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Fine Structural Evidence of Increased Endothelial Permeability in Chronic Lathyrism

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Summary. The endothelium of the thoracic aorta of Wistar rats intoxicated with Beta-Aminopropionitrile (BAPN) for 9 weeks was studied.

The animals were sacrificed at intervals, from the first to the 9th week of the treatment and 1, 2 and 3 months after the end of the treatment.

Changes in the endothelial cells were studied by electron microscopy after staining with uranyl acetate and lead citrate, after impregnation with lanthanum.

BAPN increased endothelial permeability, pinocytosis was more active in treated rats than controls, the intercellular junctions widened and cytoplasmic lesions with cell necrosis occurred. These intimal changes were comparable to those observed in man during ageing and in arteriosclerosis.

Key words: Endothelial permeability – Lathyric rats – Lanthanum – Atherosclerosis.

Introduction

Beta-Aminopropionitrile (BAPN) poisoning for nine weeks in rats resulted in lysis of the elastic framework of the aorta, modifications and multiplication of its medial smooth muscle cells with a dedifferentiation into fibromyocytes and the appearance of a subendothelial space. The BAPN induced aortic lesions were similar to those observed during human arteriosclerosis (Bouissou et al., 1978; Julian et al., 1971, 1972, 1973; Pieraggi et al., 1974).

In order to definite the role of the endothelium in the development of these lesions, this experiment was designed to assess the endothelial changes.

The endothelium is responsible for regulation of the aortic exchanges of various molecules, so the integrity of the endothelial barrier is a major factor in the development of atherosclerosis.

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Table 1

	Killed rats	E.M.	L
	1 week	2+1 C	2+1 C
	2	2+1 C	2+1 C
	3	2+1 C	2+1 C
During	4	2+1 C	2+1 C
BAPN	5	2+1 C	2+1 C
treatment	6	2+1 C	2+1 C
	7	2+1 C	2+1 C
	8	2+1 C	2+1 C
	9X	2+1 C	2+1 C
After	1 month	2+1 C	2+1 C
BAPN	2	2+1 C	2+1 C
treatment	3	2+1 C	2+1 C

1 st. column: dates of sacrifice of the animals.
2 nd. column: number of animals examined with the electron microscope (E.M.)
C=control animals.
L=animals examined after impregnation with lanthanum.
X=6 animals were killed at this time for ruthenium red staining of the cellular-coat
(4 treated with BAPN and 2 controls)

Material and Methods

Ninety male 3-week-old-Wistar rats were used in this study (58 BAPN treated rats and 32 controls).

The treated animals received 1 g/kg/day of BAPN for 9 weeks. They were killed at weekly intervals from the first to the ninth week of treatment and one, two and three months after the end of the treatment. The aortae were removed for examination. The dates and the number of animals studied at each time interval of the experiment are showed in Table 1.

For electron microscopy, the fixation of aortae started with a perfusion of Karnowsky's fixative containing 1% formaldehyde and 1.25% glutaraldehyde in a solution of 0.1 N sodium cacodylate buffer at pH 7.4 with 5% sucrose (final osmolality $\simeq$750 m osmols). Perfusion at 120 mm Hg pressure was performed under general anesthesia by intra-peritoneal injection of 0.5 mg sodium pentobarbital (nembutal). A catheter was introduced into the left ventricle of the heart at the origin of aorta. This prefixation was maintained for 10 min at a constant pressure, allowing the fixative to escape though the inferior vena cava. The thoracic aorta was then removed, minced and immersed in a fixative containing 2% freshly prepared paraformaldehyde and 1.25% glutaraldehyde in the same cacodylate buffer at pH 7.4 for 3 h at 4° C. Then the specimen was washed overnight at 4° C in a 0.1 N sodium cacodylate buffer (pH 7.4) containing 11.25% sucrose, and post fixed in OsO_4 at 2% in a cacodylate buffer containing 4.9% sucrose (pH 7.4 osmolality $\simeq$430 m osmols) for 2 h at 4° C. The tissues were dehydrated in increasing concentrations of alcohol and imbedded in Epon 812. Sections were cut with a diamond-knife in a Reichert OMU II microtome and stained with uranyl acetate and lead citrate and examined with a OPL 75 or Hitachi HU 11A electron microscope.

