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Effect of BAPN on Lipid Deposition in Rat Liver and Aorta. Studies Using 4-^{14}C-Cholesterol

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There have been numerous theories to explain the mechanism of action of Beta-amino propiononitrile (BAPN) in producing lathyrism. BAPN has been reported: to have an effect on chondroitin sulfate (1, 2), to have an action as an antimetabolite (6), to interfere with collagen synthesis (4), to cause a disturbance in the metabolism of nitrogen containing compounds (8) and also an alteration in the metabolism of individual amino acids (5).

Microscopic sections of tissue in lathyritic animals reveal definite changes in connective tissue. There is an increase in collagen (14, 9) and also an accumulation of metachromatic material (2).

A more recent explanation of the action of BAPN is that it interferes with the cross linking of collagen. It has been shown to inhibit the transformation of collagen soluble in cold 1 Molar sodium chloride to collagen insoluble in this material (13). This explanation is not incompatible with many of the above theories. These authors felt that soluble collagen differed from insoluble in that it had fewer cross links between collagen molecules. O'Dell *et al* found that BAPN also interfered with the organization of the primary peptide chains of elastin into a coherent fabric (15). These abnormalities may be related to abnormalities in aldehyde and/or lysine (16, 17, 18, 19) metabolism in collagen.

There have been conflicting reports on the effect of lathyrism in lipid deposition. Schwartz (11), using histologic techniques found that BAPN increased lipid deposition in rabbit aortas. Meyer and Vos (10), who also used histologic techniques, found no effect of BAPN on lipid deposition in rats.

In view of these conflicting findings and because of the indication that BAPN changes the nature of collagen, it was felt that it would be of interest to investigate the deposition of cholesterol in the tissues of animals fed BAPN using both quantitative chemical techniques and ^{14}C-labeled cholesterol.

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MATERIALS AND METHODS

Cholesterol deposition was studied in three groups of white, male weanling Wistar rats. Each group was started on a different experimental diet as shown in Table I at the age of three weeks.

At the end of five weeks on the diet, each group of rats was starved for 24 hours and then fed a Purina Chow pellet, to which 20 μc of 4-^{14}C-Cholesterol[1] in 95% ethyl alcohol had been added. Two days later the rats were sacrificed using intraperitoneal nembutal.

The aortas from the aortic ring to the bifurcation were removed, trimmed of adventitious tissue and washed several times in distilled water. The aortas were then minced and extracted in 20 ml of equal parts of ethyl alcohol and acetone for 24 hours. The mixture was then heated to a boil and the supernatant was poured off and saved. About 20 ml of alcohol and acetone was added to the residue; the material was again brought to a boil and the supernatant again poured off and saved. The latter process was then repeated five times. The total volume was then reduced by boiling to about 15 ml.

This extract was divided into approximately equal portions for chemical determination of cholesterol, liquid scintillation counting and chromatography.

TABLE I

Group I— High cholesterol diet:

1. Casein 10 gm
2. Corn oil 8 gm
3. Cholesterol 5 gm
4. Cholic acid .5 gm
5. Cod liver oil 2 gm
6. Sucrose 67 gm
7. Salt mixture 4 gm
8. P-aminobenzoic acid .1 gm
9. Inositol .1 gm
10. Choline chloride .2 gm

To each kilogram of diet were added:

11. Thiamine 5 mg
12. Riboflavin 5 mg
13. Pyridoxine 2.5 mg
14. Calcium pantothenate 50 mg
15. Niacin 80 mg
16. Folic acid .25 mg
17. Biotin .2 mg
18. Menadione 5 mg
19. Tocopherol 100 mg
20. Ascorbic acid 25 mg
21. Riboflavin 4.5 gm

Add 100 gm of above vitamin preparation to each kilogram of diet.

Group II—This is the normal group. Omit 3 and 4 in above diet. Calories lost by omitting 3 and 4 will be made up with corn oil.

Group III Same as Group I but add .4% BAPN (high cholesterol plus BAPN).

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Chemical Determination of Cholesterol

The extract was hydrolized by adding 3 drops of a 50% solution of KOH and incubating in a 60° water bath for one hour. The solution was then acidified to the phenolphthalein end point using 10% acetic acid solution.

