕ ГОНАДОТРОПНЫХ ГОРМОНОВ И ТИРЕОТРОПИНА НА ЯИЧКИ И НА ЯИЧНИКИ СВЕЖЕ ВЫЛУПЛЕННЫХ ЦЫПЛЯТ

У свеже вылупленных мужских и женских цыплят TSH и гонадотропные гормоны (FSH, LH) повышают число первичных семенных клеток и клеток Сертоли в семенных трубочках яичков), как и количество зернистых клеток в фолликулах яичника. Действие гонадотропных гормонов оказалось более выраженным чем действие TSH. На основе экспериментальных данных кажется, что в случае мужских и женских половых желез новорожденных цыплят TSH и гонадотропные гормоны, имеющих подобную химическую структуры, могут перекрыть или заместить действие друг друга. При применении один за другим они не повышают действия друг друга, оказанное на паренхиматозную ткань (на число семенных клеток и клеток Сертоли в яичках и числа фоллигкулов в яичниках).

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NERVE CELLS OF THE RABBIT, CAT, MONKEY AND HUMAN CAUDATE NUCLEUS: A GOLGI-STUDY

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The Golgi architecture of the nucleus caudatus in rabbit, cat, monkey and human material was analysed. The brains were prepared by Golgi—Kopsch impregnation.

The fairly divergent types of neurons practically belong to two main groups: projective or efferent neurons and local- or interneurons. The efferent neurons — as it has been confirmed by experimental studies — establish far contacts with various regions of the brain. According to the Golgi picture, their axon emits several collaterals, which produce local connections. The interneurons have only locally arborizing axon. They are mainly responsible for intrinsic connections of the nucleus.

In both main groups of caudate neurons, several types and subtypes were observed. The number of the types and subtypes of the neurons show an increasing tendency in the phylogenetic series.

Introduction

DEJERINE [3] was the first to carry out a Golgi-analysis in the striatum. Since then several authors have studied striatal cell types in various species [1, 2, 4—10, 12, 13—21, 23]. On the basis of Nissl-preparations, small and large neurons were distinguished. With Golgi-impregnation small, spiny dendritic and large, smooth dendritic neurons were initially described but subsequent studies have refined this description by demonstrating six different cell types. FOX and NICOLESCO [5] estimated the ratio of small and large cells at 20:1, whereas NAMBA [17] has shown a 53:1 and 125:1 ratio in the cytoarchitectonic areas of BROCKHAUS [1]. In SZABÓ's investigations [22] large cells were found to constitute a 5% fraction of the striatal neuron population. VOGT and VOGT [23] suggested that large neurons were the only source of striatal efferents, a claim disproved by recent findings [2, 9, 11, 21]. Golgi studies of the striatum including the caudate nucleus have been carried out in a number of species and there are some observations also in man. Therefore, the present investigations made in rabbit, cat, monkey and human material are thought to be of complementary and confirmatory value.

Method

Brains of adult animals and humans of various ages were used for Golgi-impregnation. Rabbit and cat brains were processed by the perfusion Golgi—Kopsch technique. The same

technique was applied to monkey and human brains but using immersion fixation and block impregnation. Perfused materials were cut serially. From immersion-fixed materials, blocks of the caudate nucleus were excised and embedded in celloidin. Observations were made in 120—150 μ frontal sections.

Results

Cell types of the rabbit caudate nucleus

1. *Giant neurons.* These cells possess an extremely large cell body with few dendrites (Fig. 1). They are supposedly of efferent nature. Although this type was found throughout the whole nucleus, a higher frequency of occurrence was noted towards the lateral edge. The cell body is elongated, fusiform or ovoid, 40—50 μ long, 20—25 μ thick. Large dendrites originate at the poles of the cell. They are poorly ramified and can usually be followed for a distance of 200—300 μ. Their thickness, however, suggests a much longer course. The dendritic surface is slightly irregular due to protrusions but no real spines are present. The axon originates with a large axon-hillock and can be impregnated only at its initial portion. This indicates a beginning close to the origin of the myelin sheath of the axon. Both the thickness and myelination pattern are indicative of an efferent neuron.

2. *Medium-size spiny cell with long axon.* At least two subtypes are present in the rabbit caudate. One is multipolar, 20—25 μ in diameter, with thick main-dendrites originating from the cell body. These main dendrites ramify in a tufted fashion to give rise to secondary dendrites which are densely packed with spines. The other type has more dendrites which originate with a thinner shaft making the arborization pattern radial (Fig. 2). Some of these dendrites ramify, others (mainly the thinner ones) do not ramify and after a long smooth segment they become spiny. Both sub-types have a long axon with numerous collaterals.

3. *Small, ovoid cell with spiny dendrites and long axon.* This is more frequently seen than the previous type forming thus the principal neuron-type of the nucleus (Fig. 3). A few tufted main dendrites originate from the ovoid cell body. Cells differ in the length of their dendrites but all have a fairly large arborization field. The axon has several collaterals and its morphological features (thickness, length) point to an efferent nature.

4. *Medium-size, smooth dendritic cell.* This is likely to be an efferent cell with a long axon. Several dendrites originate from the cell body. This is 20—25 μ in diameter, and slightly ovoid, although dendrite originations make it often multiangular. On the dendrites spindle-shaped varicosities of various length are seen in irregular position. The dendrites are tortuous. The axon can be followed for a short distance but has many collaterals. This is supposed to be an efferent cell.

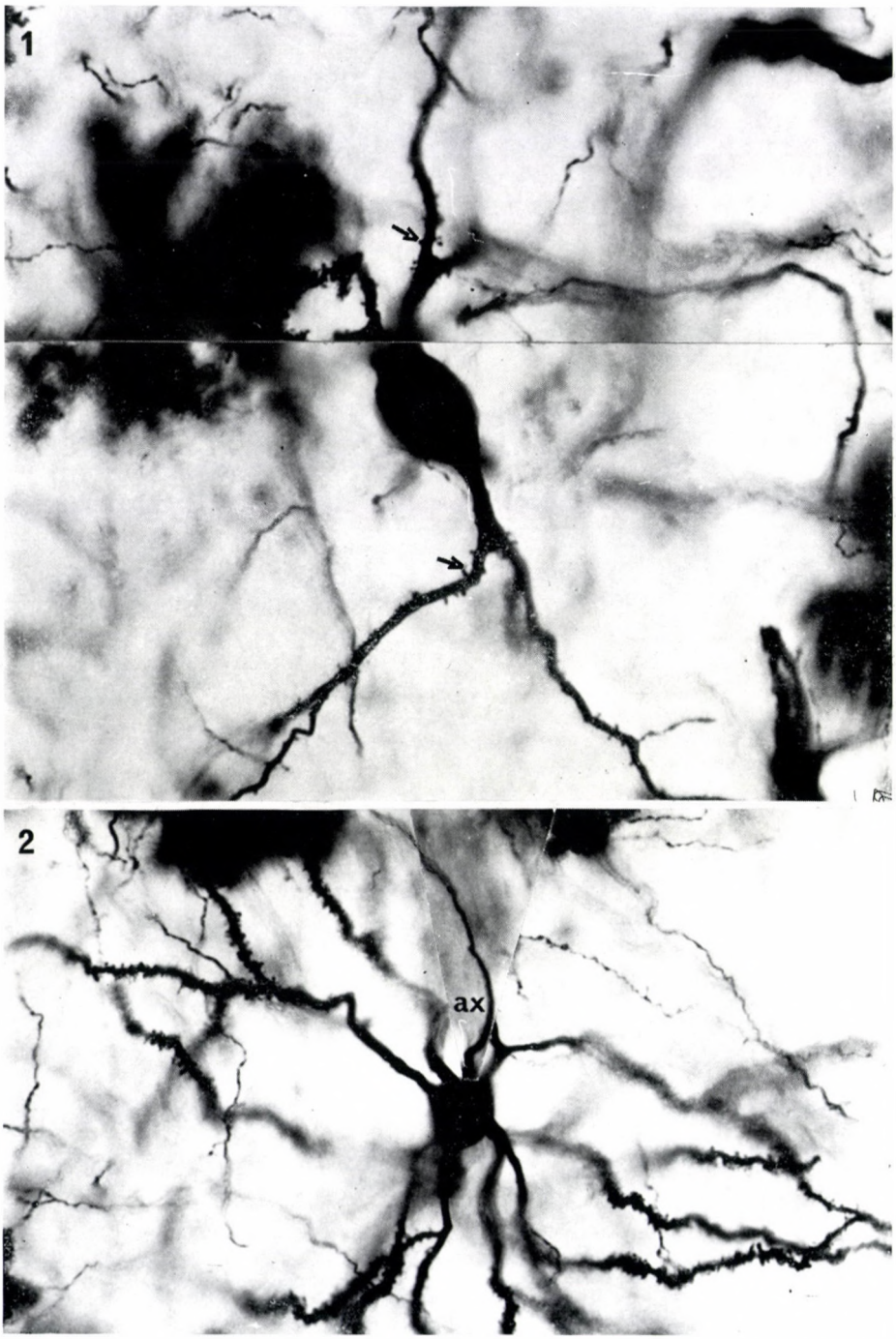


Fig. 1. Giant neuron in the rabbit caudate nucleus. Arrows point to the irregular dendritic surface. 550×

Fig. 2. Medium-size spiny neuron of radiate type in the rabbit caudate nucleus. 550×

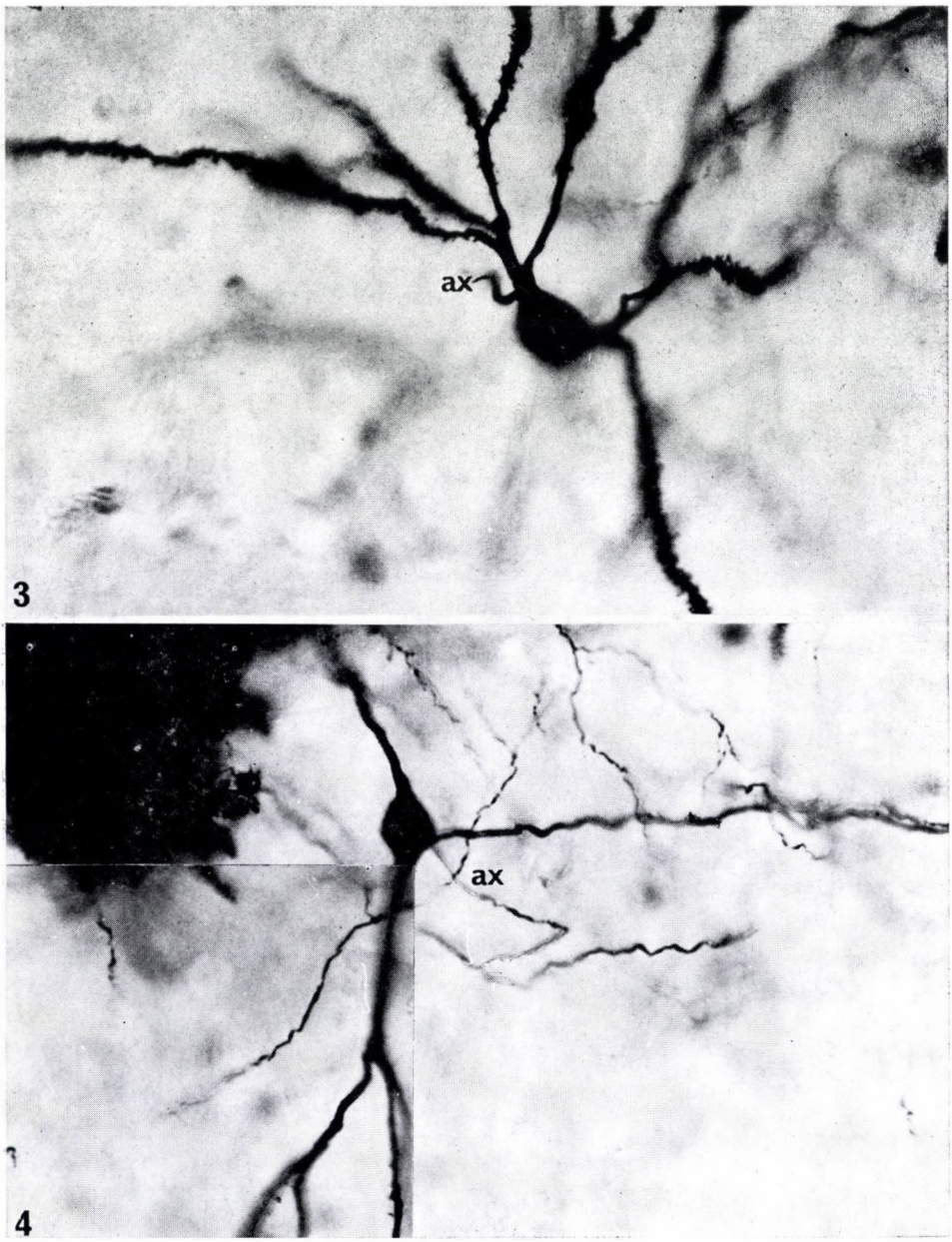


Fig. 3. Small spiny neuron of tufted type in rabbit material. 550×
Fig. 4. Small ovoid neuron with long dendrites Golgi II type cell in rabbit material 550×

5. *Small cell.* This kind of cell has long, scarcely ramifying dendrites (Fig. 4). Their initial segment is smooth and the secondary branches have some spines. Small cells are rarely impregnated, their axons are probably short.

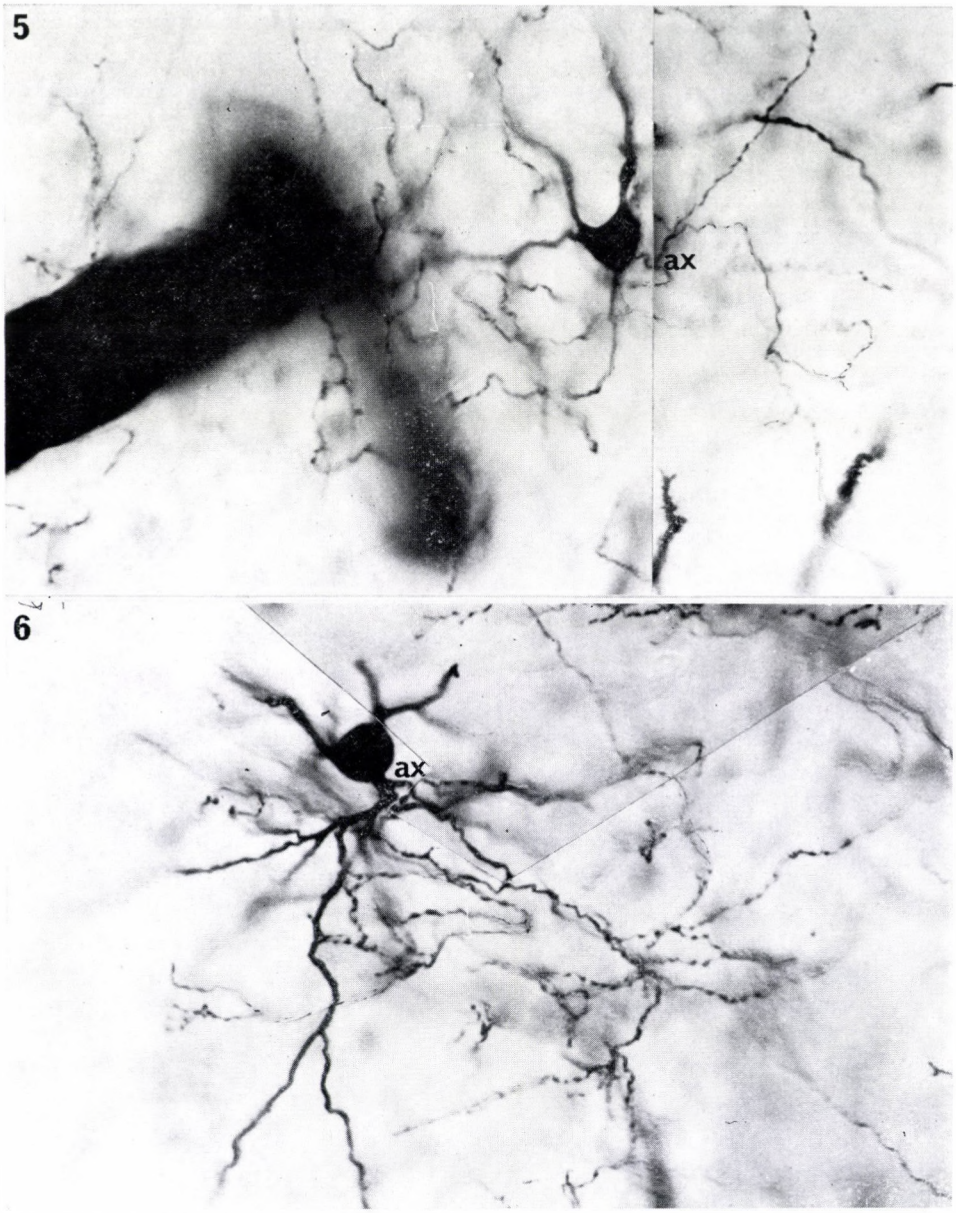
6. *Small cell with many smooth dendrites.* The dendrites are tortuous, the axon arborizes within the dendritic field (Fig. 5). Axonal branches are varicose at their preterminal and terminal portions (Fig. 6). As revealed by the electron microscopic study of other regions, such varicosities probably correspond to presynaptic loci. Accordingly, this cell type is an interneuron with a local arborization field of 200—300 μ.

Cell types of the cat caudate nucleus

1. *Giant cell.* It was impregnated more frequently than in rabbit material. The cell body is ovoid or multiangular depending on the number and position of the large main dendrites (Fig. 7). Its size is 45—50 μ. The multiangular type has tufted main denrites having thus a more abundant dendritic arborization. The dendritic surface is not smooth, protrusions and occasionally spines are seen on it. These irregularities are more frequent near the ramifications. The axon originates with an axon-hillock. It can be followed for a short distance only, but is supposed to leave the nucleus.

2. *Medium-size spiny neurons.* They have a long efferent axon. In the cat a greater variety of these neurons is present than in the rabbit. Ovoid, triangular (Fig. 8), multiangular, tufted (Fig. 9) and radiate (Fig. 10) forms were encountered with 20—30 μ average diameter. In the ovoid type, main dendrites arise at the poles of the cell; they vary in length, ramify repeatedly and have spiny secondary branches. Some triangular and multiangular neurons have tufted main dendrites. Radial cells are multiangular concerning the shape of the perikaryon. Each type has a number of secondary dendrites and therefore the dendritic arborization can be considered abundant. The dendrites are densely packed with spines. The axon originates from the cell giving off several collaterals, their termination on neighbouring cells was often observed. The number of sub-types of this cell-class is greatly increased by the variations in size of the dendritic tree and in the density of spines. Accordingly, smaller, medium and larger medium cells can be distinguished with short, long, radial and tufted dendrites and with axons having a varying number of collaterals. Due to the random nature of impregnation, their relative number is not known.

3. *Small, mainly ovoid, spiny dendritic cells.* The cell body measures 12—18 μ in diameter. As compared to the size of the perikaryon the dendritic field is large. The dendrites ramify after a characteristically long, smooth portion into more than two secondary branches. They differ in length and



Figs 5, 6. Small cell swith smooth wavy dendrites and varicose locally arborizing axon in the rabbit caudate nucleus. 550×

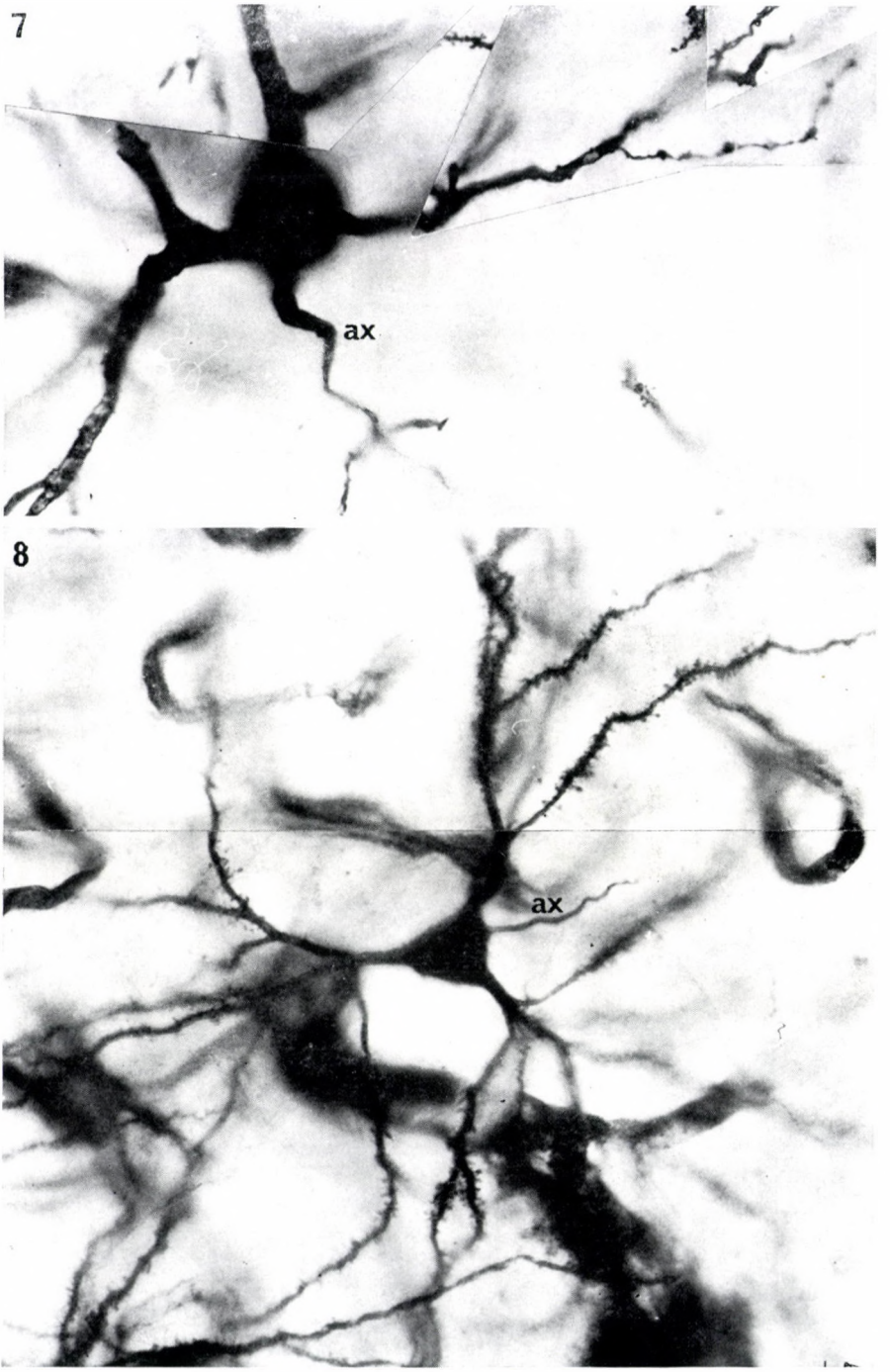


Fig. 7. Giant cell in the cat caudate nucleus. 550×
Fig. 8. Medium-size triangular projective neuron with spiny dendrites in the cat nucleus caudates. 550×

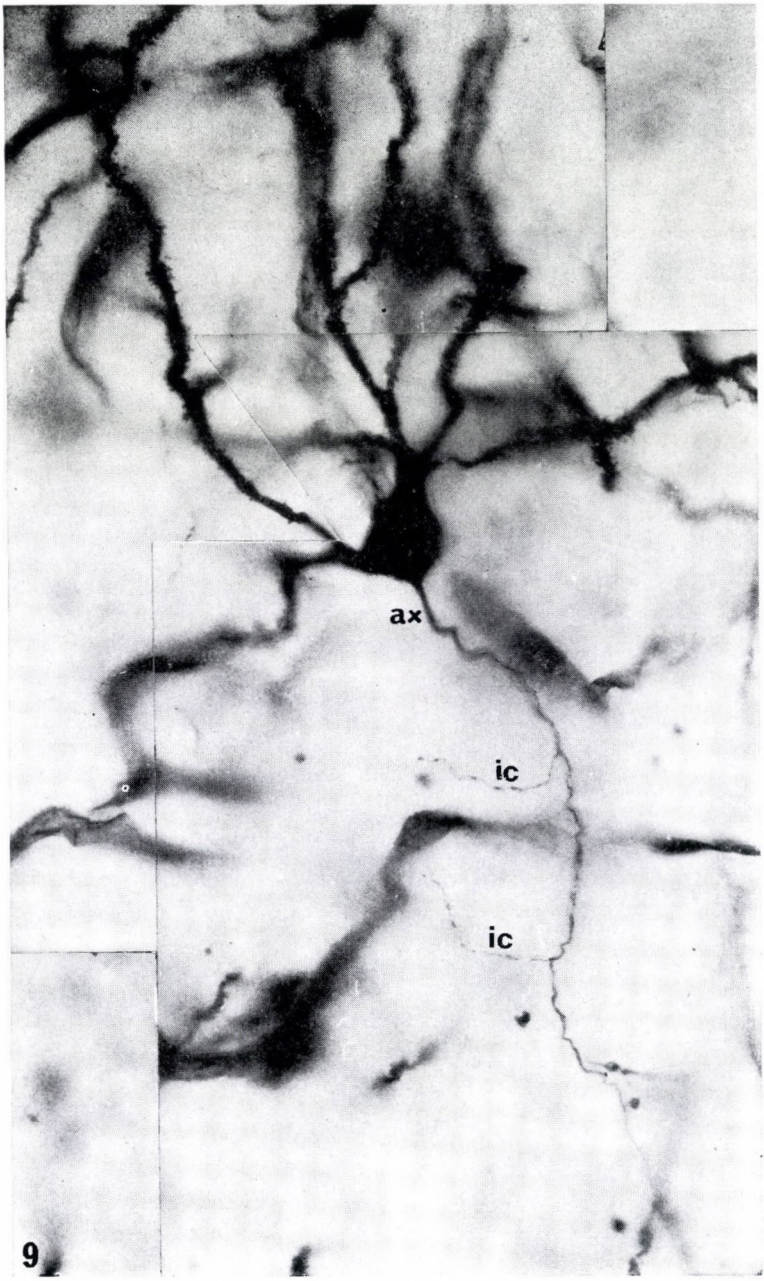


Fig. 9. Medium size, tufted type, spiny-dendritic projective neuron. Initial collaterals (ic) originate from the axon (ax). Cat material. 550×

number but are uniformly covered with spines. The bulky dendrites densely packed with spines are in sharp contrast to the small perikaryon (Fig. 12). The axon arises from the cell body, but occasionally it may originate from one of the main dendritic shafts. It has several collaterals. This cell type is thought to be efferent in nature. It has one peculier subtype, an elongated, extremely fusiform (6—7 × 18 μ in diameter) cell with polar dendrites. Its axon does not differ from that of other subtypes.

4. *Medium-size, smooth dendritic, long axonal cell.* The shape of the cell body is mostly round or multiangular (Fig. 11). It has several dendrites which repeatedly ramify and have a winded course. The dendritic surface is smooth but always displays spindle-shaped swellings. The axon arises from the cell body. Collaterals are scarce.

5. *Medium-size, smooth dendritic, short axonal cells.* The dendrites have no varicosities or swellings. The diameter of the dendritic tree is smaller than that of the previous cell type resulting in a more tortuous course of the dendrites. The axon is difficult to impregnate. It ramifies immediately after its origin and forms a delicate network within the dendritic field (Fig. 13). This cell type corresponds to the Golgi II-type interneuron.

6. *Small, round cell with varicose dendrites.* It resembles the previous type and corresponds probably to the neurogliform cell, because of its more dense axonal and dendritic tree (Fig. 14).

7. *Small, round or ovoid cell with a few long dendrites.* The dendrites are scarcely spiny. The axon is thin and arborizes in the vicinity of the cell with fine terminals (Fig. 15). It is a Golgi II-type cell.

Cell types of the monkey caudate nucleus

The monkey material, was too scarce to allow a nearly complete analysis.

1. *Giant cell.* It has a large ovoid or multiangular cell body giving rise to a varying number of main dendrites which bifurcate or ramify (Fig. 16). Some dendrites are smooth, others are covered by spines although these are spine-like protrusions rather than true spines. The axon can be followed for a short distance.

2. *Medium-size, spiny cells.* This cell type has in the monkey various sub-types such as pyramidal (Fig. 17) triangular, multiangular and ovoid in shape, determined by the number and positions of dendrites. The dendritic ramification is either radiate (Fig. 18) or of the tufted (Fig. 19) type. The spines show even greater variations in number than does the corresponding cell type of the cat. The axon originates from the cell body and has collaterals.

3. *Small, spiny dendritic cell.* This cell is mainly ovoid in shape. The dendritic spines are of varying in packing density. The axon originates from the cell body and has several collaterals (Figs 20, 21).

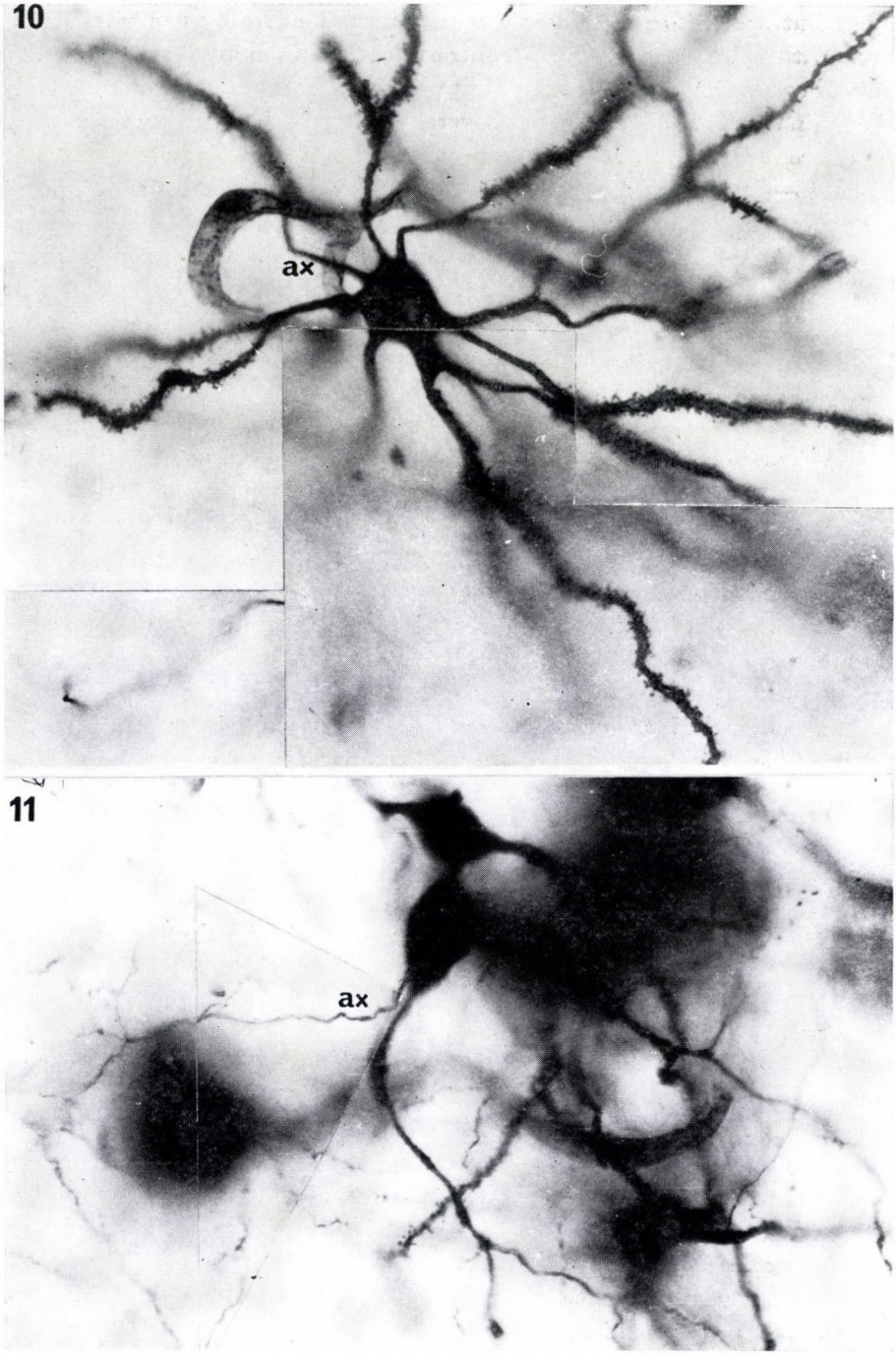


Fig. 10. Medium-size radiate, spiny-dendritic projective neuron in the cat caudate nucleus. 550×

Fig. 11. Medium-size, smooth-dendritic projective neuron in the cat caudate nucleus. 550×

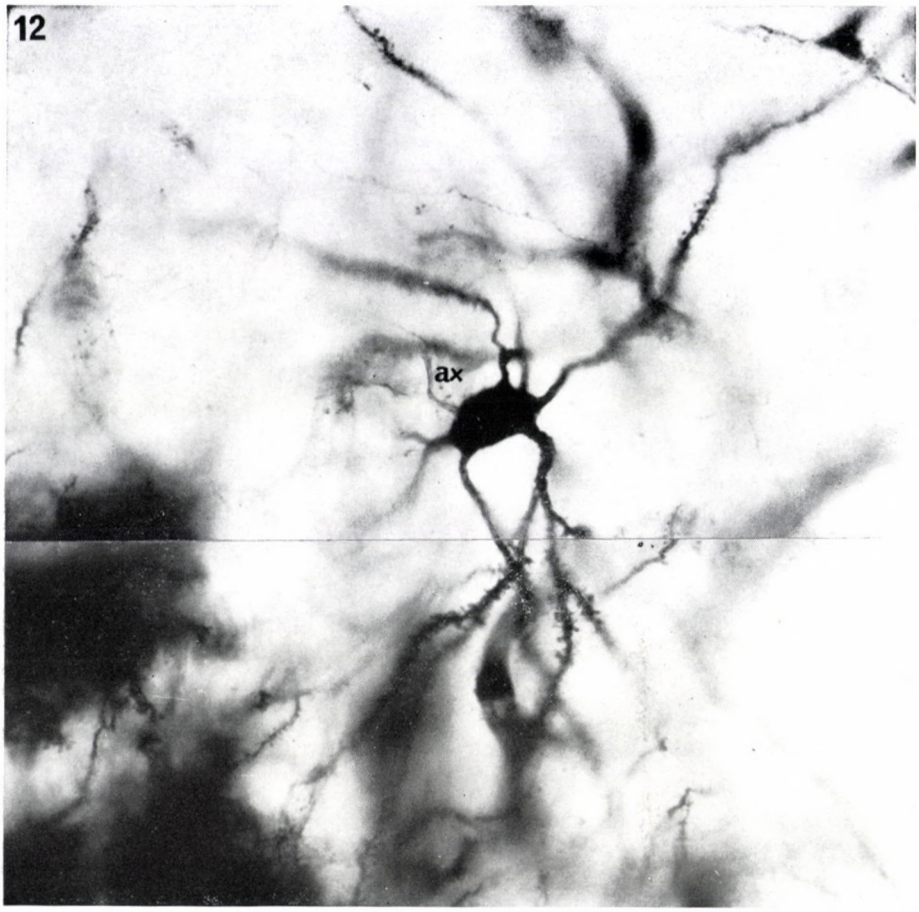


Fig. 12. Small spiny principal neuron in cat material. 550×

4. *Medium-size, smooth dendritic cell.* It has a long, presumably efferent axon and a winded or tufted dendritic tree. The axon has collaterals (Fig. 22).

