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*Original Articles*

# Chronic Lathyrism and Atheromatosis in the Rat

## Study of the V.L.D.L. and Plasma and Arterial Wall Lipids

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**Summary.** Temporary chronic administration of Beta-amino-propionitrile (B.A.P.N.) produced morphological and biochemical changes of the aortic wall of rat as well as abnormalities of the plasma lipid levels.

A hyperlipidic diet resulted in the blood plasma lipid abnormalities as B.A.P.N. intoxication.

Nine weeks of B.A.P.N. followed by 42 weeks of a hyperlipidic diet increased the aortic cholesterol level and induced an atheroma.

The diet alone produced only an endothelial lipid overload.

The structure of arterial wall played the decisive role in atherogenesis.

**Key words:** Lathyrism – Atherogenic diet – Lipoproteins – Lipids – Aorta.

In the (spontaneously and experimentally) atheroresistant rat, Beta-aminopropionitrile fumarate (B.A.P.N.) induced aortic wall lesions [23] which were characterized by alterations of the elastic fibers, penetration of proteoglycans, changes of smooth muscle cells in fibromyocytes and later fibrosis [1, 12, 13].

Pronounced atheromatous lesions were induced in the rat by chronic intoxication with B.A.P.N. (9 weeks) followed by a prolonged atherogenic diet (10 months) [1 bis] [10, 12, 14]. Thus, it was revealed that a parietal lesion (endothelial, and/or elastic and/or myocytic) was essential to the realization of a lipid accumulation within the media, as an atherogenic diet only in the normal rat brought on a lipid accumulation within the endothelium.

But, after 12 weeks of B.A.P.N., the aorta became fibrosed and after these 12 weeks the atherogenic diet (10 months) did not induce an atheroma: the lipids were deposited within a narrow band of endothelial foam cells. It is likely that, compared with 9 weeks, the more pronounced medial cicatricial fibrosis reduced the filtration of lipids.

Therefore, it appeared that the structure of the aortic wall determined and explained the penetration and deposition of lipids in the media of this artery.

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But it was known that the quantity and nature of the blood lipids played a role in atherogenesis.

In order, to determine the relative parts played by the arterial wall and the lipid abnormalities in the pathogenesis of the atheroma, it was absolutely necessary to study, in addition to the morphological aspects, the biochemical changes of blood plasma and arterial wall lipids at the same experimental intervals used in the previous studies.

This paper presents these biochemical changes in blood and aorta in chronic lathyrism and demonstrates the essential role of the aortic wall structure in the pathogenesis of the atheromatous plaque.

## Material and Methods

One hundred and sixty 3-week-old (weaned) white male Wistar rats were used.

A dose of 1 g/kg/day of a 40% aqueous solution of B.A.P.N. fumarate and the U.A.R. atherogenic diet (20% casein, 10.3% glucose, 13.4% starch, 35% animal and vegetable fat, 5% cellulose, 10% mineral mixture, 4.5% cholesterol, 1.8% sodium cholate) were given per os. The rats were divided into four groups (Fig. 1):

*Group I.* Control group consisted of 40 untreated animals. Histologically, the aorta remained normal (Fig. 2).

*Group II.* Consisted of 40 rats which treated with B.A.P.N. for only 9 weeks and then the animals were maintained on a normal diet until the end of the experiment after 10 months. They showed lesions already described in previous studies (Fig. 3) and, in addition, clinical signs of osteolathyrism.

*Group III.* Consisted of 40 rats treated with B.A.P.N. for 9 weeks, and then fed the atherogenic diet for 10 months. The atheromatous lesions described in the previous experiments were found again (Fig. 4).

*Group IV.* The 40 animals of this group were not treated during their first 12 weeks after birth. Then, the atherogenic diet was given for 10 months. Only the aortic endothelium showed lesions of lipid overload (Fig. 5).

Ten animals were sacrified each time at the following intervals: 9 weeks after the start of B.A.P.N. treatment and 3, 6 and 10 months after it was stopped.

The animals were anesthesized with chloroform and blood was collected in tubes containing E.D.T.A. at the concentration of 1 mg/ml. The aorta was removed afterwards.

### *Study of Blood Plasma*

The blood of the ten animals was immediately refrigerated and then centrifuged to obtain the plasma. They were treated in pools of ten. A sample of 2 ml plasma was used for electrophoresis of the lipoproteins and determination of the lipids. The remainder was ultracentrifuged at 40,000 rpm for 20 h at 10° cent. in a 50 TI Beckmann Rotor to obtain the V.L.D.L. according to Carlson's method [3].

