Effects of Acid Mucopolysaccharides on Growth Rates and Constituent Lipids of Tissue Cultures.* (28366)

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Although changes in the acid mucopolysaccharides of connective tissues (including the aorta) have been extensively examined, both with regard to qualitative and quantitative aspects, by Meyer(1), Kirk(2) and Dyrbye(3), as functions of increasing age by Virchow(4) and formation of atheromatous lesions by Lazzarini-Robertson(5), only recently has it been considered that the presence of acid mucopolysaccharides may actually influence the metabolisms of connective tissue (and perhaps other) cells and thus causally modify their rates of growth, cell division, morphological appearances and chemical compositions.

Our preliminary investigations and a study by Branwood(6) suggested that addition of calf aorta preparations containing acid mucopolysaccharides may result in reduction in lipid content of tissue culture cells that have been experimentally "loaded" with lipid. These findings have led to quantitative determinations of lipid changes in cultured cells following administration of acid mucopolysaccharides and aortic extracts containing them.

However, in addition to a demonstration of changes in total and constituent lipids of cells in tissue culture, a search for the mechanisms involved in the changes noted has been made, with particular emphasis on possible increases in rates of growth. The observations of Gilman(7) and Moon and coworkers(8) on the intimate relationship between vascular growth and repair and acid mucopolysaccharides especially suggest that accelerated growth may be one of the responses following administration of mucopolysaccharides.

Materials and methods. Human and chick aorta cells as well as HeLa cells have been studied in tissue culture systems. This report describes studies with the 2 latter systems; results in human arterial tissue systems will be reported later.

Chick aorta cells were prepared by stripping the intima cells from superior sections of thoracic aortas, placing them in 1% trypsin overnight at room temperature, and on the following day washing and placing them in either Eagle's minimum essential medium or Puck's N-16 medium with 10% fetal calf serum. Cells were then transferred into Leighton tubes provided with cover slips and into bottles of 1 or 2 liter capacity. They usually formed continuous sheets in both bottles and Leighton tubes within 8 days.

In the case of HeLa cells, solid sheets of cells (originally from Microbiological Associates, Inc., Albany, Calif.) were trypsinized, washed, and suspended in either Eagle's solution or Puck's N-16 medium as above. The cells formed continuous sheets in the bottles and tubes in approximately 7 days.

The HeLa cells in Eagle's medium grew rapidly, tended to adhere to the glass surface and formed piled-up areas of cells which appeared to be rather small when examined under the microscope. Cells grown in the Puck's medium appeared to be larger. They did not pile up as did the cells in Eagle's medium; large numbers of them detached from the glass and floated free in the supernatant fluid.

Actual differences in rates of growth in the 2 types of medium were not established. However, it appeared that more cells were present in the Eagle's medium. Upon "loading" the cells with lipid, it was noted that cells grown in Puck's medium appeared to take up considerably larger quantities of sudanophilic material than cells grown in Eagle's medium (Fig. 1). Consequently, Puck's medium was

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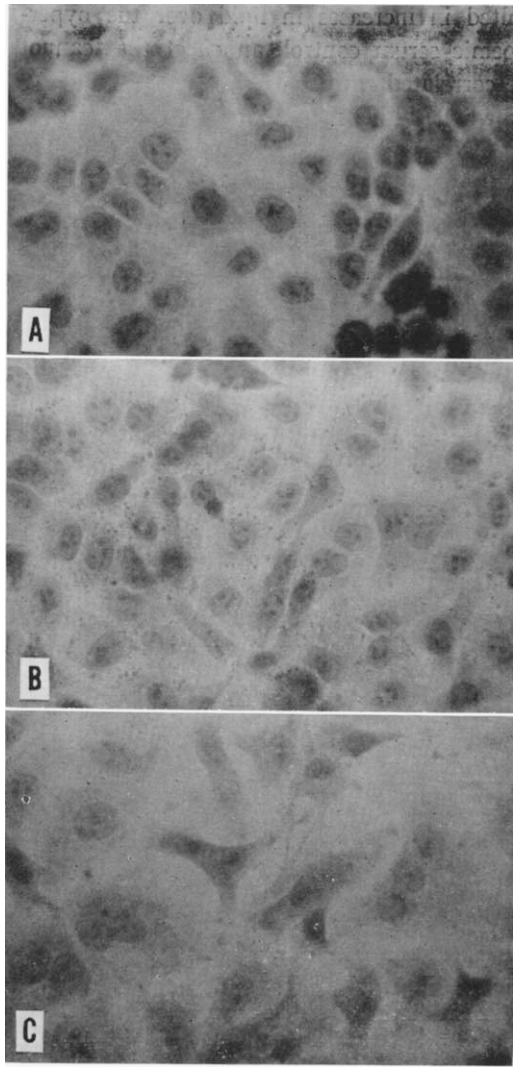

