Metabolic Parameters of the Growth-Stimulating Effect of Chondroitin Sulfate A in Tissue Cultures.* (30254)

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Chondroitin sulfate A and various extracts containing this material have been shown to act as growth-stimulating agents in cultures of HeLa cells and chick aorta intima cells (1,2). Concomitant with the provoked increase in rate of growth and cell division, a small but consistent decrease in the concentration of total lipid is noted (circa 10%). In the case of one extract(1) no measurable increase in growth occurs but a decrease in total lipids takes place.

It was the purpose of the present study to elucidate in greater detail the growth-stimulating and lipid-clearing effects of a single dose of relatively pure chondroitin sulfate A.

Comparisons were therefore made between control and experimental cultures as follows: (a) total dry weights, total RNA, total DNA and total lipids as a function of time following addition of the test material and (b) uptake of P^{32} into total RNA, total DNA and total phospholipid following these same intervals of exposure. It was reasoned that these determinations would provide additional evidence with respect to the growth promoting capacities of chondroitin sulfate A and would help clarify the speed, mode and duration of action of the compound.

Preliminary studies had indicated that the acid mucopolysaccharide was ineffective in the cultures if given during the early phases of growth and was actually deleterious to the cells if employed in conjunction with a mixture of very highly lipemic serum. Special attention was given these phenomena in the studies reported herein.

As a check on the universality of the action of chondroitin sulfate A, mouse L cells and human amnion cells were also employed as tissue culture test systems.

* Studies supported by grants from John A. Hartford Foundation and Crenshaw Research Foundation.

Material and methods. Solid sheets of HeLa cells (originally from Microbiological Associates, Inc., Albany, Calif.) were trypsinized, washed and suspended in Eagle's MEM medium(3) with 10% new born calf serum added. They were then transferred to 2-liter bottles and were allowed to grow normally for 7 days. On the 7th day human lipemic serum (containing an average of 341 mg% of cholesterol, 420 mg% in the specially enriched lipemic serum†) was added and allowed to remain for a period of 72 hours. In a few cases the lipemic serum was added to the cultures on the 2nd day and the cells were grown on this media for 3 additional days. At the time of addition of chondroitin sulfate A (CS-A) to the cultures (1 mg per 100 ml culture fluid) the human lipemic medium was replaced with the normal medium (Eagle's MEM plus calf serum). Mouse L cells were prepared in the same manner but amnion cells were allowed to grow for 2 weeks prior to addition of the human lipemic serum so as to become relatively senescent. (The original purpose of the human lipemic serum was to provide a culture in which effects on lipid metabolism might be more apparent. Actually, the cells grew more rapidly in this medium than they did in Eagle's MEM plus 10% new born calf serum.)

The test substance consisted of CS-A, deproteinized and purified in Riker Laboratories. Paper chromatography(4) reveals that the material moves as a single spot (Fig. 1) but chromatography on amino ethyl cellulose indicates the possibility of some heterogeneity (Fig. 2). This may be an artifact of the system(5). The CS-A preparation may

† This preparation consisted of 90% by volume of normal human serum plus 10% of a Beta lipoprotein fraction as obtained by addition of methyl sulfate. The final cholesterol concentration was found to be 420 mg%.

TABLE I. Lag Time and Duration of Activity Following Single Dose of Chondroitin Sulfate A in HeLa Cell Cultures.

No. of runs	Hr exposed to chon sulf A	Rel* total wt	Rel %* RNA	Rel* spec act RNA	Rel %* DNA	Rel* spec act DNA	Rel %* lipid	Rel* spec act lipid
4	3	103	103	90	100	110	106	98
5	6	*120*	*118*	*133*	100	*170*	*90*	*180*
3	12	*111*	107	*185*	96	*165*	92	*136*
5	24	*117*	105	*177*	102	109	98	*175*
5	48	108	109	96	104	96	93	97

* All controls were given a value of 100. Values appearing above are thus relative to values of the controls. Italicized values represent a probability of difference from the control that is greater than P 0.01.

contain some chondroitin sulfate C but it has previously been shown(1,2) that preparations of the latter (spotted in Fig. 1) have a lesser growth-promoting effect.