5. *Small, ovoid, spiny dendritic cell.* This cell resembles in size and dendritic arborization pattern the No. 3. efferent neuron but its axon shows Golgi II-type ramification. Axonal branches can be followed only for a short distance, so that, it can only be supposed on the basis of the axonal branching pattern that they might be local neurons (Fig. 23).

6. *Small, ovoid sparsely spiny dendrites and local axonal arborization.* The dendrites originate mostly at the poles of the ovoid cell body. On the secondary dendrites few spines may be observed. The axon arborizes in the near vicinity (Fig. 24).

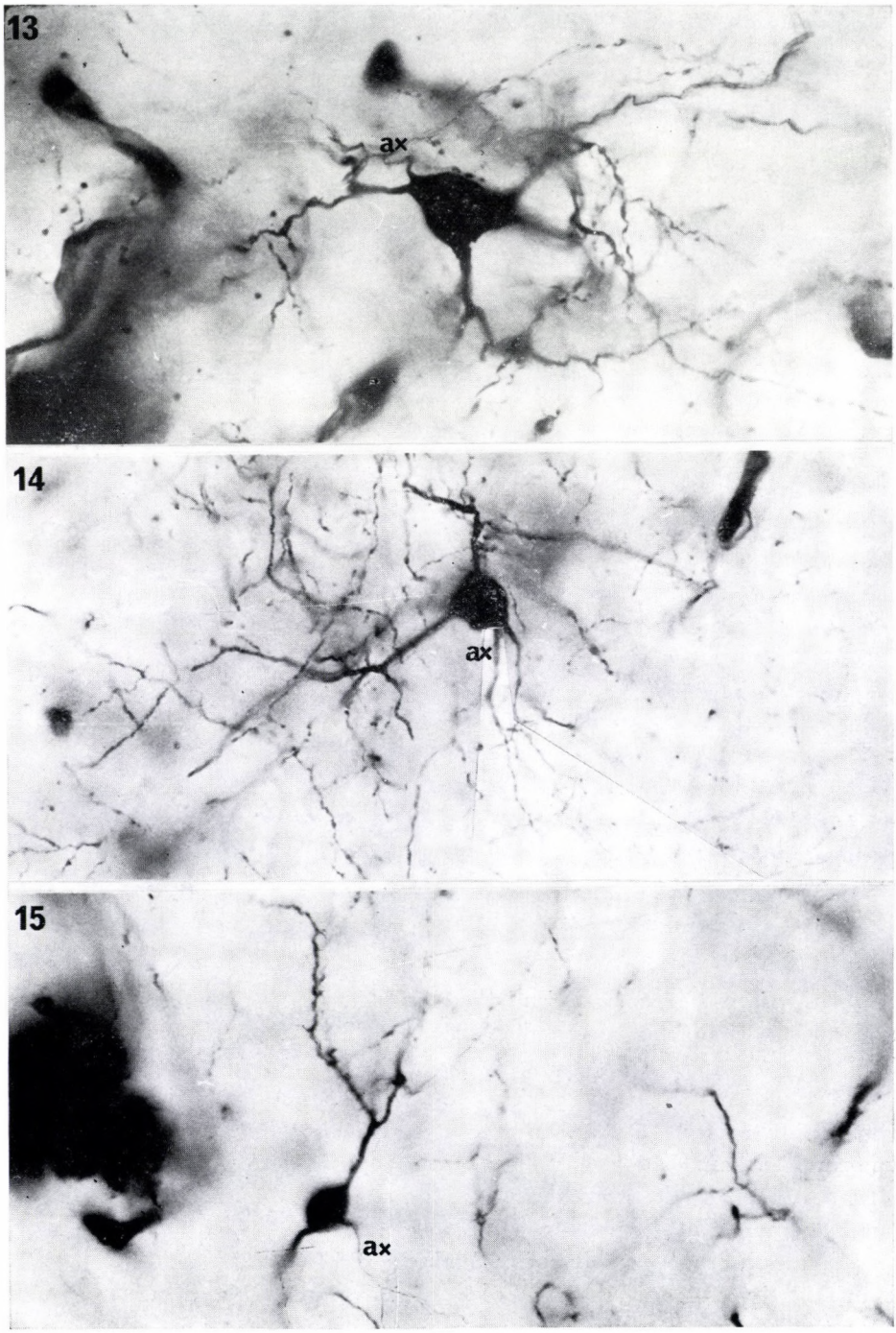
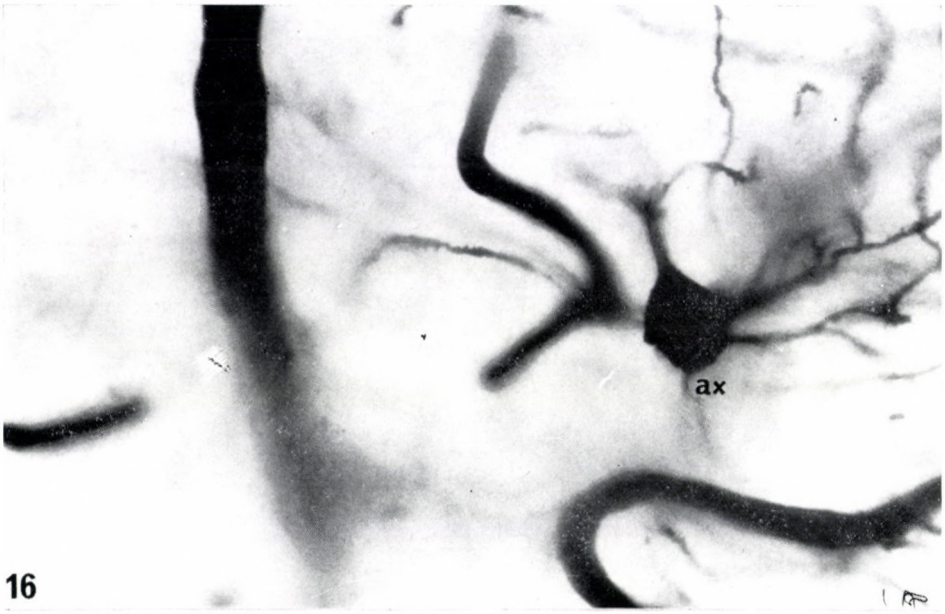


Fig. 13. Medium-size, smooth dendritic, short axonal cell in the cat caudate nucleus. 550×
Fig. 14. Small, round varicose dendritic, short axonal cell in cat material. 550×
Fig. 15. Small, short axonal cell with scarcely spiny dendrites in the cat caudate nucleus. 550×



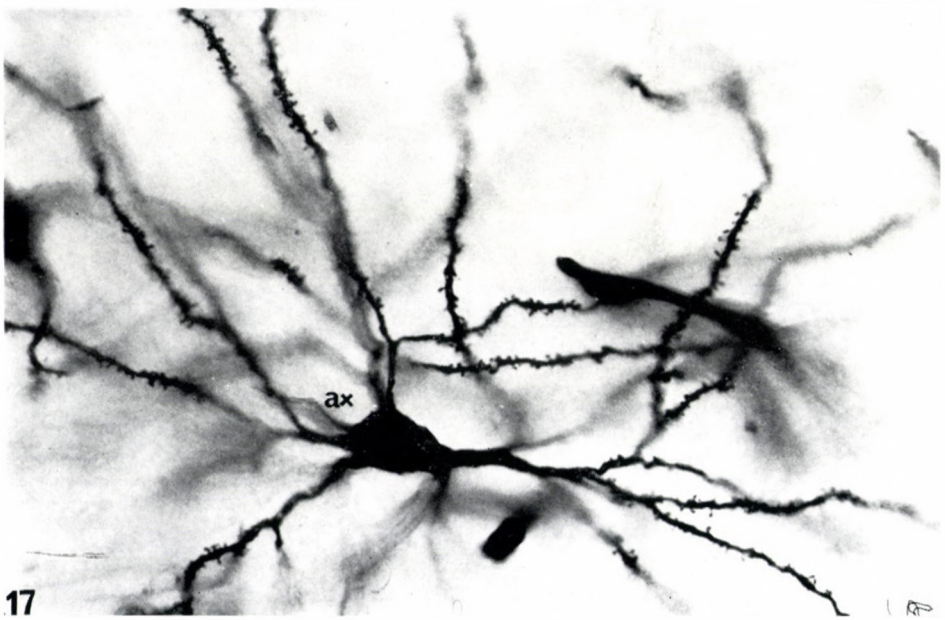
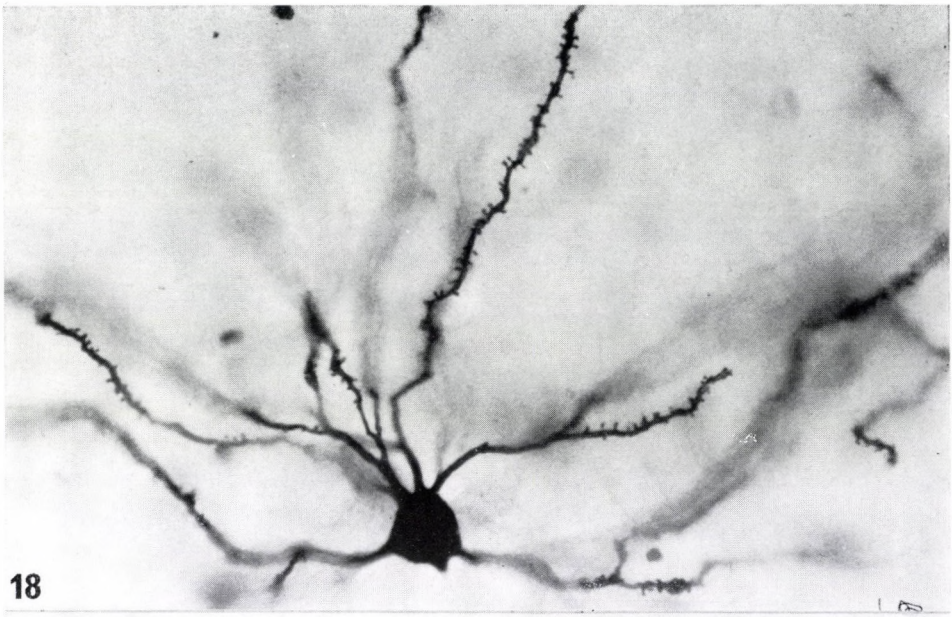


Fig. 16. Giant cell in the monkey caudate nucleus. 550×
Fig. 17. Medium-size, spiny projective neuron of pyramidal dendritic pattern in the monkey nucleus caudatus. 550×



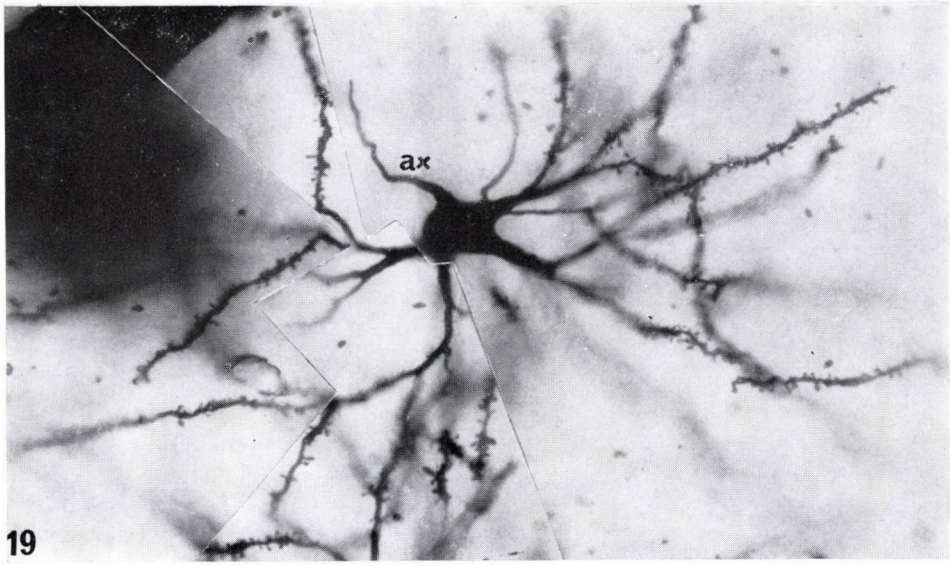
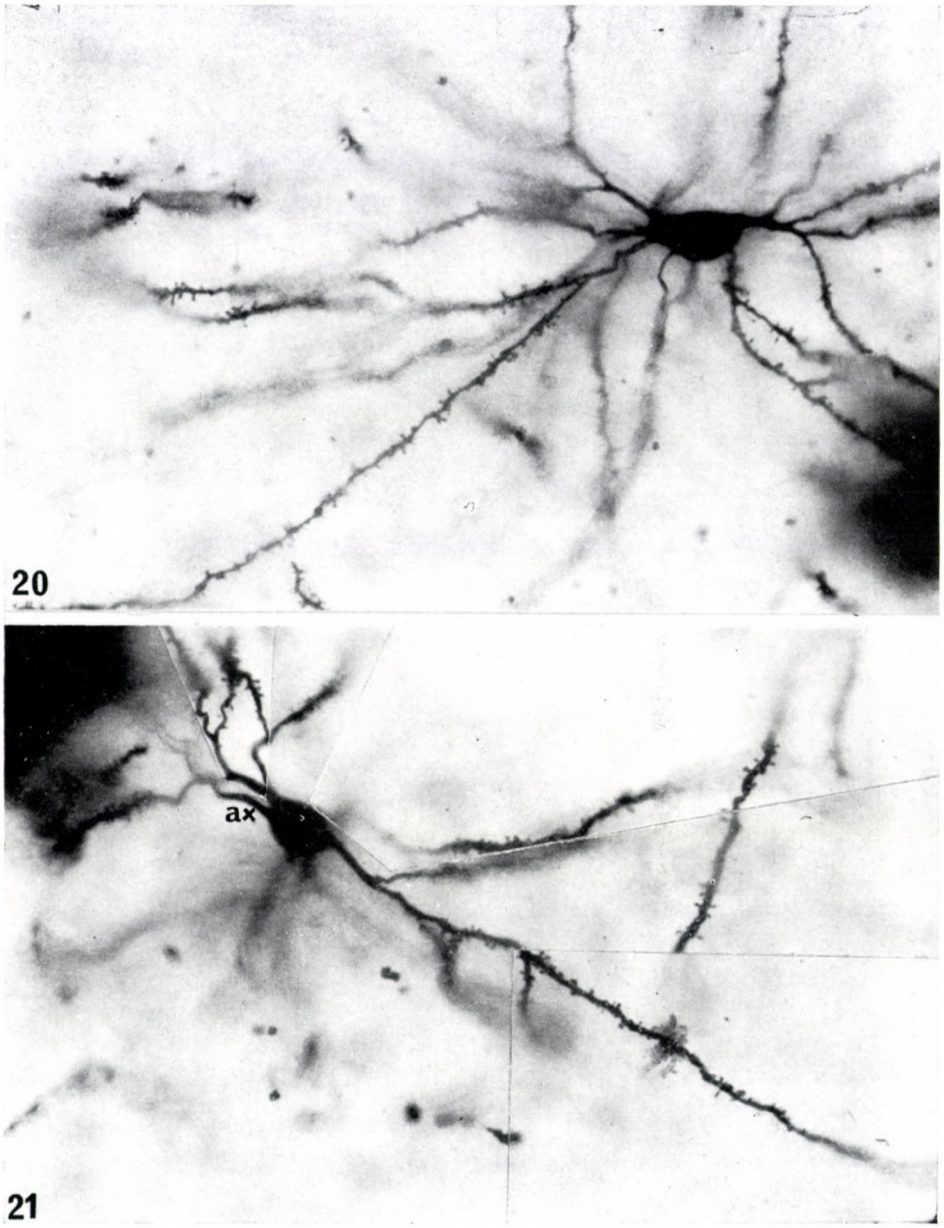


Fig. 18. Medium-size, spiny projective neuron of radiate type dendritic tree in monkey material. 550×

Fig. 19. Medium-size spiny projective neuron of tufted type in monkey material. 550×



Figs 20, 21. Small, spiny dendritic projective neurons in monkey material. 550×

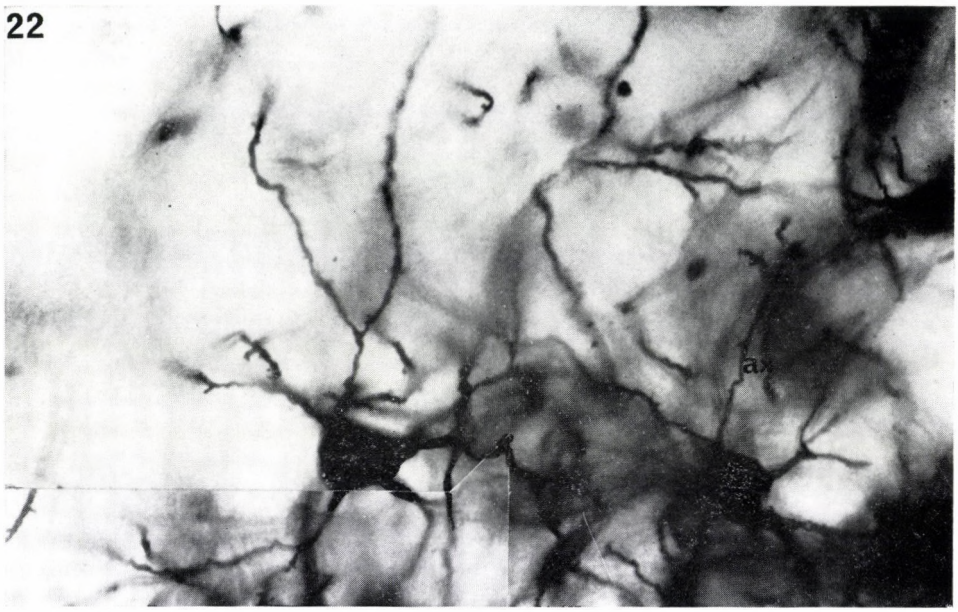


Fig. 22. Medium-size, smooth dendritic neuron with long axon in the monkey nucleus caudatus. 550×

Cell types of the human caudate nucleus

1. *Giant cell.* These cells are 40—50 μ in diameter, ovoid or multiangular in shape, depending on the number of dendrites (Fig. 25). On the basis of the dendritic surface a smooth dendritic and a spiny dendritic sub-type can be distinguished. Only the initial segments of axons are seen. The size of the cell is indicative of an efferent neuron.

2. *Medium-size, spiny dendritic cell.* According to the number and size of dendrites, several sub-types occur: smaller and larger cells (Fig. 26), ovoid, angular, multiangular forms with tufted (Fig. 27) or radiate (Fig. 28) dendritic arborizations. The number of spines on the secondary dendrites varies. The axon possesses collaterals. This cell is likely to be an efferent neuron.

3. *Small, mostly ovoid, cell;* it is occasionally fusiform (Fig. 29). Dendrites originate at the poles and are spiny. The axon having several collaterals arises from the cell. This cell type is one of the principal constituents of the cell population in the nucleus (Fig. 30).

4. *Medium-size, smooth dendiritic, long axonal cell.* It has numerous tortuous and ramifying dendrites with smooth surface but spindle-like sweillings may occur on them. The axon has collaterals (Fig. 31).

5. *Small, ovoid cell with short axon.* This corresponds to a Golgi II-type neuron. The cell body and the dendritic tree is similar to that of the small

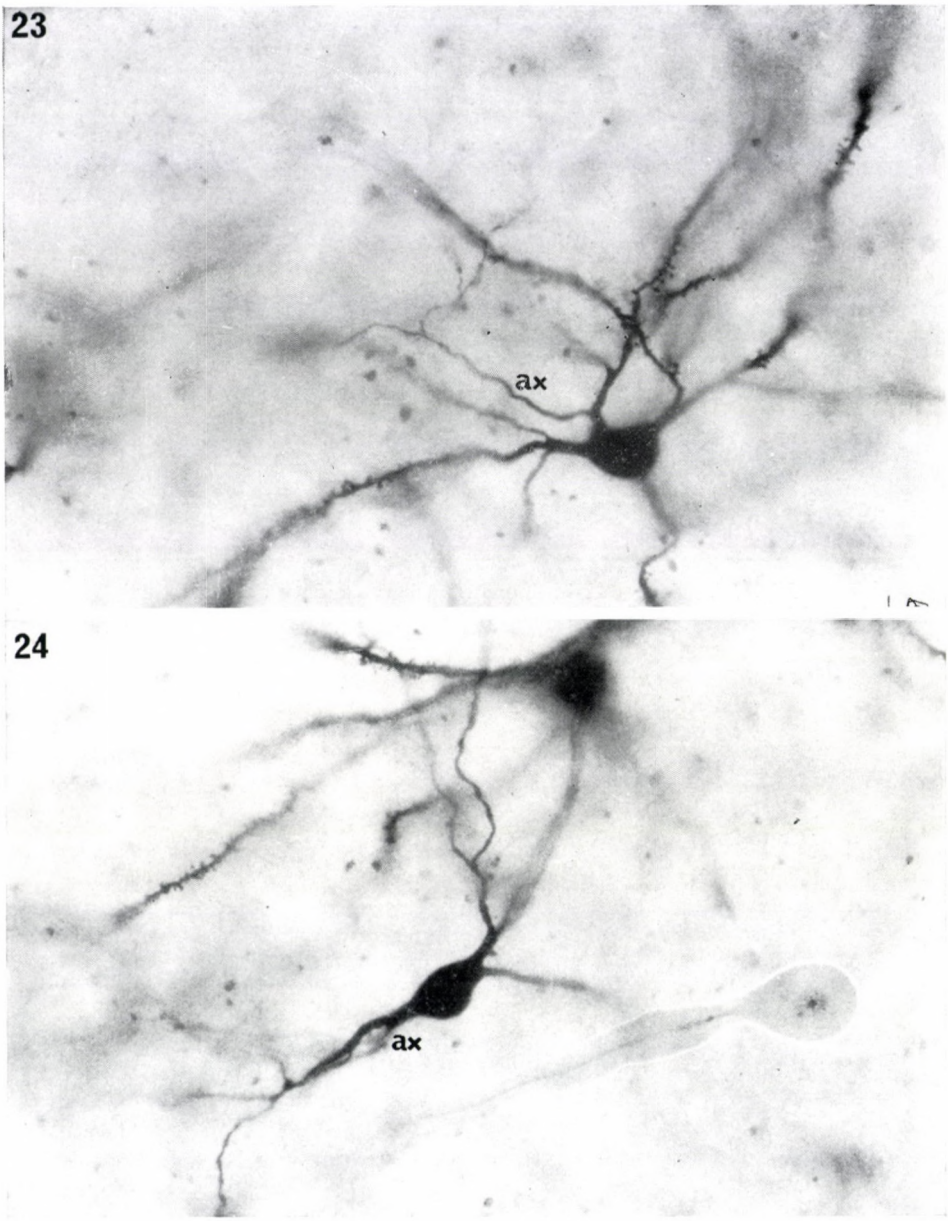


Fig. 23. Small, round, spiny dendritic neuron with short axon in the monkey caudate nucleus. 550×

Fig. 24. Small ovoid, scarcely spiny dendritic, short axonal cell in monkey material. 550×

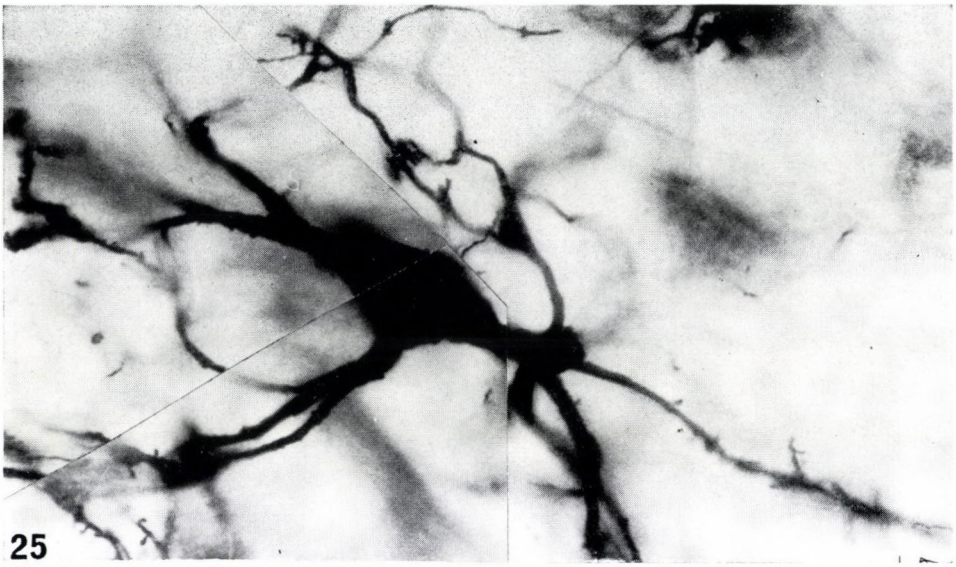
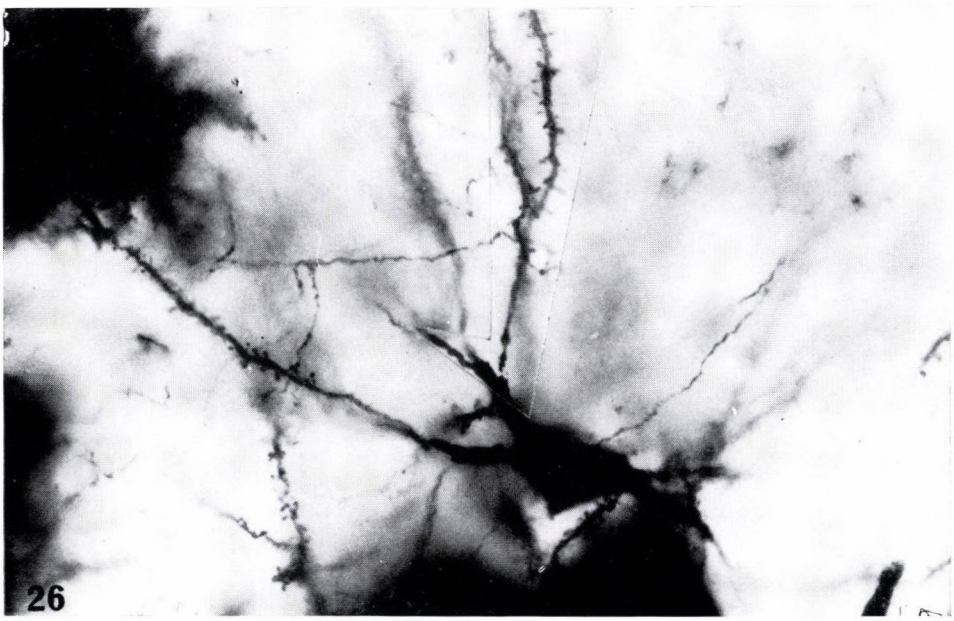
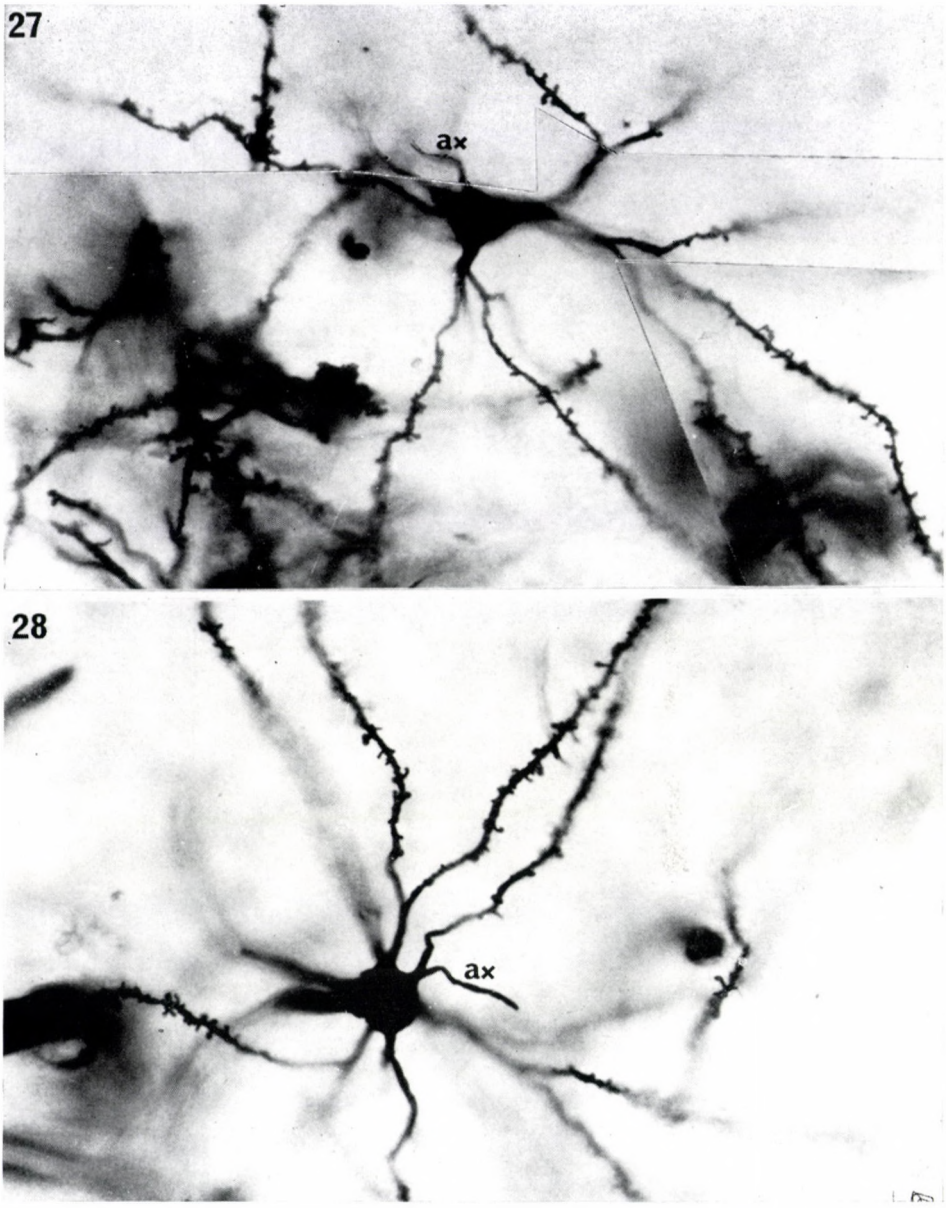


Fig. 25. Giant cell in human nucleus caudatus. 550×

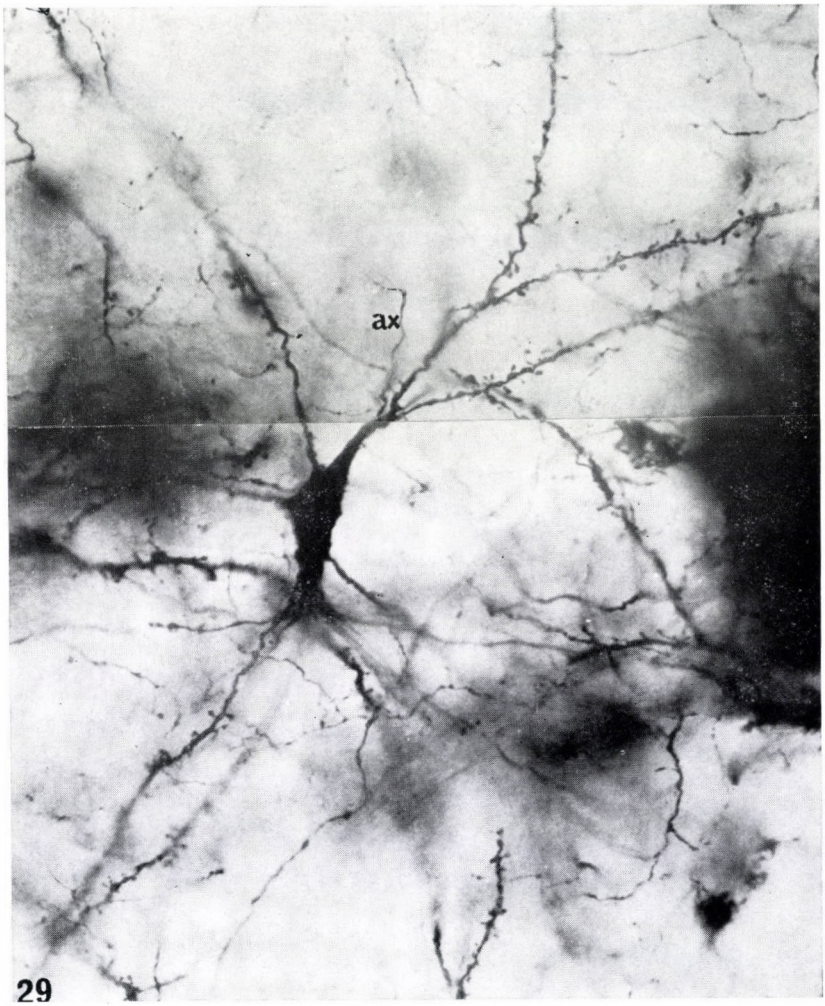


principal neuron. The axon, although thick, does not give off collaterals but divides (Fig. 32). Axonal arborization is like that of a local neuron but the total arborization could not be demonstrated.