FIG. 1. Microscopic comparison of lipid deposition in HeLa cells—Puck's medium. A. Normal control culture after 4 days growth (×268). B. Hyperlipemic serum added after 5 days growth (×268). (Note granules stained with Sudan IV.) C. Cells loaded with hyperlipemic serum. Lipids cleared by calf aorta extract after 3 days growth (×268). Stained with Sudan IV and H and E.

employed for the majority of these experiments.

In a few experiments cholesterol in alcohol was utilized as a "loading" medium. "Loading" was carried out on the fifth or sixth day from the beginning of the culture. The amount of cholesterol added to the medium was 4 mg % in alcohol. In subsequent experiments 35 mg % of cholesterol in hyperlipemic serum was fed. The cultures were considered to be "loaded" when they had been on the hyperlipemic serum diet for from 48 to 72 hours. At the end of this period all cultures were refed the standard calf serum nutrient medium (without the added cholesterol or increased quantities of hyperlipemic material).

The test substances consisted of saline extracts of acetone dried calf aorta (Extracts No. 1 and 4) or the following substances: chondroitin sulfate A (a purified preparation from Riker Laboratories, which may have contained small amounts of chondroitin sulfate C); chondroitin sulfate B (obtained through Dr. Hans Winterstein); IN 379 (pimentine HCl obtained through the courtesy of Drs. Chester Cavallito and Harold Hamilton, Irwin, Neisler and Co.).

After allowing the test substances to act for 72 hours, cells were removed from the culture bottles with the aid of trypsin and total lipids were extracted and purified according to the method of Sperry(9). Analyses of constituent lipids were carried out by silicic acid chromatography as described by Fillerup and Mead(10). Non-esterified and total cholesterol was determined by separation on silicic acid columns and by the Kingsley(11) procedure respectively. Dry weights of whole cultures were obtained gravimetrically. Fatty acid determinations were carried out in a Research Specialties Co. Gas Chromatograph (Model 60-1A) utilizing a 4-foot ethyl succinate column.

Results. Effects of the test substances on chick aorta cells are shown in Table I. When either Extract No. 1 or sodium chondroitin sulfate A was added, a diminution in total lipid content per unit dry weight of culture took place.

Similar decreases in total lipid were observed in the HeLa cell systems following administration of small amounts of the above named agents and also when Extract No. 4 was added to the cells (Table II). These changes were for the most part correlated with *an increase* in dry weight of the total culture indicating that the decrease in lipid was an effect of cell multiplication rather than a specific "clearing" effect. However,

TABLE I. Effects of Various Substances on Lipids of Chick-Aorta Cultures.

No. of exp	Test substance	% lipid*	Standard deviation	Sterol %†
5	Hyperserum control	100		10
4	Calf serum "	87	±2.7	
2	Extract 1 (.008 mg)	82	±4.1‡	6
3	" 1 (.8 ")	75		6
1	Na chondroitin sulf A (.01 mg)	88		
1	Na chondroitin sulf A (.1 mg)	81		

* Total lipid of hyperserum control in each experiment taken as 100%.