One ml of a 1% solution of digitonin (50% alcoholic solution) was added and precipitation was allowed to take place over night at room temperature. The solution was centrifuged and the supernatant carefully poured off and discarded. The precipitate was washed twice with a solution of 3 parts alcohol and 1 part ether and then again with ether.

The digitonide was suspended in equal parts (1:1) alcohol and acetone.

The solvent was evaporated off and 6 ml of saturated solution of $FeSO_4$-acetic acid followed by 2 ml of concentrated reagent grade H_2SO_4 were added according to the method of Searcy and Bergquist (12).

The color developed was compared in a Klett Colorimeter with that developed in a standard solution containing 0.4 mg cholesterol treated similarly. The cholesterol content of the extract was estimated.

The livers were also excised and treated in the same way.

Liquid Scintillation Counting

At the same time that the test pellet was impregnated with ^{14}C-cholesterol in ethanol solution, a volume equal to that added to each pellet was diluted 1/100, as a counting standard. An aliquot of each acetone-alcohol extract of tissue was evaporated to dryness and then counted in 15 ml of a solution, consisting of 5 g PPO (5-Diphenyloxazole) and .5 g POPOP [4-bis-2-(5-Phenyloxazolyl)-Benzene] per liter in a Tri Carb Liquid Scintillation Spectrometer. Following each count, 0.1 ml of the 1/100 diluted standard was added and the samples counted again. The degree of quenching was estimated by comparing the increment in the counting rate with the counting rate of an equal aliquot of dilute standard counted alone and the counting rate of the sample was appropriately corrected.

Thin-Layer Chromatography (TLC)

A portion of the extract of each tissue was concentrated almost to dryness under a stream of nitrogen and then applied to an activated silica gel G TLC plate. Standards containing cholesteryl palmitate, triolein, cholesterol and lecithin were also applied to each plate, following which, the plates were developed in a mixture of solvents consisting of petroleum ether: diethyl ether: acetic acid: 85:15:1 in which the cholesteyl esters had an Rf of apporximately 0.9 triglycerides 0.6; cholesterol 0.2; and phospholipids remained at the origin. Following development, the plates were sprayed with dichlorofluorescein, the lipid spots were located under ultraviolet light and the spots corresponding to the cholesteryl esters, the triglycerides and the phospholipids were scraped off into separate screw-top test tubes. To each tube, 1 ml methanol:H_2SO_4, 98:2, V/V, and an aliquot of a solution of margaric acid in methanol, as an internal standard, were added. The tube was sealed air-tight with a teflon-lined screw cap and

TABLE II

GROUP II NORMAL

Rat #	*Aorta Cholesterol (mg)*	*Liver Cholesterol (mg)*	*Liver Chol. / Aorta Chol.*	*Rat Original Weight (gm)*	*Rat Final Weight (gm)*	*Food Consumption (gm)*
1.	.13	1.1	8.4	62	132	513
2.	.11	2.5	22.8	54	115	355
3.	.13	1.2	8.8	52	133	496
4.	.14	.7	5.1	56	137	504
5.	.11	1.4	13.3	58	149	568
6.	.12	1.1	8.7	63	169	609
7.	.13	1.4	10.9	49	121	481
8.	.12	1.0	8.6	61	160	575
9.	.08	1.2	15.7	56	147	539
10.	.24	1.6	6.7	63	136	443
Average	.13 ± .04	1.3 ± .5	10.9 ± 5.2	57	140	508
				% gain = 243% (Orig. wt. 100%)		