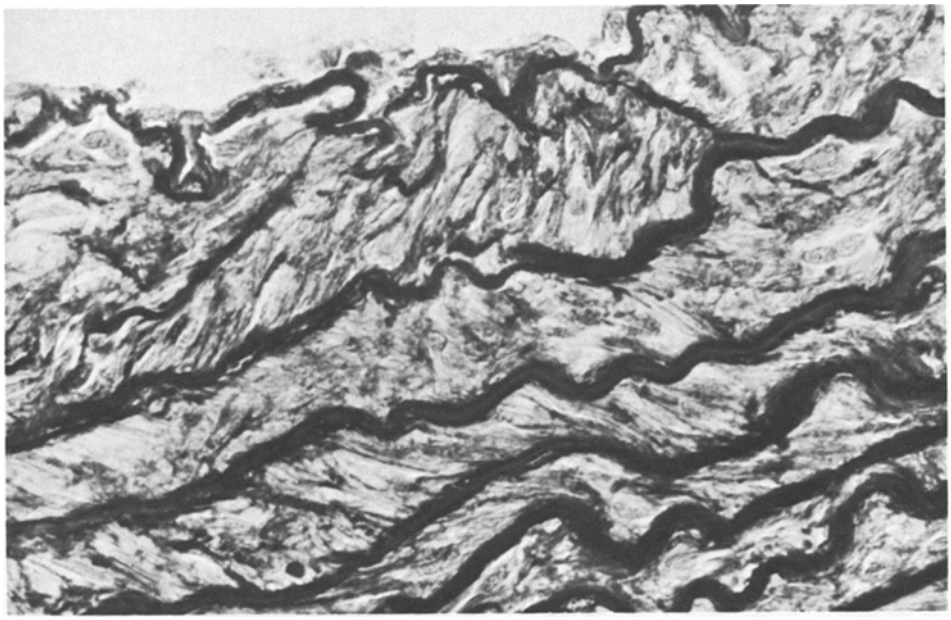

Fig. 1. Rat aorta treated for nine weeks with BAPN. Lysis of innermost elastic lamella, and enlargement of interlamellar spaces. Verhoeff stain $\times 40$