As indicated in Table I, CS-A was allowed to act in cultures for periods of 3, 6, 12, 24 and 48 hours. Ten minutes prior to trypsinizing the cells and removing them from the bottles, 100 μg of P^{32} was added to each culture. The cultures were then washed 3 times in ice-cold magnesium and calcium free saline solution (GKN)(3) and frozen simultaneously. Thawing was carried out in either Delsal's solution (one part methanol to 4 parts methylal) or in one part of methanol to 2 parts of chloroform so as to stop enzymic action and extract lipids in one step.

The non-lipid solids were dried by evaporation under nitrogen, were ground up and washed 3 times in ice-cold 5% perchloric acid to remove low molecular weight organic phosphorus and the total RNA and DNA extracted by the method of Schmidt and Thannhauser(6). Determinations of total RNA and DNA were carried out on a Cary recording spectrophotometer. Samples of RNA, DNA and total lipids were applied to planchets and counts of P^{32} disintegrations were made on a Nuclear Chicago low background counter (Model 202). Addition of P^{32} one hour prior to removal of the cells resulted in excessive labeling of both RNA and lipid components. The period of exposure in the culture was reduced to 10 minutes but it is very probable that some labeling occurred during the washing procedure. Great care was taken, therefore, to assure that each culture was treated in identical fashion while preparing the cells for analysis.

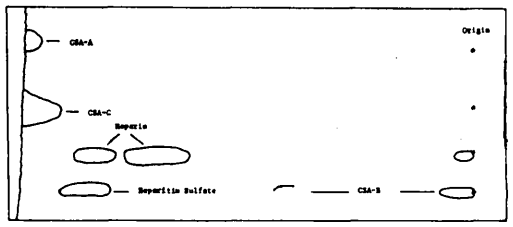

FIG. 1. Paper chromatogram of various mucopolysaccharides.
CSA-A = chondroitin sulfate A.
CSA-B = chondroitin sulfate B.
CSA-C = chondroitin sulfate C.

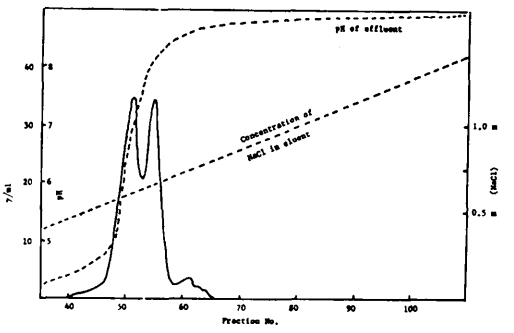

FIG. 2. Diagram showing elution of chondroitin sulfate A preparation from aminoethyl-cellulose.

Results. As can be determined from Table I, no discernible effect of CS-A occurred within 3 hours following its addition to HeLa cell cultures. By 6 hours, however, a 20% increase in total weight of cells had taken place and this was matched biochemically by an increase in total RNA. Uptake of P^{32} into both of the nucleic acids was increased and uptake of P^{32} into phospholipid was almost twice as great in the case of the cultures exposed to CS-A as compared to control cultures. A small decrease in the per-

TABLE II. Effect of Single Dose of Chondroitin Sulfate A in Mouse L Cells and Human Amnion Cells.

Material	No. of runs	Hr exposed to chon sulf A	Rel* total wt	Rel* % RNA	Rel* spec act RNA	Rel* % DNA	Rel* spec act DNA	Rel %* lipid	Rel* spec act lipid
L cells	2	6	133	113	152	100	172	98	120
L "	1	24		112	137		105		295
Human amnion	1	24							190

* All controls were given a value of 100. Values appearing above are thus relative to values of the controls.

TABLE III. Deleterious Effect of Single Dose of Chondroitin Sulfate A in Various Cultures Fed with Hyperlipemic Serum.