All lipid-extractions were carried out with a chloroform-methanol (7/11) solvent systems as used by Rose and Oklander [21]. The different lipids were separated by thin layer chromatography on Merck 0.25 mm silicagel in different solvent systems. The separation of free cholesterol from cholesterol-ester was performed in petroleum-ether, ethylic-ether, acetic acid (80/20/1). Each band was scraped and the cholesterol determined by Lieberman's method [17] (acetic anhydride, sulfuric acid).

The different classes of lipoproteins were determined by electrophoresis with a tube polyacrylamide gel method according Feliste's [5] modified Davis method [4]. Three different acrylamide

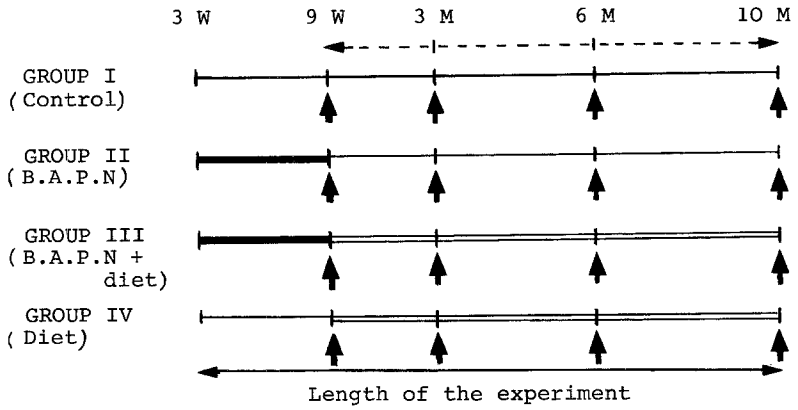



**Fig. 1.** Experimental design. ▲ Killed, ▬B.A.P.N., = Atherogenic diet, – Normal diet, ◄--►Observation after B.A.P.N. and during atherogenic diet

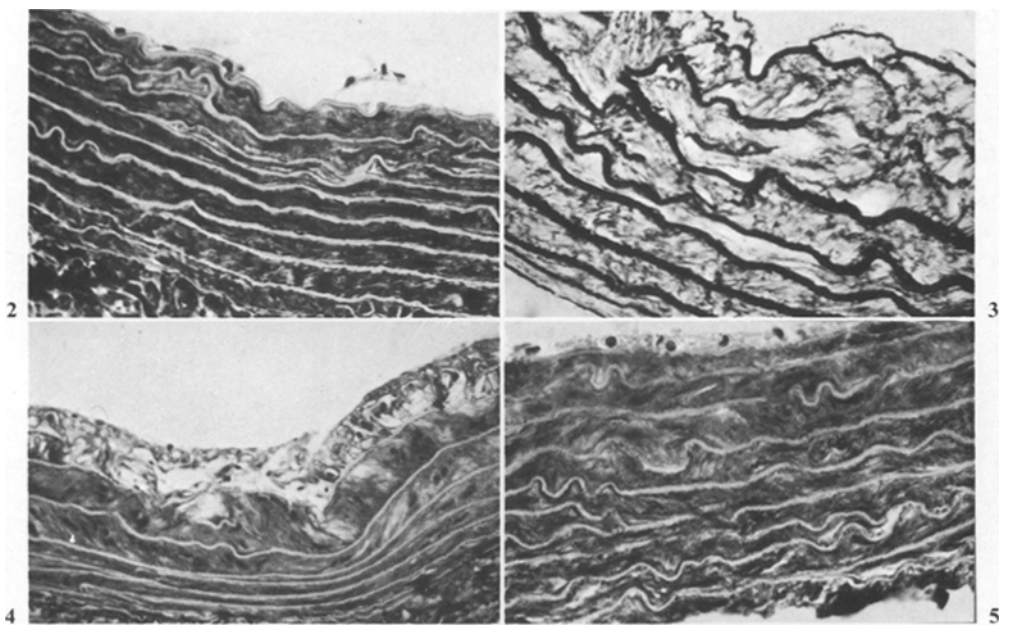



**Fig. 2.** Group I: Normal aorta. The endothelial cells abut against the inner elastic lamina. The thick parallel elastic lamellae of the media enclose regular spaces containing muscle cells. Masson ×25