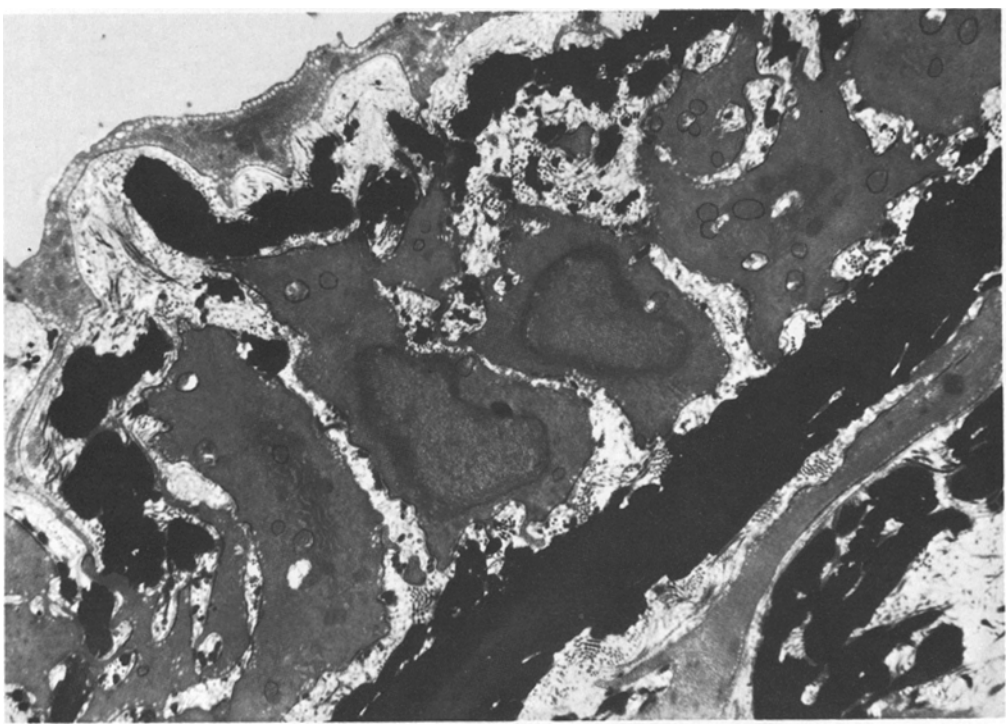

Fig. 2. Rat aorta treated for nine weeks with BAPN. The first elastic lamella is broken. In the subendothelial space, small and short collagen bundles were observed. Uranyl acetate-lead citrate stain $\times 13{,}500$

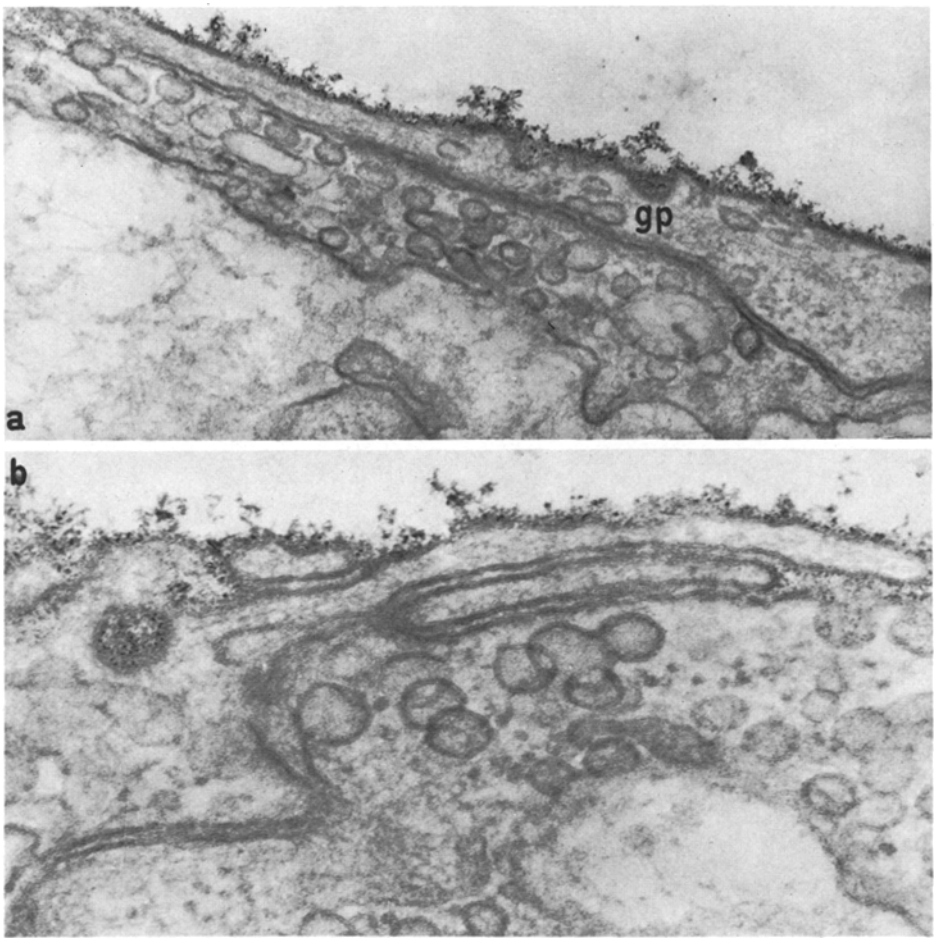

Fig. 3a and b. Endothelial cells of thoracic aorta in a control rat. Two aspects of intercellular junction. **a** Gap junction (*GP*). Lanthanum stain ×25,000. **b** Intercellular cleft. Lanthanum stain ×38,000

