Acknowledgments

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Heparan Sulfates of Cultured Cells. II. Acid-Soluble and -Precipitable Species of Different Cell Lines*

Paul M. Kraemer

ABSTRACT: Six established mammalian cell lines, all adapted to growth in suspension culture, as well as a diploid mouse embryo lung strain grown as monolayers on glass, have now been studied for their capacity to synthesize heparan sulfate. The established cell lines are CHO, Don C, L5178Y, BHK-C13, L-929, and HeLa, and they differ in their species of origin, their morphology on glass, and their ability to synthesize differentiated cell products. Each was examined for both directly acid-soluble heparan sulfate and heparan sulfate rendered acid-soluble by papain digestion of the acid precipitate. All seven cell types synthesized heparan sulfate during exponential cell growth. In each case, heparan sulfate appeared in both directly acid-soluble and precipitable cell fractions. In fact, similar amounts of radioactive precursors were incorporated into the acid precipitate in all cells studied. The results suggest that heparan sulfate is a general cellular constituent rather than a differentiated cell product. The possibility is raised that all mammalian cells can synthesize this material and that it is vital to some general life process at the cellular level.

The term heparan sulfate designates mammalian glycosaminoglycans that structurally resemble heparin but which are less highly sulfated and lack significant anticoagulant activity (Brimacombe and Webber, 1964). That is, the sugar polymer chain consists largely of alternating glucosamine and glucuronic acid units; both linkages are $\alpha 1 \rightarrow 4$; the molecules contain both *O*-sulfate and *N*-sulfate residues, and in their native state are covalently linked to polypeptide by means of a galactosyl–galactosyl–xylosyl–*O*-serine linkage region (Knecht *et al.*, 1967). It appears that heparin and heparan sulfate are the only mammalian complex carbohydrates that contain N-sulfated hexosamine residues; hence, the pronounced lability of these *N*-sulfate groups can be exploited in a number of ways for studies of the N-sulfated glycosaminoglycans in the presence of large amounts of other complex carbohydrate species.

The structural distinctions between heparan sulfate and heparin appear to be solely quantitative rather than qualitative [*e.g.*, it now seems clear that both types contain at least some N-acetylated hexosamine units (Linker *et al.*, 1958; Jaques *et al.*, 1966; Lindahl, 1966; Cifonelli and King, 1970)], raising the possibility that animal tissues contain a continuum of N-sulfated glycosaminoglycan species. If this is true, the further possibility must be considered that all of these species are metabolically and functionally related and that the anticoagulant activity of the more highly sulfated varieties is essentially fortuitous and physiologically irrelevant.

The N-sulfated glycosaminoglycans are widely distributed in animal tissues (Brimacombe and Webber, 1964); however, this tissue ubiquity leaves unanswered the question of which cells possess synthetic capability. Indeed, it has been widely believed that the synthesis of these molecules is restricted to certain highly differentiated cells, especially mast cells. Thus, the previous report from this laboratory that demonstrated heparan sulfate production by an established line of Chinese hamster fibroblasts (Kraemer, 1968) encouraged a reexamination of this question. In the following report, evidence is pre-

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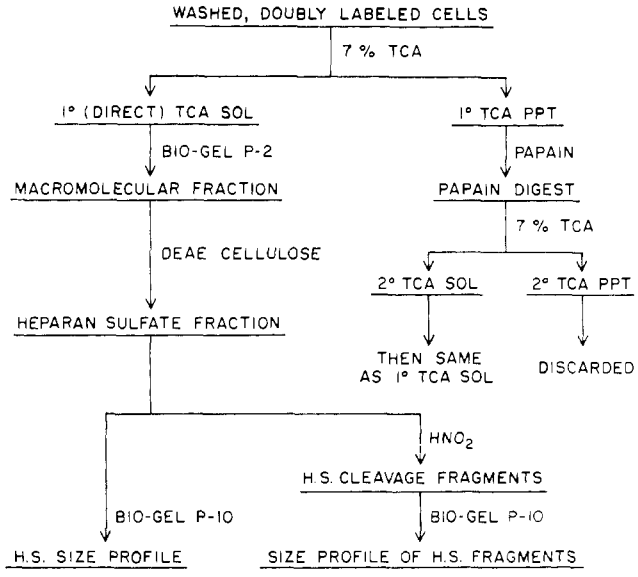

FIGURE 1: Diagram of protocol used for the isolation and identification of heparan sulfate from cultured cells.

sented showing that heparan sulfate is made by cultured cell lines and strains of widely different characteristics.