Figs 26, 27, 28. Medium-size, spiny projective neurons triangular, tufted and radiate in type in human nucleus caudatus

6. *Small, fusiform neuron with polar dendrites.* On the secondary dendrites some spines are found. The axonal arborization is of a local nature therefore this cell is considered to be a Golgi II-type neuron (Fig. 33).



Figs 29, 30. Small fusiform and ovoid neurons with spiny dendrites and long axon in human caudate nucleus. 550×

7. *Small, round cell with long, sparsely spiny dendrites and local axonal arborization.* The initial segment of dendrites is smooth. After bifurcation a few spines appear. The axon ramifies near the cell body (Fig. 34).

8. *Medium-size, smooth dendritic and short axonal neuron.* The cell body is mostly multiangular due to dendritic shafts. It is similar to the efferent neuron of the same size. On the smooth surface of repeatedly branching dendrites, spindle-shaped swellings occur. The axon is often visible showing an abundant arborization within the dendritic field (Fig. 35). Axonal branches are thin with terminal varicosities.

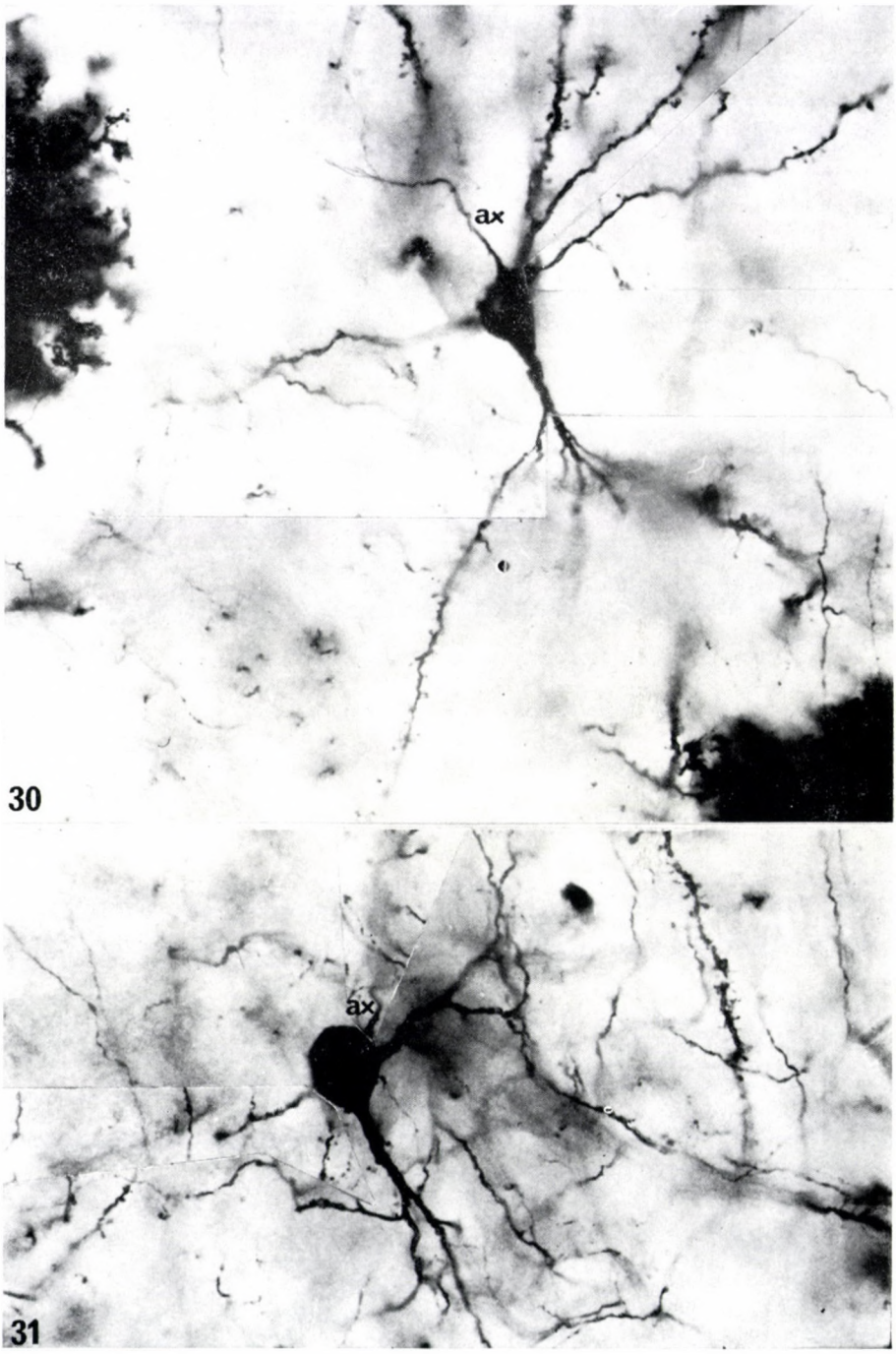


Fig. 31. Medium-size neuron with smooth dendrites and long axon in human caudate nucleus. 550×

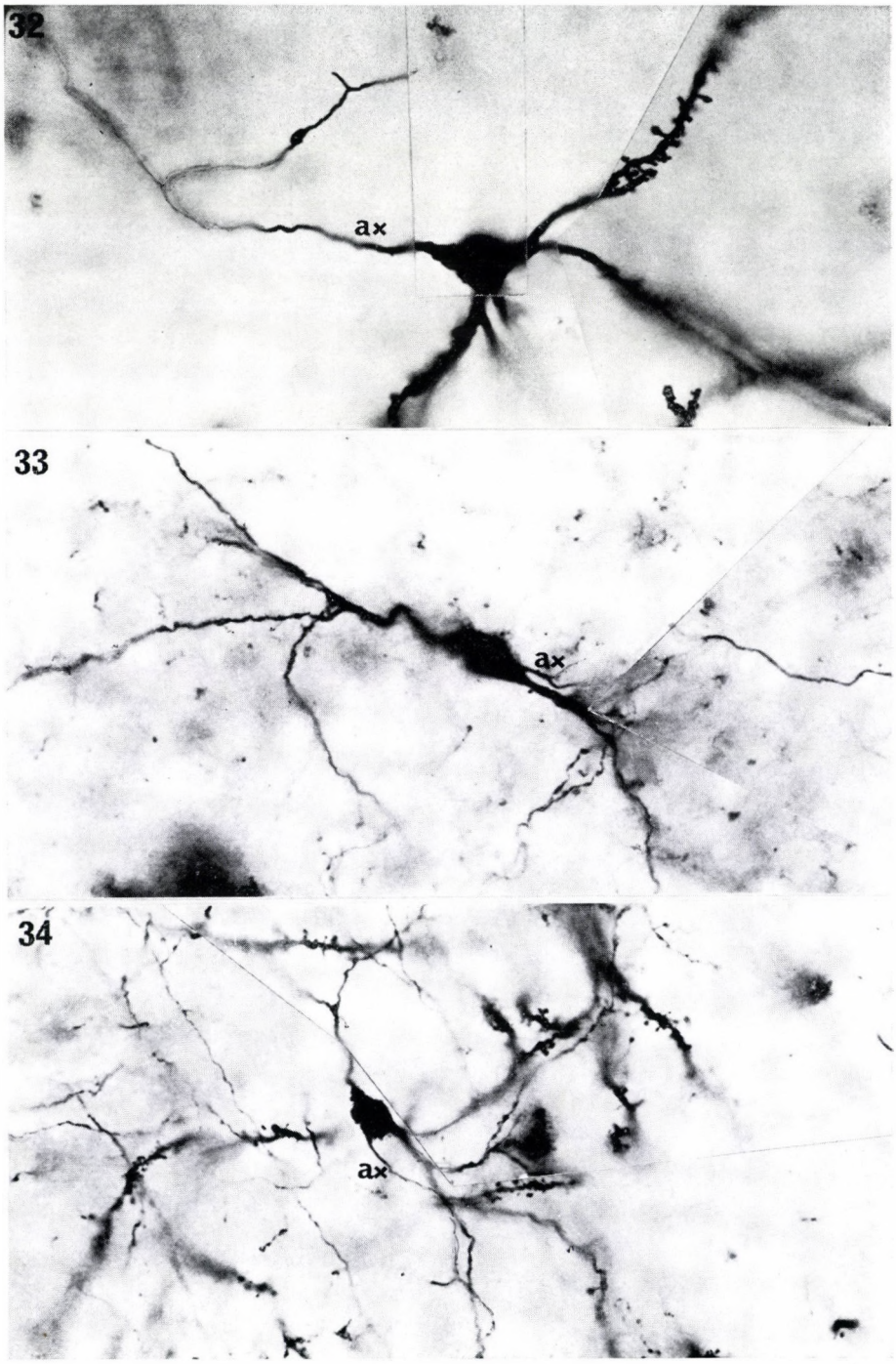


Fig. 32. Small ovoid neuron with spiny dendrites and short axon in human material. 550×
Fig. 33. Small, fusiform neuron, with short axon in human material. 550×
Fig. 34. Small round cell with sparcely spiny dendrites and short axon in human caudate nucleus. 550×

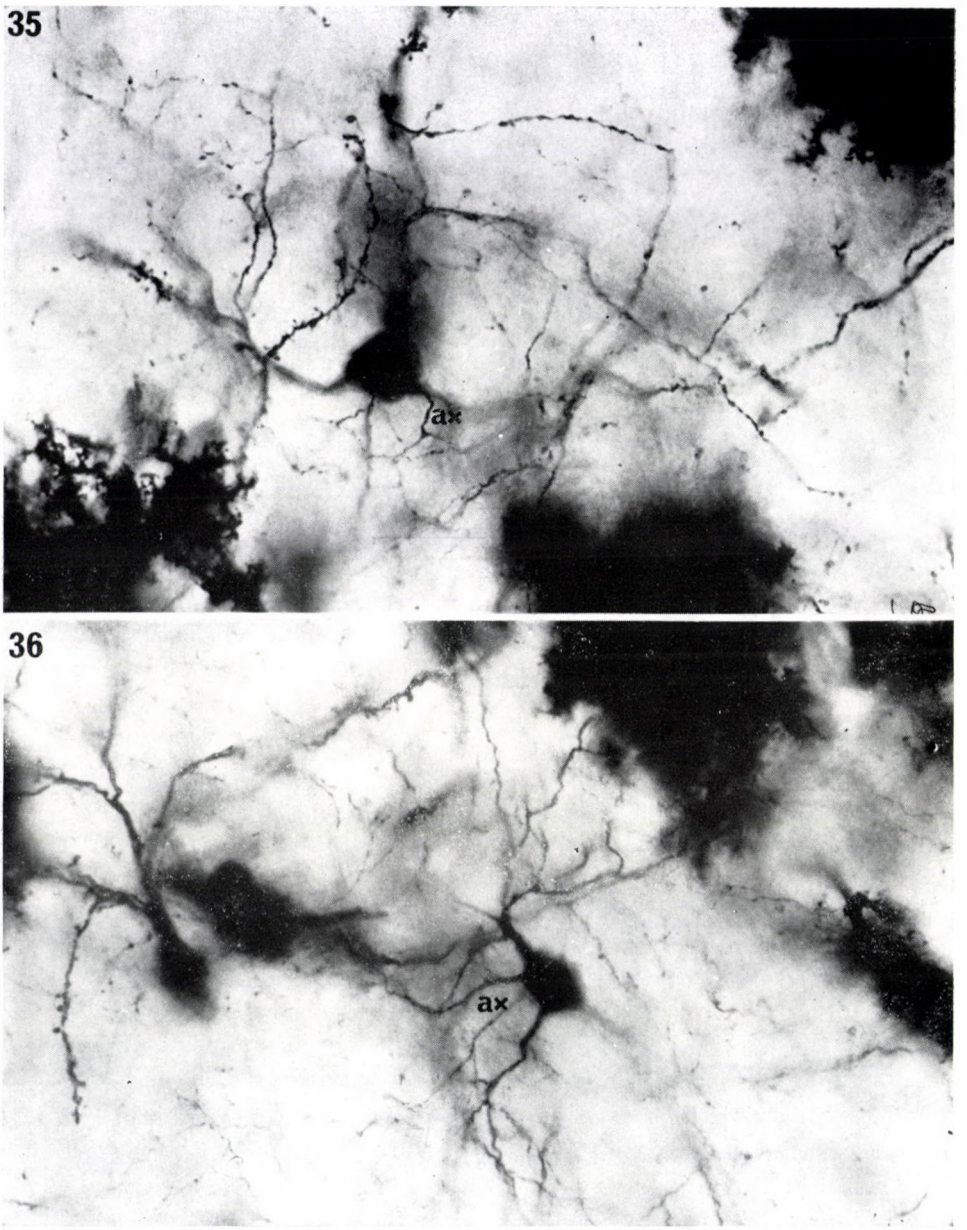


Fig. 35. Medium-size short axonal neuron with smooth dendrites in human material. 550×
Fig. 36. Small ovoid neuron with smooth dendrites and richly arborizing short axon. Human caudate. 550×

9. *Small, ovoid smooth dendritic neuron with short axon.* The arborization and course of dendrites gives the impression of a spider-net. The axon ramifies within the dendritic tree. It probably corresponds to the neurogliform neuron (Fig. 36).

Discussion

As to the classification of the cell types of the caudate nucleus, owing to limitations of our material, and especially of our monkey material, some conclusions are only preliminary.

In the striatum, two types of large neuron are found: one with numerous spiny dendrites and another with a few, smooth dendrites. Both types have long axons [4, 7, 8, 12, 16, 19, 20]. CAJAL [20] called these cells 'giant cells'. Its sub-types are referred to as 'large spiny type II neuron' [4] and 'large neurons with fewer dendrites' [19]. The cell body of these neurons is 30—50 μ in diameter, and fusiform or triangular in shape. They were described by RAFOLS and FOX [19] in the ventral neostriatum and by GROFOVA [9] in the posterior putamen. The latter author using the horseradish peroxidase method has shown that the large efferents of the neostriatum project to the retrorubral nucleus. We could identify this cell type in all four species studied but no preferential localization has been observed. The difference between its sub-types seems not to be significant in terms of function.

The other large neuron type of the caudate nucleus was called 'medium' by CAJAL [20]. He divided the medium-size spiny neurons of the rabbit and human striatum into long and short axonal cells. This was corroborated in dog and human material by LEONTOVICH [16]. FOX et al. [6], however, pointed out that the axon of these cells can not be followed for a distance longer than 100 μ, and this was the reason why a 'short axonal' class was established. Cells seen in their preparations corresponded to those shown by DEJERINE [3]. In the cat, medium-size spiny neurons were found to have either a short or a long axon [13]. These cells too, were classified according to the number of dendritic spines. The term 'medium neuron with many dendritic spines' [19] is used as a synonym for 'spiny type I neuron' [4]. In our material an increasing number of subtypes of medium-size spiny neurons has been found in the species studied.

Among the variety of medium-size cells, the medium smooth dendritic, long axonal type is rather distinct. This cell type was not encountered in the rabbit while it was frequently seen in cat, monkey and human material. This type may correspond to the third long axonal neuron group.

The fourth group of efferent, long axonal cells is constituted by the small, spiny dendritic neurons. It was present in all the species studied. Together with the medium spiny dendritic cells they form the principal cell group of

the caudate nucleus, as it has been described in a number of species [4, 12, 13, 20].

A further group of neurons belongs to the Golgi type II interneuron class. Data concerning this type are rather controversial; in our material, different species contained different forms of this cell.

Medium, aspiny dendritic, short axonal neurons were particularly frequently impregnated in human material whereas they seemed to be absent in the rabbit, and were less frequent in the cat and the monkey.

Small Golgi II-type neurons with few spines and smooth dendrites were present in all the studied species. The small, spiny dendritic fusiform and ovoid, tufted dendritic Golgi II-type neurons were seen in human material only.

CAJAL [20] described a neurogliform neuron in the human striatum. LEONTOVICH [16] could also identify similar short axonal cells. For some time these findings could not be confirmed. FOX et al. [7] studied in detail the problem of neurogliform neurons. In their opinion the oligodendrocytes of the monkey striatum are very similar to neurons but high resolution light microscopy and electron microscopy clearly shows that these cells are attached to myelinated fibres and correspond, according to the del Rio Hortega classification, to type I oligodendrocytes. Recently, however, the existence of a neurogliform neuron has gained new support [4, 13, 19]. We have found spidery type neurogliform neurons in the human caudate nucleus; they differed slightly from the cortical neurogliform neurons.

In conclusion it can be stated that in spite of a limited success in its impregnation, the primate caudate nucleus showed a greater variety of cell types than the rabbit and cat caudate nucleus. This phylogenetic increase in number of neuronal types applies equally for relay- and interneurons. The numerical increase of types of both relay- and interneurons in the phylogenetic series refers to the more and more complicated connections and intrinsic organization which point to the refined function of the caudate nucleus.

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UNTERSUCHUNG DER GOLGI-ARCHITEKTUR IM NUCLEUS CAUDATUS DES GEHIRNS VON KANINCHEN, KATZEN, AFFEN UND MENSCHEN

MÁRIA ÉDER, T. VIZKELETY und T. TÖMBÖL

Die Golgo-Architektur des Nucleus caudatus von Kaninchen, Katzen, Affen und Menschen wurde besonders im Hinblick auf die Zelltypen an Serienschnitten untersucht, die mit der Imprägnation nach Golgi—Kopsch hergestellt wurden.

Die ziemlich unterschiedlichen Neuronen gehören zwei großen Gruppen an: Projektions- oder efferente Neuronen und lokale oder Interneuronen. Die efferenten Neuronen stellen die Verbindung mit entfernten Körperregionen her, wie dies zahlreiche Experimente beweisen. Die Axone dieser efferenten Neuronen entsenden zahlreiche Kollateralen, die an der Ausbildung der lokalen Verbindungen beteiligt sind. Die Interneuronen besitzen lokal verzweigte Axone, und diese Neuronen sind es, die in erster Linie die inneren Verbindungen des Kerns zustande bringen.

Innerhalb beider Gruppen lassen sich zahlreiche Typen und Subtypen unterscheiden. Die Zahl der Typen und Subtypen zeigt in der phylogenetischen Reihe eine zunehmende Tendenz.

ИЗУЧЕНИЕ КЛЕТОК ХВОСТАТОГО ЯДРА В МОЗГЕ КРОЛИКА, КОШКИ, ОБЕЗЬЯНЫ И ЧЕЛОВЕКА

МАРИА ЕДЕР, Т. ВИЗКЕЛЕТИ и Т. ТЁМБЁЛ

Авторами была изучена архитектура Гольджи в хвостатом ядре кролика, кошки, обезьяны и человека с особым вниманием на типы клеток, в серийных срезах, изготовленных импрегнацией по Гольджи – Кропш.

Невроны довольно различного типа относятся к двум большим группам, а именно: в группу проекционных или эфферентных невронов и в группу местных или интерневронов. Как доказывает ряд экспериментальных данных эфферентные невроны создают связь с отдаленными областями тела. Аксоны этих эфферентных невронов отправляют многочисленные колатерали, участвующие в создании местных связей. Интерневроны имеют разветвляющиеся на месте аксоны. Внутренние связи ядра образуются главным образом этими невронами.

В пределах обеих групп можно различать многочисленные подтипы. Число типов и подтипов показывает в генетической линии повышающуюся тенденцию.

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RETINAL PROJECTIONS IN THE ADULT XENOPUS LAEVIS: A STUDY WITH COBALT FILLING TECHNIQUE

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Cobaltic-lysine complex was used to show retinal projections in adult Xenopus laevis. Retinal fibres partially decussate in the chiasma, and terminate on both sides in the nucleus ovoidalis, lateral geniculate complex, pretectal area, optic tectum, and in the basal optic nucleus. Cobalt filled fibres were found in the preoptic area, dorsal hypothalamic area, and in the periventricular region close to the wall of the third ventricle.

Key words

Retinal projections, Xenopus, cobalt-filling

Introduction

Our knowledge on the anatomy of the frog's central visual pathways has recently been summarized [1, 5, 10, 13]. The African Clawed Toad, Xenopus laevis, which is extensively used in experiments concerning the development and physiological properties of the visual system, received little attention from morphologists. Salter [9] described the diencephalic visual centres in normal animals. Using ^{3}H-proline as tracer, Levine [6] gave a brief account of the retinal projections. In larval Xenopus the cobalt filling technique proved to be a useful tool to disclose fine anatomical details of the visual pathways [12]. We obtained similar results in adult Rana esculenta [4], therefore it seemed wort-while applying the technique in adult Xenopus. Our aim was to provide a normal anatomical basis for experimental studies on the course of fibre bundles or even individual axons of retinal origin, and the mode of their termination.

Materials and methods

Thirteen, 1.5—2 years old toads, Xenopus laevis, were used. Under urethan anaesthesia (0.2 g/100 g body weight intraperitoneally) the mucous membrane of the palate was incised in the midline. The optic nerve was exposed and transected close to the eyeball. The central

stump of the nerve was introduced into a small plastic tube filled with a cobaltic solution. The tube was fixed with vaseline.

Preparation of the cobaltic solution. One hundred ml of distilled water containing 4.4 g of L-lysine HCl (Reanal) and 13 g cobaltic oxide (Co_2O_3, Merck) was boiled for 12 hours. The solution was made up to the original volume every hour with distilled water, and after cooling it was filtered through a fine filter paper. Excess water was evaporated at 80° C to reach a final volume of 10 ml, and then the pH was adjusted to 7.0—7.2 with L-lysine HCl.

Postoperative treatment, precipitation of cobalt into insoluble CoS, embedding and sectioning of the brains were the same as described before [4]. Cobaltous-sulphide precipitate was intensified with the physical developer introduced by GALLYAS [2].

Preparation of Gallyas intensifier Stock solution A: 80 g sodium acetate (3 H_2O), 5.6 ml concentrated acetic acid, 1 g silver nitrate, and 10 ml of 1% cetylpiridinium chloride were dissolved in 600 ml distilled water. After 24 hours, the solution was filtered and 60 ml of 1% Triton-X 100 was added to the filtrate. The final volume was made up with distilled water to 800 ml. Stock solution B: 2.5% sodium tungstate. Solution C: 0.2% ascorbic acid, freshly prepared from an ampoule of vitamin C injection. The working solution was prepared immediately before use by mixing 8 parts of solution A, 1 part of solution B, and 1 part of solution C. The duration of intensification was 5—10 min. at room temperature depending on the amount of precipitated CoS.

The intensified precipitate was stabilized in a 0.2% solution of yellow gold chloride (1 min), followed by a 2 min. rinse in 1% sodium thiosulphate. Finally, the sections were washed in distilled water, counterstained with toluidine blue, dehydrated and mounted in Permount.

Results

The optic tracts. The optic nerves enter the ventral wall of the brain just in front of the infundibular recess and form the chiasma. The crossing optic fibres occupy the ventral one third of the diencephalon at the level of the posterior margin of the chiasma (Fig. 1). On the contralateral side, like in other Anuran amphibians, the optic fibres are arranged in three tracts. The marginal optic tract (MOT) covers the lateral wall of the diencephalon (Figs 1, 4) to split at the mesodiencephalic border into a lateral and a medial division which embrace the tectum.

Fibres crossing the midline in the dorsal half of the chiasma form the axial optic tract (AOT). This tract cannot be separated clearly from the marginal tract. We describe here as AOT the fascicles of retinal fibres at the medial part of the optic tract penetrating the lateral forebrain bundle, and partly the lateral geniculate complex (Fig. 1). The AOT can be followed to the pretectal region as a dorsocaudally directed stream of fibres in the lateral diencephalic neuropil (Figs 2, 4).

The basal optic tract (BOT) consists of sparsely scattered fibres originating at the caudal margin of the chiasma, and travelling caudalwards in the hypothalamus. A few fibres bypass the basal optic nucleus (BON), and can be followed into the medulla (Fig. 3). We could not observe branching of these medullary optic fibres, nor the exact site of their termination.

The calibre of fibres falls into the range of 0.5—2.0 μm in the three optic tracts in cobalt preparations.

A large number of retinal fibres do not cross and establish ipsilateral connections. In the MOT, ipsilateral fibres occupy the anterior edge of the tract.

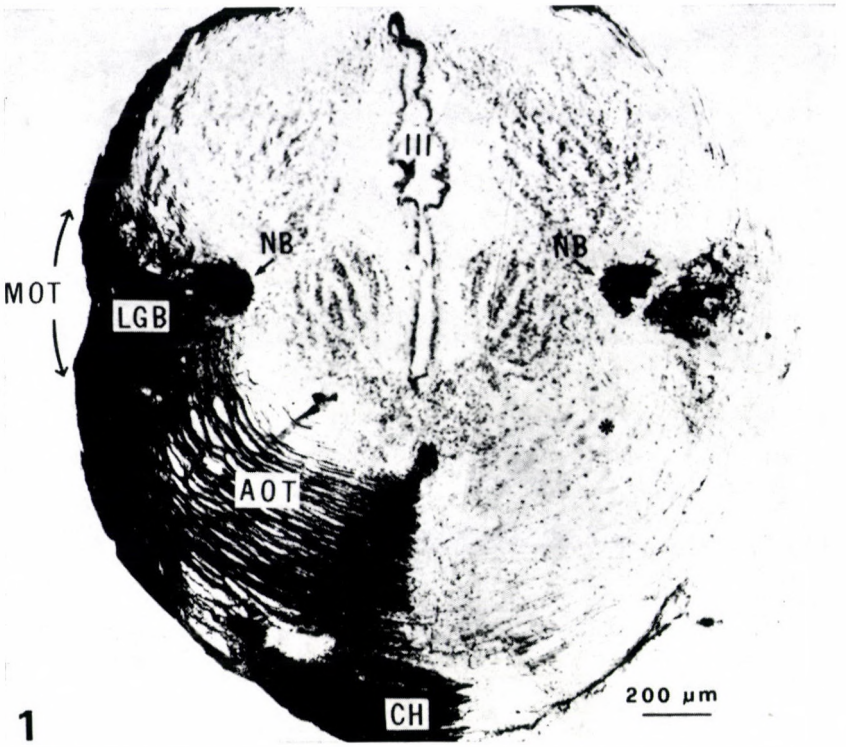


Fig. 1. Cobalt filled retinal fibres in the diencephalon. Cross section through the middle one-third of the lateral geniculate complex. The left side is contralateral to the filled optic nerve. AOT — axial optic tract; CH — chiasma; LGB — lateral geniculate body; MOT — marginal optic tract; NB — neuropil of Bellonci; III — third ventricle; asterisk shows the site of the lateral forebrain bundle

They are scattered throughout the AOT, but only few can be detected in the BOT.

Contralateral projections

The first place where cobalt filled fibres terminate is the nucleus ovoidalis (Fig. 2). This is a small cluster of cells with its long axis tilted slightly rostralwards at the telencephalo-diencephalic border. The retinal fibres enter the nucleus ventrolaterally and surround it with a dense network of thin axons. Only few fibres intrude among the densely packed neurons.

Lateral geniculate complex

The majority of fibres terminate in two neuropils: the neuropil of Bellonci, the dorsal part of the lateral geniculate complex of SALTER [9], and in the later-

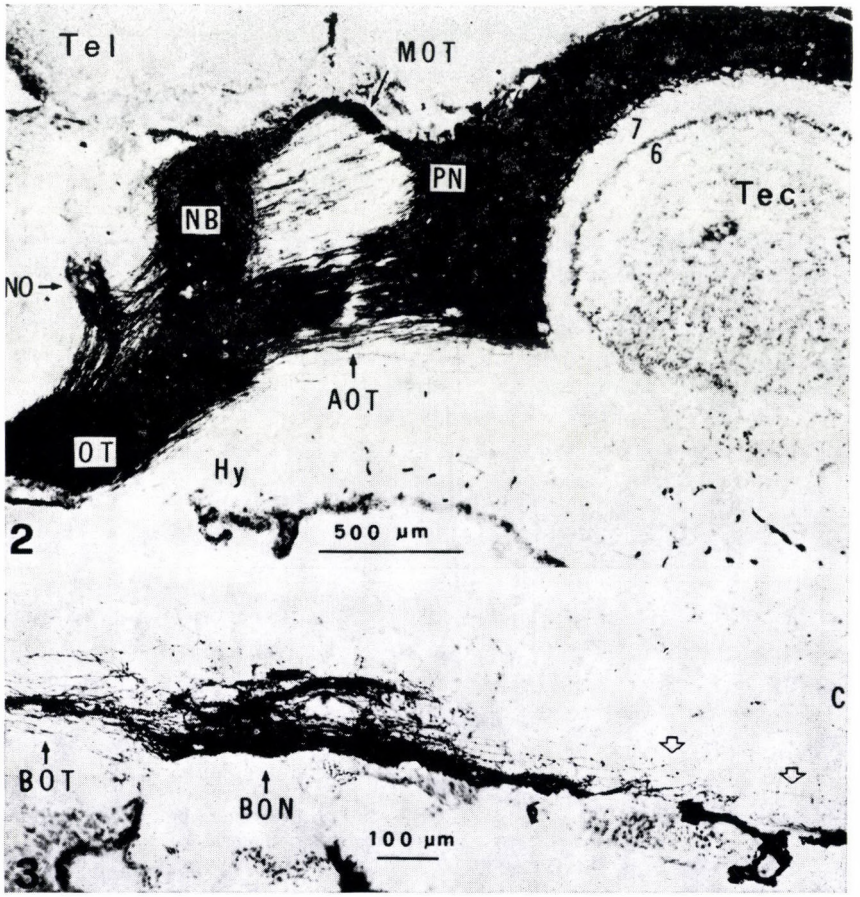
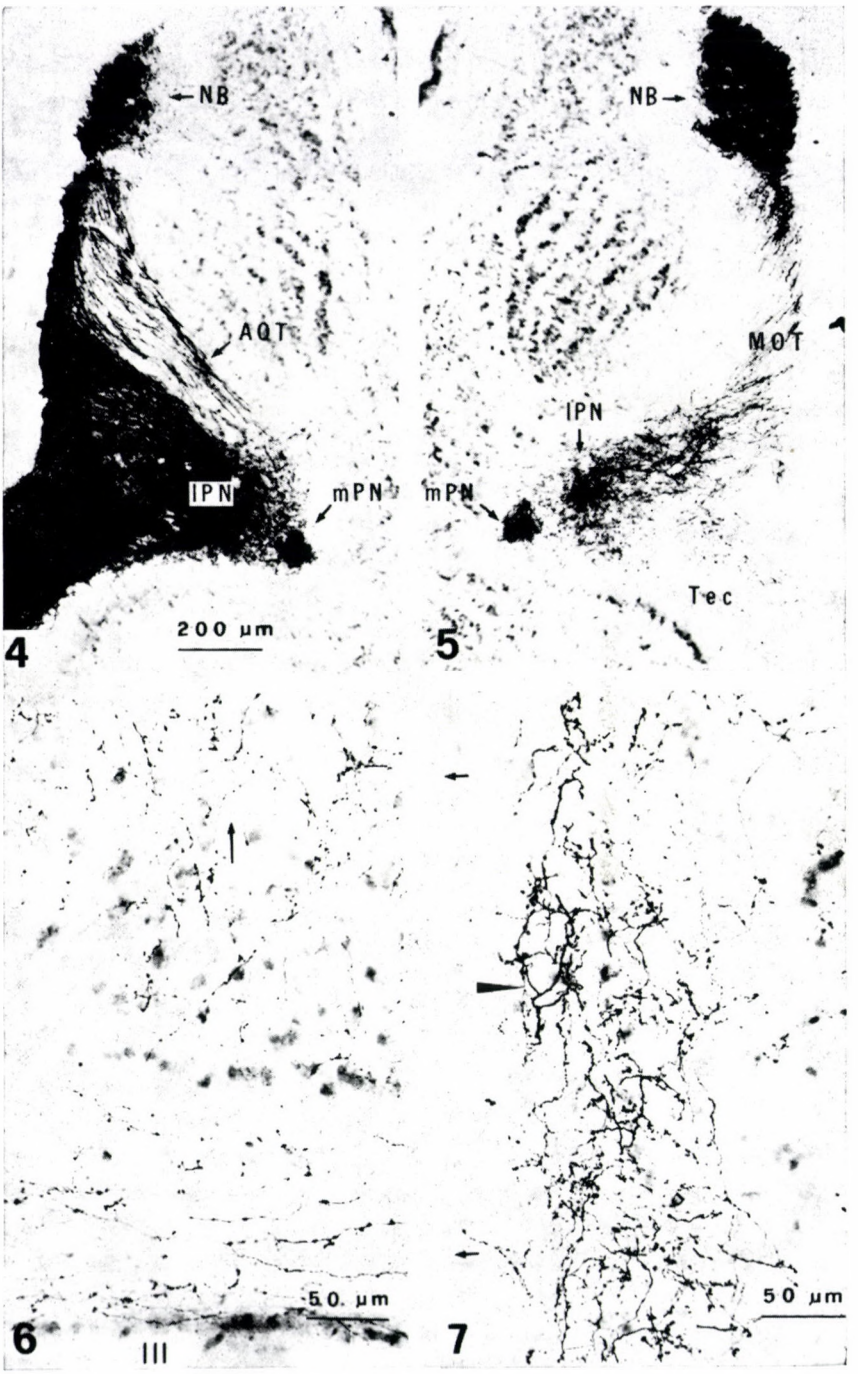


Fig. 2. Cobalt filled retinal fibres in the diencephalon and the optic tectum. Parasagittal section contralateral to the filled optic nerve. AOT — axial optic tract; Hy — hypothalamus; MOT — marginal optic tract; NB — neuropil of Bellonci; NO — nucleus ovoidalis ; OT — optic tract ; PN — pretectal neuropil; Tec — tectum opticum; Tel — telencephalon; 7.6 — tectal layers

Fig. 3. Cobalt filled fibres in the basal optic tract (BOT), and in the basal optic nucleus (BON). Open arrows point to fibres entering the medulla. Parasagittal section. C— caudal

al geniculate body (ventral part of the lateral geniculate complex). The hapes and position of the two neuropils are very similar to that described by SCALIA et al. [11] in Rana pipiens (Fig. 2). Most of the fibres terminating in the dorsal neuropil originate from the AOT, and the ventral neuropil is supplied mainly by the MOT. Besides, a number of very thin, slightly beaded axons can be found among the cells of the lateral geniculate nucleus, nucleus rotundus, posterior entopenduncular nucleus, and in the ventrolateral area.