Preparation for the permeability study with lanthanum: Arterial tissue was permeated with fixative containing neutralized lanthanum which was prepared by dissolving lanthanum nitrate in water (4% solution) and slowly adding 0.01N NaOH until pH 7.7. This solution was added to equal volumes of aldehyde fixatives (perfusion and immersion fixations). Tissues were post-fixed with a 1/1 mixture of 2% OsO_4 in sodium cacodylate buffer and neutral lanthanum.

Study of the cellular coat by ruthenium red. Six rats (4 treated with BAPN for nine weeks and 2 controls of the same age) were stained by ruthenium red (RR). A 0.2% RR solution was prepared in cacodylate fixative buffer (0.4 M), this buffer with RR was used to prepare aldehyde fixation and post fixation (OsO_4) solutions and also to wash the aortae.

Control issue specimens of each animal of experimental group were examined by light microscopy to check for the presence of the elastic lesions induced by BAPN; the sections were stained with Masson's trichrome and with iodinated iron hematoxylin of Verhoeff.

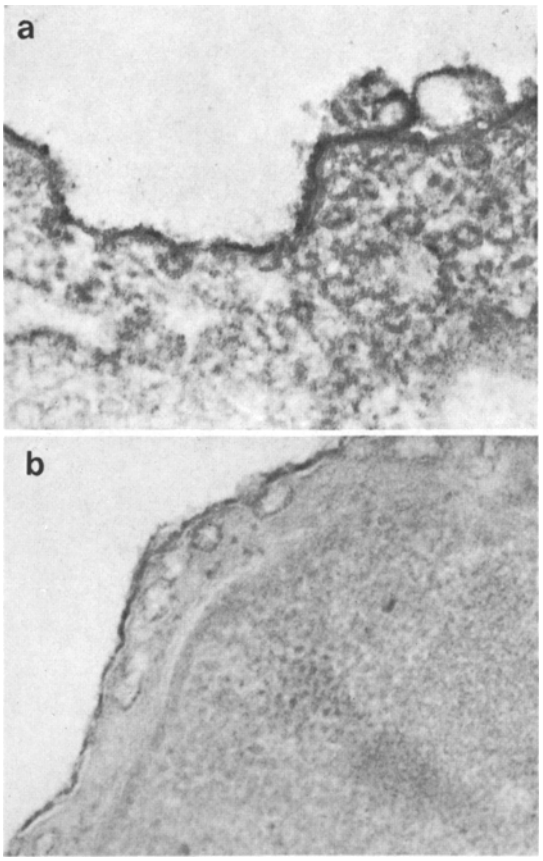

Fig. 4a and b. Comparative aspect of cell-coat in control rat and nine-week treated rats.
a control rat is thick, regular continuous and homogenous. Ruthenium red stain ×38,000.
b treated rat with BAPN: cell coat is thin irregular. Ruthenium red stain ×38,000

Results

The lesions of chronic lathyrism are illustrated in Figs. 1 and 2: lysis of innermost elastic fibers, and enlargement of interlamellar spaces by an accumulation of proteoglycans (in histochemical reactions this substance was metachromatic after staining with toluidine blue, alcian blue positive and PAS negative).

Studies of endothelial modifications after BAPN treatment.

From the first to the sixth week of BAPN treatment, the endothelium showed the same structure as the controls. Endothelial cells were elongated and flattened forming a continuous layer. Their cytoplasm contained few organelles (except for a small quantity of rough endoplasmic reticulum, a few mitochondria and scattered ribosomes; a Golgi complex was found in some cells).