Materials and Methods

Cell Lines and Culture Conditions. Most experiments reported here concern exponentially growing suspension cultures of established cell lines. In each case, 0.1 μCi/ml of glucosamine-6-*t* (New England Nuclear, sp act. (Ci/mole) 1150) and 1.0 μCi/ml of $Na_2{}^{35}SO_4$ (New England Nuclear, about 300) were added to the growing cells, and the cells were harvested 16–30 hr later when the cell count had doubled.

CHO Chinese hamster fibroblasts were grown as previously described (Kraemer, 1971). Don C Chinese hamster cells were obtained from Dr. R. R. Klevecz, City of Hope, Duarte, California, and were grown in F-10 medium as were CHO cells. HeLa cells were provided by Mr. Charles James, University of New Mexico School of Medicine, mouse fibroblast L-929 cells were obtained from Dr. Angus Graham, Wistar

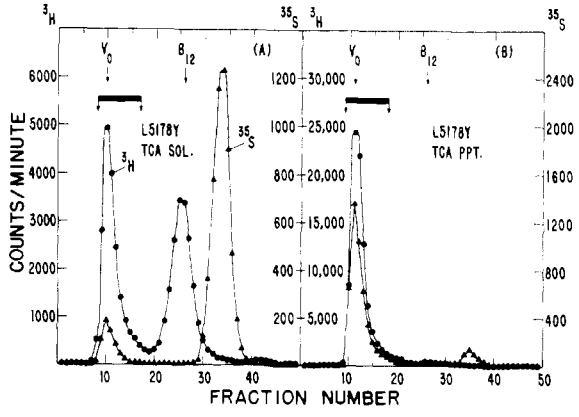

FIGURE 2: Bio-Gel P-2 chromatography profiles of the initial cell fractions from 6 × 10^8 L5178Y cells grown for one generation in medium with 0.1 μCi/ml of glucosamine-6-*t* and 1.0 μCi/ml of $Na_2{}^{35}SO_4$. Aliquots of 0.1 ml from each 2-ml fraction were counted; then the remainder of those fractions indicated by the bar were pooled and lyophilized.

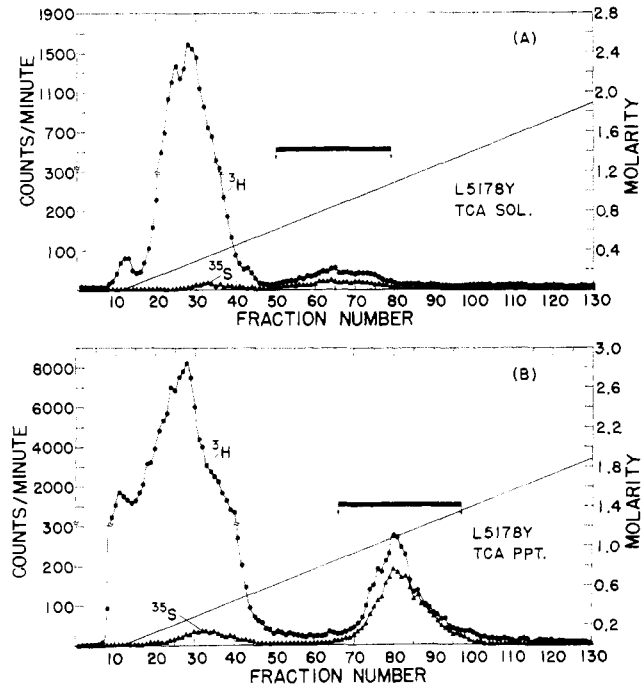

FIGURE 3: DEAE-cellulose chromatography profiles of material excluded from Bio-Gel P-2 and harvested as indicated in Figure 2. Aliquots (0.5 ml) of each 3.0-ml fraction were counted, and the remainder of fractions covered by the bar were pooled and lyophilized.