Figs 4—5. The optic tracts and their termination in the diencephalon, and in the optic tectum (Tec). Horizontal section through the dorsal one-third of the neuropil of Bellonci (NB). Fig. 4. shows the contralateral, Fig. 5. the ipsilateral retinal projections in the same section. AOT — axial optic tract; lPN — lateral pretectal neuropil; mPN — medial prectectal neuropil; MOT — marginal optic tract

Pretectal area

Fibres of the MOT and several fascicles of the AOT terminate in a dorsoventrally oriented cylindrical area, the lateral pretectal neuropil (posterior thalamic nucleus). The optic terminals form a dense network which is traversed by several fibres running towards the optic tectum (Figs 2, 4). At the dorsomedial margin of the lateral neuropil the density of terminating fibres is higher than in other parts of the neuropil. In more ventral levels, this area is separated from the lateral neuropil, and becomes the medial pretectal optic neuropil, the uncinate field of Scalia [10]. This neuropil is supplied with fibres by the AOT (Fig. 4). Several fibres enter the pretectal nucleus and the postrotundal grey (nomenclature according to SALTER [9]), and reach the periventricular fibrous layer (Fig. 6). Occasionally, 1 or 2 cobalt filled fibres could be found in the posterior commissure.

Optic tectum

The rest of the MOT and AOT terminate in the tectum mesencephali. The outermost part of layer 9 is occupied by thinner fibres, and they are less numerous than in the deeper layers. The rest of layer 9 is densely packed with fibres and black granules of silver precipitate (Fig. 2). Three plexuses of thicker fibres separated by two layers of thinner ones can be recognized. The thickest fibres invade layer 8. Below this layer only a few thin fibres appear. Some of them leave this area, and terminate among cells in the periventricular tectal layers.

The basal optic nucleus is an ill-defined group of cells in the anterior half of the tegmentum mesencephali surrounding a neuropil with a few cells in it. Cobalt labelled fibres delineate its borders rather well, but it can hardly be recognized in normal material. Even in cobalt preparations the anterior and posterior ends of the nucleus cannot be determined exactly (Fig. 3). The majority of retinal fibres reaches the BON at its ventral part and bends upwards in loosely spaced fascicles. The terminal branches do not fill the neuropil completely, leaving empty spaces among the fascicles.

Ipsilateral projections

In all neuropil areas where crossed retinal fibres terminate, cobalt filled fibres can be detected also on the ipsilateral side. In the lateral geniculate

Fig. 6. Cobalt filled fibres in the periventricular region. The arrow points toward the protectal neuropil. Horizontal section. III — third ventricle

Fig. 7. Retinal fibres and their terminals in the optic tectum, ipsilateral to the cobalt-filled optic nerve. Horizontal section. Arrows point toward the surface of the tectum. Terminal branches of a thicker fibre are indicated by an arrow-head

body the optic terminals form a relatively loose network, so that individual fibres and their terminals can be observed. Most of the preterminal fibres originating from the MOT are collaterals and those of the AOT seem to be direct fibres. The branching pattern of terminals is similar as that described with the Golgi technique in larval Xenopus [3]. The number of cobalt filled fibres terminating in the neuropil of Bellonci is almost as high as on the contralateral side (Fig. 1).

At the pretectal region, the lateral optic neuropil contains few terminating fibres. The density of retinal fibres in the medial neuropil, however, is comparable to that on the contralateral side (Fig. 5).

In the optic tectum the ipsilateral fibres are distributed throughout the inner half of layer 9. The thinnest fibres give origin to few branches, while the thick fibres have a well-defined terminal arbor (Fig. 7). The exact size of these terminals could not be measured, but their length is usually over 80 μm.

The number of cobalt filled fibres in the BON is negligibly small.

Hypothalamic and periventricular terminals

In the preoptic and the dorsal hypothalamic area, a few, very thin beaded axons arborize among the perikarya. Their number is higher on the contralateral side. From the dorsal hypothalamic area similar fibres can be followed to the periventricular neuropil. Here they travel caudalwards, parallel with the lateral wall of the third ventricle. At the level of the lateral geniculate complex and the pretectal area, these fibres intermingle with other ones distributed among the periventricular cellular layers. A few cobalt filled fibres continue their way caudalwards and reach midtegmental levels. Here and then a few branches originate. The branches either keep their direction or bend towards the laterally located layers of neurons (Fig. 6). Cobalt labelled neurons were not found in those parts of the brain where retinal fibres terminate.

Discussion

In a recent study [4] cobaltous-lysine complex was applied to show retinal projections in Rana esculenta. In the present investigations the cobalt technique was further modified. The cobaltous-lysine complex was replaced by cobaltic lysine complex. This compound remained stable for several months, and the intensity of filling was higher than with cobaltous-lysine. The use of the new Gallyas developer improved the quality of the preparations. Even very thin cobalt containing fibres could be detected, and they appeared against a colourless background. The developer is insensitive to light, and the time to reach sufficient intensification is less than 10 min. The short treatment in gold

chloride and in sodium thiosulphate prevented a diffusion of the precipitate, which occurred when the sections were not gold toned.

Our observations on the contralateral retinal projections in Xenopus are in good accordance with those reported by LEVINE [6]. We found, however optic fibres not only in neuropils, but also among perikarya in the periventricular cellular layers. Autoradiography is apparently not capable of showing these fine sparsely distributed fibres. In the contralateral BON we found projections heavier than those described by Levine, and a few fibres could be followed from this nucleus to the level of the emergence of the trigeminal nerve. STEEDMAN et al. [12] described these fibres in larval Xenopus. Fibres of similar destination were mentioned by NOMOKONOVA [7] in Rana temporaria.

Ipsilaterally we found in the diencephalon more fibres than did LEVINE [6], and succeeded in showing the ipsilateral retinotectal projection.

Generally, retinal projections in Xenopus correspond well to those in Rana esculenta as revealed by the cobalt filling technique. Minor differences, however, could be observed. In Xenopus, heavy bilateral projections were found in the nucleus ovoidalis [6]. Reinvestigating our Rana preparations, we found a few fibres terminating in a group of cells just in front of the lateral geniculate body. These cells might correspond to the nucleus ovoidalis (LÁZÁR, unpublished observation). In Xenopus, the ipsilateral retino-geniculate projection is heavier than in R. esculenta.

In the optic tectum, the arrangement of fibrous layers is similar to that in Ranidae. The layers which may correspond to POTTER's [8] layers A, B, C, D, E and F can be recognized. Below layer 8, only a few fibres can be found and thus Potter's layer G is virtually absent. Layer 8, however, is invaded by thick fibres, which intermingle with similar ones terminating in the depth of layer 9. We suppose that the fibres in layer 8 are analogous to the fibres of layer G in the frog. Electrophysiological analysis is required to verify this supposed analogy.

The calibre of fibres in cobalt preparations falls into the range determined by WILSON [14] for adult Xenopus. We could, however, measure only those fibres which do not form compact bundles, and their number is only a fraction of the whole. The thinnest fibres had a diameter of 0.5 μm. This may mean that fibres thinner than 0.5 μm were not filled, or else the real thickness is less than 0.5 μm but they were thickened by silver precipitate during intensification. Recently, we could demonstrate in Rana (LÁZÁR, unpublished observation) that thin unmyelinated optic fibres could be filled with cobalt. This may be the case in Xenopus, too.

A remarkable finding was the presence of cobalt-labelled fibres in the periventricular neuropil in the diencephalon. We do not know whether these fibres are the axons of retinal ganglionic cells, or belong to another system,

and had been labelled accidentally by diffusion of the cobaltic solution into the hypothalamus. The intensity of label in these fibres suggest that they might have been filled directly together with other axons of the optic nerve. If they are retinal fibres, they may have some special, yet unknown function. Conversely, they may be stray optic fibres, which did not find the target area during development, and have no definite function. Further studies are required to establish their role in the function of the visual system.

Addandum: After the manuscript of the present paper was submitted for publication, R. L. LEVINE described the retinal projections of Xenopus in detail (An autoradiographic study of the retinal projections in Xenopus laevis with comparisons to Rana, J. comp. Neurol., **189,** 1—29, 1980). His results are similar to ours, but he did not report periventricular projections.

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DIE RETINALEN PROJEKTIONEN BEI ADULTEN XENOPUS LAEVIS: UNTERSUCHUNGEN MIT KOBALT-AUFFÜLLUNG

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Zum Nachweis der bei Xenopus laevis aus den retinalen Ganglienzellen entspringenden Fasern und deren Endungen wurde ein Kobalt-Lysin-Komplex angewandt. Die retinalen Fasern kreutzen sich partiell im Chiasma opticum und endeten an beiden Seiten im Nucleus

ovoidalis, im Complexus corpus geniculatum laterale, im prätektalen Bereich, im Tectum opticum sowie im basalen Opticus-Kern. Die mit Kobalt gefüllten Fasern kamen auch im präoptischen Gebiet, in der Area dorsalis hypothalamica und im periventrikulären Bezirk sowie auch in der Nähe der 3. Gehirnkammerwand vor.

РЕТИНАЛЬНЫЕ ПРОЕКЦИИ У ВЗРОСЛОГО XENOPUS LAEVIS ИЗУЧЕНИЕ С МЕТОДОМ КОБАЛЬТОГО НАПОЛНЕНИЯ

П. ТОТ, Д. ЛАЗАР и Т. ГЁРЧ

Для выявления волокон, исходящих из ретинальных ганглионных клеток Xenopus laevis и их окончаний авторы применили комплексное соединение кобальт-лизин. Ретинальные волокна частично перекрещиваются в перекресте зрительных нервов и оканчиваются на обеих сторонах в nucleus ovoidalis, в комплексе corpus geniculatum laterale, в претектальной области, в зрительном покрове и в базальном ядре зрительного нерва. Волокна, наполненные кобальтом, наблюдались в предоптической области, в дорсальном гипоталамическом поле и в перивентрикулярной области, вблизи стенки третьего желудочка мозга.

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FUNCTIONAL STRUCTURE IN LYMPHOEPITHELIAL COOPERATION*

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With the help of electron microscopic investigations the morphological and functional connection of the epithelial and lymphoid elements was examined in the lymphoepithelial organs (thymus, bursa of Fabricius, tonsils, Peyer's patches, gland of Harder). In all these organs special receptor cells were demonstrated, which may play a role in the uptake and transfer of the antigen. In the cytoplasm they all contain characteristic flattened vacuoles, with walls similar to the structure of the cell membrane. Formation of these bodies is identified with the striated bodies of micropinocytosis vermiformis and it was observed that the endocytosis start with a disintegration of the cell membrane which enters with the absorbed antigen in characteristic form into the cytoplasm of the epithel cell and induces the process of cellular immunity. Besides the function of lacrimal secretion the Harder's gland, as a lymphoepithelial organ is full of plasma cells, which constitutes the main lymphoid elements of this organ.

In an attempt to classify the lymphoid organs according to their structure, their cellular content carefully must be studied to reach a better understanding of their identical of different function. When investigating the cellular composition of these organs, we have found that only the percentual combination of the mature or immature lymphoid cells or their subpopulations show any significant change [19]. A striking difference is that in one group of these organs, in addition to the lymphoid cells, epithelial cells also take part in their construction. Therefore these lymphoid organs may be classified as lymphoepithelial organs. These are of course the thymus, the bursa of Fabricius of birds, the intestinal lymphoid organs, Peyer's patches, the vermiform process and, finally, the coecal tonsil and gland of Harder in birds.

These organs may be divided into three groups. In the first group the epithelium is found on the surface, as in the case of the tonsils; in the second group the epithelium lies within the parenchyma but does not reach the surface, as in the thymus; and in the third group the organ is covered by epithelium, but some epithelial tissue is found also inside the organs, as in the bursa of Fabricius, and gland of Harder.

Each of these epithelia are of entodermal origin and glandular in character. Each represent functionally similar but morphologically different cell

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types, being active as a receptor cell in the uptake and transmission of antigen, initiating immunobiological events.

Among the lymphoepithelial organs, the thymus is the only one that has lost its connection with the endoderm, thus [15, 24] antigen can reach the binding and recognition site only via the circulation. Thymic epithelial cells produce the so called thymic factors [4, 14] and secrete them into the circulation [4]. The antigen receptor and transferring cell is located in the thymic medulla at the cortico-medullary border [18, 28]. The scheme of the cell is seen in Fig. 1. The stretched cell body is formed by large gyrose lobes containing some structure-poor ectoplasm and highly structured, centrally located endoplasm with a cytocentre and radially placed microtubuli.

The variegated nucleus is excentric. Scattered among the tubuli are the tonofibrils, Golgi's apparatus and some characteristic granules of these cells which based on granular size, may be grouped into cells A and B the last

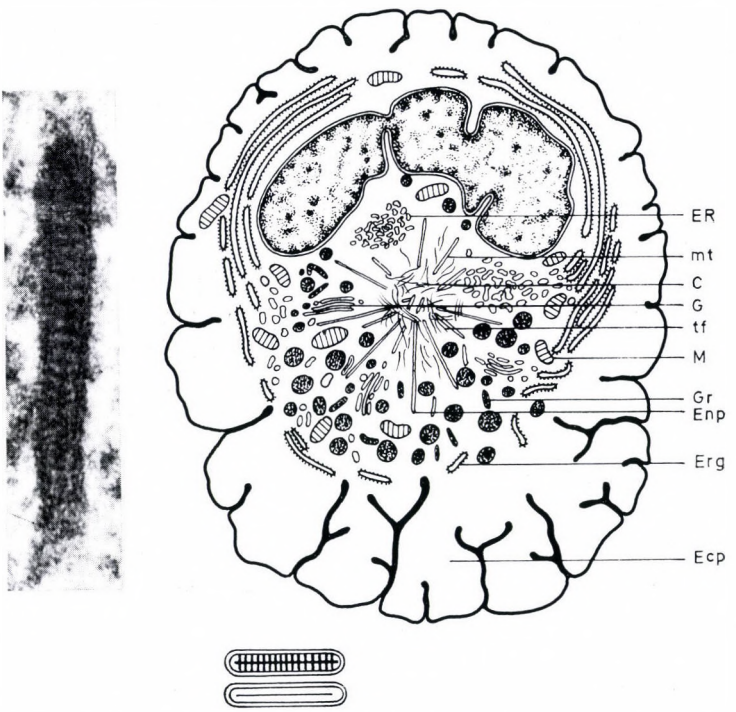


Fig. 1. Structure of the special cells. The endoplasm (Enp) is rich, the ectoplasm (Ecp) poor in organelles. The centriole (C) in the centre of the endoplasm is radially surrounded by microtubules (mt), tonofibrils (tf). Golgi apparatus (G) and characteristic granules (Gr). The element of the smooth endoplasmic reticulum (ER) usually forms groups. The ectoplasm is indented by gyri of the cell-membrane. High-power magnification on the left: striated body (sb) under 151.800 magnification. Unit membrane of 90 Å thickness on the surface of a striated body and in its interior, a system of transverse lamellae about 100 Å periodicity. The intercellular space is filled with a substance inferior in density to that of the granules

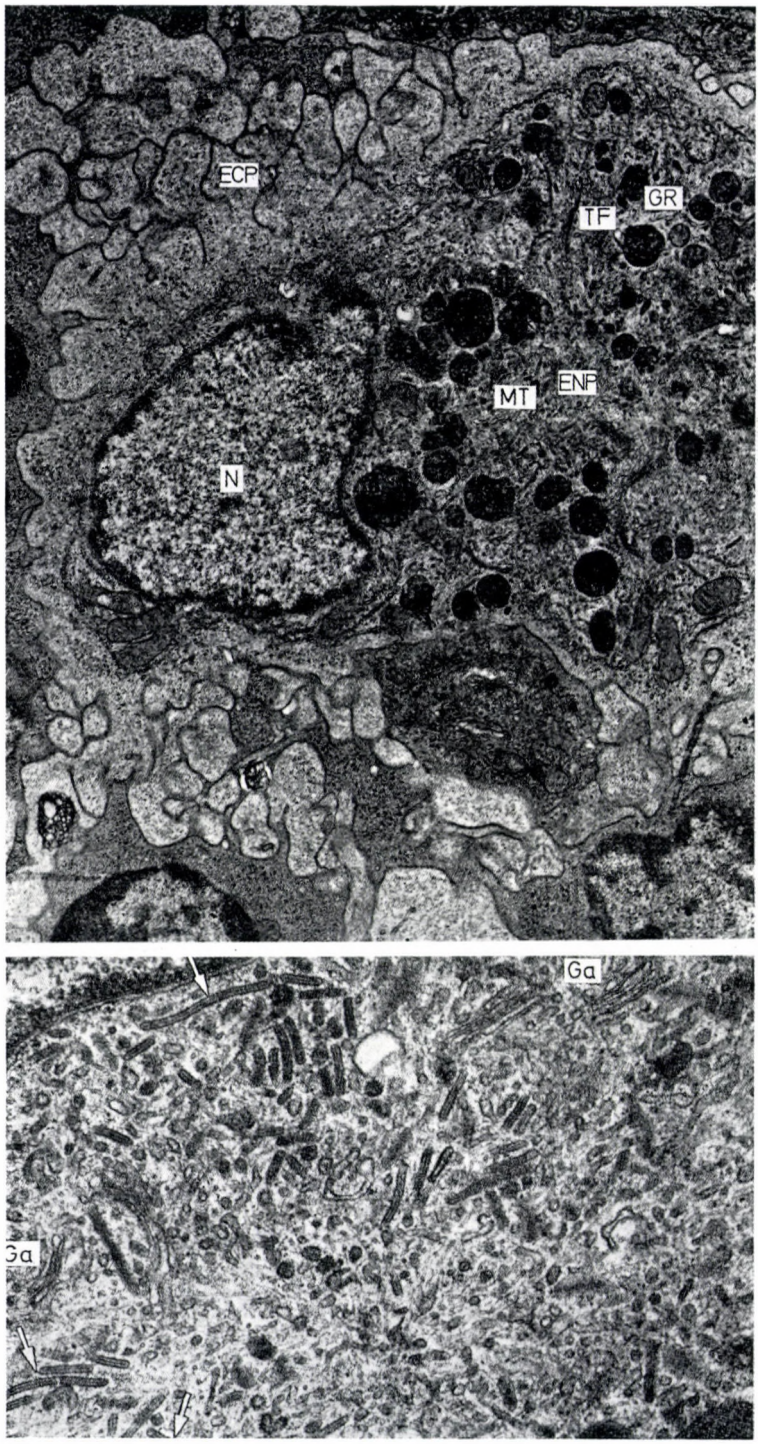


Fig. Ia. The gyrose light ectoplasm of a B cell, in the form of a cauliflower is sharply outlined (secretion), surrounding the endoplasm and the granules. N = nucleus, GR = granules, ECP = = ectoplasm, ENP = endoplasm with organelles, TF = tonofibrils, MT = microtubules. Magnification: ×12.200

Fig. Ib. Part of cytoplasm in the special cell under higher magnification. The arrows indicate striated bodies. Ga = Golgi apparatus. Magnification: ×41.000

group has larger granules and frequently contains tennis-racket-like bodies, reaching 0.5 μ in leagth and 400—500 Å in diameter with a 100 Å wide, darkly coloured stripe running through the centre. The striated bodies show numerous cross streaks in rhythmic intervals.

Table I. Fig. I/b. shows a transmission electronmicrograph of the cell with the details mentioned above. Figure shows part of that B cell under higher magnification. We see many granules of moderate density. From with end of the rough endoplasmic reticulum some smooth walled vesicles, the secretion is separated. They touch the cell membrane and subsequently release the humoral thymic factor into the intercellular space. The striated bodies resemble Bierbek's granules [28]. The striated bodies are surrounded by a 90 Å unit membrane. The transverse lamelle found within the bodies revealed a 100 Å periodicity.

Table I. Fig. I/a. exhibits a clear and finely granulated filamentous field, characteristic of gyrose ectoplasm. The localization of these cells indicate that they are identical with the thymosin containing cells identified by rabbit antithymosin [17]. It is most probably that thymosin is produced by these cells [4]; the secretory process may be observed in monolayer tissue cultures of human and guinea pig thymic epithelial cells.

Table II. Fig. I/a. showes a secreting epithelial cell from the monolayer tissue culture of a five month old human fetus.

The expected cooperation of the epithelium and the lymphoid cells [31] is demonstrated in tissue culture [25, 26, 30], forming a rosette-like cellular aggregation and embracing either an epithelial cell or a macrophage in the centre encircled by thymocytes (Table II. Fig. II/b). The cooperation of lymphoid and epithelial cells manifests the phenomenon called peri- and emperipolesis [29] shown in Table II. Figs II/c, d. In the rat thymocyte tissue culture, cellnests are formed. These nests or cell pockets have some epithelial cells in their centre that are tightly surrounded by thymocytes. In monolayer cell cultures this appears as follows. Around and within the epithelial cells vivid thymocytes are seen and the thymocytes are enclosed in a vacuole but are able to set themselves free from the cell (Table II. Fig. II/c). Similar emperipolesis is shown in Table II. Fig. II/d, where the mouse epithelium is loaded with some living and some destroyed thymocytes.

Fig. 2. Displays the electron micrograph of an emperipolesis. The intact thymocyte is well visible within the cytoplasm of the epithelial cell. Such tight symbiosis between the epithelial and lymphoid cells may play a significant role in the function of the thymus [27].

A schematic-structural picture of the bursa of Fabricius or rectal thymus is shown in Fig. 3. The bursa is covered by a characteristic layer of epithelium [6, 7] that continues into the medullar epithelial reticulum [10, 11, 13]. The surface epithelium of the bursa may be divided into two main layers, the

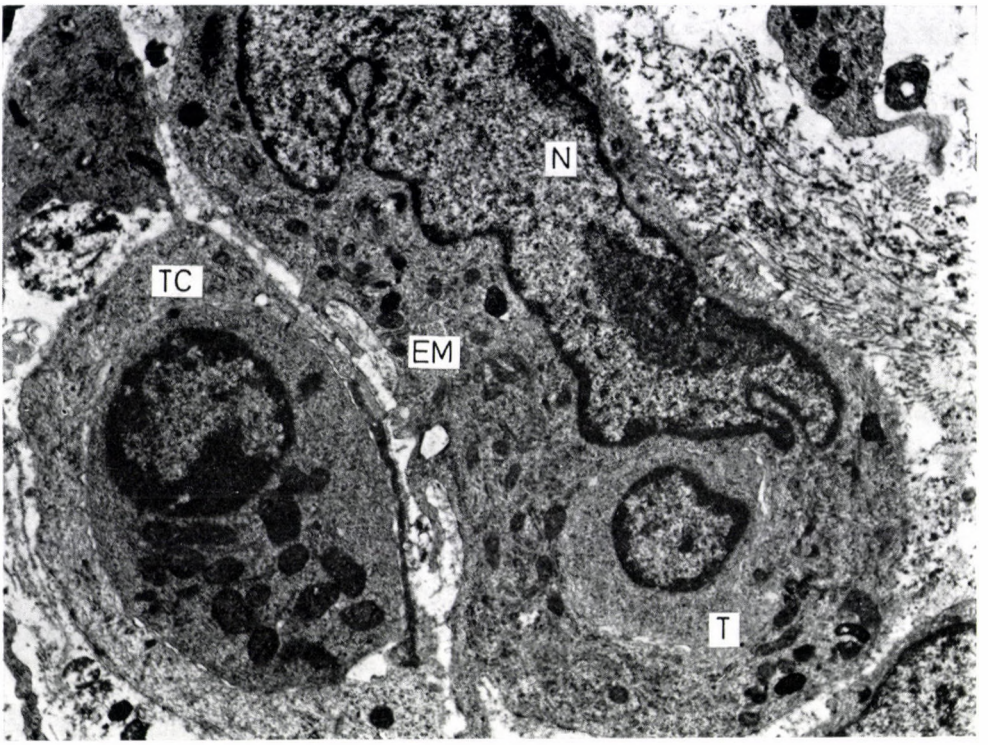
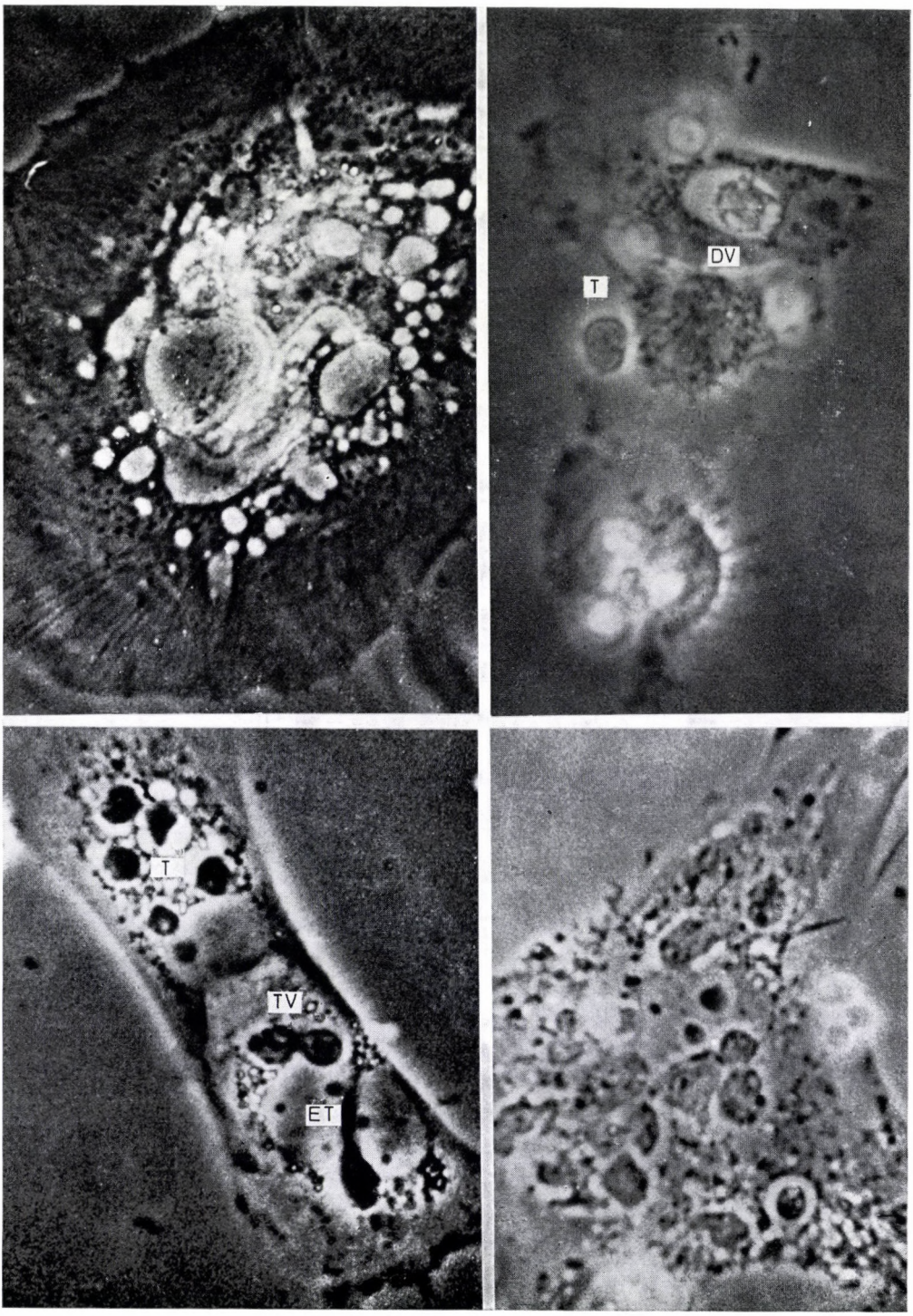


Fig. 2. Emperipolesis in tissue culture of a human fetal thymus. T = thymocyte, EM = = epithelial macrophage, N = nucleus, TC = transitorial cell

suprafollicular and the interfollicular epithelium. The epithelium is arranged on the basal membrane at the cortico-medullary boundary (Table III. Fig. III/a) through which the migration of lymphoid cells between cortex and medulla takes place [19].

The interfollicular epithelium is glandular in type while the suprafollicular epithelium is the receptor cell (Table III. Fig. III/b) in which, numerous vesicles, are seen containing medium dense material. The role of these epithelial cells is best demonstrated by the fact that if one places a few drops of India ink or ferritin onto the bursal surface, some minutes later the carbon granules adhere, thus making it possible to count the folliculi (Table III. Fig. III/c). Somewhat later these granules are found deeper intercellularly.

The structure of the palatine tonsils is shown in Fig. IV. The expected structural appearance of the stratified epithelium covering the tonsils is blurred by the lymphoid and plasma cells and macrophages embedded within the passages of the reticular area [20]. The passages found in the epithelium perforate the basal membrane and continue in the interfollicular channels of the tonsillar parenchyma, while on the surface the aperture of the passage is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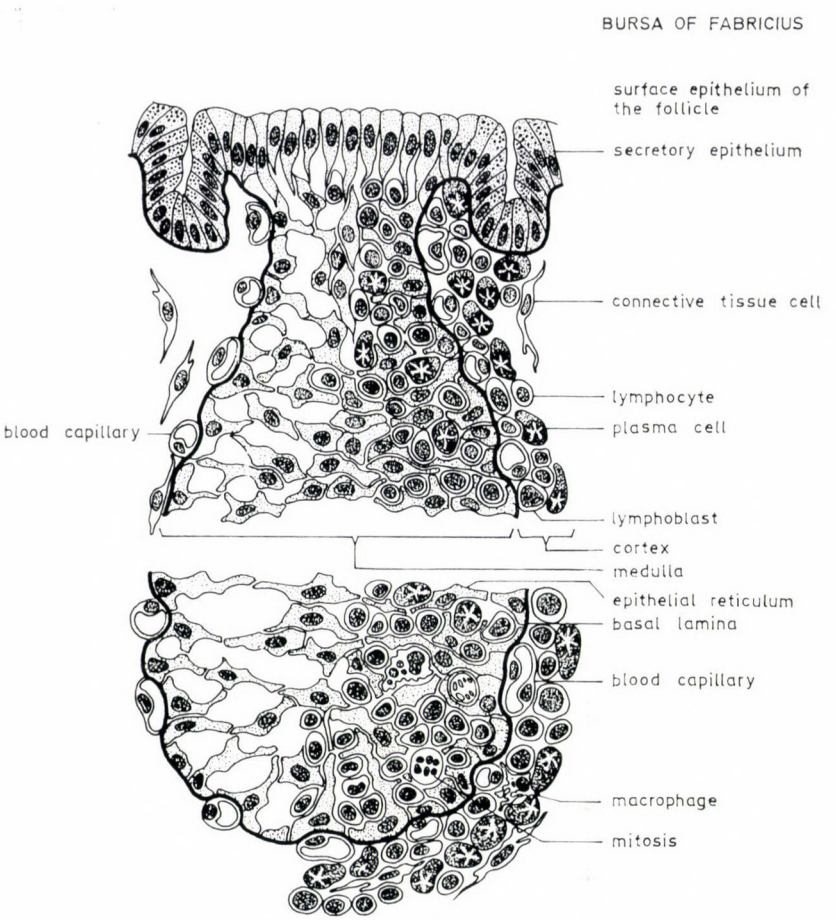


Fig. 3. Scheme of the bursa of Fabricius. Lymphoid cells have been omitted on the left in order to show the framework of the organ

Table II.