**Fig. 3.** Group II: B.A.P.N. administration for 9 weeks. Typical vascular lesion of chronic lathyrism. The elastic lamellae are thin, disrupted, separated in the inner half of the media. Verhoeff ×25

**Fig. 4.** Group III: B.A.P.N. administration for 9 weeks and after atherogenic diet for 10 months. Aortic atherosclerosis: disruption of the elastic network, fibrosis and foam cells are observed in the inner half of the media. Masson ×25

**Fig. 5.** Group IV: Atherogenic diet for 10 months. The lipids are deposited within a narrow band of endothelial vacuolated cells. Masson ×25

concentrations, 3%, 3.5% and 4.6% were used in successive layers. Thus, the chylomicrons, V.L.D.L., L.D.L., H.D.L. and lipo-albumins were isolated. The lipoproteins were pre-stained with Sudan Black B in glycol propylene [7].

The V.L.D.L. were delipidated as described by Shore [24]. An aqueous solution of lipoproteins (~5–10 mg/ml) was extracted twice with diethyl ether at 4° C, then dialysed against cold degassed water and re-extracted three times at pH 4 with ethanol/ether mixtures (10/90, 20/80, 30/70, v/v respectively).

The apoproteins were subjected to disc gel electrophoresis in 7.5% polyacrylamide gel containing 8 M urea. The gels were prepared as described by Kane [15].

After pre-incubation of samples in 8 M urea at room temperature for 30–60 min, specimens containing 100–120 µg of protein were applied to each gel. The upper tank buffer was Tris-glycine, pH 8.91, and the lower tank buffer Tris-Tris HCl, pH 8.07. Gels were fixed and stained with Coomassie Blue in 7% acetic acid.

All lipid essays, the different classes of lipoproteins and apolipoproteins were carried out in duplicata.

*Study of the Aorta*

All lipid extractions of the aorta were done according to the same methods as for the blood. The aortas were also treated in pools of ten.

## Analytic Results

### *A. Study of the Plasma*

### Total cholesterol (g/l)

**Table 1.** Total plasma cholesterol level at 9 weeks, 3, 6 and 10 months in four groups of animals

| | | For groups III and IV, start of atherogenic diet | | |
|---|---|---|---|---|
| | 9 weeks ↓ | 3 months | 6 months | 10 months |
| I (Control) | 0.600 | 0.675 | 0.720 | 0.950 |
| II (B.A.P.N.) | 0.630 | 0.660 | 0.730 | 1.06 |
| III (B.A.P.N. + diet) | 0.630 | *8.6* | *8.5* | *11.55* |
| IV (Diet) | 0.600 | *8.1* | *8.2* | *8* |

I: The plasma cholesterol level increased with age

II: B.A.P.N. alone did not modify the plasma cholesterol level

III–IV: In animals maintained on the atherogenic diet, the plasma cholesterol level is high. After B.A.P.N. treatment the administration of the atherogenic diet induced a much higher cholesterol level

Study of the lipoproteins.

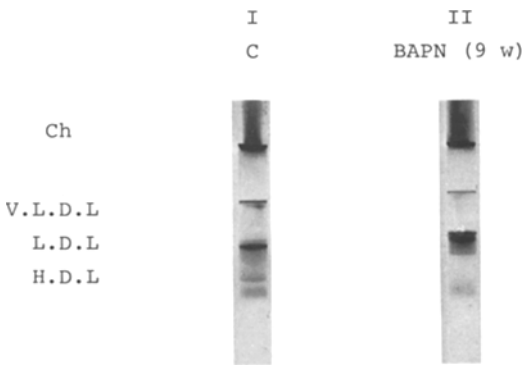



**Fig. 6.** Electrophoresis of lipoproteins. After B.A.P.N., disturbance of the mobility of the lipoproteins. Decrease of H.D.L. Increase of L.D.L., *C*: Controls