TABLE III
HIGH CHOLESTEROL

GROUP I

Rat #	Aorta Chol. (mg)	Aorta C^{14} (cpm)	Liver Chol. (mg)	Liver C^{14} (cpm)	Liver Chol. / Aorta Chol.	Liver C^{14} / Aorta C^{14}	Rat Orig. Weight (gm)	Rat Final Weight (gm)	Food Consumption (gm)
301	.4	5840	3.6	1,190,183	9.3	204	122	183	453
302	.5	7728	6.2	1,270,084	13.1	164	121	168	361
303	.4	10400	3.4	448,275	9.5	43	117	147	307
304	.4	14032	3.1	320,659	7.0	23	106	127	281
305	1.2	7296	6.4	621,840	5.4	85	120	178	377
306	.2	8496	3.6	1,140,682	17.8	135	85	104	388
307	.4	9728	3.2	906,240	7.3	93	97	126	360
308	.4	8192	7.2	651,790	20.6	80	94	133	302
309	.4	5008	4.0	451,114	11.8	91	90	146	429
310	.3	12976	3.2	355,884	10.0	27	82	94	282
Average	.5 ± .3	8970 ± 2886.0	4.4 ± 1.5	735,751 ± 362,911.4	11.2 ± 4.8	82 ± 58.8	103	141	354
						% Gain = 136% (Orig. Wt. 100%)			
GROUP III				HIGH CHOLESTEROL + BAPN					
401	.06	1372	1.1	52,778	18.3	38.4	75	102	250
402	.06	1700	.3	19,788	5.0	11.6	91	91	273
403	.06	2536	.7	57,999	11.0	23.0	83	81	197
404	.07	3664	.2	25,129	3.1	6.8	82	85	259
405	.07	2012	.6	13,357	9.3	6.6	85	81	235
406	.08	2056	1.3	6,220	16.8	3.2	90	100	188
407	.09	2272	.6	43,962	6.7	19.3	47	52	257
408	.06	1508	1.2	15,656	21.1	10.4	81	76	160
409	.06	1388	1.6	23,717	25.6	17.1	56	59	220
Average	.07 ± .01	2060 ± 725.1	1.0 ± .5	28,700 ± 18,367.8	13.0 ± 7.8	15.1 ± 10.9	69	73	204
						% Gain = 107% (Orig. Wt. 100%)			

heated at 60° C overnight. Two ml. of water were then added to each; the methyl esters were extracted into petroleum ether. This solution was then concentrated and an aliquot was injected into the gas-liquid chromatography (GLC) column.

Gas-Liquid Chromatography (GLC)

The GLC column was glass, five feet long, 4 mm I.D. and was packed with ethylene glycol adipate polyester, 15%, coated on Chromosorb W 85% (Analabs, Inc., Hamden, Conn.). At 180° C, methyl stearate had a retention time of approximately 12 minutes. Each analysis was carried out until methyl arachiodonate emerged. The detector was a hydrogen flame ionization detector calibrated for quantitative accuracy by the injection of standard mixtures of known composition. The areas under each of the major peaks corresponding to the methyl esters of: 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 20:4 acids (the first number is the chain length, the second the number of double bonds) were measured by triangulation and summed. The sum itself was then compared with the area of the methyl margarate (17:0), corresponding to the known quantity of margaric acid that had been added, form which ratio the total fatty acid present in each fraction was estimated.

The summarized data are in Tables II and III. The results are expressed in terms of cholesterol content and cholesterol ^{14}C-cpm in the total organ. The animals on BAPN and high cholesterol gained much less weight than the high cholesterol or normal group. Their liver cholesterol was also slightly less than that of the normal group and markedly less than the high cholesterol group. Also, there was less cholesterol in their aortas. ^{14}C-cholesterol uptake studies were not done on the normal group. In the group fed BAPN the ^{14}C-uptake was reduced in both aorta and liver compared to the high cholesterol group. The ^{14}C deposition in the livers of the BAPN group was reduced out of proportion to the reduction in the aortas.

The results of analysis by gas chromatography are shown in Tables IV and V. There was no difference in the fatty acid composition of the cholesterol ester or phospholipid fractions between the high cholesterol and high cholesterol-BAPN groups. There was insufficient triglyceride in the BAPN-high cholesterol group for analysis.

In Table V, results on the analyses of the aortas of the high cholesterol group are presented. The quantity of fatty acids in the BAPN-fed group was so low that the methyl esters could not be distinguished from solvent impurities.