Fig. IIa. Secreting epithelial cell from the monolayer thymus culture of a five months old human fetus. Confluent secretion granules filling the cell body

Fig. IIb. Peri- and emperipolesis in the thymus culture of a five month old human fetus. The thymocytes are partly attached to membranes of the reticulum cells. They may enter into the interior of the cell, where they arrive at the digesting vacuole. T = thymocyte, DV = = digesting vacuole

Fig. IIc. Emperipolesis from the monolayer thymus culture of a human fetus. Microcinematographic picture. On the left pole of the flattened epithelial cells dead thymocytes (T), while on their right pole an elongated thymocyte (ET) located intracellularly and forming an eight (TV) in the centre of the cytoplasm. The indentation of the eight is caused by the vacuole membrane just perforated by the cell leaving the vacuole

Fig. IId. Monolayer mouse thymus culture. Epithelial cell full of living and dead thymocytes

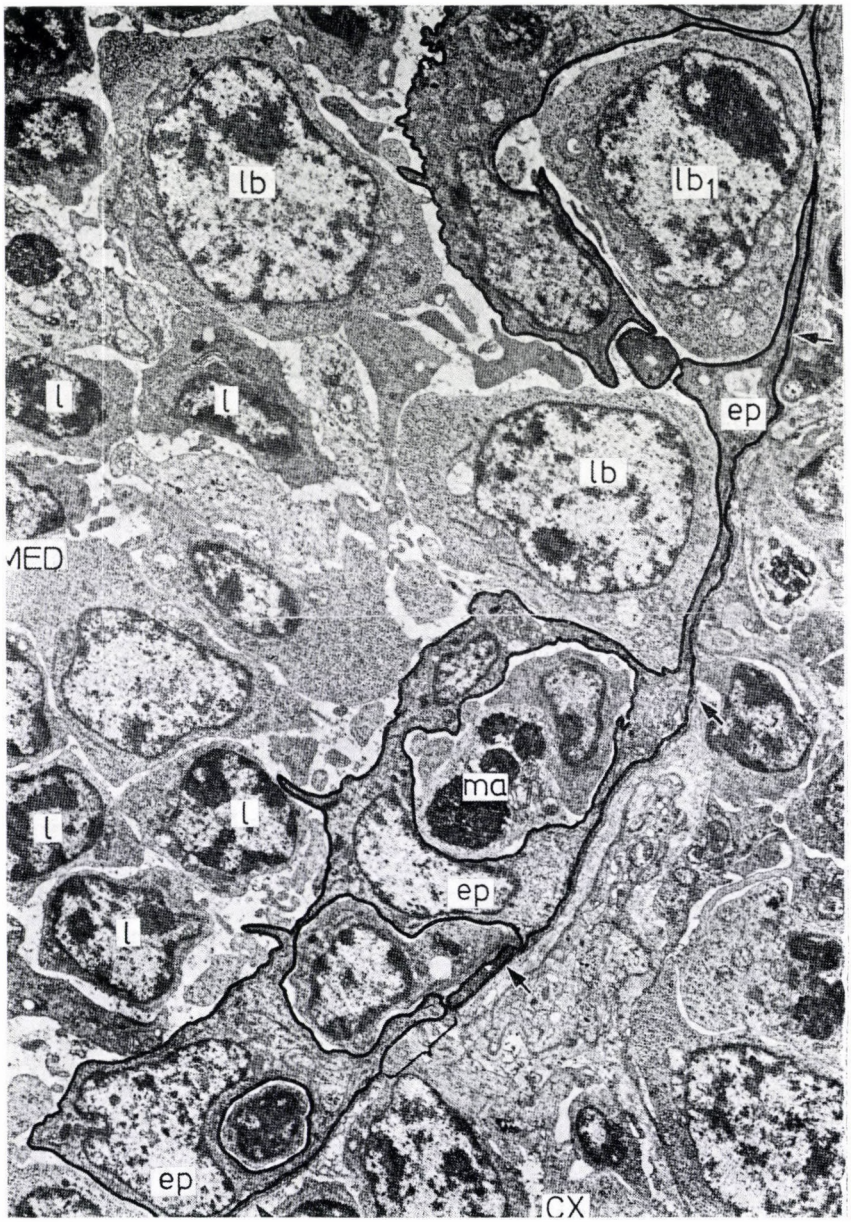
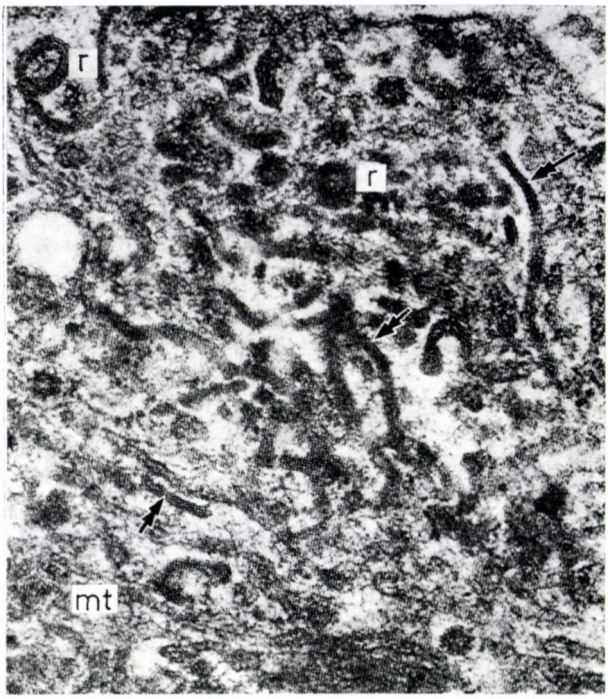


Table III

Fig. IIIa. Chicken bursa of Fabricius: cortico-medullary border. The epithelial layer between cortex and medulla is drawn. India ink. The arrow indicates the basal membrane. The secreting epithelial layer and the basal membrane are perforated by lymphoid cells, l = lymphocyte, lb = lymphoblast, ma = macrophage, ep = incomplete epithelial layer owing to perforating cells. CX = cortex, MED = medulla. Magnification: ×7.000



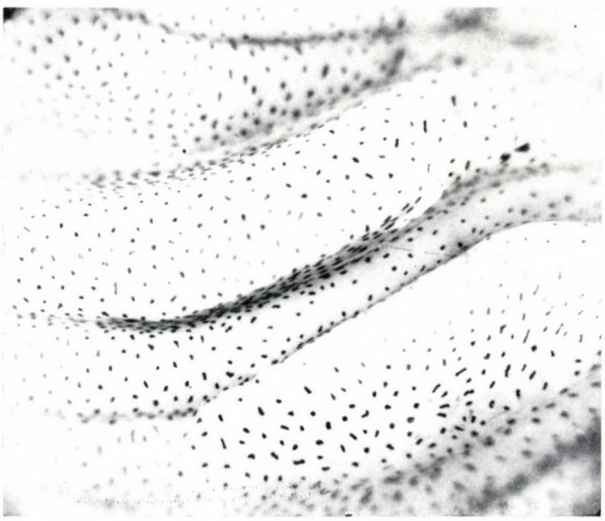


Fig. IIIb. Receptor cells from the bursa of Fabricius. In the cytoplasm indicated with double arrow there are longer and shorter, sometimes ringlike bodies (r). mt = microtubules. Magnification: ×7.000

Fig. IIIc. Surface of the chicken's bursa of Fabricius. The India ink arrives from the cloaca in the lumen of the bursa of Fabricius and the follicular epithelium phagocyted it. The areas in the follicular epithelium appear as black dots. Magnification: ×9

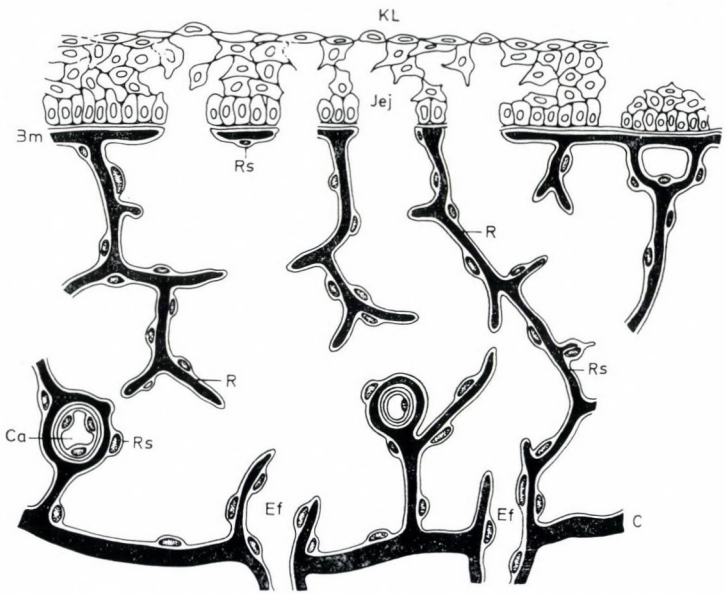


Fig. IV. Rough scheme of the tonsil's structure. Jej = intraepithelial ducts closed towards the lumen by a single cell layer. Bm = basal membrane, Rs = reticulum cells, R = reticular fibre, Ca = capillaries, Ef = the capsule perforating a vas efferens, C = capsule, Kl = lumen

blocked by some large fungiform cells (Table IV. Fig. IV/a). If these cells are detached, the aperture becomes free, opening the way for the bacterial antigen from the oral cavity toward the mobile cells of the passage. If peroxidase is dropped onto the tonsil the tracer will arrive within five minutes in the inter-epithelial passage and subsequently also in the macrophage.

Under the scanning electron microscope, two types of cells can be seen on the surface (Table IV. Fig. IV/b). The fungiform cells may also be called receptor cells. They reveal numerous mitochondria, a number of ergastoplasm tubuli and massive bundles of tonofibrils. Some unusual bodies resembling those found in the receptor cells of the thymus and bursa, and microvesicles are seen in the cells (Table IV. Fig. IV/c). In our opinion these formations are flattened vesicles, and this membranes correspond the cell membrane. The trilaminar structure confirms this finding, as it contains two closely adjacent unit membranes turning towards each other at both ends, like in the zonula occludens which also comprises five layers. Some of them resemble a tennis-racket. The peculiar structure of the fungiform cells is indicative of active cellular transport.

In the Peyer's patches [6, 7], the lymphoid elements that had penetrated into the epithelium, distort the epithelial cells having microvilli (Fig. 6). Goblet cells are absent. The epithelial cells lend a bizarre shape to the lymphoid

nests. Lymphoblasts and plasma cells lying here are girdled by a network of narrowed basal parts of epithelial cells (Fig. 5). The picture shows the epithelium deformed by lymphoblasts and plasma cells. A major role in the formation of this network is played by an epithelial cell described by OWEN [21] as a "microfold" cell, which when flattened, permits the approach of lymphoid cells at 0.3 μm from the lumen.

These cells include a prominent nucleolus, a supranuclear Golgi apparatus and numerous vacuoles and are characterized as receptor or transport cells. The microfold cells with knotted and demaged microvilli also contain a large number of mitochondria and fibrils.

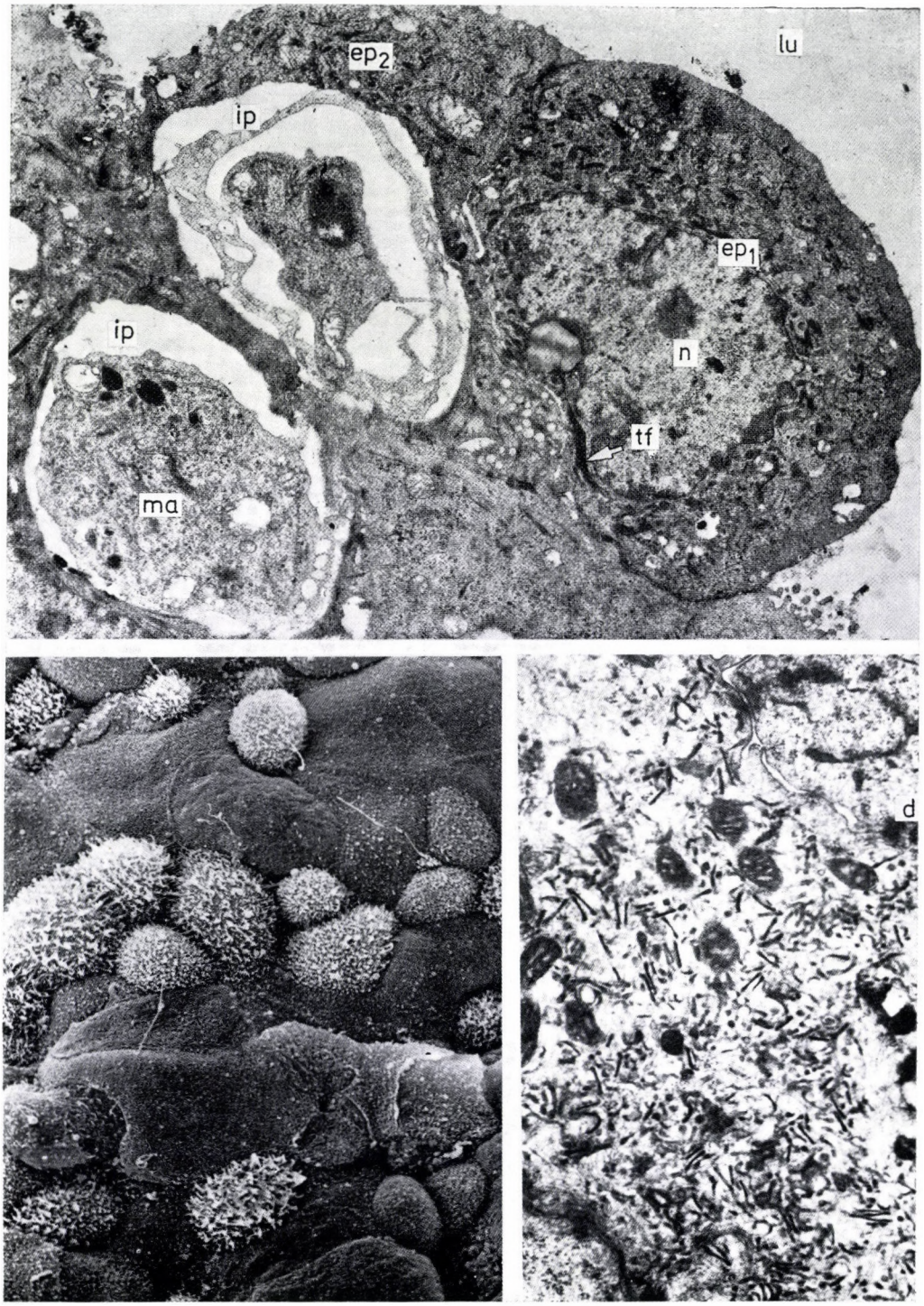
When Indian ink or ferritin is dropped onto this area, it is soon found within the vacuole of the cell [13]. The M-cell may produce IgA, discharging it into the lumen together with its secretion. This is the place of maturation of the small lymphocytes into plasma cells.

Included in the lymphoepithelial organs is the so-called accessory lacrimal gland of Harder with its central collecting duct and stellated lumen [5]. It is a tubuloalveolar gland with cylindrical glandular epithelium without a basal membrane (Table V. Figs V/a, b). The surface epithelium is connected with the deep epithelium well provided with tonofilaments [12]. Their netlike processes surround the numerous plasmoblasts (Table V. Fig. V/c) and plasma cells, frequently separated by thin epithelial cytoplasmic streaks from the acinar lumen [2]. A homogeneous mass was found between the cells; its function remains unknown.

The lymphoid elements are represented almost exclusively by mature plasma cells [1, 3]. The interrelationship between the epithelial and lymphoid elements resembles that of the thymus and bursa, i.e. plasma cells intend epithelial ones and cause considerable deformation. The luminal folds of the glandular duct are coated by another kind of epithelium, as the acinus possessing a number of microvilli (Table V. Figs V/b, d) and lacking any secretory vacuole, while the acinus itself is loaded with excreting vacuoles.

Labelling the thymus and the bursa cells with ^{3}H-thymidine, it can be established that plasma cells glands of Harder's are derived to three-quarters from the bursa and to one-quarter from the thymus [16]. The microvilli-laden cells covering the duct are true receptor cells, carrying out also transport function. They catch the antigens [8, 9] and they deplet the tear together with the IgA also produced by the plasma cells.

Bursectomy has verified that the plasma cells in the glands of Harder are independent of the B cell population [22]. Administering the antigen intravenously, the antibody merges in the tears and in the serum, but if dropped directly into the eyes, the antibodies are found only in the tears [7, 9].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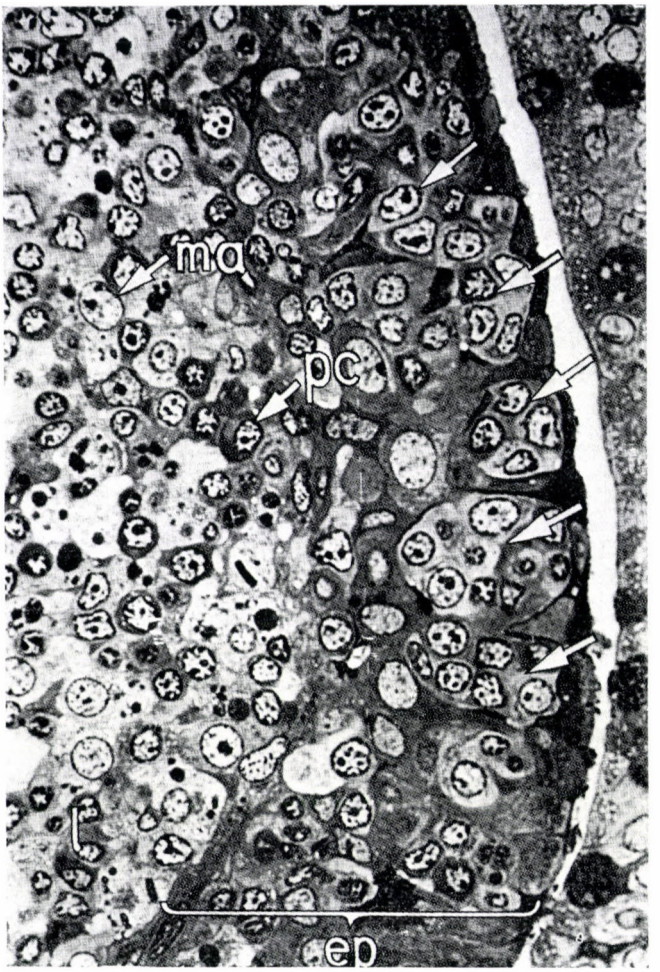

Fig. V. Surface layer of rabbit's Peyer's patches. Lymphoid cells deform the epithelia cells (ep). No goblet cells appear. Arrows point to lymphoid immigrated into the epithelium. l = = lymphocyte, ma = macrophage, pc = plasma cell. Magnification: ×1000

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Table IV

Fig. IVa. Part of the tonsillar epithelium. Fungiform cell (ep. 1, ep. 2) closing the intraepithelial duct. n = nucleus, tf = tonofibril, ip = intraepithelial duct, ma = macrophage in the intraepithelial duct, lu = lumen. Magnification: ×11.000

Dig. IVb. Scanning micrograph of rabbit tonsil reveals two types of epithelial cell. One of them is cobblestone-like, while the other is fungiform in shape having many microvilli. Magnification: ×6000

Fig. IVc. Bodies in the fungiform epithelial cell of the tonsils. They resemble the form of bodies in the special cells of the thymus and in the bursa. Magnification: ×26.000

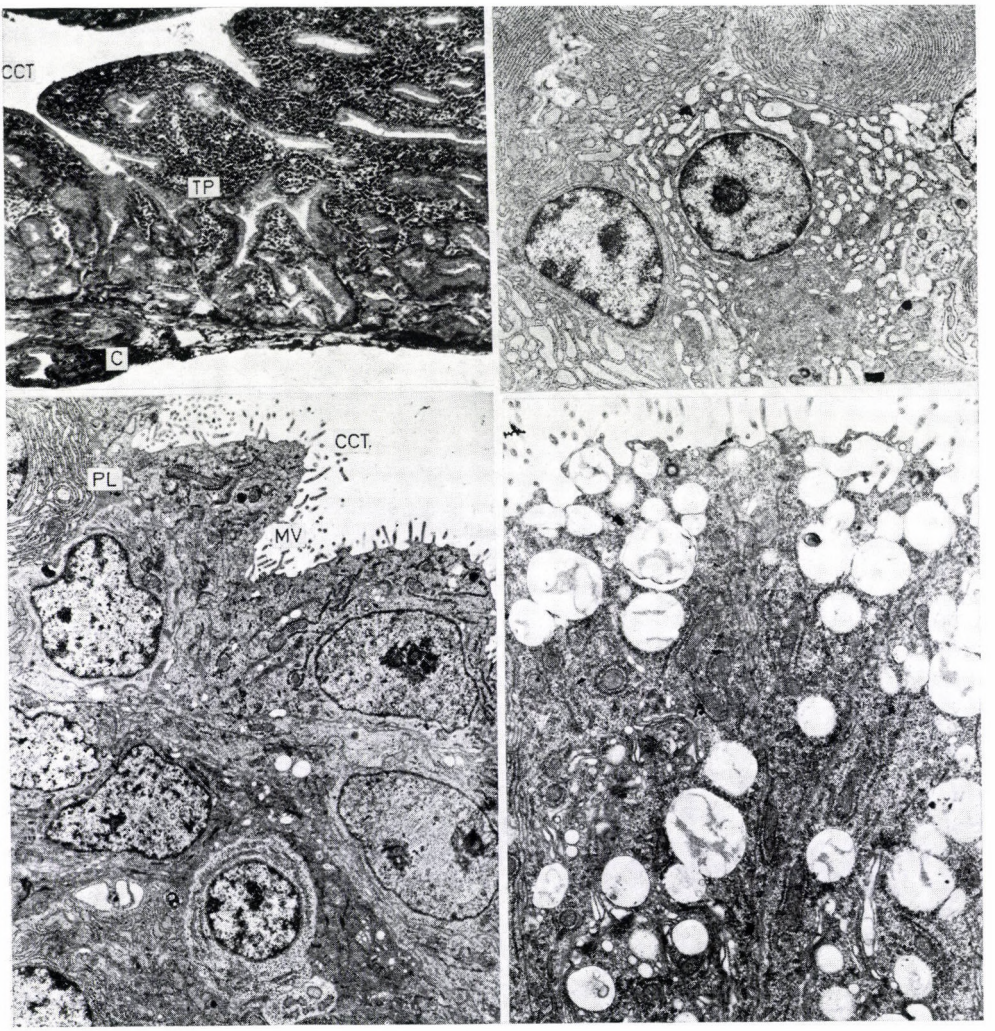

Table V

Fig. Va. Light micrograph of Harder's gland. Its acini are located close to the capsule (C), while the tunica propria (TP) around the central collecting tubule (CCT) is crowded with plasma cells. Magnification: ×240

Fig. Vb. Electron micrograph of the surface epithelium of the central collecting tubule (CCT). The epithelial cells have microvilli and developed ergastoplasmic cisternal. Under these cells there is another type of epithelial cell which is stellate in shape and its cytoplasm produces tonofibrils. The intercellular spaces are filled with plasma cells. MV = microvilli. Magnification: ×10.700

Fig. Vc. Survey picture of the tunica propria of the central collecting tube. Young and mature plasma cells are intermingled. Magnification: ×9200

Fig. Vd. The acinar cells of Harder's of gland elaborate many secretion granules discharged by exocytosis from the cells. Magnification: ×20.000

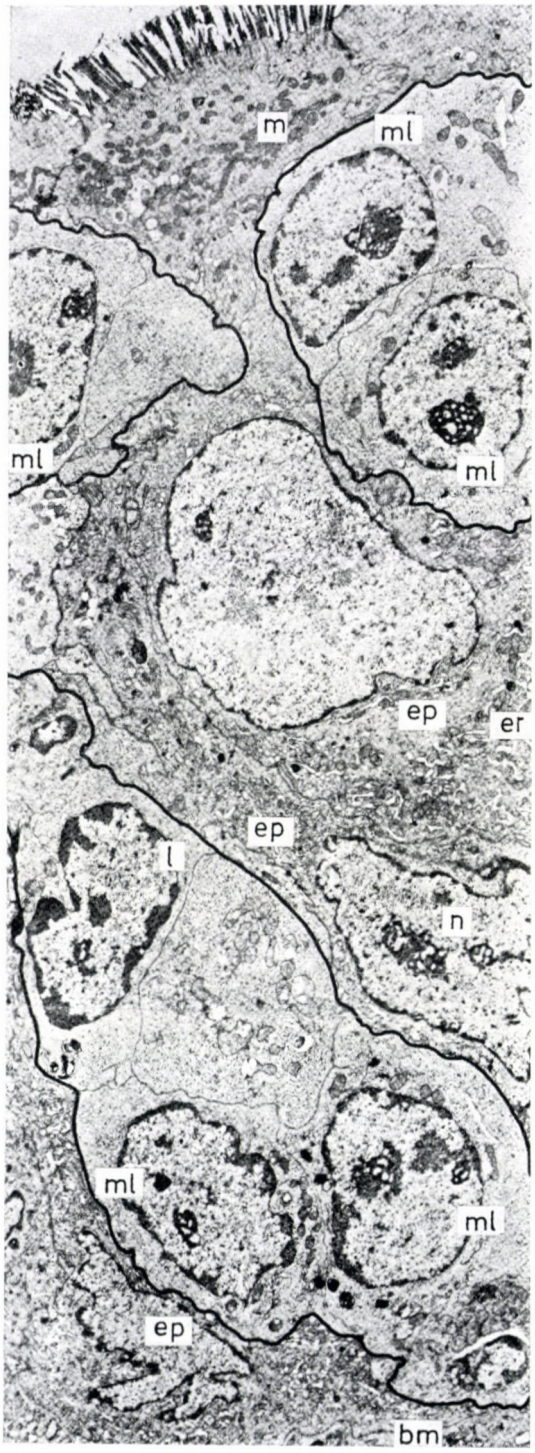

Fig. VI. Surface epithelium of Peyer's patches (rabbit). In the epithelial cell nests, lymphocytes of medium size. The contour of the cell nest is outlined. The lymphoid nests deform the epithelial cells (ep). ml = lymphocytes, n = nuclei, er = endoplasmic reticulum, bm = basal membrane, m = mitochondrium. Magnification: ×5000

We have confirmed that the lymphoepithelial organs possess a special receptor and transporting cell, in which a cytoplasmic formation indicating a micropinocytotic function. [23, 24].

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WECHSELWIRKUNG DER EPITHEL- UND LYMPHOIDZELLEN IN DER FUNKTIONALEN STRUKTUR DER LYMPHOEPITHELIALEN ORGANE

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Der morphologische und funktionelle Zusammenhang der epithelialen und lymphoiden Elemente in den lymphoepithelialen Organen (Thymus, Bursa Fabricii, Tonsillen, Peyersche Plaques, Herder-Drüsen) wurde elektronenoptisch untersucht. In all diesen Organen werden spezielle Rezeptor-Transfer-Zellen beschrieben, die in der Aufnahme und im Transfer der Antigene eine Rolle spielen. Diese Zellen enthalten in ihrem Zytoplasma kürzere-längere abgeflachte Vakuolen, deren Wände sich aneinanderschmiegen und so unterschiedlich geformte Stäbchen bilden. Die Struktur der Stäbchenwand ist dem Aufbau der Zellmembran ähnlich und die Stäbchen erinnern an die gestreiften Körper bei Mikropinozytosis vermiformis. Ihre Entstehung hängt mit der Desintegration der Zellmembran beim Beginn der Endozytose zusammen. Die Zellmembran gelangt zusammen mit dem ihr anhaftenden Antigen im Zuge der Endozytose in das Zytoplasma der Epithelzellen und induziert so die Zellimmunität. Die Hardersche Drüse ist als lymphoepitheliales Organ, neben ihrer Thränenflüssigkeit ausscheidenden Funktion, mit zu 3/4 aus der Bursa und zu 1/4 aus dem Thymus stammenden Plasmazellen gefüllt und in ihr geht eine den anderen Organen ähnliche lymphoepitheliale Kooperation vor sich.

ВЗАИМОДЕЙСТВИЕ ЭПИТЕЛИАЛЬНЫХ И ЛИМФОИДНЫХ КЛЕТОК В ФУНКЦИОНАЛЬНОЙ СТРУКТУРЕ ЛИМФОЭПИТЕЛИАЛЬНЫХ ОРГАНОВ

И. ТЁРЁ, И. ОЛАХ, ЖУЖА КИТТНЕР

В лимфоэпителиальных органах (зобная железа, Фабрициева сумка, миндалина, Гардера железа) с помощью электронного микроскопа была изучена морфологическая и функциональная связь эпителиальных и лимфоидных элементов. Во всех этих органах дается описание специальных рецепторно-трансферных клеток, играющих роль в приеме и передачи антигенов. В цитоплазме этих клеток имеются более короткие — длинные уплощенные вакуолы, стенки которых прилегают друг к другу и образуют таким образом палочки разнообразной формы. Строение стенки этих палочек такое же как и структура клеточной мембраны, они подобны полосатым телам при micropynocytosis vermiformis и их возникновение связано с дезинтеграцией клеточной мембраны в связи с началом эндоцитоза. Клеточная мембрана вместе с прилипающим к ней антигеном проникает посредством эндоцитоза в цитоплазму эпителиальных клеток и наводит начало клеточного иммунитета. Гардера железа — кроме ее слезоотделяющей функции —, будучи лимфаэпителиальным органом, наполнена плазматическими клетками, 3/4 которых происходит из сумки, а 1/4 из зобной железы. Подобно как и в других органах, в железе Гардера также осуществляется лимфоэпителиальная кооперация.

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PROMOTING EFFECT OF FEEDER CELLS IN MAINTENANCE OF ADULT RAT HEPATOCYTES

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A procedure is described for maintaining primary cultures of adult rat hepatocytes for prolonged periods of time on a layer of irradiated mouse fibroblast cell line (C3H/10T1/2) and on a secondary lung fibroblasts obtained from Sprague Dawley rats. Morphologically and ultrastructurally the cocultivated hepatocytes retained many characteristics of hepatocytes *in vivo*. Within 24 hours after seeding, the individual cells were attached on the feeder cell layer and the *in vivo* polarity of the liver cells reappered. Electron microscope studies demonstrated the appearence of newly developed bile ducts and junctions between hepatocytes as well as between hepatocytes and feeder cells. Histochemically, these cells were positive for glucose-6-phosphatase and for glycogen. After 14 days in culture the hepatocytes could be reseeded onto fresh C3H10T1/2 cells.

In contrast, hepatocytes maintained on plastic substrate lost their glycogen content and the epithelial character of the liver cells after 5 days in culture, and by day 10 this culture became predominantly fibroblastic.

It is suggested that hepatocytes maintained on an irradiated fibroblast feeder layer provide a valuable approach for studying the morphogenesis, cytotoxicity, or the metabolism of different chemicals *in vitro*.

Introduction

Short or long-term maintenance in culture of epithelial liver cells as a means to evaluate cellular events in response to chemical treatment or to other biologically active factors, has several advantages over whole animal studies. Liver cells cultivated *in vitro* are valuable for studies of morphogenesis, enzyme induction, cytotoxicity of hepatotoxic agents or of neoplastic transformation by liver carcinogen [8, 13, 27, 28, 32, 33, 44, 46]. Hepatocytes, however, like other well-differentiated epithelial cells, are not easily maintained in culture without the loss of many of their functions *in vivo* [6, 7, 14, 17, 44, 46]. The major disadvantage of most cell culture methods presently available is that hepatocytes rapidly dedifferentiate and become fibroblast-like cells permitting only a non-specific response of eukaryotic cells [9, 18]. Therefore the development of a new cell-culture method was thought to be essential to facilitate the long-term maintenance of high-yield epithelial-like liver cells in culture. Such a new method would be useful for the identification of environmental contaminants which are potential aetiological agents of human cancer or mutations.

Several techniques have been developed to isolate liver cells for growth or maintenance in culture, although these techniques are not widely used in experimental toxicology or oncology. Most methods for obtaining large numbers of functional hepatocytes from adult animals are based on perfusion of the liver through the portal vein with different enzymes [1, 2, 4, 13, 16] like collagenase-hyaluronidase, protease, trypsin-collagenase, etc. [19, 20, 36, 43, 45, 47]. Different cell lines and strains of propagable epithelial cells have been developed in several laboratories [5, 10, 11, 24, 27]. An essential requirement for such epithelial cultures which could be used to test toxicity or carcinogenecity of chemicals is that they retain important function-specific capabilities like albumin synthesis [21] and also that they remain metabolically responsive to activation by protoxins or procarcinogens [25, 26, 28, 37, 38]. In long-term culture the normal *in vivo* functions of liver cells have been difficult to achieve. The effects of different culture media and serum concentrations on cell seeding and viability or phenotypic expression of liver cells have been studied by several authors [15, 18, 23, 24]. The presence in the medium of hormones such as insulin or glucagon, has enhanced the plating efficiency of hepatocytes [3, 18, 23, 35], and helped to maintain the normal hepatocyte ultrastructure, prolonged glycogen synthesis and hepatocyte survival. When the liver cell culture was maintained on floating collagen membranes [30, 31], during the first few days the collagen gel shrank to about one-sixth of its original volume. The liver cells remain functionally viable up to 20 days, maintaining a measurable level of cytochromes such as cytochrome P-450. This method has, however, certain disadvantages for morphological or biochemical studies because the number of surviving cells is rather small.

Long-term culture of adult liver cells with inducible tyrosine aminotransferase was achieved by growing the cells on a feeder layer [26, 37, 38]. We have been using primary adult hepatocytes to study the effects of metabolic activation of different liver carcinogens into their active mutagenic or carcinogenic intermediates [25]. The present paper reports on the light microscopic and ultrastructural characteristics of hepatocytes maintained on different feeder cells in long-term culture.