Institute, and BHK-C13 Syrian hamster fibroblasts from Dr. Alexander Kisch, University of New Mexico School of Medicine. The HeLa, L-929, and BHK-C13 cells were all grown in Joklik-modified MEM medium purchased from Grand Island Biochemical Company. The murine lymphoma cells, L5178Y, were obtained in 1965 from Dr. Lionel Manson, Wistar Institute, and were grown in Fischer L-1 medium obtained from Schwarz Biochemical Company. In addition to these established cell lines, experiments were also done with diploid mouse embryo lung fibroblasts (strain MEL) grown as monolayers in F-10 medium. These cells were in the fifth passage of *in vitro* growth when labeling medium was added. All cells were negative for PPLO by the criteria routinely used in this laboratory (Kraemer, 1971).

General Protocol Used for Isolation and Identification of Heparan Sulfate. The same protocol, derived from experience with CHO cell line (Kraemer, 1971), was used for experiments with all of the cultured cells studied and is summarized in Figure 1. In each case, heparan sulfate from two cellular fractions was isolated and identified: (1) heparan sulfate of the direct trichloroacetic acid soluble fraction of whole cells, and (2) heparan sulfate of the trichloroacetic acid precipitate, rendered trichloroacetic acid soluble by papain digestion.

Following growth of the cells in medium containing glucosamine-6-*t* and $Na_2{}^{35}SO_4$, the cells were harvested by centrifugation (or scraped off the glass with a rubber policeman in the case of MEL cells), washed three times with Earle's balanced salt solution, and resuspended in cold water. Cold 40% trichloroacetic acid was then added to a final concentration of 7%. After 30 min in an ice bath, the preparation was centrifuged for 10 min at 2000 rpm, and the water-clear, trichloroacetic acid soluble fraction was decanted, ether-extracted three times (residual ether removed with a

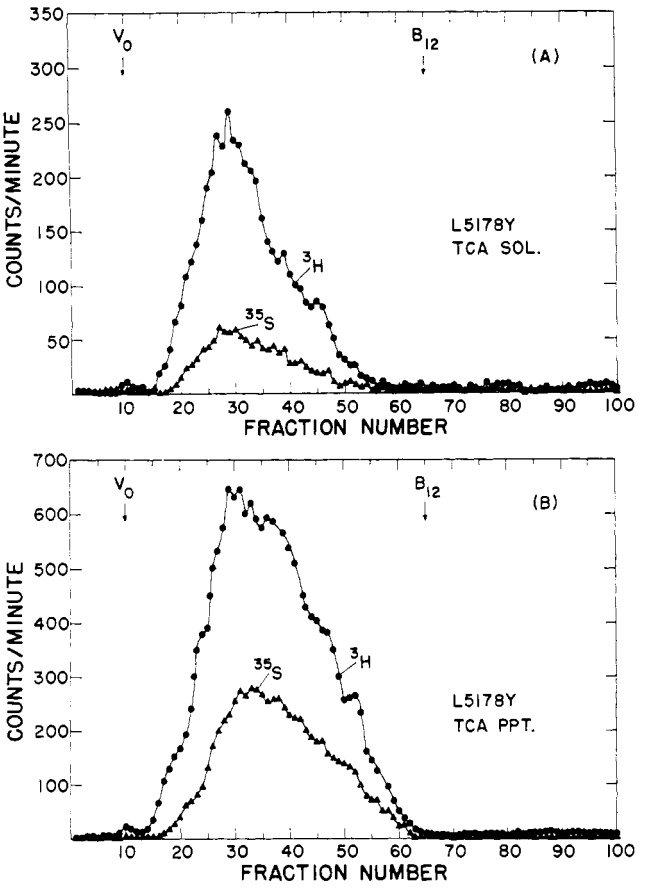

FIGURE 4: Bio-Gel P-10 chromatography profiles of one-half of the material harvested as indicated in Figure 3. All of each 2.0-ml fraction was counted.