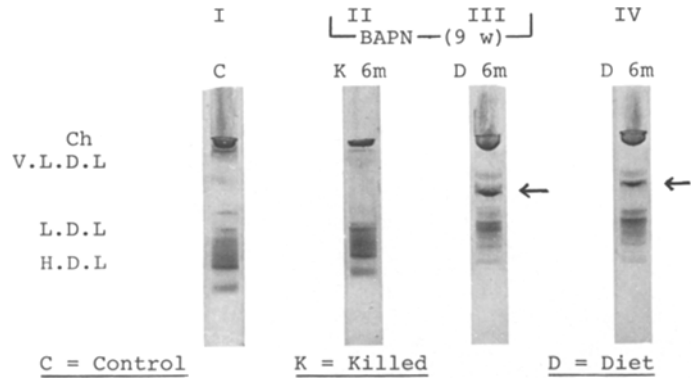



**Fig. 7.** Electrophoresis of lipoproteins. Six months after 9 weeks of treatment with B.A.P.N., the lipoproteins bands were again the same as in the controls. In groups III and IV after 6 months of the hyperlipidic diet, the same changes were observed as after administration of B.A.P.N. for 9 weeks (Fig. 6), but a new band appeared between the V.L.D.L. and L.D.L. (←)

*a) Electrophoresis.* The rats of group II showed already changes in the migration of the different lipoproteins after 9 weeks of treatment with B.A.P.N. (Decrease of H.D.L. and increase of L.D.L.) (Fig. 6). Three months after the B.A.P.N. treatment was stopped and without an atherogenic diet, the lipoproteins bands were again the same as in the controls. These findings were similar 6 months after the termination of B.A.P.N.-treatment (Fig. 7).

In groups III and IV, respectively, after 3 and 6 months of the atherogenic diet, the same changes were observed (Fig. 7), but, additionally, after 6 months of the atherogenic diet, a new band appeared between the V.L.D.L. and L.D.L. [16, 18].

*b) Ratio (F ch/ch E) in the V.L.D.L.*

**Table 2.** Ratio of (F ch/ch E) of V.L.D.L. in the four groups of animals

| | 9 weeks | ↓ Start of atherogenic diet for groups III and IV | | |
|---|---|---|---|---|
| | | 3 months | 6 months | 10 months |
| I (Control) | 45/*55* = 0.81 | 42/58 = 0.72 | 43/57 = 0.75 | 45/55 = 0.80 |
| II (B.A.P.N.) | 38/*62* = 0.61 | 35/65 = 0.53 | 35/65 = 0.53 | 48/52 = 0.92 |
| III (B.A.P.N. + diet) | 39/62 = 0.61 | 18/*82* = 0.21 | 22/*78* = 0.28 | 29/*71* = 0.40 |
| IV (Diet) | 45/55 = 0.81 | 26/*74* = 0.35 | 24/*76* = 0.31 | 25/*75* = 0.33 |

I: The ratios remained similar during the length of the experiment
II: After 9 weeks, B.A.P.N. produced an increase of the esterified cholesterol level (from 55 to 62%), but, at the end of 10 months, this ratio was again the same in the control group (from 55 to 52%)
III–IV: The atherogenic diet resulted in a greater and constant increase of the esterified cholesterol level, namely a significant decrease of the (F ch/ch E) ratios as early as 3 months after the administration of the diet

*c) Apoproteins.* Electrophoresis of the apoproteins in the V.L.D.L. of 6 months old control rats (group I) identified two groups of bands, namely those of C and $R_2$ and $R_3$ (arginine rich) apoproteins according to Shore's classification [24].

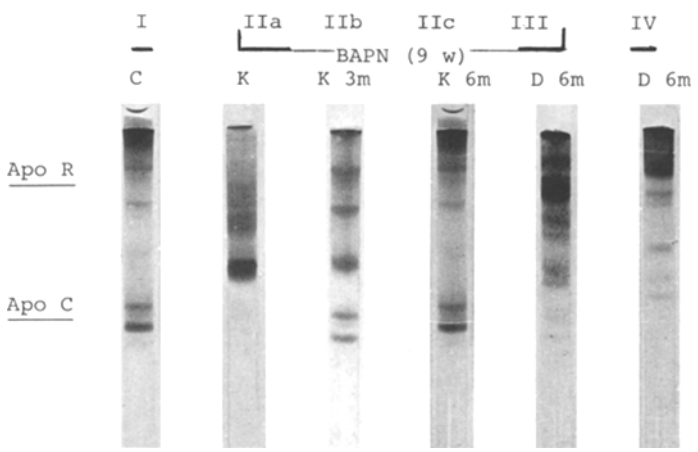



**Fig. 8.** Apoproteins of V.L.D.L. in four groups. I = 2 groups: apo C, $R^2$ $R^3$. IIa = Disappearance of B bands. IIb–IIc = Reappearance of C bands. III–IV = Disappearance of C bands, increase of apo R. *C* Controls, *K* Killed, *D* Atherogenic diet. B.A.P.N. (9 weeks) = Group II at different times (IIa killed immediately after B.A.P.N., IIb killed 3 months, IIc killed 6 months after B.A.P.N.), and group III killed 6 months after B.A.P.N.