Material and methods

Cells and cell culture. The feeder cells, C3H/10Tl/2 cells [34] and lung fibroblasts [39] were grown in Williams E medium (Flow Laboratories) and in heat-inactivated fetal calf serum (Gibco, Grand Island, N. Y.) supplemented with 2mMole L-Glutamine (Flow Laboratories), penicillin 100 U/ml, streptomycin 100 μg/ml and fungizone 1 μg/ml. Cells were maintained in 25 cm^2 T-flasks (Falcon) in humified incubators with 5% CO_2 in the air at 37° C. When fibroblasts were grown to confluency the medium was changed and 24 hours later the cells were irradiated lethally with 500R from a cobalt source. The cells were used as feeder cells 1 to 3 days after irradiation.

Primary liver cells from 6 to 10 weeks old male Sprague-Dawley (SD) rats were prepared by the method of WILLIAMS [45, 47] *in vitro*. The viability of cells determined by trypan blue exclusion was at least 90% in four consecutive experiments. Three hours after initial

cell seeding the medium was changed to 8 ml fresh nutrition medium to remove unattached cells and cell debris. The medium was changed thereafter every 48 hours. *Light and electron microscopy*. Liver cells for light microscopy and for Periodic Acid Schiff (PAS) staining were fixed in a dish with 95% methanol for 2—24 h. The glycogen content of hepatocytes was determined by the method of THIERY [40]. After fixing in Karnovsky solution, the glucose-6 phosphatase activity was detected by the method of WACHSTEIN and MEISEL [42] and TICE and BARNETT [41]. For general morphology studies the cells were maintained on Melanex sheets (Transiluran Co., Kansas City, MO.). The feeder cells were first grown to confluency and were irradiated prior to seeding of hepatocytes. After various growth periods the cultured cells were fixed and embedded for electron microscopy as follows. The cells were washed with phosphate buffer (PBS) pH 7.4 and then fixed for 90 min. at 4° C with 2.5% glutaraldehyde solution in veronal buffer, pH 7.2. After dehydration the cells on Melanex were embedded in Durcupan ACM (Fluka) and ultrathin sections parallel and perpendicular to the Melanex sheet were cut by LKB ultramicrotome. Thin sections were stained with uranyl acetate and lead citrate. Observations were made with a JEM 100 C electron microscope.

Results

Primary hepatocytes were gained in four different experiments by collagenese perfusion of male SD rats. In each experiment cells from one liver were used. The average number of hepatocytes was $6.4 \times 10^8 \pm 10\%$ per liver. A single cell suspension of liver cells was inoculated on an irradiated confluent monolayer of mouse embryo fibroblast (C3H/1OTl/2) cells, or on irradiated primary adult rat lung fibroblasts. This helped to determine whether such cocultivation could prolong the maintenance of the epithelial character of liver cells as compared to those seeded directly on plastic.

6×10^6 liver cells in 8 ml medium in 25 cm^2 T-flasks were used in each experiment. Three hours after seeding the liver cells were attached in a cluster-like arrangement with 30—40% plating efficiency (see Table I). After three hours of inoculation, the number of liver cells was 2.5×10^6 when attached to C3H/1OTl/2 cells, and 2.8×10^6 when cocultivated with lung cells. After 24h in culture, the number of viable, well attached and spread hepatocytes was 1.2×10^6 on the plastic and 2.2×10^6 or 2.5×10^6 on the C3H/1OTl/2 cells or on lung cells respectively. After 48h in culture the colony types were slightly different on each substrate. The liver cells maintained on plastic did not spread well and only 0.8×10^6 cells remained attached from the original 6×10^6 inoculated hepatocytes. At the same time the hepatocytes on feeder cells showed a much higher viability. The number of viable hepatocytes as calculated by the trypan blue exclusion test was 1.9×10^6 when seeded on the C3H/1OTl/2 cells and 2.1×10^6 when maintained on lung cells. After 5 days in culture on feeder cells the liver cells exhibited a morphology definitely different from that of hepatocytes cultures on plastic dish. The freshly seeded liver cells were attached usually together in a mosaic-like arrangement on each substrate. After 5 days in culture the liver cell colonies on plastic were small and the border of liver cell groups was uneven, surrounded by elongated and pale, flattened cells (Fig. 1). Generally, 40—60 cells made up one colony; the number of viable hepatocytes per T-flask was 0.7×10^6. Hepatocytes seeded on irradiated fibro-

Table I

Long-term culture of hepatocytes on different substrates

Time	Plastic	Cell No $\times 10^6$	Irr. C3H/10T1/2	Cell No. $\times 10^6$	Irr. lung cells	Cell No. $\times 10^6$
3 h	no attachment	—	partially attached	2,5	well attached	2.8
24 h	partially attached	1.2	well attached	2.2	flattened	2.5
48 h	well attached	0.8	flattened	1.9	flattened	2.1
5 days	flattened small colonies	0.7	mosaic-like, large colonies	1.6	elongated large colonies	1.9
14 days	confluent fibroblast-like culture	1.1	—elongated colonies —degenerated areas	1.9	detachment	—
24 days	confluent fibroblast-like culture	~	fibroblast like and mosaic-like colonies, more degeneration	~	—	—
32 days	—	—	detachment	—	—	—

blastic feeder cells produced much larger colonies (200—600 cells per colony) and the total number of hepatocytes was 1.6 or 1.9×10^6 per dish on C3H/10T1/2 cells or on lung cells, respectively (Figs 2 and 7). At the same time the liver cells were elongated and usually formed dense and irregular colonies on the irradiated lung cells. In some cases the liver cells cocultivated with lung fibroblasts became more fibroblast-like than those cultured together with C3H/10T1/2 cells (Fig. 7).

Fig. 1. Primary hepatocytes maintained on plastic after 5 days in culture. The PAS positive cell number was 0.7×10^6 cells/T-flasks; $\times 180$

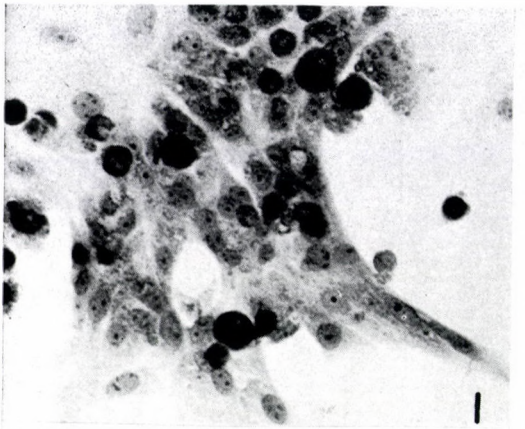
Fig. 2. Primary hepatocytes maintained on C4H/10T1/2 cells 5 days after seeding. In this large colony liver cells preserved the cubic shape of epithelial cell types. The PAS positive cell number was 1.6×10^6 cells/T-flasks; $\times 180$

Fig. 3. Confluent liver cell monolayer maintained on plastic, 14 days after seeding. The cells are growing in a fibroblast-like pattern. They exhibit only amylase resistant PAS staining. PAS-staining; $\times 180$

Fig. 4. Liver cells cocultatived with C3H/10T1/2 cells 14 days after seeding. The epithelial characteristics still persist. PAS-staining- $\times 120$

Fig. 5. Long-term culture of liver cells without feeder layer. These cells are rich in microfilaments and they reemble fibroblast-like culture. PAS-staining; $\times 120$

Fig. 6. Long-term culture of liver cells maintained on feeder (C3H/10T1/2) cells, after 24 days in culture. PAS-staining; $\times 80$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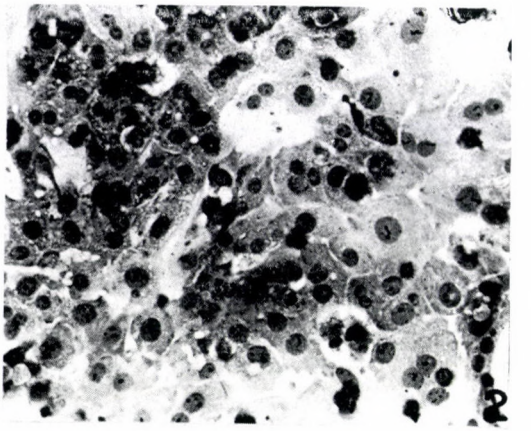
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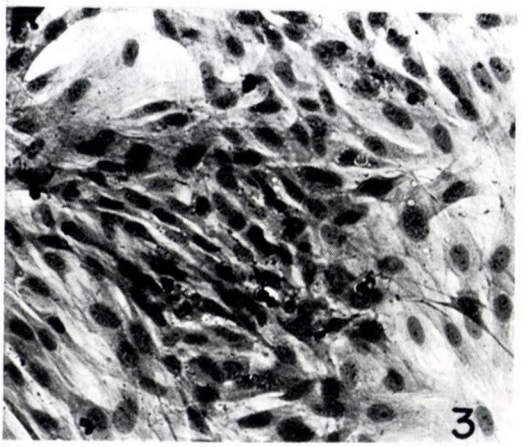
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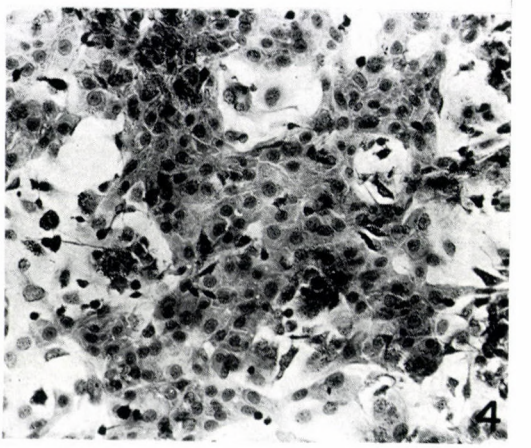
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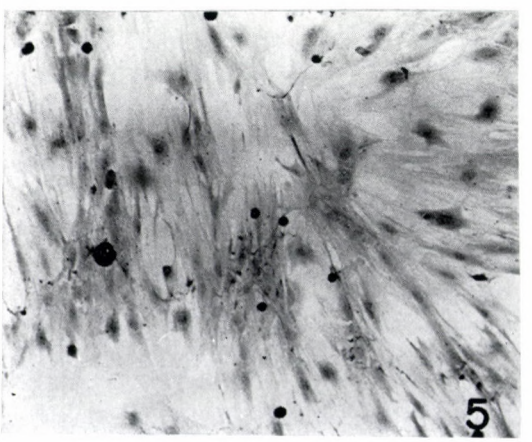
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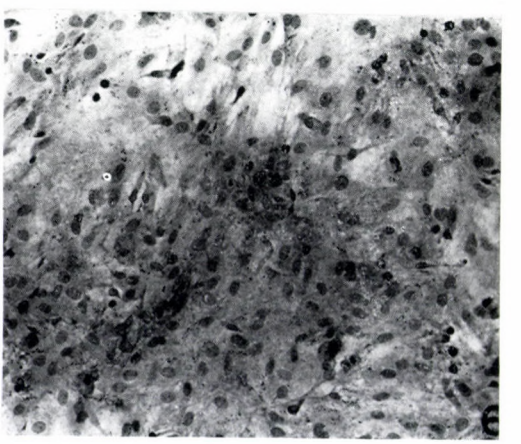
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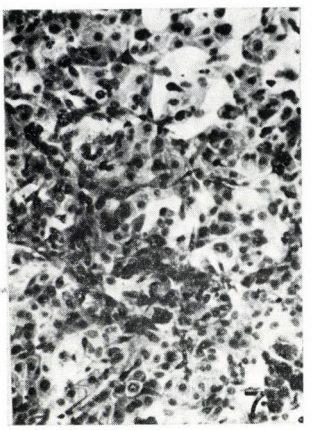
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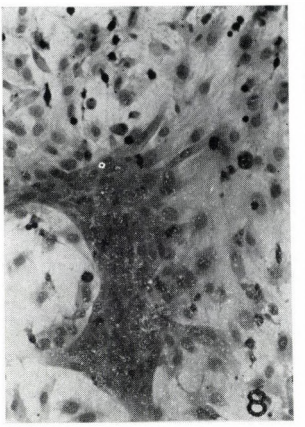
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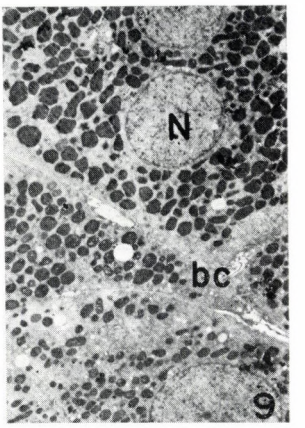
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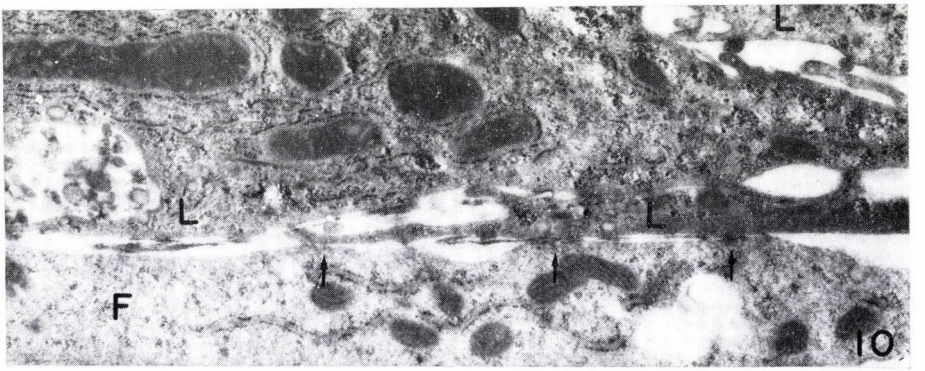
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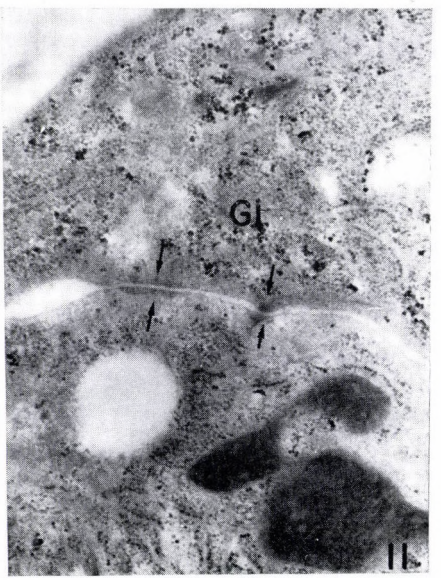
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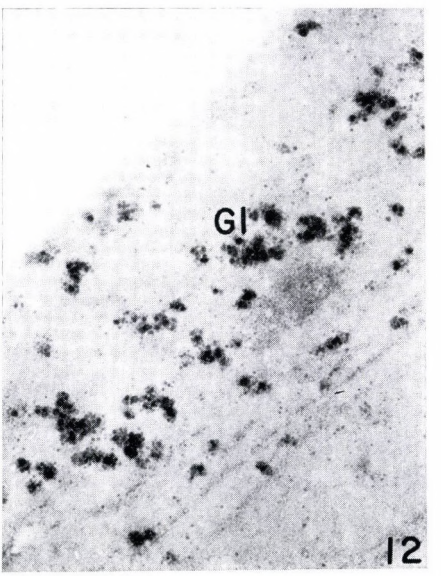
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After 14 days the liver cell colonies on each substrate became confluent (Figs 3 and 4), moreover, on lung cells the liver cells necrotized and the monolayer became detached from the bottom of the dish. The cause of this rapid necrotizing process is not known.

The epithelial character of liver cells, nevertheless, was well preserved on feeder C3H/10Tl/2 cells even after 14 (Fig. 4) or 24 days (Fig. 6) in culture. The longest survival time in cocultivation with C3H/10Tl/2 cells was 32 days. By this time most liver cells degenerated and began to develop multinucleated giant cells, but they did not necrotize. Those cocultivated with lung cells began to degenerate and necrotize about 14 days in cocultivation, however, in this case even the feeder cells detached themselves from the plastic dish. A possible explanation is that the irradiated feeder cells had an increasingly insufficient nutrition and metabolism caused by the degenerating liver cells. The liver cell culture maintained on plastic dish became more and more fibroblast-like after 14 (Fig. 3) or 24 days (Fig. 5). The feeder cell monolayers cocultivated with liver cells were tested by PAS-staining to demonstrate their glycogen or neutral mucopolysaccharide content, but they were always negative in this respect. They were elongated with pale cytoplasm but rich in microfilaments. The liver cells maintained on plastic did not show PAS positivity even after 5 days in culture. Those liver cells which were cultured on feeder cells, on the other hand, have preserved some glycogen during the whole culturing. Glycogen was detected in the liver cells, even in 32 days cultures.

Subculture

After 14 days in culture, the hepatocytes maintained on C3H/10Tl/2 cells were successfully removed by collagenase (0.05%) treatment and reseeded on freshly irradiated feeder cells. After 24h these subcultured hepatocytes developed small groups of attached liver cells and they anchored to the sub-

←

Fig. 7. Liver cells maintained on lung feeder cells after 5 days in culture. The cells are in a dense cord-like arrangement; the colony has an irregular border. PAS-staining; ×80

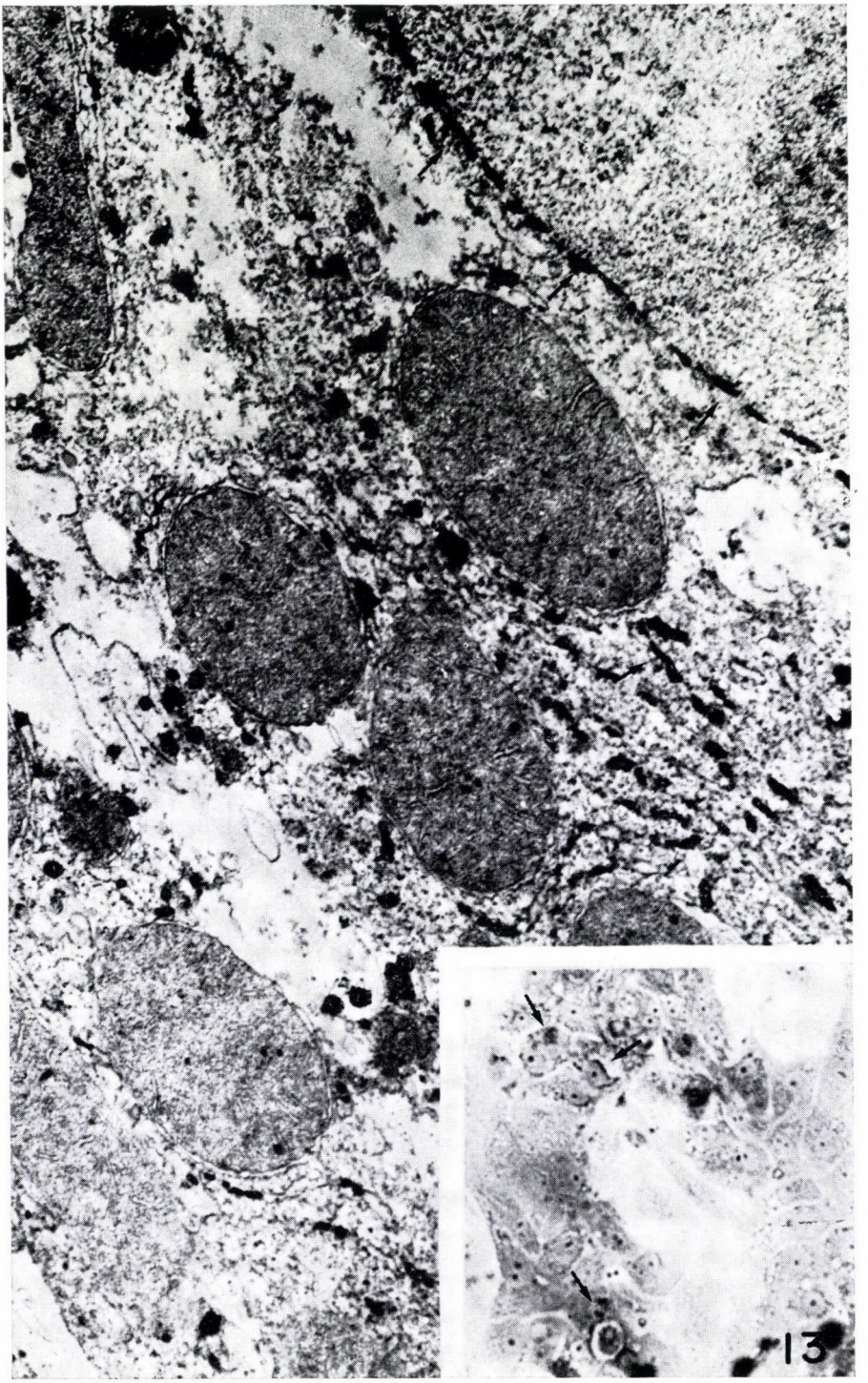
Fig. 8. Subculture of liver cells reseeded after 14 days in primary culture. Cells are forming an epithelial type colony 5 days after reseeding into secondary culture. PAS-staining: ×80

Fig. 9. Electron micrograph of liver cells after 24h of seeding. The polarity of liver cells is restored, many electron dense mitochondria are seen. *Abbr.:* bc — bile canaliculi, M — mitochondrium, N — nucleus, v — vascular pole, — ×6480

Fig. 10. Liver cells and feeder (fibroblast) cells form contacts, developing intermediate junctions (arrow) after 10 days of cocultivation. L — liver cells, F — fibroblast. — ×36.400

Fig. 11. Two neighbouring liver cells developed an intermediate junction. The cell membranes are thickened on both sides of the contact. In the cytoplasm many glycogen rosettes are seen (arrow). Gl — glycogen. 14 days after cocultivation. ×64.000

Fig. 12. Glycogen rosettes as demonstrated by Thiery-staining 14 days after cocultivation. ×116.000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strate with a very low (3—5%) plating efficiency. After 5 days in subculture (Fig. 8), the liver cells spread out and their viability was 95% as demonstrated by the trypan blue exclusion test.

Electron microscopy

Electron microscopic observations were made on liver cells cocultivated with the C3H/10T1/2 cells and maintained on Melanex film. Samples were fixed in the flask and embedded in Durcupan. The blocks were cut perpendicularly to the Melanex sheet so that the topographical relation between feeder cells and liver cells could be studied. Liver cells restored their polarity in 24h after seeding, resulting in newly developed bile canaliculi and junctional specializations (Fig. 9). At low magnification (Fig. 9), the liver cells were seen to contain numerous dark mitochondria. This tissue-polarisation of the hepatocytes remained unchanged from 5 to 14 days after seeding. There were intimate intercellular junctions between hepatocytes and feeder cells (Fig. 10) exhibiting at places intermediate, although not specialized, junctions [12]. The cellular contacts between the two types of cell were occasionally focal, or point-like, sometimes arranged longitudinally to the sinusoidal surface of the liver cells. Contacts which developed between the hepatocytes were a result of cell collision, in which the membranes of hepatocytes developed a very close relationship, but well matured desmosomes or gap junctions were not observed by the routine methods used. In Fig. 11, two neighbouring liver cells are shown after 14 days culture. The cell membranes are thickened on both sides but typical desmosomes have not developed. At the same time, 14 day old cultured hepatocytes contained glycogen rosettes staining by the Thiery method (Fig. 12), corroborating the result of light microscopic PAS staining.

The glucose-6-phosphatase activity of liver cells was demonstrated cytochemically [41]. The endoplasmic reticulum membranes and the nuclear membranes were strongly positive in the liver cells cocultivated with C3H/10T1/2 cells for 5 days (Fig. 13). The insert of Fig. 13 demonstrates the intensity of the enzyme activity in monolayer by light microscope.

Discussion

The present observations demonstrate the ability of feeder cells to preserve hepatocellular morphology and other typical features of adult liver cells in long-term culture. The presence of feeder cells helps to maintain glycogen synthesis and glucose-6-phosphatase activity of the cultured liver cells. During the cocultivation of feeder and liver cells, after three hours of seeding,

Fig. 13. Glucose-6-phosphatase reaction of liver cells maintained on C3H/10T1/2 cells after 5 days in culture. ×24.900
Insert: Glucose-6-phosphatase activity in the same liver under the light microscope. ×200

40—50% of liver cells were able to anchor to the feeder cells. Liver cells maintained on a feeder layer had a slower rate of dedifferentiation than those cultured on the rigid plastic dish. This new cocultivation method helped to increase the number of those viable hepatocytes, which produce glycogen and participate in gluconeogenesis as deduced from their glucose-6-phosphatase activity. Liver cells maintained on plastic became transformed predominantly to fibroblast-like cells without any glycogen content after 5 days in culture, while liver cells plated on feeder cells started to lose their glycogen content only after 14—20 days in culture.

MICHALOPOULOS et al [30, 31] compared the liver cell morphology and biochemical characteristics maintained *in vitro* on rigid (plastic or collagen coated dish) and elastic (floating collagen membranes) substrates. According to their results the elastic substrate preserved a better epithelial morphology and different functions of the liver cells, than did the rigid material. This finding was fully corroborated by our observations. The most likely explanation for these differences between "rigid" and "elastic" substrates can be summarized as follows.

1. Attachment of liver cells to any substrate occurs only when the surface of the host substrate is uneven enough to allow the liver cell microvilli to anchor to the substrate.

2. The close contact between feeder cells and liver cells ensures a possibility for metabolite or material exchange (29, 29a). Indeed, circumstancial evidences by LANGENBACH et al. [25] and by KUROKI and DREVON [22] showed that during cell mediated mutagenesis, an enhanced material exchange can take place between identical and non-identical cell types.

3. Because of the uneven surface of the feeder layer, the establishment of a multilayered three-dimensional liver cell culture has more probability than on rigid (plastic) substrates. A quasi-3 dimensional culture is more convenient than a monolayer for normal functioning of epithelial cells.

4. As a consequence of the quasi-3 dimensional organization, cocultivated liver cells on a feeder layer retain more of their original cubic shape than those maintained on pure plastic. Cells on rigid membranes became thin and elongated, a handicap for normal functioning of the liver cells.

5. Finally, the uneven surface of fibroblasts results in an enlarged surface of the contacting liver cells which in this way have an increased oxygen and nutritonal uptake. This, in turn prolongs the cell's lifetime and diminishes intracellular degenerative processes.

Two problems which emerge from the present observations cannot be answered with certainty. (i) We do not know why C3H/10Tl/2 are more suitable as feeder cells than lung cells in cocultivation with liver cells. (ii) Likewise, it is also difficult to explain why liver cells appear to be normal until the 10th day after seeding on lung cells but after this period they necrotize

rapidly and the lung cells detach from the dish. One possible explanation is that the isolated lung fibroblasts which are not monoclonic, represent a variety of different cell type, exhibiting a differential sensitivity to the 5000 R dose applied. The problem is hoped to be solved in future experiments utilizing monoclonic lung cells as feeder layer.

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DIE FÖRDERNDE WIRKUNG DER NÄHRZELLEN IN DER AUFRECHTERHALTUNG DER LEBERZELLEN VON ERWACHSENEN RATTEN

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Ein Verfahren wird beschrieben, mit dessen Hilfe die aus adulten Sprague-Dawley-Ratten isolierten und unter In-vitro-Bedingungen lange Zeit überlebenden Leberzellen an röntgenbestrahlten Fibroblasten (C3H/10Tl/2) und Lungenzellen aufrechterhalten wurden. Aufgrund morphologischer und ultrastruktureller Untersuchungen wurde festgestellt, daß die auf diese Weise gezüchteten Leberzellen geraume Zeit (32 Tage) ihr In-vivo-Eigenschaften behalten.

24 Stunden nach der Aussaat haben sich die Leberzellen auf die Oberfläche der Nährzellen abgesetzt und ihre in vivo charakteristische Polarität hatte sich erneut ausgebildet. Elektronenoptisch gelang es, am biliären Pol dieser Zellen neugebildete Gallengänge zu erkennen, und zwischen den Leberzellen sowie zwischen deren Membranen und den Nährzellen sowie zwischen deren Membranen und den Nährzellen waren verschiedene Zellkontakte entstanden. Bei der histochemischen Analyse gaben diese Zellen in bezug auf den Glukose-6-phosphatase- und Glykogengehalt positive Reaktionen. Die bereits 14 Tage gezüchteten Leberzellen war es gelungen auf eine frische (C3H/10Tl/2) Nährzellenschicht überzuimpfen. Versucht man dagegen die Leberzellen ohne Nährzellenschicht nur am Boden des Plastikgefäßes zu züchten, so verlieren sie schon nach 5 Tagen stürmisch ihre für die Leber kennzeichnenden Eigenschaften und bis zum 10. Tag wird ihre Mehrzahl den Fibroblasten ähnlich. Aus all dem läßt sich folgern, daß die Züchtung der Leberzellen auf röntgenbestrahlten Nährzellen ein geeignetes Verfahren zur In-vitro-Untersuchung der Morphogenese, der Zytotoxizität oder des Metabolismus gewisser chemischer Stoffe darstellt.

ПРОДВИЖАЮШИЕ ДЕЙСТВИЕ КОРМЯШИХ КЛЕТОК НА ВЫЖИВАНИЕ КЛЕТОК ВЗРОСЛЫХ КРЫС

АННА ТОМРА, Р. ЛАНГЕНБАХ

Дается описание метода, с помощью которого печеночные клетки, изолированные из взрослых крыс штамма Спрейг—Доулей, длительное время выживающих в условиях in vitro, содержали на облученных рентгеновскими лучами фибробластах (C3H/1 0T1/2) и легочных клетках. На основе морфологических и ультраструктурных исследований было установлено, что при таком способе культивирования печеночные клетки длительное время (в течение 32 дней) сохраняют свои приживленные свойства.

24 часов после высева печеночные клетки осаждались на поверхности слоя кормящих клеток и заново появилась полярность, типичная для их прижизненного состояния. В электронном микроскопе удалось выявить на билиарном полюсе новообразовавшиеся желчные пути и между печеночными клетками, как и между мембранами печеночных клеток и кормящими клетками образовывались клеточные связи. При гистохимическом анализе эти клетки показали в отношении содержания глюкоз-6-фосфатазы и гликогена положительную реакцию. После 14 дневного культивирования печеночные клетки удалось перевить на поверхность свежего слоя кормящих клеток (C3H/1 0T1/2). В противоположность этому, при культивации без кормящих клеток на дне пластмассого сосуда печеночные клетки уже на 5-й день потеряют свой характерные для печени свойства и на 10-й день их преобладающее большинство становится подобным фибробластам. Из сказанного можно заключить, что выращивание печеночных клеток на облученных кормящих клетках представляет собой пригодный метод для изучения морфогенеза и цитотоксичности печеночных клеток или для изучения метаболизма определенных химических веществ in vitro.

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PATHOMORPHOLOGY OF ACUTE LYMPHOBLASTIC LEUKAEMIA IN CHILDHOOD: CONVOLUTION OF LYMPHOBLAST NUCLEI

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In the period between 1960—1978 36 children dead with acute lymphoblastic leukaemia (ALL) were studied. Nine cases were found to be in pathological remission, i.e., without any leukaemic infiltrations (7 of these were seen in the last 5 years). The remaining 27 could be divided into two groups on the basis of nuclear structure: in 20 cases the lymphoblastic nuclei were pycnotic, "highly convoluted", or lobulated. in 7 the nuclei were round or oval and their convolution was slight. The survival was 10.8 months in the first and 3.6 months in the second group. Although the origin and the degree of differentation of the cells of these two groups are not clearly known, it seems that the two types represent different stages of T-lymphoblast evolution.

Recent progress in the diagnosis, staging and especially in the therapy of acute lymphoblastic leukaemia (ALL) in childhood resulted in a prolonged survival of the patients, and even the possibility of a permanent cure seems to be close at hand [5, 15).

The influence on the survival of a large number of factors was studied. Initial lower leukocyte and higher (above 100.000/μl) platelet counts, age between 2—8 years, female sex, the absence of hepatosplenomegaly and of mediastinal tumour, a coarsely granular PAS positivity of the lymphoblasts and their "non-T, non-B" character ("O-cells") seem to be associated with a more favourable prognosis.

The pathomorphological characteristics of ALL-s and of T-lymphoblastomas (non-leukaemic at the time of diagnosis) were first appreciated by Barcos and Lukes [1] who described the convoluted apperance of the surface of T-lymphoblast nuclei ("convoluted or cerebriform nuclei"). Recognition of this cytological feature besides others (focal acid phosphatase reaction, immunocytological tests) enable the pathologist to diagnose a more or less immature T-cell tumour (ALL, T-lymphoblastoma) with a high degree of certainty and at the same time to exclude a number of other possibilities (Burkitt-tumour, immature myeloid and monocytic infiltrates). The relationship between nuclear convolution and immunological and other markers (T-cell, "non-T, non-B" ALL-s) has not yet been established. Since, however, the number of cells with convoluted nuclei and especially the degree of nuclear

convolution of the lymphoblasts in ALL and T-lymphoblastoma is variable, there is a possibility to characterize them by these parameters. This seems all the more justified since immunocytological data indicate that T-ALL, "non-T, non-B" ALL'-s and some other unfrequent types [2] represent various stages of blocked differentation of the same cell lineage, i.e., of T cells [3].

For assesing the degree of nuclear convolution, bone marrow and blood smears, organ imprints and even cytocentrifuge preparations are not optimal probably because of the flattening of the cell nuclei. Owing to this circumstance and the fact that few cases have been studied pathomorphologically, a histological study of necropsied ALL cases seemed to be warranted.

In the present work ALL cases necropsied in the last 18 years were raexmined and the relationship of age, sex and survival and of the pathoemorphological features was analysed.