† % of total lipid; isolated by silicic acid chromatography.

‡ Note that this standard deviation was obtained by combining results obtained from experiments in which 2 different dosages were employed. In this table the authors confine their statements to effects obtained *within the range* of .008 to .8 mg of Extract 1. Although .8 mg of Extract 1 may appear to be more effective in these cultures, the amount of data does not justify this conclusion.

Probability of a difference in lipid content between hyperserum controls and Extract 1 treated cells is greater than 99%.

in the case of Extract No. 4 it appeared that a lipid "clearing" effect took place in the absence of a proportional increase in weight of culture.

Chondroitin sulfate B did not cause significant changes in either total lipid or dry weight. However, addition of IN 379 resulted in increases in lipid over the hyperlipemic serum control, an effect that cannot be correlated with rate of growth (or lack of growth) of the cultured cells (Table II).

Analysis of constituent lipids showed that both free and total cholesterol per unit dry weight of culture and cholesterol per unit weight of lipid were reduced with a single application of the effective test substances (Tables II and III). Repeated applications appeared to be only slightly more effective, if at all.

Gas chromatograms of methylated fatty acids from the cultures (Fig. 2) revealed that an effect of lipid "loading" was to increase the saturated fatty acids (palmitic and stearic) over those of so-called normal cultures (maintained on standard calf serum). However, when cells were induced to grow faster and to decrease their lipid, as they were by treatment with Extracts No. 1 and 4, it was the unsaturated fatty acids which appeared to display the largest decreases.

Discussion. Although a superficial survey of the data presented may suggest that the potency of certain agents to reduce the amount of lipid per unit tissue is primarily a function of that agent's capacity to stimu-

TABLE II. Effects of Various Substances on Lipids and Dry Weights of Hela Cultures.

No. of exp	Test substance	% lipid*	Standard deviation	Free sterol, %†	Standard deviation	% dry wt‡	Standard deviation
17	Hyperserum control	100		8	±1.6	100	
6	Calf serum control	85	± 9.5	7		84	
1	Extract 1 (.0008 mg)	89		9			
2	Extract 1 (.008 mg)	82		4			
3	Extract 1 (.08 mg)	87	± 2.2§	5	±1.5	209	
5	Extract 1 (.8 mg)	87		7		181	±34.0
1	Extract 1 (1.6 mg)	89		5			
5	Extract 4 (.33 mg)	76	± 2.7			116	± 9.2
6	Chond sulf A (.01 mg)	86	± 8.3	7		136	±29.0
3	Chond sulf A (.01 mg)	79		6		154	
6	Chond sulf B (.01 mg)	96	±11.0			140	±34.0
3	IN 379 (10^{-4})	112	±27.0			122	

* Total lipid of hyperserum control in each experiment taken as 100%.

† % of total lipid; isolated by silicic acid chromatography.

‡ Total dry weight of hyperserum control in each experiment taken as 100%.

§ In several instances standard deviations are calculated from results of experiments in which several different dosages of the same material were given. The authors thus confine their statements to effects obtained *within the ranges* of the doses given and do not attempt to distinguish an optimum dose or effective dose, for this system.

Probability of a difference in lipid content between hyperserum controls and Extract 1 and 4 treated cells is greater than 99%; between hyperserum controls and Chondroitin sulfate A is greater than 95%. Probability of a difference in free sterol content between hyperserum controls and extract treated cells is greater than 90%.