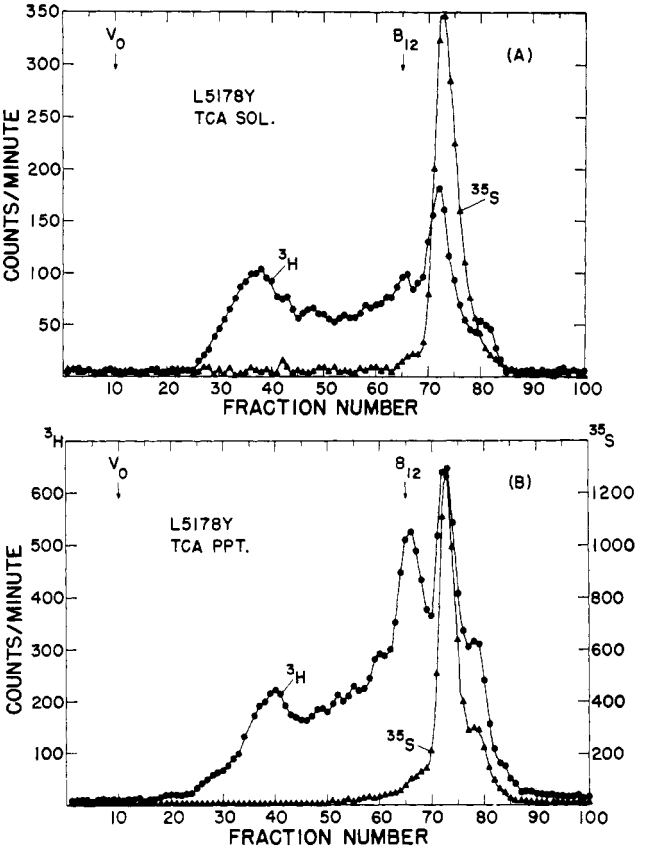

FIGURE 5: Bio-Gel P-10 chromatography profiles of one-half of the material harvested as indicated in Figure 3 and then treated with nitrous acid. All of each 2.0-ml fraction was counted.

nitrogen stream), and lyophilized. The trichloroacetic acid precipitate was resuspended in 0.1 M ammonium acetate and ether-extracted, residual ether was blown off, and then papain (Worthington), activated with 0.005 M cysteine-HCl and 0.001 M EDTA, was added to 1 mg/ml. Papain digestion was done at 56°, 16 hr, then the digest was put in an ice bath, and after 10 min, cold tricholoracetic acid was added to 7%. After a further 30 min in the ice bath, the 2° trichloroactic acid soluble fraction was harvested, ether-extracted, and lyophilized.

Bio-Gel P-2 chromatography (1.2 × 140 cm, eluted with 0.1 M ammonium acetate) was used to isolate the macromolecular material from the direct trichloroacetic acid soluble and 2° trichloroacetic acid soluble fractions. The excluded material from these runs was lyophilized and run on DEAE-cellulose columns and eluted with a linear salt gradient, 0.01 M to 2.0 M ammonium acetate. The combined putative heparan sulfate harvest from each of these column runs was divided into two equal portions and lyophilized. One portion was run on Bio-Gel P-10 directly, the other was first treated with nitrous acid and then run on Bio-Gel P-10. Aliquots of fractions of each of the three types of column runs were examined for ^{3}H and ^{35}S radioactivity by liquid scintillation counting methods. In general, the details of these methods were identical with those used in the previous report (Kraemer, 1971).

Results

Murine Lymphoma Line L5178Y. Results of the entire protocol procedure for isolation and identification of heparan sulfate of L5178Y cells are illustrated in Figures 2–5. Figure 2 illustrates the initial isolation of acid-soluble, macromolecular, doubly labeled material as defined by exclusion from Bio-Gel P-2. Both the direct acid-soluble cellular fraction (Figure 2A) and the acid-soluble material liberated from the trichloroacetic acid precipitate by papain digestion (Figure 2B) included such material and, in the main, these Bio-Gel P-2 chromatography profiles had the same characteristics as those previously reported for Chinese hamster cell line CHO. That is, prominent amounts of ^{3}H-labeled UDP-*N*-acetylhexosamine and inorganic $^{35}SO_4$ were retained on the column run of the direct acid-soluble cell fraction, while material from the trichloroacetic acid precipitate was almost entirely macromolecular.