Material	No. of runs	Hr exposed to chon sulf A	Rel* total wt	Rel* % RNA	Rel* spec act RNA	Rel* % DNA	Rel* spec act DNA	Rel %* lipid	Rel* spec act lipid
HeLa	3	6	91	106	80	80	98	94	57
"	2	12	95	86	91	91	107	110	86
L cells	2	24	89	66	32			113	17
Human amnion	1	6	91					110	76

* All controls were given a value of 100. Values appearing above are thus relative to values of the controls.

cent of lipid in the total dry weight was noted.

Although differences from controls were not quite as striking, the same general changes were apparent in cultures exposed to CS-A for 12 hours. At the end of the 24-hour interval, however, uptake of P^{32} into DNA had slowed. By 48 hours, the specific activities of RNA and phospholipid as well as DNA had reverted to those of control cultures even though the total dry weight of CS-A treated cultures and the relative RNA and DNA remained slightly higher.

In mouse L cells and human amnion cells exposed to CS-A for 24 hours, uptake of P^{32} into phospholipid was greatly accelerated in the 2 experiments undertaken (Table II). Uptake of P^{32} into DNA and RNA during this time interval was not measured. However L cells exposed for 6 hours showed marked increases in uptake of P^{32} into RNA, DNA and phospholipid (Table II).

When CS-A was applied following feeding of the cultures with the specially prepared hyperlipemic serum, inhibition of growth was observed in mouse L cells and human amnion cells as well as in HeLa cells. The uptake of P^{32} into phospholipid appeared to be especially retarded in all these cultures (Table III).

No effect of CS-A was detected in HeLa cell cultures (exposed to the substance for 24 hours) if only 5 days had elapsed since the start of the culture (Table IV).

Discussion. Because of the brief exposure of the cultures to P^{32} (10 minutes in the nutrient medium) it is highly probable that uptake of isotope into the various substances measured represents a true index of the relative rate of turnover of these materials in an experimental culture as compared to those in a suitable control culture.

There would thus seem to be little doubt that if conditions are right, RNA, DNA and phospholipid syntheses are increased in CS-A treated cultures. A lag period in the action of CS-A (of at least 3 hours) may, in part, represent the time required for entry into the cells (or nuclei). Further studies are, however, required to ascertain that the material actually enters the cell.

The overall activity appears to be considerable after 6 hours exposure but the peak activity for one or all of the metabolic parameters measured may be just before or after this time interval. By 24 hours the turnover of DNA *per se* has apparently stopped but the cells appear to show a lag in the decrease of metabolic pathways involving RNA and phospholipid. The results sug-

TABLE IV. Lack of Effect of Single Dose of Chondroitin Sulfate A in HeLa Cell Cultures Grown for Only 5 Days in Culture Flasks.

No. of runs	Hr exposed to chon sulf A	Rel* total wt	Rel %* RNA	Rel* spec act RNA	Rel %* DNA	Rel* spec act DNA	Rel %* lipid	Rel* spec act lipid
4	24	101	102	90	90	95	97	98

* All controls were given a value of 100. Values appearing above are thus relative to values of the controls.

gest that the CS-A has been rendered inactive or has been diluted by this time. This view seems to be borne out by the fact that all measured metabolic parameters have returned to control levels by 48 hours but in the latter case the possibility is present that many of the originally treated cells may have been released into the medium.

Overall growth in these cultures was measured in terms of the actual dry weights of the cells adhering to the glass sides of the containing vessels. It is possible that these results could be misleading due to differential release of cells from the glass. However, the concomitant increase in specific activity of DNA, RNA and phospholipid in cultures showing increases in dry weights of attached cells, indicates that the rate of growth is indeed increased. An earlier investigation(2) showed greater individual cell volumes in CS-A treated cultures.

The increase in relative amount of total RNA (at least at the 6-hour interval) and the increased specific activity of total RNA in CS-A treated cultures is consistent with the requirements for increased protein synthesis in growing cells (more ribosomal RNA as well as messenger RNA); and the heightened turnover of phospholipid and decreased total lipid in the cells suggests that this material (phospholipid) is being produced for utilization by the mitochondria, thus providing energy to drive the cell in the direction of growth.