Pinocytic vesicles were found under the cell membrane, arranged in one or two rows over the entire surface of the endothelium which sent a few processes of cytoplasm into the subendothelial space. The cells lay directly on the first elastic lamella or were separated from it by a narrow space consisting of loose

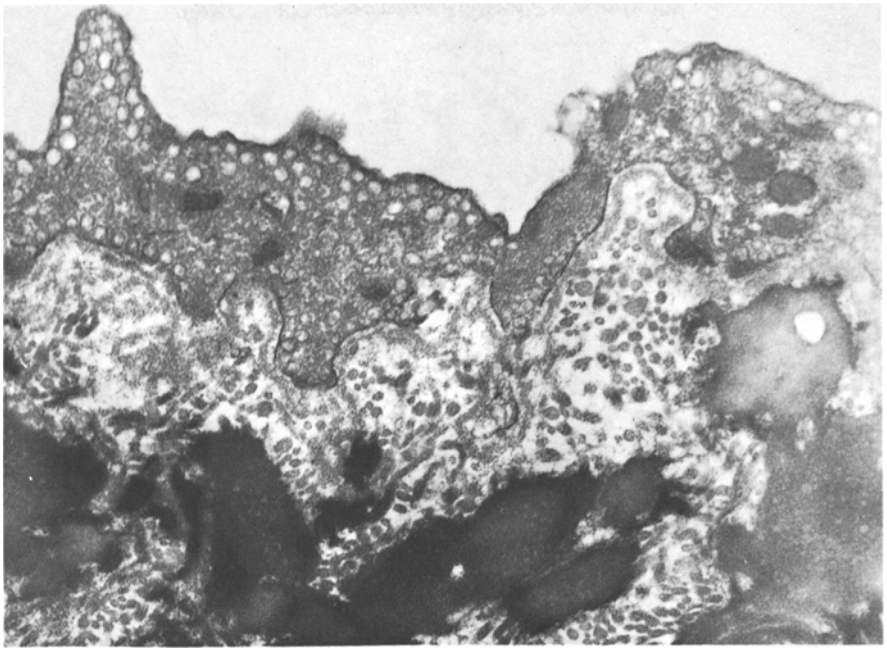

Fig. 5. Thoracic aorta rat treated for nine weeks with BAPN. Swelling of the endothelial cells with increased pinocytic vesicles. Uranyl acetate – lead citrate stain ×15,000

connective tissue containing a few microfibrils and a few isolated short collagen fibers. After lanthanum staining, the intercellular junctions were of the "gap type" (Fig. 3a, 3b) but one also found points of closer cellular contact representing very short zones of tight junctions (Huttner et al., 1973a, 1973b). This staining method also defined a cell coat which appeared as a regular, dense and thick border. The cellular coat was also stained with ruthenium red, and appeared regular, thick and continuous (Fig. 4a).

Endothelial lesions were first seen at the seventh week of treatment and became well established at the eighth and ninth weeks.

Several types of changes were observed:

a) Endothelial swelling and pinocytosis: endothelial cells were enlarged many pinocytic vesicles were seen in the cytoplasm. At the ninth week of BAPN treatment the pinocytic vesicles occuped almost the intire cytoplasm (Fig. 5). The other organells were scattered.

b) Cell necrosis: at 8th and 9th weeks of BAPN treatment cellular necrosis was observed. The necrosis occasionally affected entire cells which lost their normal cytoplasmic structural components.

c) Widening of the intercellular junctions. A clear space appeared and became sharply defined between two cell membranes forming "true channels" which were stained with lanthanum (Fig. 6a–b).

d) Irregularity of the cellular coat. The rats treated with BAPN presented lesions of the cell coat, which appeared thinned or discontinuous and irregular;