As illustrated in Figure 3, highly charged, doubly labeled material was isolated from both cell fractions on DEAE-cellulose. These column runs showed several differences from comparable runs with CHO cells. One prominent difference was the abundant amount of directly acid-soluble, ^{3}H-labeled macromolecular material with lower charge found in L5178Y cells (Figure 3A). In fact, of all cell lines studied, L5178Y cells synthesized the most prominent amounts of directly acid-soluble macromolecular complex carbohydrates eluting between 0.01 and 0.5 M salt on DEAE-cellulose. It is speculated that these materials represent partially glycosylated cell sap immunoglobulins (such as those reported by Swenson and Kern, 1967, 1968) and thus reflect the differentiated nature of L5178Y lymphoma cells.

The DEAE-cellulose runs for L5178Y cells also differed from CHO cell material in that the former runs completely

TABLE I: Incorporation of Glucosamine-6-*t* and $Na_2{}^{35}SO_4$ into Heparan Sulfate and into Total Macromolecular Material of Various Established Cell Lines.[a]

		L5178Y	BHK-C13	L-929	CHO
Total macromolecular incorporation, cpm	^3H	170,165	56,172	46,805	68,315
	^{35}S	8,737	13,514	6,992	13,011
Trichloroacetic acid soluble macromolecular incorporation, cpm	^3H	31,143	1,106	1,395	9,539
	^{35}S	1,082	1,436	947	7,921
Trichloroacetic acid soluble macromolecular incorporation, % of total	^3H	18	1.8	3	14
	^{35}S	12	11	14	61
Heparan sulfate, trichloroacetic acid soluble, cpm	^3H	2,240	932	1,278	7,098
	^{35}S	1,092	1,050	1,438	7,262
Heparan sulfate, trichloroacetic acid precipitate, cpm	^3H	6,882	3,730	2,841	5,004
	^{35}S	3,516	3,850	2,947	3,000
Heparan sulfate, total, cpm	^3H	9,086	4,662	4,119	12,102
	^{35}S	4,608	4,900	4,385	10,626
Heparan sulfate, trichloroacetic soluble, % of total	^3H	24	24	31	59
Heparan sulfate – total, % of total macromolecular incorporation	^3H	5.3	8.3	8.8	18
	^{35}S	52	36	63	82
Heparan sulfate, $^{35}S/^3H$, trichloroacetic acid soluble		0.49	1.13	1.13	1.07
trichloroacetic acid precipitate		0.49	1.13	1.13	.60
total		0.51	1.05	1.06	0.88

[a] Data are normalized to 10^8 cells that had doubled in cell number during growth in labeling medium.

lacked material provisionally identified as hyaluronic acid and chondroitin sulfate. [Thus, as shown in Figure 3B, only a single, highly charged, labeled peak eluted from DEAE-cellulose above 0.6 M salt, while comparable runs for CHO and BHK-C13 cells (Figure 6) contained the A and C peaks that were degradable with testicular hyaluronidase. None of the L5178Y cell macromolecular radioactivity was degradable with testicular hyaluronidase.] As reported for CHO cells, some of the incorporated $^{35}SO_4$ coeluted with the latter half of the glycopeptide material, but a major part of total incorporated $^{35}SO_4$ eluted in the regions characteristic for heparan sulfate. Also, as reported for CHO cells, the putative heparan sulfate of the direct acid-soluble material eluted earlier than the heparan sulfate released from the acid precipitate.