Our observations are indicative of two types of nuclear configuration in ALL: (a) "highly convoluted" nuclei and (b) round or oval nuclei with only a slight degree of irregularity in the nuclear profile ("slightly convoluted" nuclei). In a given case one of the two types is predominant. The survival of the patients with "highly convoluted" nuclei proved to be significantly longer than that of the patients with "slightly convoluted" nuclear profiles.

Material and methods

Between January, 1960, and June, 1978, 39 cases under 14 years of age, with the clinical diagnosis of ALL, were autopsied. The distribution of the cases in the above period was not even, 3/4 of them were observed in the second half of the period. This may be due to the fact that since 1971, the Paediatric Department is a regional centre for the management of ALL patients. In 3 of the 39 cases paraffin blocks of tissues were not available. These were excluded from the study. The age and sex distribution of the remaining 36 cases is given in Table I. Although in 21 cases cytochemical and immunocytological examinations were performed, in the present study our chief interest was the pathohistological features of the cases.

Table I

Distribution of the 36 histologically reevaluated ALL casses according to age and sex

Age (yrs)	Number of cases	Average age (yrs)	Male : female ratio
0— 2	12 (33.3%)	1.6	8:4
3— 5	12 (33.3%)	4.2	10:2
6— 8	7 (19.5%)	7.3	6:1
9—11	3 (8.3%)	11.3	1:2
12—14	2 (5.6%)	12.8	2:0
Total	36 (100%)	5.1	27:9

For the pathohistological reevaluation (HE, PAS, Giemsa, reticulin stainings) formol-paraffin blocks were available from the bone marrow (35 cases), liver [34], spleen [32], lymph glands [28], kidney [29], lung [22], brain [16], adrenal glands [9], ovaries or testes [5]. In a few cases tonsils, thymus heart, intestines, stomach, skin samples were also examined. The naphthol-AS-D-chloroacetat esterase reaction was performed in each case in order to exclude immature myeloid leukaemia and to demonstrate the presence of myeloid metaplasia.

To exclude the influence of subjective factors in the assessement of the degree of nuclear convolution, the coded sections were evaluated by two independent observers and the results were compared. This was repeated several times with a high degree of reproducibility and agreement in opinion of the two observers.

Observations

In Table II, survival of the 36 ALL patients in relation to the year of diagnosis is shown. In the second half of the period 1960—1978 there was a slight increase in survival. Taking into consideration the survival of the patients of the last few years still under treatment, a more prolonged survival was apparent.

Table II

Mean survival time of the 36 ALL cases

Year of diagnosis	Number of cases	Survival (mean, months)
1960—1965	5	5.3
1966—1971	12	8.8
1972—1978*	19	9.6

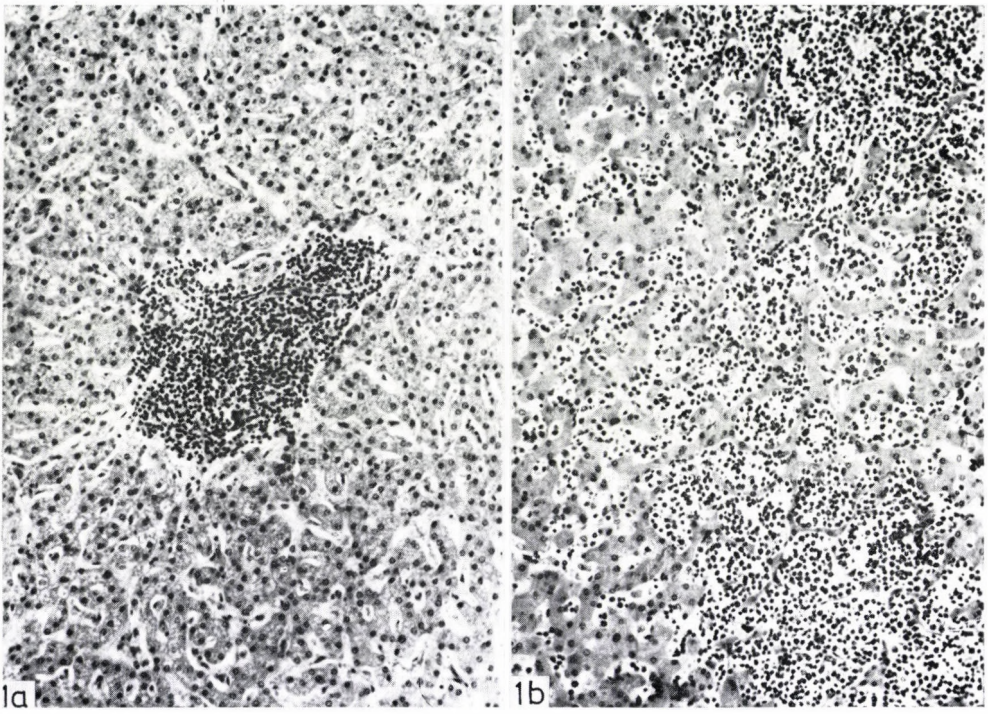
*up to 30. June, 1978

Histological reexamination of the 36 cases revealed no leukaemic infiltration in 9 patients. Of these, 8 died in the last 5 years, which clearly shows the efficiency of newer therapeutical regimens and also its dangers: all of them died in consequence of infection and/or haemorrhage.

Out of the 27 deaths in relapse, 26 showed lymphoblastic infiltrations of the bone marrow, a further patient seemed to be in remission (blood and bone marrow), but at necropsy a meningeal leukosis was found. Concerning the other organs, the presence of leukaemic infiltrations was as follows: liver 21/34, spleen 21/32, lymph gland 18/28, kidney 17/29, lung 6/22, brain 9/16, adrenal gland 4/9, gonads 0/5.

Not dealing with the well known autopsy findings of ALL [13], we shall describe our observations on the leukaemic infiltrations (a) of the liver; (b) of the lymph glands; and (c) on the convolution of ALL lymphoblast nuclei.

(a) In 13 cases we did not find any leukaemic infiltrations in the liver (average survival 9.5 months). Among the remaining 21 cases in 11 only peri-

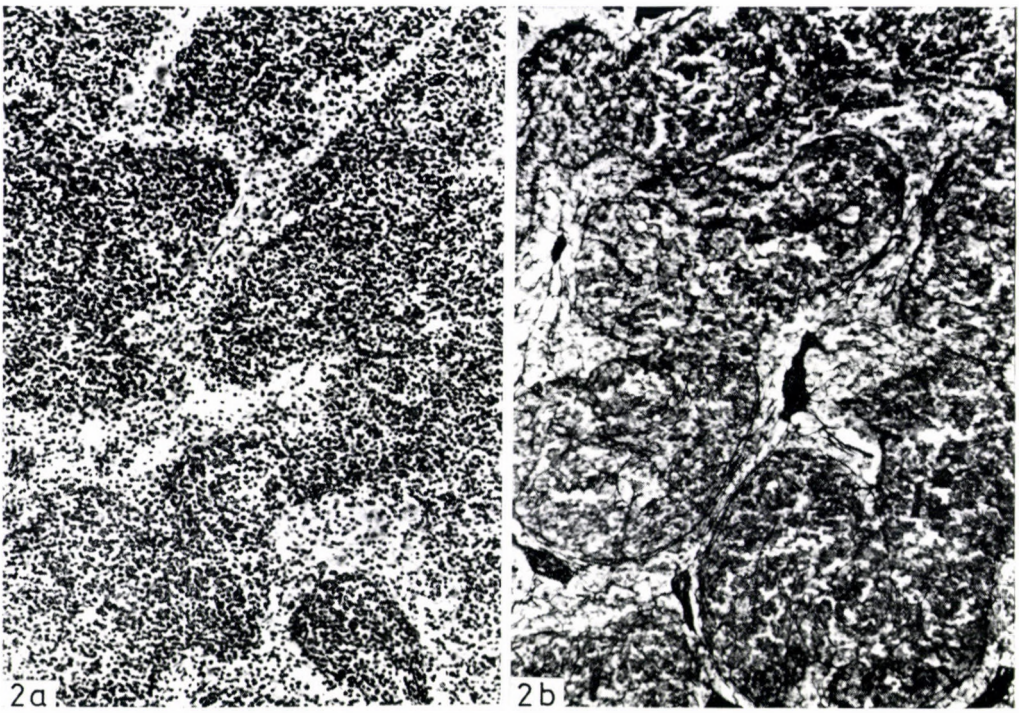


Figs 1a—b. ALL, liver, Giemsa, approx. ×180 (*a*) Sharply outlined periportal infiltration ("lymphocytoma"), (*b*) periportal infiltration extending into the sinusoids

portal (survival 9 months) and in 10 others, periportal and sinusoidal (survival 8.5 months) accumulations of lymphoblasts were present (Figs 1. a—b). There were sharply circumscribed periportal infiltrations in two cases with the clinical diagnosis of ALL, in whom, however, the cells in the periportal fields proved to be naphthol-AS-D-chloroacetat esterase positive, thus these cases, considered to be acute myeloid leukaemias, were excluded from the study. This observation has some differential diagnostic significance, since it is generally believed that periportal infiltrations ("lymphocytomas") may occur only in haemoblastoses of lymphoid origin. As far as haemoblastoses of adults are concerned, we have not seen exceptions to this rule. It appears, however, that the situation is different in children.

(b) In the lymph glands in ALL, in contrast with the observation of RAPPAPORT [12], the sinuses and sometimes the primary follicles were preserved. Due to this type of involvement, the lymph glands exhibited some degree of nodularity or rather lobulation (Figs 2. a—b). In a few cases there were masses of leukaemic cells in the paracortical areas and medullary cords.

(c) In the 27 ALL cases who died in relapse we thoroughly studied the appearance of nuclei of lymphoblasts, mainly in Giemsa-stained sections.



Figs 2a—b. ALL, lymph gland (*a*) Giemsa, (*b*) reticulin, approx. ×180. In the infiltrated lymph gland the sinusoids are well preserved, the appearence of the lymph gland is somewhat nodular (lobulated)

According to the appearance of nuclei the cases fell into two types. In 20 cases (74%) the small nuclei were highly convoluted, even lobulated ("strongly convoluted group"). The lymphoblast nuclei of the further 7 cases (26%) were easily distinguished from the previous group, their large nuclei with dispersed chromatin being only slightly convoluted, even uniformly round or oval ("slightly convoluted group"). Inclusion of the cases to one of these two groups was decided by two independent observers with a high degree of reproducibility in repeated tests.

Nuclear convolution in the "strongly convoluted group", unlike the fine gyri and foldings of the nuclear membranes in biopsy material, was pronounced, the nuclei appearing somewhat shrinked, deeply stained and lobulated. Even if the high degree of nuclear convolution may be partly the result of postmortal changes, the time elapsed between death and necropsy did not differ significantly in the two groups. That means that the nuclei in the "slightly convoluted group" are less sensitive to artificial effects than the nuclei of the other group. There was a significant difference in survival between the two groups, being 10.8 months in the "highly" and only 3.6 months in the "slightly convoluted

Table III

Distribution of cases according to the degree of nuclear convolution (age, sex, survival)

Nuclear configuration	Number of cases	Age (means, yrs)	Male:female ratio	Survivsl (mean, months)
"highly convoluted"	20 (74%)	4.6	15:5	10.8
"slightly convoluted"	7 (26%)	6.9	4:3	3.6
Total	27	5.2	19:8	8.9

group" (Table III). The occurrence of cases of both groups was fairly even in the period of time studied.

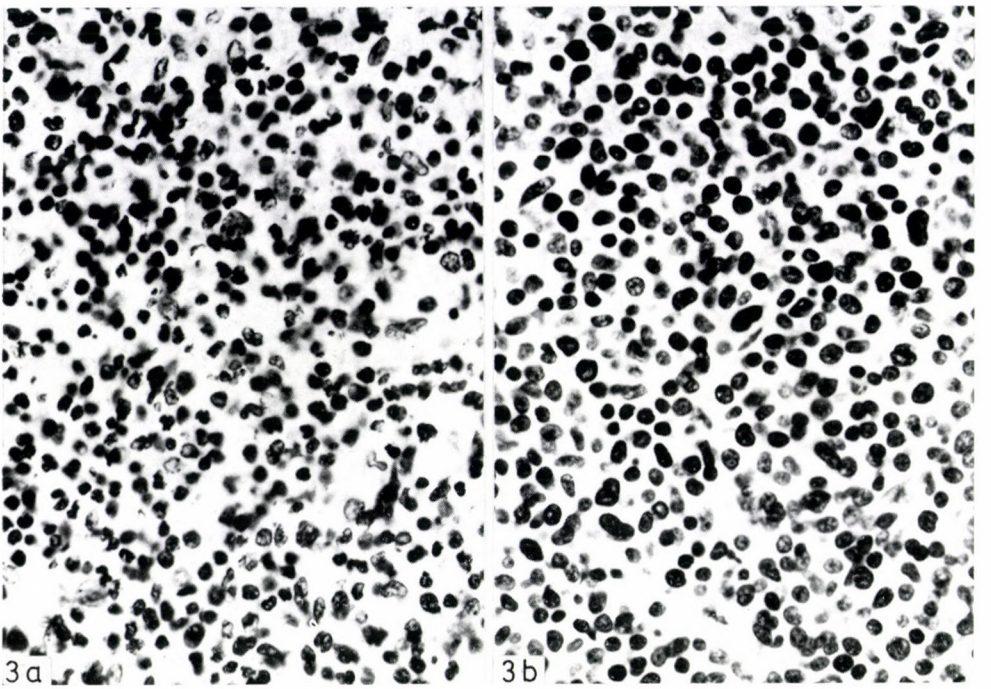
In two cases an interesting phenomen, termed type switchover was observed. In these two patients after long remissions a terminal relapse developed, uninfluenced by the previously effective cytostatic regimens (survival 22 and 23 months). In both cases there were infiltrations of "highly convoluted" cells in some organs (liver, kidney), but in the vessels, in the bone marrow, spleen and lymph nodes the "slightly convoluted" cells were predominant (Fig. 4). The simultaneous occurrence of the two cell types in one tissue indicates a significant difference in the sensitivity of their nuclei to postmortal effects.

Discussion

The diagnosis of ALL is based on cytochemical and immunocytological typing of blood and bone marrow cells and biopsies are rarely done in this malignant lymphoma. A biopsy is done only in order to clarify the nature of lymph gland enlargement, of skin infiltrations or to prove or exclude the presence of testicular infiltrations. In cases of childhood lymphomas without leukaemia the histologic examination of lymph glands is of diagnostic importance.

In recent years, ALL was a further addition to the increasing number of haemoblastoses in which long, permanent remissions may be achieved and post mortem experience clearly indicates that the predominant part of the patients die in "pathological remission", due mainly to infections or haemorrhagic complications. In recent years the number of cases with relapse has decreased, a fact which underlines the importance of studying cases with massive organ infiltrations in order to learn more about the natural history of ALL.

Concerning liver infiltrations there are certain differences, one group showing infiltrations strictly limited to the periportal fields, and the other showing infiltrations involving also the sinusoids. Although there is no significant difference in survival of the patients belonging to these groups, neverthe-



Figs 3a—b. ALL, liver, periportal fields, Giemsa, approx. ×800. (*a*) Nuclei irregular in size and configuration ("strongly convoluted"); (*b*) nuclei more or less round, a few convoluted nuclei ("slightly convoluted")

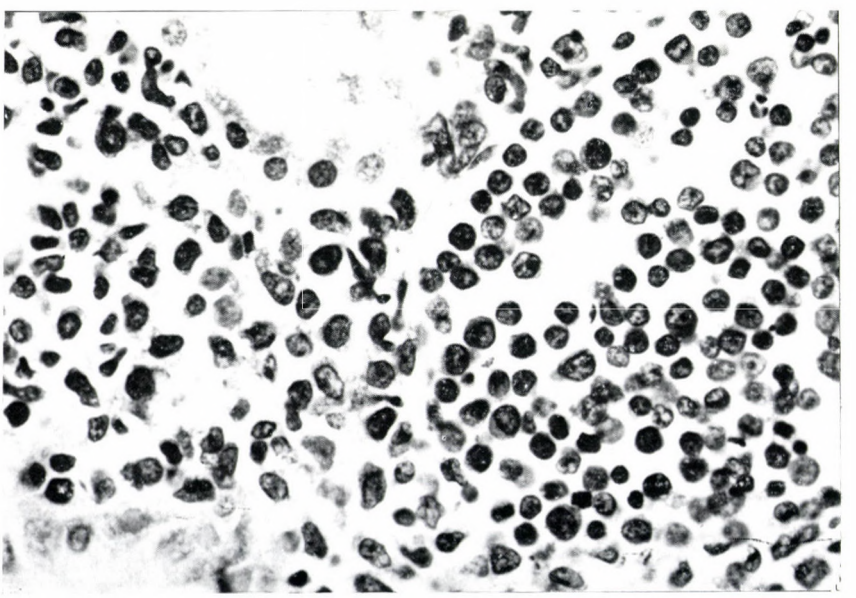


Fig. 4. ALL, kidney, Giemsa, approx. ×800. Lymphoblast nuclei in the interstitial spacesy are "strongly convoluted", those in the lumen of the vessel (at left), round or oval ("slightl convoluted")

less it would be interesting to know the causes of this difference. The naphthol-AS-D-chloroacetate esterase reaction was very useful in two cases, displaying sharply limited periportal accumulation of cells of myeloid origin. This observation stresses the importance of performing the reaction of doubtful periportal cell infiltrations are seen in biopsy material.

Our observations on lymphoblast nuclei in ALL agree in some respects with those of NATHWANI et al. [11] and of LENNERT [9]. In 20 cases (74%) the cell nuclei were uniformly "strongly convoluted", but in 7 cases (26%) round or oval "slightly convoluted" nuclei were predominant. In addition, there was an important difference in survival between the two groups, being three times longer in the "highly convoluted group" than in the other group. On the basis of this finding, in initial cases a study of the nuclear convolution seems warranted. Since nuclear convolution can more reliably be judged in sections than in smears or imprints, histological examination of the small bone marrow pieces remaining after making smears of aspiration material is a useful method, routinely done in cases of ALL at our Department. Furthermore, our findings show that in autopsy material the nuclei undergo changes such as pycnosis and lobulation. This transformation, the causes of which are unknown, is seemingly independent of the period between death and autopsy and may be observed sometimes also in biopsy material. According to GLICK et al. [7] lymphoblast nuclei in "non-T non-B" ALL show folds and bridges, which probably become more accentuated in post mortem material.

The origin of the "slightly convoluted" cells is uncertain. It is unlikely that they should correspond to Burkitt-tumour with acute leukaemia, this being an infrequent occurrence: in the material of FLANDRIN et al. [6] it represented about 2% of the cases, and its histological appearance is quite different from that in our cases. The occurrence of a few unambigously convoluted cells, furthermore the positive focal acid phosphatase reaction, a T-cell marker [4, 15] is suggestive of their being immature T-lymphoblasts. In addition, one of the two cases with type switchover was initially acid phosphatase (focal) positive which indicates the original T-cell nature of the cells. In a recent study, WILLIAMS et al. [17] wrote that in lymphoblastic lymphoma and ALL one rarely finds nuclear convolutions. Their ALL cases were, however, studied in smears or cytocentrifuge preparations, but not in sections. Although their material can hardly be compared with ours, their cases with cells forming EAC rosettes and without surface Ig (9 of 36) might have been T-cell precursor leukaemias (negative E rosette, absence of surface Ig and presence of complement receptors [16], and they may thus corresponded to our "slightly convoluted group". There are also observations [8, 18] indicating that "non-T non-B" ALL-s might be of B-cell origin. Nevertheless, it is clear that further studies are needed for the cytochemical-immunocytological identification of the two cell types found in our ALL material.

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PATHOMORPHOLOGIE DER KINDLICHEN AKUTEN LYMPHOBLASTOIDEN LEUKÄMIE ÜBER DIE KONVOLUTION DER LYMPHOBLASTENKERNE

T. PAP, IBOLYA RETIH, P. KAJTÁR und G. KELÉNYI

Im pathologisch-anatomischen Institut der Universität Pécs wurden die zwischen 1960 und 1978 beobachteten 36 Fälle von autoptisch gesicherten lymphoblastoiden Leukämien analysiert. In 9 Fällen wurde eine pathologische Remission festgestellt, d. h., bei den hystopathologischen Untersuchungen liessen sich keine leukämische Infiltrate nachweisen (7 dieser Fälle stammten aus den letzten 5 Jahren). Die übrigen 27 Fällen lassen sich aufgrund der Kerne der leukämischen Zellen zwei Gruppen zuordnen: in 20 Fällen waren die Kerne pyknotisch und zeigten ausgeprägte Konvolution oder waren gelappt. Demgegenüber wurden in 7 Fällen runde oder ovale, mäßig zusammengeballte Kerne nachgewiesen. Die Überlebenszeit lag in der ersten Gruppe bei 10,8 Monaten, in der zweiten bei 3,6 Monaten. Obwohl der Ursprung und Differenzierungsgrad der Zellen der beiden Gruppen nicht mit Sicherheit beurteilt werden kann, läßt sich mit hoher Wahrscheinlichkeit annehmen, daß sie verschiedene Differenzierungsphasen der T-Zellen darstellen.

ПАТОМОРФОЛОГИЯ ДЕТСКОЙ ОСТРОЙ ЛИМФОБЛАСТОИДНОЙ ЛЕЙКЕМИИ О СВЕРТЫВАНИИ ЯДЕР ЛИМФОБЛАСТОВ

Т. ПАП, ИБОЙА РЕТИ, П. КАЙТАР и Г. КЕЛЕНЬИ

Авторами было изучено 36 аутоптических случаев острой лимфобластоидной лейкемии, наблюдавшихся от 1960 до 1978 г. в Патологоанатомическом институте Медицинского университета г. Печ. В 9 случаях установили патологическую ремиссию, то есть в ходе патологоанатомических исследований не было выявлено лейкемических инфильтратов. (7 из 9 случаев наблюдались в последних 5 лет). Остальных 27 случаев на основе ядер лейкемических клеток можно было отнести к двум группам. В 20 случаях ядра оказались пикнотическими, показали выраженное свертывание или дольчатость. В противоположность этому в 7 случаях были выявлены круглые ядра с умеренной конволюцией. В первой группе выживаемость длилась 10,8 месяцев, а во второй группе 3,6 месяцев. Хотя происхождение и степень дифференциации двух групп нельзя определить с полной уверенностью, кажется вероятным, что они представляют различные фазы дифференциации Т-клеток.

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„DICLONAL" GAMMOPATHIES

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Eleven patients with "diclonal" gammopathies have been followed clinically and cytologically in relation to the changes in their serum protein composition. It was observed that the malignant transformation became faster after a second protein had appeared with the appearance of immature lymphoid elements and plasmoblasts. Malignant transformation may occur after long periods of stagnation, therefore a continuous observation of such cases is mandatory.

Introduction

Malignant proliferation of immunologically active lymphoid-plasma cells may be accompanied by some specific clinical, cytological and immunological manifestations. In these cases each homogeneous (monoclonal) immunoglobulin (Ig) formed is derived from one, but occasionally from two or more cell groups. These latter are termed di- or oligoclonal gammopathies.

In the past decade, several observations have called for a revision of the "one immunoglobulin, one cell clone" theory. The increasing number of diclonal gammopathies described in recent literature provide data concerning the clonal origin and genetic control of Ig-synthesis and the background of lymphoproliferative diseases [1, 3, 10, 11, 15, 29, 47—57, 74, 75, 89, 93, 95].

The incidence of diclonal gammopathies cannot be estimated realistically. Most authors mention a 1% incidence but values of 1—5% have also been reported [7, 17, 20, 31, 32, 39]. There are altogether 160 cases in the literature in which the joint occurrence of two or more monoclonal proteins was observed in the same patient's serum [17, 20, 39, 61]. In the case of a joint occurrence of two monoclonal proteins they usually have identical kappa (or lambda) types of light chains, while Ig-classes (heavy chains) do not show such a preferential linkage. For example, IgG may equally associate with IgA or IgM, with a random coupling.

Preferential linkage of identical light chains and the frequency of its occurrence suggest a connection between the synthesis of the two proteins which argues in favour of monoclonal derivation. Although immunofluorescence studies support the existence of different sources of origin [10, 13, 14, 21, 26, 55, 93] rather than a dual synthetic activity of one single clone [11, 28],

the resemblance in primary structure of the two proteins still points to the existence of a common precursor cell [12].

The two most frequently encountered combinations, IgG + IgA and IgG + IgM, amount to 40% of all cases [77], whereas the IgM + IgA combination is scarcely observed [24]. In normal cases the number of double positive cells containing the IgM + IgA combination is extremely low (0.25%). This finding [23, 24] supports the view that the number of malignant clones is similar to their "counterparts" in healthy individuals.

It is not clear how internal or external factors can lead to the formation of cells of dual immune-capacity and how such a cell-line can develop in the presence of a clonal producing a monoclonal protein.

To study these questions, 11 patients with double gammopathy were collected. We have attempted to follow the changes in their clinical symptoms in relation to those of cytological and immunological patterns, the dynamism of the whole process and, where this was possible, the connection of therapy and clinical manifestations.

Material and methods

The material was selected from 201 patients whose sera contained monoclonal protein (M-component). Selection was made by testing the sera and possibly urine.

Grouping of cases (Table I.) was made according to the clinical manifestations. The first group contained "non-malignant" cases one of which *(I. D.)* proved to be non-malignant in the long run, while the other *(P. J. I.)* after ten years turned from a typical non-malignant "benign monoclonal gammopathy" (65) to a malignant lymphoma-like pattern as judged by its a transition to te second group. The second group consisted of relatively non-malignant cases *(P. J. II., I. K.)* showing a very slow progress till the appearance of a new monoclonal protein. In the third group of patients clinical symptoms were from the beginning characteristic for a malignant process *(F. J., P. S., P. M.)* but the time course of the process still allowed to control the immunological and cytological events. In the fourth group, observation of the progress and of the changes was difficult *(S. J., K. J.)* or impossible *(G. G., O. J.)*.

In 7 patients *(F. J., G. G., I. D., K. J., O. J., P. M., S. J.)* a double gammopathy was detected at the first examination. In two of them *(G. G., O. J.)* changes could not be followed due to the short survival and the lack of continuous observation. In four patients *(I. K., P. J. I., P. J. II., P. S.)* diclonal gammopathy developed from an earlier monoclonal stage during the period of observation.

The histology could be examined in 7 cases *(F. J., I. K., K. J., P. J. II., P. M., P. S., S. J.)*; samples of bone marrow of 4 patients *(F. J., I. D., I. K., P. J. I.)* were processed for electron microscopy.

Study of the protein was carried out by immunoelectrophoresis [78], agar- and agarose-gel electrophoresis [37, 38, 100], titration immunoelectrophoresis [33] and dilution-comparative immunoelectrophoresis [60]. The latter was adapted for the demonstration of double IgG gammopathies by the modification of the method of OSSERMAN [58]. In the diluction of experimental material, IgG precipitation bands were compared with those of immunologically pure "normal" IgG. Antigen-deficiency of IgG variants was studied using immune sera readily reacting with IgG.

For electrophoresis, DIFCO agar (Special Agar Noble) and SERVA agarose were used. Immune sera were obtained from HUMAN, PHYLAXIA, HYLAND, BEHRINGWERKE and NORDIC DIAGNOSTIC [30]. Gel electrophoretic plates were evaluated by KIPP-ZONHEN/SKALAR device. Ultracentrifugation was performed in PHYWE-57 ultracentrifuge. Gel filtration was made through PHARMACIA Sephadex G_{200} material [85] by an LKB ULTRORAC 7000. For electronmicroscopic studies were used JEM 100B and 100C electronmicroscopes. Bone marrow samples obtained by puncture were fixed in glutaraldehyde and osmic acid, and embedded in Araldite. Ultrathin sections were cut with REICHERT ultramicrotome and stained with uranyl acetate and lead citrate [REINOLDS, 72].

Results

I. "*Benign monoclonal gammopathies*" are rarely transformed to malignant disease [4—6, 65]. An intercurrent event is supposed to cause such a transformation. In the case of *I. D.*, the protein anomaly was indicative of a malignant process, in sharp contrast with the non-malignat clinical appearance and, except for a primary chronic polyarthritis, no other symptoms occurred. Rheumatoid arthritis may occur together with monoclonal gammopaties [18, 43, 92]. Occasionally free light chains may appear in the concentrated urine but they form electrophoretically a heterogeneous zone and the kappa/lambda ratio is usually 2:1, similarly to the heterogeneity of polyclonal Ig. In this patient, however, a homogeneous M-component was found in the less concentrated urine (1:8) and with immunoelectrophoresis both free light chains could be demonstrated in the native urine.

The initial polyclonal hyperplasia of rheumatoid arthritis may later be limited; the wide-spectrum immune-response becomes restricted to "oligo- di- or monoclonal". Although this is a possible sequence of events, the search for a malignant lymphoproliferative process as a background of symptoms must not be given up. This has been justified in other cases [71].

Table I

Summary of cases

Name	Age	Sex	Clinical diagnosis	First observation	Serum M-proteins	Urine M-proteins	Cytost. treatment	Death	Pathologic findings
I.D.	52	Male	PCP	1975. X.	IgAL + IgAL	BJK + BJL + Fc	No	Alive	
P.J. I.	67	Male	CAD	1968. III.	IgMK + IgGL	BJK	No	Alive	
P.J. II.	80	Male	MM	1970. V.	IgAK + IgG_2K	BJK + fragments	Yes^{x}	1974. XII.	MM
I.K.	74	Male	WMG	1967. III.	IgMK + IgG_1K	BJK + Fc(γ_1)	Yes^{xx}	1971. VIII.	WMG ? ML
F.J.	62	Male	MM	1975. IV.	IgG_1K + IgG_1K	BJK	Yes	1977. VII.	PBS
P.S.	47	Male	MM	1969. I.	IgG_1L + IgG_2K	?	Yes	1972. XII.	MM*
P.M.	56	Male	WMG?MM?	1966. XII.	IgMK	BJK + BJL	Yes	1971. (?)	WMG ? ML*?
S.J.	76	Female	MM, ML?	1970. IV.	IgMK + IgGK	?	Yes	1971. X.	PBS
K.J.	64	Female	MM	1976. II.	BJK	BJK + BJL	Yes	1977. III.	MM*
G.G.	66	Female	MM	1969. I.	IgAK + IgGK	BJK	Yes	1969. VII.	MM
O.J	68	Female	WMG?	1966. IV.	IgMK + IgGK	?	No	1966. V.	ML, MM?

PCP primer chronic polyarthritis
CAD chronic cold agglutinin disease
x, xx in the final stage
MM multiple myeloma
WMG macroglobulinemia Waldenström
ML malignant lymphoma
PBS plasmoblastic sarcoma
*, *, * with many immunoblasts or plasmoblasts

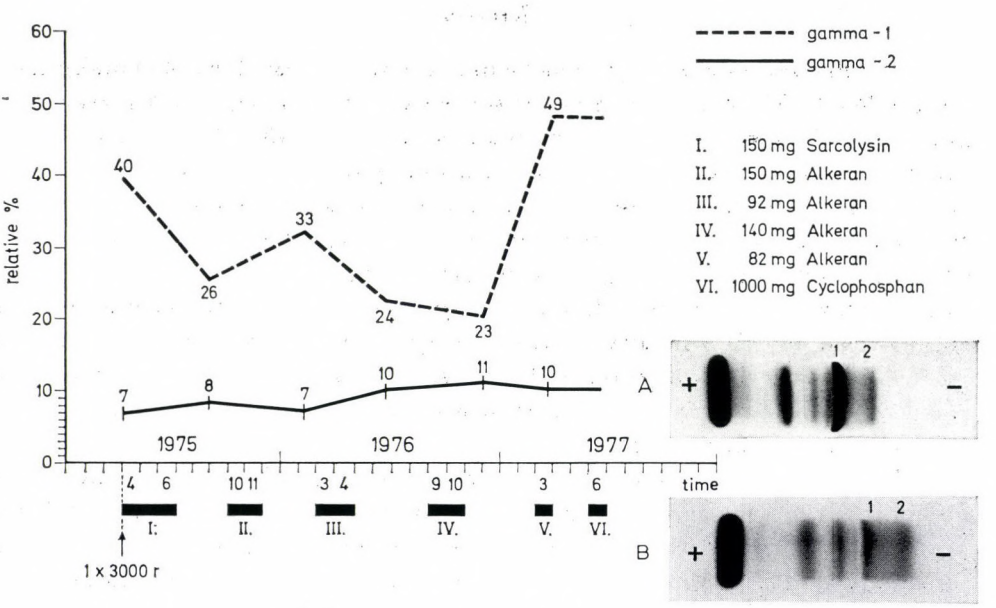


Table II

Relative concentrations and their changes during cytostatic therapy of two IgG_1K M-components of *F. J.* Agar-gel electrophoretic picture prior to cytostatic therapy *(A)* and after treatment IV. *(B)*

Long-term observation of non-malignant cases and the detection of malignization is not frequent [39, 93]. *P. J. I.* had a 10 years clinical history starting with a chronic cold-agglutinin disease following an atypical pneumonia. Malignization occurred during the observation period. In the lymphoid-plasma cells of the bone marrow, lipid-like inclusions were found [65]. Subsequent immunological test have shown besides IgMK the incipient production of an IgGL protein with the excretion of Bence Jones kappa protein. As the biological relationship between controlled and extensively proliferating cell types is obscure, the documentation of each single case may substantially add to the understanding of the question whether or not a postinfection immune-response is capable of inducing a benign monoclonal gammopathy; can this turn into a malignant form; may clones exist whose parent cells were transformed by a virus?