In the rats of group II, an important change in the apoproteins characterized by the disappearance of the C bands was observed; but, 3 months after the treatment, these reappeared and the $R_2$ $R_3$ bands were even accentuated. At 6 months, the apoproteins C and R were again normal.

In groups III and IV, one noted a disappearance of the apoproteins C and an increase of apoproteins R (Fig. 8).

### *B. Study of the Aorta*

Total cholesterol (in percent of wet weight).

**Table 3.** Total cholesterol level in the aortic wall of four groups of rats (g/100 g g wet weight)

| | 9 weeks | ↓ Start of atherogenic diet for groups III and IV 3 months | 6 months | 10 months |
|---|---|---|---|---|
| I (Control) | 0.105 | 0.104 | 0.104 | 0.118 |
| II (B.A.P.N.) | 0.166 | 0.122 | 0.152 | 0.182 |
| III (B.A.P.N. + diet) | 0.166 | *0.402* | *0.491* | *1.074* |
| IV (Diet) | 0.105 | *0.340* | *0.345* | *0.705* |

I: In control animals, the total cholesterol of the pool-sample (10 aortas) did not vary
II: B.A.P.N. increased the total cholesterol in the aortic wall
III: The atherogenic diet rapidly induced an increase of the cholesterol level in the aortic wall. Nevertheless, the cholesterol level always remained higher in group III than in group IV

Ratio of freee cholesterol to esterified cholesterol.

**Table 4.** Ratio of (F ch/ch E) of the aortas examined at 6 and 10 months

| | 6 months | 10 months |
|---|---|---|
| I (Control) | 86/14 = 6.14 | 84/16 = 5.25 |
| II (B.A.P.N.) | 89/11 = 8.09 | 80/20 = 4 |
| III (B.A.P.N. and diet) | 70/30 = *2.33* | 73/27 = *2.70* |
| IV (Diet) | 71/29 = *2.44* | 71/29 = *2.44* |

I: There was no change in group I between 6 and 10 months
II: With time, B.A.P.N. increased the level of esterified cholesterol
III–IV: In less than 6 months, the aterogenic diet decreased the (F ch/ch E) ratio

## Discussion

To the best of our knowledge, no biochemical study of blood plasma had been performed in B.A.P.N. treated rats. The following observations could be made on the basis of our experimental findings:

The B.A.P.N. alone (Group II) did not modify the total plasma cholesterol level. Changes in the migration of the different lipoproteins were seen: disappearance of H.D.L., accentuation of L.D.L. An increase of the esterified cholesterol level in the V.L.D.L. was found and, simultaneously, an important change in the apoproteins was observed, particularly the disappearance of the C bands.

All these changes in plasma were time related to the administration of B.A.P.N. They regressed and the various parameters were normal 6 or 10 months after the treatment.

In the aortic wall, the B.A.P.N. increased the total cholesterol levels. This change was observed 10 months after the end of the treatment.

In animals (group IV) maintained solely on the atherogenic diet the total plasma cholesterol-level was high. This datum was different of those of B.A.P.N.

The atherogenic diet induced changes in the migration of the different lipoproteins (disappearance of band H.D.L., accentuation of bands L.D.L.) and a high and constant increase of the esterified cholesterol level in the V.L.D.L. The atherogenic diet alone induced an important change in the apoprotein electrophoresis characterized by the disappearance of the C bands and increase of the R bands. These changes were also found in samples obtained from the B.A.P.N. treated rats.

In the aortic wall of animals maintained on the atherogenic diet a constant and marked increase of the total cholesterol level and decrease of the (F ch/ch E) ratio were found. In animals treated with B.A.P.N., changes of the aortic wall were similar to those obtained from the animals maintained on the atherogenic diet.