DISCUSSION

Animals fed BAPN ate less, gained less weight, had lower quantities of cholesterol in their aortas and in their livers and following the administration of labeled cholesterol, had a lower uptake of radioactive cholesterol into both liver and aorta than did the animals on otherwise similar diet. Since all the animals fed the labeled cholesterol were observed to have eaten it, the BAPN may have depressed the absorption of cholesterol by the intestinal tract. While deposition of labeled cholesterol in the aorta and in the liver was depressed in

TABLE IV

LIVERS	*Palmitic*	*Palmitoleic*	*Stearic*	*Oleic*	*Linoleic*	*Arachidonic*
Cholesterol Esters	*16:0*	*16:1*	*18:0*	*18:1*	*18:2*	*20:4*
Group I (High Cholesterol)						
(10 samples)	11.2 ± 1.0	9.9 ± 1.2	1.6 ± 0.3	51.0 ± 2.3	26.2 ± 1.9	
Group III (High Cholesterol + BAPN)						
(10 samples)	9.0 ± 0.3	7.3 ± 1.2	2.0 ± 0.4	53.1 ± 1.2	28.7 ± 1.7	
Phospholipid						
Group I						
(10 samples)	30.9 ± 2.6		25.7 ± 2.6	15.9 ± 5.6	21.3 ± 1.7	6.9 ± 2.1
(0.15 mg/100 mg)						
Group III	25.2 ± 3.2		27.1 ± 3.4	11.3 ± 2.8	24.4 ± 3.6	12.9 ± 3.2
(0.13 mg/100 mg)						
Triglycerides						
Group I						
(6 samples)	24.8 ± 7.2		4.2 ± 4.0	38.6 ± 7.6	26.5 ± 6.8	3.
(0.05 mg/100 mg)						
Group III	Present in very small quantities—QNS.					

TABLE V

AORTA	*16:0*	*16:1*	*18:0*	*18:1*	*18:2*	*20:4*
Cholesterol Ester						
Group I (0.03 mg/sample)	35 ± 4.5		8 ± 7.6	39 ± 5.6	17 ± 11	
Group III QNS				QNS		
Phospholipid						
Group I (0.5 mg/sample)	26 ± 5.7		30 ± 5.1	15 ± 2.1	21 ± 6.2	8 ± 6.8
Group III (0.4 mg/sample)	27.3 ± 5.3		34 ± 3.3	15 ± 1.5	21 ± 3.4	1.7 ± 9.9
Triglyceride						
Group I (0.7 mg/sample)	23 ± 3.4	5 ± 2.4	7 ± 2.4	37 ± 2.1	26 ± 5.2	
Group III (1.5 mg/sample)	24 ± 9.6	3.1 ± 20	11.9 ± 5.2	30.7 ± 1.5	29.6 ± 16.9	

the BAPN fed animals, the effect on the liver seemed relatively more pronounced. There was certainly no increase in lipid deposition in the BAPN group.

There was no significant difference in the fatty acid composition of the liver lipids in the animals in the two groups. If fat absorption were seriously impaired in the BAPN group, one might expect a decrease in the content of essential fatty acids. Since the linoleic acid content in the livers of the BAPN-fed groups was not markedly diminished, it is unlikely that there was marked impairment of fatty acid absorption.

These results agree with the previous studies which concluded that BAPN did not increase lipid deposition in the aorta. They also agree with the results of an *in vitro* study by Milch *et al* (20) showing that altering the aldehyde cross links in the collagen of the bovine aorta did not alter the pattern of lipid deposition.

In addition, results are presented for lipid deposition in the cholesterol-fed rat aorta. These appear to differ from those in the liver, in that the cholesterol esters containing palmitic acid are disproportionately increased, almost reaching the elevation of oleic acid esters. The reasons for this are unclear.

SUMMARY

1. The effect of BAPN on lipid deposition in the rat aorta was studied.

2. BAPN produced a marked reduction in chemical cholesterol deposition in the aorta, but this was not out of proportion to the reduction in liver chemical cholesterol or rat weight.

3. Studies with ^{14}C-labeled cholesterol confirmed the above reduction.

4. ^{14}C-cholesterol showed a much more marked reduction in the livers of the cholesterol-BAPN fed animals than in the aortas as compared with those fed the high cholesterol diet alone. Possible reasons for this are discussed.

5. There was little difference in the distribution among the various lipid fractions in the livers of the cholesterol-BAPN fed animals as compared with the cholesterol fed animals.

6. For the first time results of studies of lipid distribution in the aorta of rats on high cholesterol diets are presented.

7. These studies reveal, that as in most organs studied, cholesterol oleate predominates. However, unexpectedly, cholesterol palmitate is quite elevated in the aorta.

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