II. Within the group of *initially relatively non-malignant but later malignantly transformed diseases,* the serum of *P. J. II.* displayed the sudden appearance of a new paraprotein IgG_2K, in addition to the earlier IgAK, accompanied by the excretion of kappa-type Bence Jones protein and Ig-fragments (Plate II. D. E.). The bone marrow became rich in cells and focally lymphoid cells appeared in the mass of mostly immature plasma cells (Plate

Plate I.

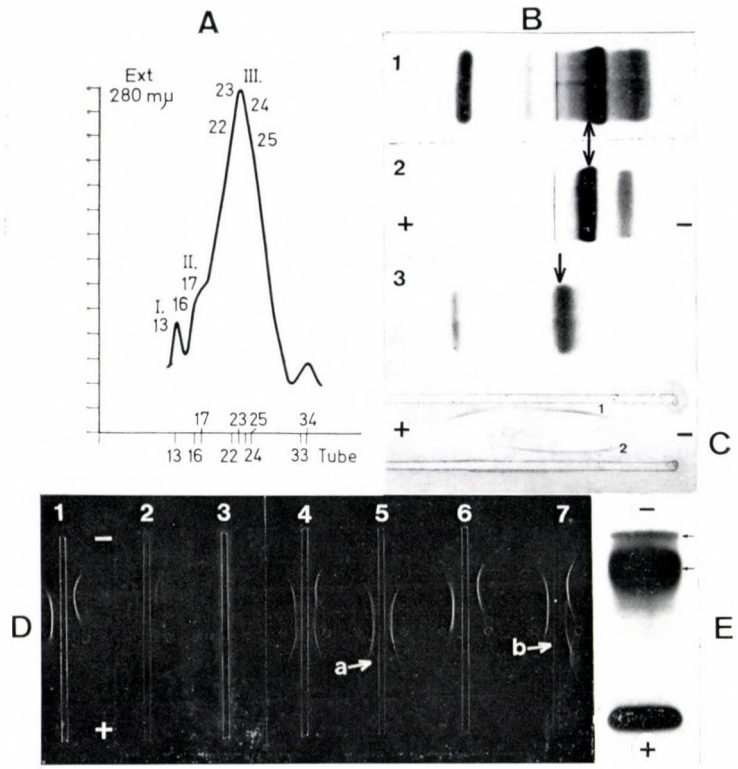


Plate I. Agar-gel electrophoretic (A) and ultracentrifugal picture (B) of serum-samples of *I. K.* Sample 1 had a macroglobulin concentration of 31% comprising several polymer fractions. Two years later (2) the amount of 15 S was only measurable (24%). Arrow points at M-components. *Fig. C* shows the immunoelectrophoretic examinations of the euglobulin fraction and supernatant of the native serum of *I. K.* In the first trough anti-IgG (gamma); in the second and fifth, anti-IgM (mü); in the fourth, anti-kappa, and in the sixth, polyvalent immune sera were poured. *Figs D, E, F* show the separation of the two monoclonal proteins. Components purified by Sephadex G_{200} (preparative column, 100×10 cm, elution volume 120, 300, 500 ml) can readily be recognized by agar-gel electrophoresis *(D)* besides M-component appearing in the 1st and 2nd peaks *(F)*. In the latter, a substantial amount of normal IgG is present. This M-component shows an antigen-deficiency with dilution-comparative immunoelectrophoresis *(E, arrow)*

VII, E.). In the case of *I. K.*, a sudden malignization was encountered. The formerly non-malignant form of Waldenström macroglobulinaemia was replaced by a rapidly progradiating form: in addition to IgMK an IgG_1K monoclonal protein appeared. In the urine, kappa type free light chain and Fc ($gamma_1$) fragment was excreted. With the appearance of IgG_1K, the amount of IgMK decreased (Plate I). As heavy chains of the two proteins were not analysed immunochemically, the genetic mechanism of switching over from the synthesis of one heavy chain to another [22, 67, 86, 87, 95—98] could not

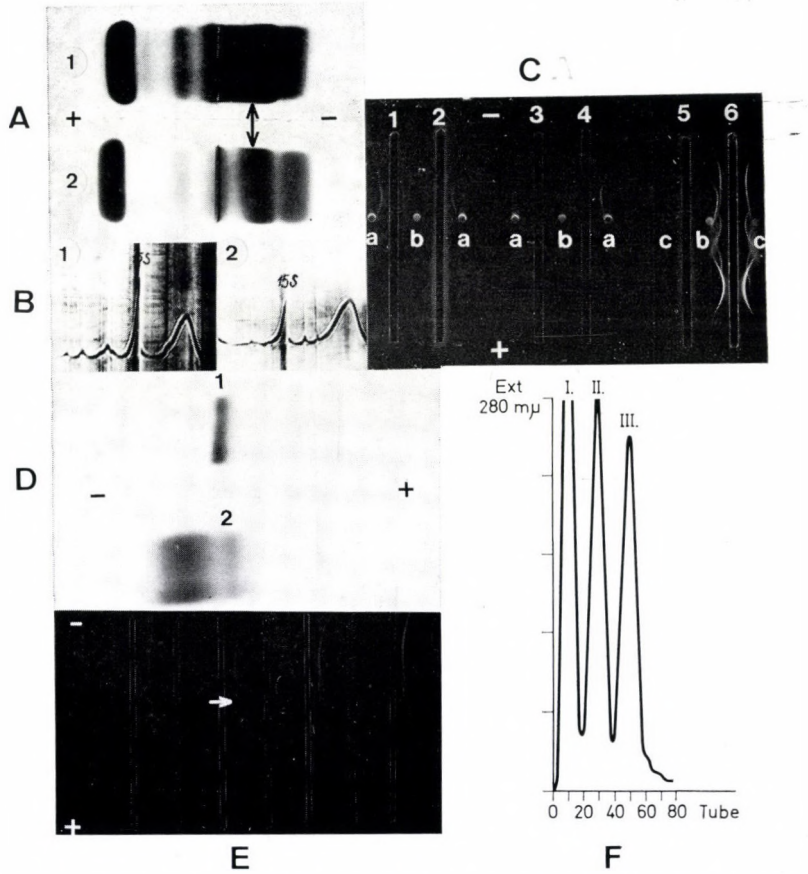
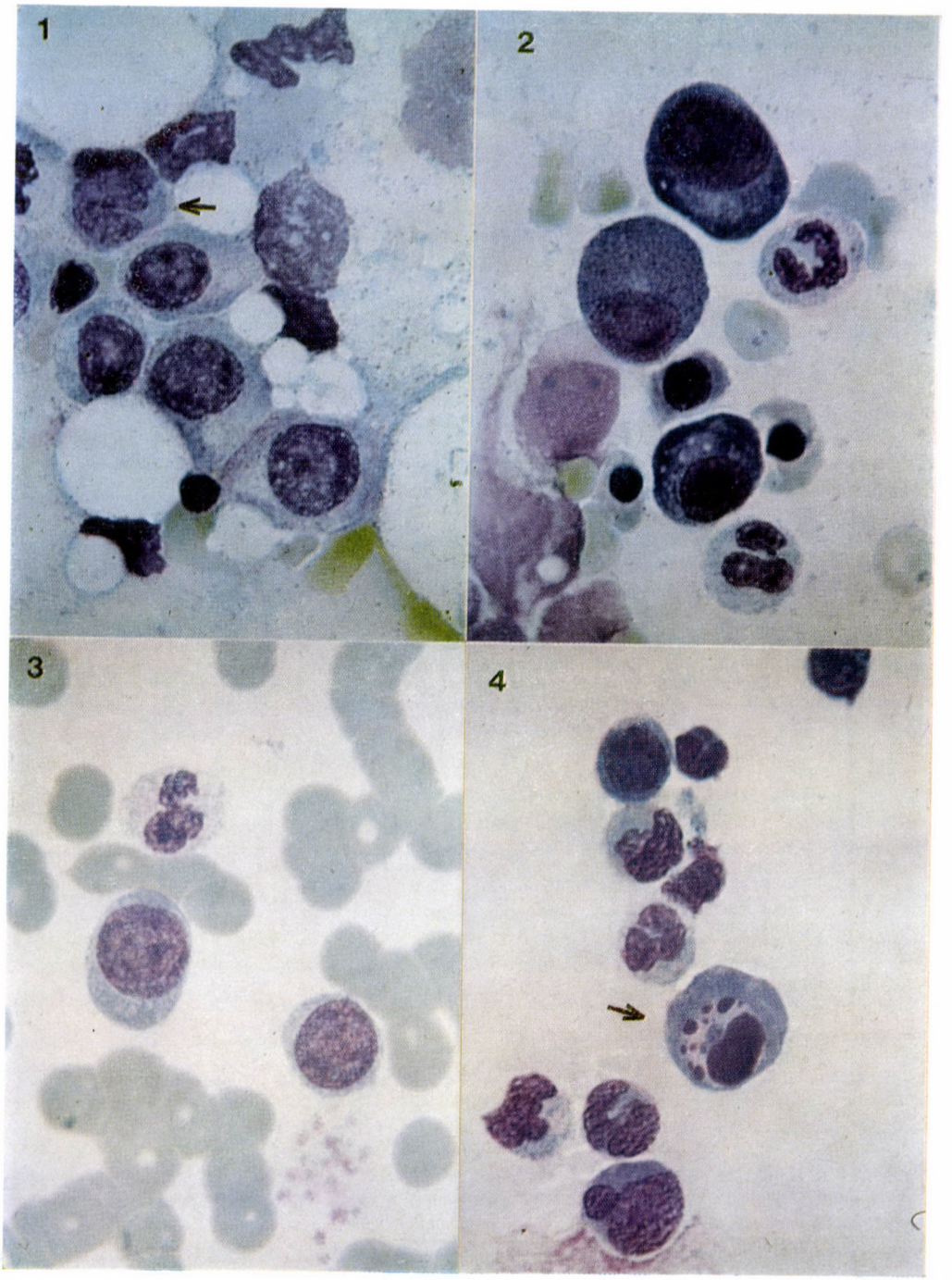


Plate II. Fig. A shows the fractionation by Sephadex G_{20}, gel filtration (column size 70×1.5 cm; elution volumes, 52, 68, 92 ml) of the urine concentrated to 1/10 of *P. M.* In fraction *III*, heavy chain determinants were absent. With anti-kappa and lambda immune sera, strong precipitation arhes were obtained *(C. 1, 2)*. *Fig. B* shows the agar-gel electrophoresis of the M-components of the serum (1), euglobulin fraction (2) and urine concentrated (3) to 1/10 of *P. M.* These fractions, investigated by immunoelectrophoresis, in *Fig. D:* 1, anti-Fc; 2, anti-Fab; 3, anti-lambda; 4, anti-kappa; 5, anti-IgA (alpha); 6, anti-IgG (gamma); 7, polyvalent immune serum. On the right side of the troughs is seen the serum, on their left side, the urine. Arrow "a" has a double point while "b" can be placed in the position of IgA but can hardly be seen with polyvalent immune serum near the through. *Fig. E* shows the agarose-gel electrophoresis of the serum and double M-components (arrows) of *P. J. II.*

Plate III. Bone marrow smear of *F. J.* Cells with light cytoplasm and nuclei containing nucleoli *(1)*. Arrow points at a cell having a lobulated, large nucleus. The other cell population *(2)* has excentric nuclei and basophilic cytoplasm. In the peripheral blood, lymphoid-plasma cells with narrow cytoplasm and well visible nucleoli are conspicuous *(3)*. Amoung isolated cells *(4)* the arrow indicates an atypic lymphoid-plasma cell possessing a fragmented nucleolus. In its vicinity, cells with finger-like nuclear lobulation can be observed (separated by Ficoll-Uromiro). May-Grünwald-Giemsa, $\times 1000$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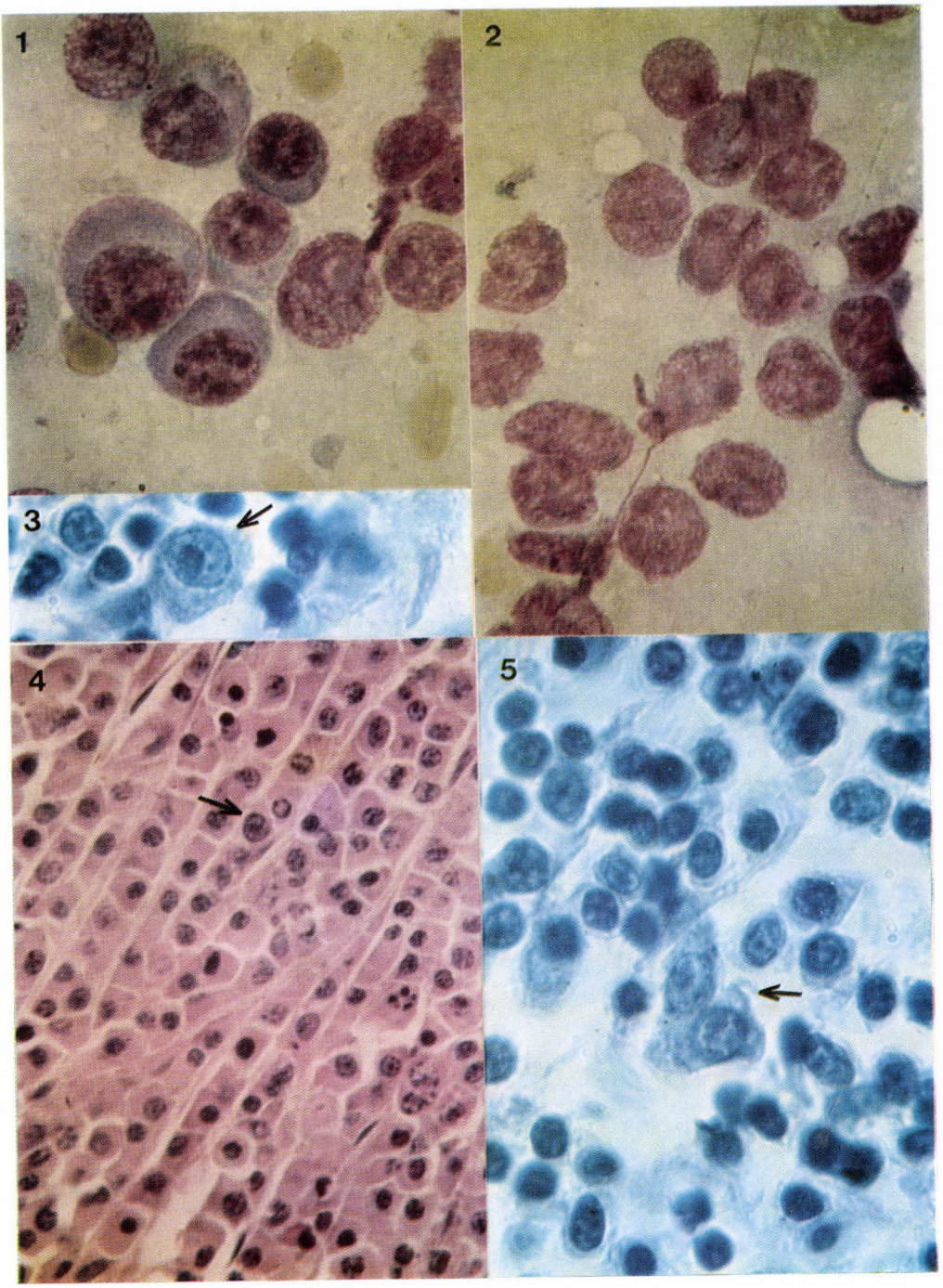
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be verified. Still, the decrease of IgMK on the appearance of IgG_1K suggests that inhibition of the production of one monoclonal protein is coupled with the onset of production of the other. Cytologically, the bone marrow displayed a colourful and hitherto unknown picture. All cells of the lymphoid-plasma cell-line were present. Immature lymphoid cells occurred in a large number accompanied by plasmablasts differing in maturity (Plate VI).

Mature plasma cells were rarely encountered and electron microscopy of the cell-line revealed a similar nuclear structure supporting the view that *these cells belong to the same line but are stopped at different stages of maturation.* Thus, the two different monoclonal proteins may have originated from one single monoclonal precursor by its daughter cells of different maturity.

III. In the group of *initially malignant and continuously controlled cases, F. J.* possessed two identical heavy chain class and light chain type (IgG_1K + + IgG_1K) proteins which, however, differed in electrophoretic mobility and concentration. There are two circumstancial evidences of the different origin of two monoclonal proteins: (i) two cell types can be seen in bone marrow smears (Plate III, 1, 2, and Plate V); (ii) during treatment, the concentration of the M-components changes in the opposite direction: the concentration of the protein of lower mobility slightly increases, while that of the other decreases. This may be an adequate indicator of the drug-sensitivity or resistance of the individual clones (Table II). The clinical pattern changed into plasma cell leukaemia and administration of a new cytostatic was without effect. This form, in fact, can hardly be influenced by drugs [46, 59, 66, 93]. Atypical cells with fragmented nuclei present in the blood (Plate III, 4) also indicate a high degree malignancy [66]. The transitional nature between myeloma multiplex and lymphoma was supported by the histological examination of lymph nodes (Plate VII, C). This has several therapeutic and nomenclatural implications.

The double gammopathy of *P. S.* developed from a monoclonal form during continuous high-dose cytostatic treatment (melphalan) light and heavy chains differed from those of the previous protein (IgG_1L + IgG_2K).

The cause(s) of diclonal gammopathy formation are not known. *Prolonged cytostatic treatment* [36, 94, 99], *cell fusion or hybridisation can be held responsible* [16, 48, 83, 84]. The cae of *P. S.* accompanied by the appearance of neoplastic cervical lymph nodes (Plate VII, D) together with the new monoclonal protein suggests that cytostatics may induce malignant cell proliferation or

←

Plate IV. In the bone marrow smear of *K. J.* most plasma cells are of nucleolated and immature, with a narrow cytoplasmic rim *(1)*. In addition to plasma cells clusters of another lymphoid cell type lacking a cytoplasmic rim are present *(2)*. May-Grünwald-Giemsa, ×1000 *Figs. 3—5.* Histologic sections of the spleen. Arrows point to pale immuno- and plasmoblasts. Giemsa, ×600. Costal tumour *(4)* with numerous immature plasma cells (arrow). Haematoxylin-eosin, ×400

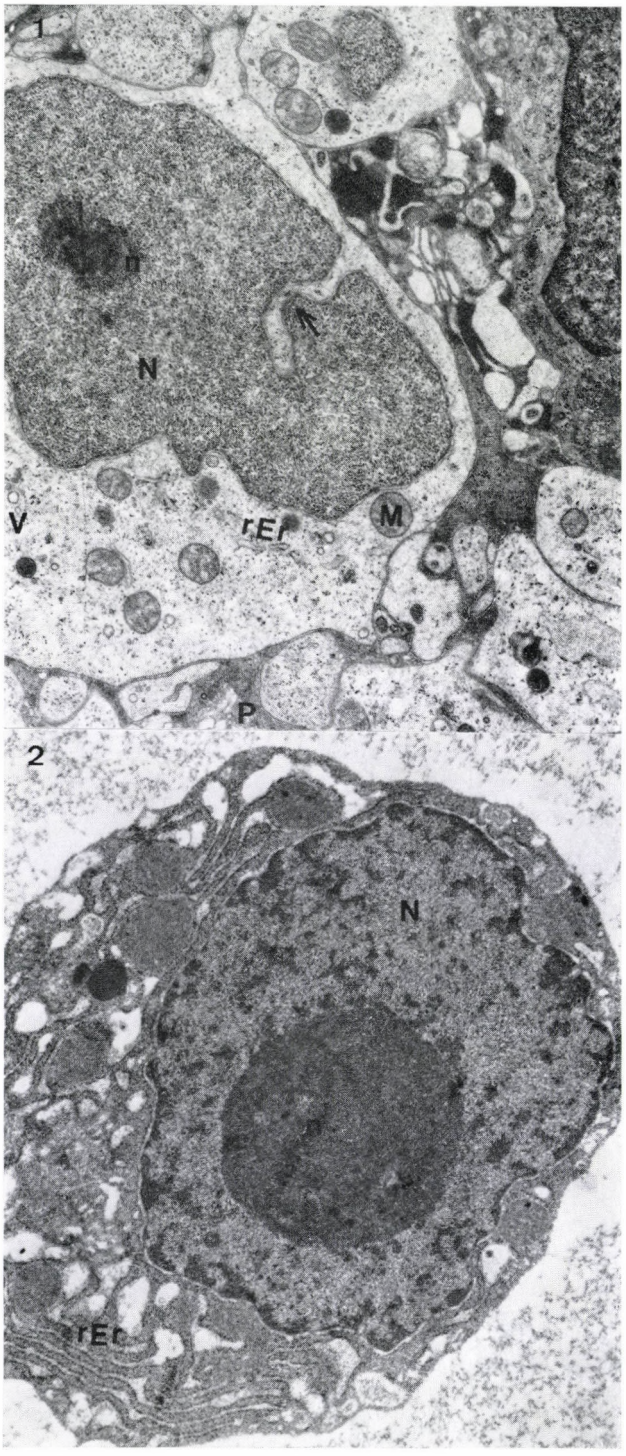

Plate V. In *Fig. 1* immature lymphoid cell surrounded by processes of reticular cells. The cytoplasm is poor in organelles; a few mitochondria (M), vesicles (V) and immature granular endoplasmic reticulum (rEr) are visible. The nucleus is intented (arrow) with evenly distributed chromatin and prominent nucleolus (n). Electronmicrograph from the bone marrow of *F. J.* ×13.500. The other cell type *(2)* has a large nucleus with an extraordinarily large nucleolus. Well developed granular endoplasmic sacs. ×16.500

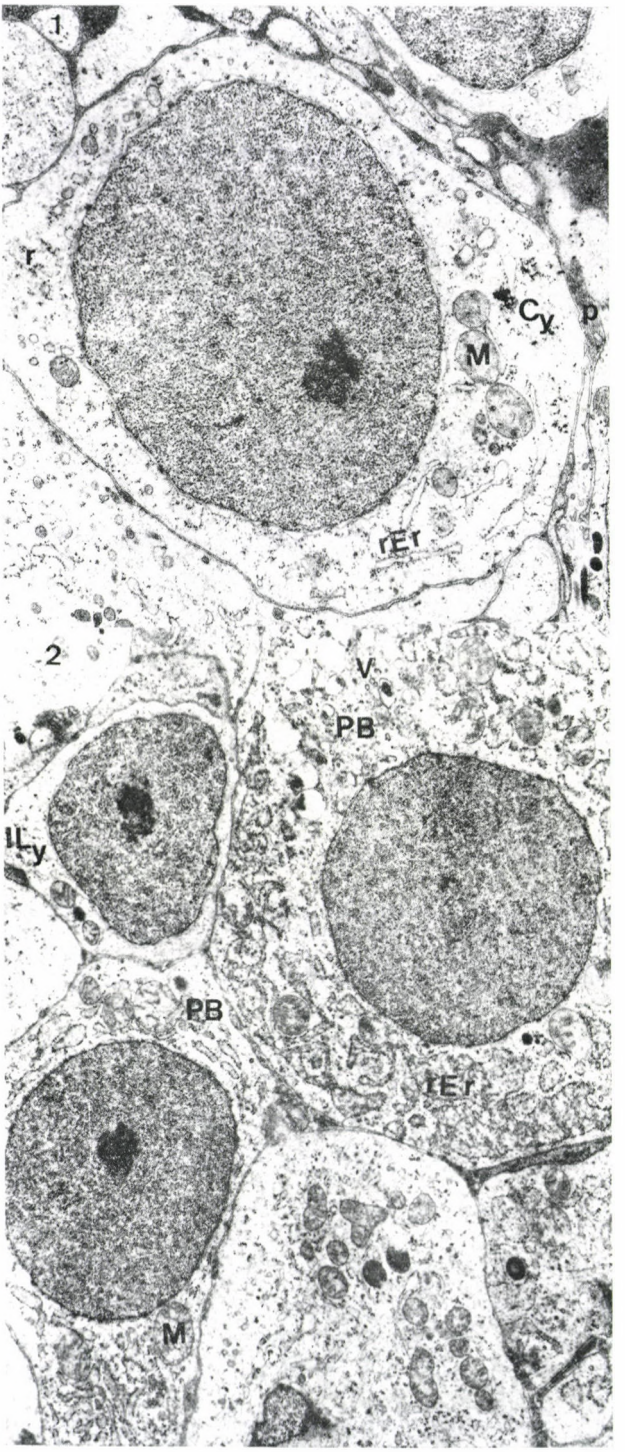

Plate VI. Electronmicrograph *(Fig. 1)* of immature lymphoid cell with poorly developed endoplasmic reticulum and scattered ribosomes (r). Bone marrow of *I. K.* ×15.500. *Fig. 2.* Immature lymphoid cell (ILy) and plasmoblasts (PB) differentiating into young plasma cells. ×7750

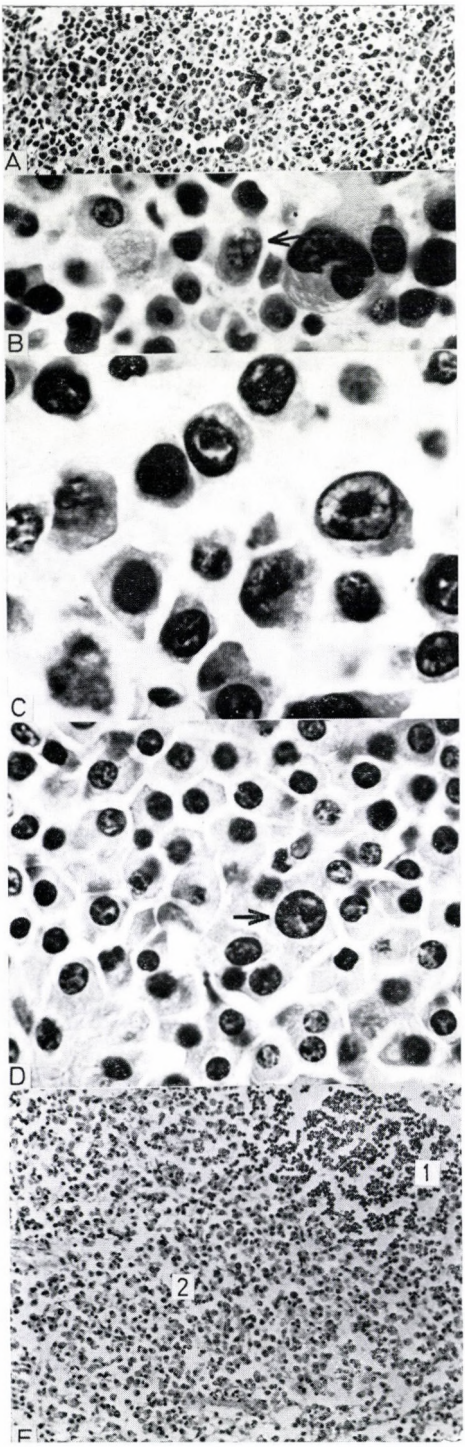

Plate VII. In *Fig. A* a neoplastic lymph node of *S. J.* is shown. Multinuclear giant-cells (arrow) are seen in the group of lymphoid-plasma cells. The same is shown in *Fig. B*. The arrow points to a plasmoblast having a nuclear inclusion. Haematoxylin-eosin, ×110 and 600 (photo by Dr. G. Kelényi). *Fig. C:* immuno- and plasmoblasts from the neoplastic lymph node of *F. J.* Haematoxylin-eosin, ×600. *Fig. D:* the arrow points to a large plasmoblast from a neoplastic lymph node of *P. S.* Haematoxylin-eosin, ×400. *Fig. E* bone marrow of *P. J. II.* Two cell types are seen: lymphoid cells in clusters (1) surrounded by plasma cells (2) different in maturity. Haematoxylin-eosin, ×160

the malignant transformation of the cells. The case of *P. M.* is remarkable with regard to the transitional efficiency of combined cytostatic treatment (melphalan, cyclophosphamide) following an ineffective melphalan therapy in the case of a mixed picture of double Bence Jones proteinuria, myeloma, Waldenström macroglobulinaemia and immunoblastic lymphoma.

IV. In this group *the short observation* of the disease has made it impossible to perform controls similar to those in the previous groups. Recording of these cases may, however, provide a number of useful data on lymphoid-plasma cell tumours (Plate IV, and Plate VII, A, B).

Discussion

The diagnosis of diclonal gammopathies is often difficult [9, 19, 40, 57, 60, 73, 79, 82, 91]. The clinical picture is not uniform [2, 44, 71, 77], cytological data are scarce, and appropriate investigations could be done in a few of the cases reported [2, 8, 44, 45, 75, 77]. Ultrastructural data are also scarce [75] in spite of the fact that determination of the degree differentation and maturation of the cells has a considerable value in assessing the dynamism of the process. Relatively more data are available with the immunofluorescent antibody technique [11, 26, 42, 68, 69, 74, 75, 77, 88, 90, 101]. This approach however, has serious limitatios [26—28, 68, 69, 89, 90, 101].

Proliferative diseases of B-cells, in particular their diclonal gammopathies, often show a complex pattern causing several problems in diagnosis and classification [8, 23, 24, 34, 41, 71, 77, 93]. The nature of neoplastic clonal proliferation is largely dependent on the developmental stage of the malignant cell. Mixed patterns of appearance are determined by the degree of differentiating capacity of malignantly transformed cells and by the reaction of clonal proliferation to factors affecting the development of the normal immune system. The appearance of two malignant cell types or the formation of a second clone adds to the difficulties of classification.

In our 11 cases, the clinical appearance of the disease was correlated with protein and cytological anomalies and throughout the course of the process. Observation of these cases over a considerable lenght of time was thought to elucidate questions related to the dynamism of these "immunocytomas" and to early malignant changes.

The present findings suggest that diclonal gammopathies (IgG-varieties) can be diagnosed by dilution-comparative immunoelectrophoresis [60]. The comparatively high incidence (5.4%) of double gammopathies in our material is attributed to the routine performance of this "screening" examination and of others in cases of monoclonal gammopathy every second or third month. Examination of the urine is of particular importance.

Changes of the clinical picture and protein spectrum (appearance of new paraproteins) call attention to the light and electron microscopic histology of the bone marrow [63, 64]. In these cases, acceleration of the malignization is faster than in the "usual" cases of monoclonal gammopathy [25, 76]. During these periods, the appearance of a new protein can be expected which points to a trigger mechanism. Immature lymphoid elements and plasmoblasts dominate the cell population with frequent nuclear and cytoplasmic inclusions. These cells appear soon in the lymphatic organs and at other sites.

When a new monoclonal protein appears, the disease may become resistant to the former therapy. As malignant transformation may occur after long periods of stagnation, continuous and close observation is mandatory in such cases.

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BIKLONALE GAMMOPATHIEN

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Die Verfasser beobachteten 11 Fälle von biklonalen Gammopathien und versuchten die klinischen, morphologischen Veränderungen sowie die Veränderungen des Eiweißbildes und der Kinematik des Prozesses zu verfolgen.

Ihren Beobachtungen nach ist in solchen Fällen das Tempo der malignen Umwandlung erheblich schneller als bei den "üblichen" monoklonalen Gammopathien. "Explosionsartig" können neue Proteine in Begleitung von juvenilen, unreifen lymphoiden Elementen und Plasmoblasten auftreten.

Die maligne Umwandlung kann auch plötzlich, nach einem längere Zeit stationären Zustand auftreten, deswegen ist bei diesen Krankheitsbildern die regelmässige Kontrolle unerläßlich.

БИКЛОНАЛЬНЫЕ ГАММОПАТИИ

С. ОТТО, М. БЁРЖЁНЬИ Ш. ЭКХАРДТ

Авторы собрали 11 случаев биклональных гаммопатий и изучали изменения клинической, цитоморфологической и белковой картины, кинетику процесса.

По их наблюдениям в таких случаях темп злокачественного перерождения гораздо быстрее, чем при «обыкновеных» моноклональных гаммопатиях. Взрывообразно может появиться новый белок в сопровождении молодых, незрелых лимфоидных элементов и плазмобластов.

Злокачественное перерождение может внезапно начинаться после длительного стационарного состояния и поэтому непремнно нужен систематический контроль этих патологических процессов.

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RECENSIO

Dietrich STARCK: *Vergleichende Anatomie der Wirbeltiere auf Evolutionsbiologischer Grundlage.* 2: Das Skeletsystem: Allgemeines, Skeletsubstanzen Skelet der Wirbeltiere einschliesslich Lokomotionstypen. Springer Verlag, Berlin—Heidelberg—New York, 1979, pp. 775 with 567 figures. Price: DM 248,—

After the first volume dealing with animal phylogeny and the basic principles of comparative anatomy and outline of systematics, this second part of D. STARCK's three-volume book is devoted to the description of the vertebrates' skeletal system and the types of their locomotion on an evolutionary basis.

Turning over the pages of the more than a thousand citations of mostly modern books and articles, the reader can find pieces of the author's works in the field of osteology which perhaps may explain the full-scale representation of the subject within this comprehensive treatise. (The third volume is planned to contain the anatomy of muscle, the nervous and vascular systems, sense organs and viscera.)

The first chapter of the second volume consists of a brief histological survey dealing with cartilage, bone, dentin and some other mineralized tissues and the connections of the elements of vertebrate skeleton. The description is based on light microscopical observations and practically no data on the ultrastructure of these tissues are included.

The subsequent chapters which, according to the main purpose of the book, deal with the macroscopic anatomy of the whole skeleton and of its parts, are excellently written. The text and the beautiful illustrations give ample information concerning both the mechanical and statical features of the skeleton as well as the anatomical connections of the skeletal elements with the viscera, nerves and blood vessels. A number of interesting data for homologies of bones are presented to demonstrate phylogenetic relations between different vertebrate groups.

All chapters contain detailed lists of references and the book is supplemented with a subject index and with a register of the species mentioned in the text.

This comprehensive work is a very good source of data and information on the subject and may be useful for all those interested in research and teaching of comparative anatomy.

Dr. J. KOVÁCS

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