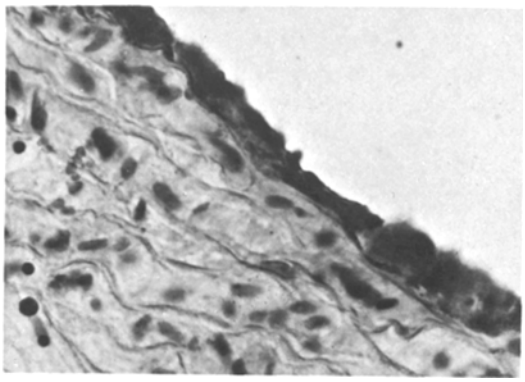
In experimental an human atheromatosis, in type III hyperlipoproteinemia [8] and in hypothyroidism [20] several or all of theses changes in plasma lipoproteins have been observed: modification of the mobility of the lipoproteins, presence of an abnormal lipoprotein [16, 18] and a relative increase of the esterified cholesterol in the V.L.D.L. [2]. The latter was already noted by Rodriguez [19, 20] in the rabbit maintained on a atherogenic diet. The increase of esterified cholesterol confirmed that the V.L.D.L. were deposited in the arterial wall. This was shown already using $I^{125}$ labelled V.L.D.L. [19].

The changes of apoproteins (a disappearance of the C and intensifications of the R bands) [19, 20] were also found in rabbits treated with an atherogenic diet.

In man and other mammals, C peptids were co-factors of lipoprotein lipase [9] and, possibly, of L.C.A.T. [22]. Their decrease might be the cause of the important lipid metabolic changes which have been observed.

An accumulation of total cholesterol and particularly cholesterol ester in aortic wall is classic in experimental and human atherosclerosis [6].

B.A.P.N. in combination with a atherogenic diet (group III) increased the plasma aortic lipid abnormalities which were observed in animals treated with either an atherogenic diet or B.A.P.N.



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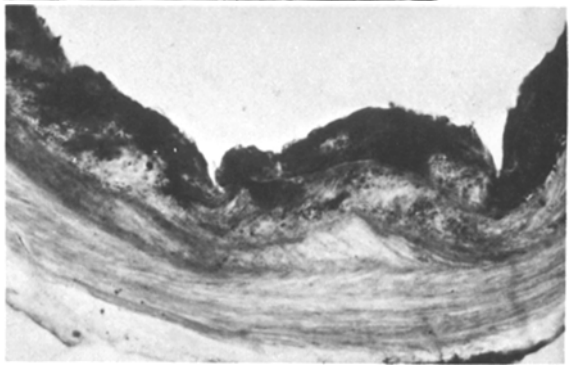

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**Fig. 9.** Group IV: Atherogenic diet for 10 months. Endothelial lipid overload. Sudan Black ×40

**Fig. 10.** Group III: B.A.P.N. administration for 9 weeks and after atherogenic diet for 10 months. Aortic atherosclerosis. Destruction of inner elastic lamellae. Fibrosis. Foam cells and lipid deposition in the interstitium. Masson ×40

**Fig. 11.** Group III: B.A.P.N. administration for 9 weeks and after atherogenic diet for 10 months. Deposition of lipids in the inner media. Sudan Black ×10

The atherogenic diet alone (group IV) did not induce atheromatous lesions in the rat (Fig. 9) although the blood lipids were modified, but produced severe atheromatosis when it was given at the time of formation of the B.A.P.N. aortic lesions, that was at the 9th week of the treatment by B.A.P.N. (group III) (Figs. 10–11).

Therefore, it is likely that the lesion of the aortic wall was necessary for the formation of the atheroma. Variations in the level of the blood lipids alone never resulted in the formation of atheromatous plaques in a normal aorta.

These findings were also confirmed by the total aortic cholesterol levels which were always higher in group III (Rats receiving B.A.P.N. + atherogenic diet).

## Conclusion

In the rat, chronic, but temporary administration of B.A.P.N. (9 weeks) induced morphological (alteration of elastic fibers, penetration of proteoglycans, late fibrosis with changes of smooth muscle cells in fibromyocytes) and chemical changes in the aortic wall (increase of the total and esterified cholesterol) as well as plasma lipid level fluctuations (disturbances in the mobility of the lipoproteins, increase of esterified cholesterol in the V.L.D.L.; in the apoproteins, modifications of the C and R bands). The morphological changes were definitive but the chemical changes were transitory.

In the rat, an atherogenic diet resulted in the same blood plasma lipid abnormalities as in B.A.P.N.-intoxication, except for the total plasma cholesterol level.

A nine weeks treatment with B.A.P.N. followed by 42 weeks of an atherogenic diet increased the aortic cholesterol level more than the diet alone and induced an atheroma.

The diet alone produced only an endothelial lipid overload.

*It is likely that the structure of the arterial wall playes a decisive role in atherogenesis.*

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