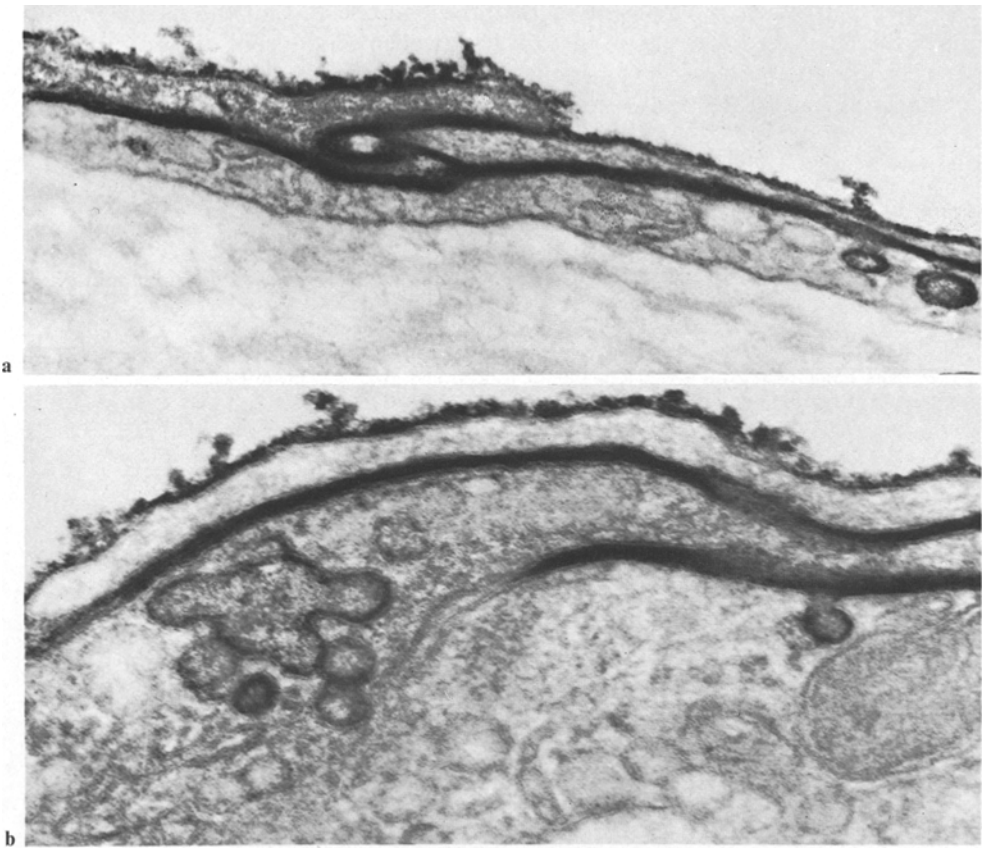

Fig. 6a and b. Thoracic aorta of rat treated for nine weeks with BAPN. **a** the junction between the two cells was deeply penetrated by lanthanum. Lanthanum stain ×38,000. **b** marked permeability of an intercellular junction to lanthanum indicated widening of specialized junctions. Lanthanum stain ×52,000

this finding was observed after lanthanum stain and was confirmed with ruthenium red staining. In the two control animals a regular dense and homogenous layer was seen, but in the four treated animals (nine weeks of BAPN treatment) an irregular appearance with alternating dense zones of variable thickness and complete absence of staining in some areas was found. Thus BAPN produced significant changes in the glycocalix (Fig. 4b).

e) Mitoses were not observed and myofilaments never seen.

f) Appearance of subendothelial space. In the BAPN treated animals a space of variable thickness appeared interposed between the endothelial cells and the first continuous elastic lamella of the media (Fig. 2).

This space contained large quantities of granular material, some collagen fibrils and fragments of elastic substance.