Just why the HeLa cells do not respond to treatment with CS-A in early phases of growth is not known. There is no indication that the cells are in any special state of growth at 5 days when kept on normal media.

The effect of feeding the cells with special hyperlipemic serum was to reduce the weights of both control and CS-A treated cultures. However, the cultures treated with CS-A showed accelerated declines. Metabolically, the treated cultures displayed a considerable decrease in phospholipid turnover. The effect simulates that which would be expected from a regulatory agent. It seems possible that in these cultures CS-A shifted an already stressed cellular economy into a deleteriously imbalanced condition, *i.e.*, the cells were stimulated in excess of available energy.

It was found that CS-A, when employed under proper conditions, accelerated the growth of all cells tested. These include HeLa cells, chick aorta intima cells, mouse L cells and human amnion cells. (Preliminary studies on human aorta and rabbit aorta slices indicate that CS-A provokes increased metabolic activity in these tissues as well.) From these data and from results obtained by Meyer and others(7) (including stimulatory effects on hair follicle cells) it is suspected that a wide spectrum of cells will be found to be sensitive to the action of related compounds. Schiller (8) has shown that CS-A is normally present in human plasma in an amount approximating 1.5 mg per liter. Since earlier studies by the authors(1) have shown that both protein bound and deproteinized CS-A are effective in tissue cultures at concentrations which are even less than this value (a level which is apparently kept constant in the plasma) it appears quite possible that acid mucopolysaccharides and CS-A in particular, may be normal metabolic agents.

Other growth promoting substances such as the hormone ecdysone(9) and pituitary growth hormone(10) have been shown to exert their ultimate effects on the genetic mechanisms which regulate the production of messenger RNA. It appears that a variety of such messengers must be elaborated for the complicated processes accompanying growth and cell division, but a single stimulating substance is apparently capable of starting

a chain reaction which proceeds in the direction of growth. CS-A may be such a substance. Its effects on the genetic mechanism will be more closely investigated.

Summary. Evidence is presented which further indicates that under specific conditions chondroitin sulfate A will act as a growth stimulating agent. Effective in tissue cultures in concentrations as low as those ascertained for several hormones, production of RNA, DNA and phospholipid is increased simultaneously within a few hours after application (the total extractable phospholipid is reduced indicating an increased rate of turnover), but the stimulating effect is gone by 24-48 hours. The demonstrated action of small amounts of chondroitin sulfate A in several types of cells, together with the finding that this material is normally present in human plasma in such amounts suggests that it may be a metabolic agent in the regulation of cell processes.

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Received April 1, 1965. P.S.E.B.M., 1965, v119.

Hydroxyproline in Human Blood: Forms in Which It Is Present.* (30255)

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The amino acid hydroxyproline is present essentially only in collagen of all the body proteins, but it cannot enter into this protein except through proline. It has, therefore, merited considerable interest as a guide to the metabolism of collagen. Much is known of its excretion in urine, but until very recently little was known of its presence in blood. For some time we have been studying the forms in which hydroxyproline is excreted in urine and have found that about half is in the form of the dipeptide prolylhydroxyproline. We wanted, therefore, to determine this dipeptide in blood serum and to estimate how much of the hydroxyproline of serum is in this form. Keiser *et al*(1) had shown that the method of Prockop and Udenfriend(2) cannot be used to determine total serum hydroxyproline, but that much of the hydroxyproline of alcohol precipitates can be extracted with hot trichloroacetic acid. We attempted to estimate the protein hydroxyproline of serum by this means(3). Later, however, we found that a considerable portion of the material containing hydroxyproline in the hot TCA extracts is dialyzable. LeRoy *et al*(4) have recently produced data to indicate that the hydroxyproline of alcoholic precipitates is largely protein. Further study by us is consistent with this view since it indicates that dialysis of the hydroxyproline in hot TCA extracts of alcoholic precipitates is undoubtedly due to hydrolysis under the conditions of the hot acid extractions. In the present paper we report values of the

* Supported in part by grants from Inst. of Arthritis and Metab. Dis., N.I.H., and Muscular Dystrophy Associations of America, Inc.