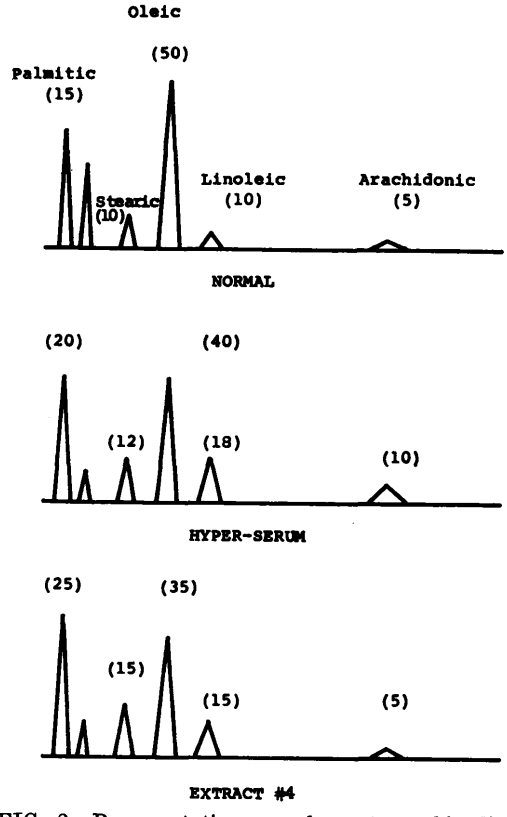

FIG. 2. Representative gas chromatographic distributions of major fatty acids in normal, hyperserum and Extract 4 treated cultures. Percentages of occurrence are indicated in parentheses above each peak and, with one exception, are rounded off to the nearest 5%. The second peak from the left in each pattern represents palmitoleic acid. Treatment of "loaded" cell cultures with Extract 1 or chondroitin sulfate A results in a pattern similar to that shown for Extract 4.

late cell division and thus to decrease the unit lipid by dilution, the action of Extract 4 may be more specifically that of clearing the cell of lipid. Why a difference in growth promoting potency should occur between Extracts 1 and 4 (and also the perhaps unique capacity of Extract 4 to clear lipid in lieu of growth) is not clear, since both extracts were prepared in the same manner. Similar variations in lipid clearing effects in the rabbit and the rat were previously noted by Morrison *et al.*(12). These variations were then considered as probably due to intra-species biologic variations. Another explanation may be that the tissues extracted are not uniform in their content of active substances. However, the somewhat variable responses of the individual cultures to chondroitin sulfate B attest to the complex nature of the interactions being studied.

One of the possible mechanisms which may be operating in the reduction of cellular lipid in tissue cultures, however induced, is suggested by the concomitant reduction of both cholesterol and unsaturated fatty acids in extract treated cultures. Since no mechanism is known for the breakdown of cholesterol in such cultures, it may be that the sterols combine with unsaturated fatty acids (for which they display an affinity) and leave the cells in the form of soluble lipoproteins. The production and secretion of lipoproteins by tumor cultures has been noted and preliminary findings in this laboratory indicate that C-14 labelled cholesterol leaves the tumor cells both intact and in considerable quantity. However, it may also be that cholesterol synthesis is somehow slowed or altered.

The observations discussed above should be viewed in the light of data by Gilman *et al.*(13) which reveal an intimate relationship between the acid mucopolysaccharides and the growth of arterial tissue cells. They are also pertinent to the findings of Murata(14) which indicate that injection of sulfated polysaccharides (obtained from seaweed) into rabbits causes a fall in serum lipids and inhibits development of atherosclerosis.

Summary. Lipid analysis of chick aorta intima and HeLa cell tissue cultures confirms histological studies indicating that total lipids and cholesterol are significantly reduced per unit volume of cells when very

TABLE III. Relative Total Cholesterol in HeLa Cultures Treated with Various Extracts (Kingsley Method).

Exp No.	Test substance	%* total cholesterol
1	Hyperserum control	15
1	Na chond sulf A (0.1 mg) × 1	14
1a+1b	Extract 1 (.8 mg) × 1	12.5
1	Na chond sulf A (.01 mg) × 1	13
1	Chond sulf B (.1 mg) × 1	14
2	Calf serum control	16
2	Hyperserum control	19
2	Na chond sulf A (0.1 mg) × 1	14
2	Extract 1 (.8 mg) × 1	12

* % of total lipid.

small amounts of calf aorta extracts or the mucopolysaccharide, chondroitin sulfate A are added to the cultures. The lipid clearing effect noted in cells is often associated with stimulation of cellular growth and with a process of lipid dilution by cellular multiplication. However, in the case of one aortic extract, lipid clearing took place without significant stimulation of cellular growth. The latter suggests a specific cellular clearing effect as distinguished from lipid dilution by cell division and growth.