The experiment groups killed, 1, 2 and 3 months following the completion

of BAPN treatment demonstrated that the endothelial and intimal changes described at 8 and 9 weeks persisted. The swollen endothelial cells contained many pinocytic vesicles. The junctions appeared less permeable after lanthanum staining especially at 2 and 3 months.

Discussion and Interpretation

BAPN produced marked changes in the elastic framework of aorta. These have been known since the experimental production of dissecting aneurysms by Ponsetti and Baird, 1952; with the toxic chemical. This work had been repeated by several workers, Bachhuber et al., 1954; Doerr, 1960; Doerr et al., 1960; Lalich, 1956; Walker et al., 1956; Walker, 1957; and lately by Kadar et al., 1978. The mode action of BAPN upon the elastic framework is controversial. It was thought to act as an inhibitor of lysine oxidase, an enzyme required for the formation of SH bridges in the polymerisation of the elastic lamellae (Van der Hoof et al., 1959; O'Dell et al., 1966; Walker et al., 1956) or result in an overproduction of soluble collagen. According to Kadar et al., 1978; Alper et al., 1968; Bhatnagar et al., 1965; Dasler, 1968; and Levene et al., 1968. BAPN has an effect on the intramolecular bonds of the collagen peptide chains. Chronic BAPN poisoning produces a significant changes in the arterial wall without rupture; it is characterized by lysis and fragmentation of the innermost elastic lamellae (Julian et al., 1972; Larrue et al., 1974; Pieraggi et al., 1974). It is also associated with increased ground substance in the sub-endothelial space.

The lesions described above were also observed in the present experiment. One could surmise that the appearance of the sub-endothelial space was the result of the action of BAPN on the endothelial cells which, showed an increased pinocytic function and widened intercellular junctions. These endothelial lesions were accompanied by a change in the cellular coat which became disorganized and focally tended to disappear altogether. It is well known that the cell coat plays an important role in cellular exchanges in general and in endocytosis in particular. This alteration of the cellular coat was probably related to the observed increase in pinocytosis.

The action of BAPN on the endothelial cells was supported by the finding of identical lesions produced experimentally by arterial hypertension (Huttner et al., 1970–1973c) hypoxia (Kjeldsen et al., 1955), the injection of vaso-active substances (Huttner et al., 1970, 1973d) and acute sickness (Sharma et al., 1977).

The cellular changes observed during chronic BAPN poisoning are believed to be related to increased intercellular exchanges, increased permeability of the junctions results in the penetration of small and perhaps large molecules. The greatly increased pinocytosis facilitates the passage of the large molecules. This hypothesis is supported by various studies carried out in capillaries containing an uninterrupted endothelium, since the aortic endothelium has the same histological appearance (Florey, 1967 and Florey et al., 1970). Lanthanum impregnation (Huttner et al., 1973b), peroxidase (Huttner et al., 1973c and 1973d); Karnowsky, 1967; Stein at al., 1972; Vegge et al., 1977) and ferritin (Burns et al., 1968) particle markers have shown that the exchanges take place through pinocytic vesicles and intercellular junctions. Small molecules cross the junctions and large molecules are "handled" by the cytoplasmic versicles (Bachhuber et al., 1954; Karnovsky, 1967; Schwartz et al., 1972; Stein at al., 1972).

This experiment and those mentioned above indicate that endothelial per-

meability is the determining factor in the formation of the sub-endothelial space. The destruction of the elastic lamellae, a characteristic effect of BAPN, and the newly created intimal space resembled the changes observed at the intimal-medial junction in human arteries with ageing (Sendrail-Pesque et al., 1969); with ageing, endothelial permeability does indeed increase.

The endothelial changes induced by BAPN were clearly evident. Are they the result of a direct effect of this toxic chemical on the cell or of an indirect effect by a qualitative change of plasma lipoproteins? It is known that BAPN produces variations in the mobility of the different lipoproteins with disappearance of the high density lipoproteins band and accentuation of the low density lipoproteins and very low density lipoproteins bands (Bouissou et al., 1978).

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