1. Meyer, K., Davidson, E., Linker, A., Hoffman, P., *Biochim. et biophys. acta,* 1956, v21, 506.

2. Kirk, J. E., Dyrbye, M., *J. Gerontol.,* 1957, 12:23.

3. Dyrbye, M. O., *Aging of Human Arterial Tissue. Biochemical Studies of the Acid Mucopolysaccharides,* Munksgaard Co., Copenhagen, 1959.

4. Virchow, R., *Gensammelte Abhandlungen zur wissenschaftlichen Medizin,* Meidinger, Frankfurt, 1958, p492.

5. Lazzarini-Robertson, A., Jr., *Angiology,* 1961, v12, 525.

6. Branwcod, W., personal communication.

7. Gilman, T., *Connective Tissue, Symposium,* Blackwell Scientific Publications, Oxford, 1957, p120.

8. Moon, H. D., *Connective Tissue Thrombosis and Atherosclerosis, Connective Tissue Reactions in the Development of Arteriosclerosis.* Academic Press, N. Y., I. H. Page, Ed., 1959, p33.

9. Sperry, W. M., *J. Biol. Chem.,* 1953, v118, 377.

10. Fillerup, D. L., Mead, J. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 574.

11. Kingsley, G. R., Schaeffert, R. R., *J. Biol. Chem.,* 1949, v180, 315.

12. Morrison, L., Bergman, H., Rosenthal, M., unpublished data.

13. Gilman, T., *Arch. Path.,* 1959, v67, 642.

14. Murata, K., *J. Gerontol.,* 1962, v17, 30.

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Role of Skeleton and Kidney in Fluoride Absorption in Rat. (28367)

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Considerable work has been done in an attempt to identify those factors which regulate or modify the absorption of fluoride from the gastro-intestinal tract. Several different inorganic elements, such as calcium(1, 2), magnesium(3,4), phosphorus(5), and molybdenum(6-8), may alter the rate of fluoride absorption. Metabolism studies have suggested further that the age of the animal(9), especially with respect to rate of skeletal growth, and its previous exposure to fluoride affect fluoride absorption(10). The metabolic equilibrium suggested by Fig. 1 indicates that if the kidneys are damaged or seriously impaired, thus removing a major source of fluoride detoxification, fluoride which is absorbed must either be deposited in the skeleton or remain in the soft tissues and circulating body fluids. Carlson and co-workers (11) have shown in the nephrectomized rat using isotopically-labeled fluoride that the tissue fluoride concentration is elevated. Since it has been shown that the metabolism of fluoride may be significantly affected in renal failure(12), and an increase in soft tissue fluoride content results in nephrectomized rats, these studies were conducted to provide more information concerning the specific efforts of nephrectomy and previous exposure to fluoride upon rate of fluoride absorption in the rat.

Experimental. Series I. A total of 18 male Wistar strain albino rats ranging in age from 55 to 60 days were divided into 2 groups of 8 each and a third group of 2 animals according to body weight. All animals were housed in pairs in an air-conditioned room and received a low-fluoride stock corn

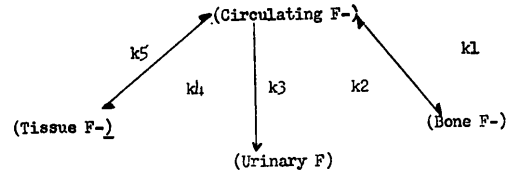

FIG. 1. Schematic diagram of metabolic pathways of fluoride.