Figure 4 illustrates that the behavior of the putative heparan sulfates of L5178Y cells isolated from DEAE-cellulose was similar on Bio-Gel P-10 to the behavior previously reported for CHO cell heparan sulfate and authentic heparan sulfate from bovine lung (Kraemer, 1971). Figure 5 illustrates the direct nitrous acid cleavage products of this material run on Bio-Gel P-10 and establishes the identification of these heparan sulfates. The Bio-Gel P-10 profiles of the cleavage products of direct nitrous acid treated heparan sulfates of L5178Y cells were identical with those previously reported for CHO cells (Kraemer, 1971).

Chinese Hamster Line CHO. Results for CHO cells, using the protocol illustrated in Figure 1, are tabulated in Table I. In addition to these data, the heparan sulfates of this cell line have been previously studied by a variety of other procedures (Kraemer, 1968, 1969). Taken together, the identification of these heparan sulfates is clearly established and can be summarized as follows. The heparan sulfates of CHO cells contained incorporated radioactivity from supplied glucosamine-*6-t* and inorganic sulfate-^{35}S and eluted from DEAE-cellulose columns in a broad band between hyaluronic acid at about 0.5 M acetate and chondroitin sulfates at above 1.2 M acetate. They were distinguishable from the latter species by their partial retention on Bio-Gel P-10 columns and by their resistance to degradation by testicular hyaluronidase. CHO cell heparan sulfates contained equimolar amounts of hexosamine and uronic acid (orcinol method) but yielded a high carbazole:orcinol ratio. A large proportion of the sulfate could be removed by extremely mild acid hydrolysis, and this resulted in the formation of free amino groups and reduced the net charge of the molecule. Enhanced degradation of the molecule with heparin-grown *Flavobacterium heparinum* (compared to unadapted *F. heparinum*) was demonstrated; this reaction resulted in the formation of small mobile fragments similar to those formed from bovine lung heparan sulfate. The CHO cell heparan sulfates also behaved as did bovine lung heparan sulfate upon cellulose acetate electrophoresis and Bio-Gel P-10 chromatography before and after direct nitrous acid degradation.

Other Cultured Cells. The same procedures as used for L5178Y and CHO cells were also used for isolating and identifying heparan sulfates of four other established cell lines: (1) Don C, a Chinese hamster "fibroblastic" cell line; (2) BHK-C13, a Syrian hamster line derived from baby hamster kidneys but having a "fibroblastic" morphology; (3) L-929, murine fibroblasts; and (4) HeLa cells, originally derived from a human cervical carcinoma and possessing "epithelioid" behavior when grown on glass. In addition, diploid mouse embryo lung fibroblasts grown on glass were studied. In each case, directly acid-soluble material and material rendered acid-soluble by papain digestion of the acid precipitate were studied separately. These fractions were, in each case, derived from cells grown exponentially in suspension for approximately one doubling time in medium containing 0.1 mCi/l. of glucosamine-*6-t* and 1.0 mCi/l. of $Na_2{}^{35}SO_4$.

A number of features of these runs were basically the same in all cells tested. For instance, all of the initial Bio-Gel P-2 separations showed prominent retained peaks of ^3H-labeled UDP-*N*-acetylhexosamine and inorganic $^{35}SO_4$ for the direct

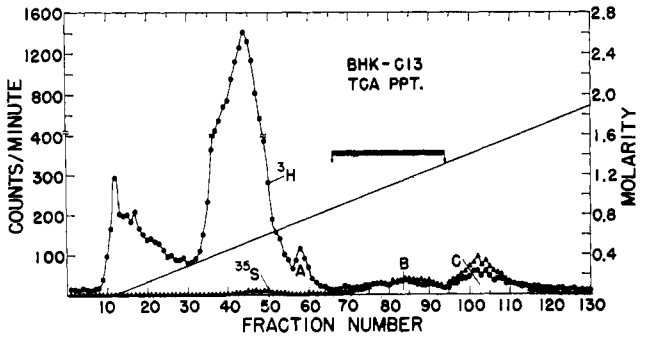

FIGURE 6: DEAE-cellulose chromatography profile of macromolecular material of acid-precipitable material of doubly labeled BHK-C13 cells rendered acid-soluble by papain digestion. This profile is procedurally analogous to Figure 3B.

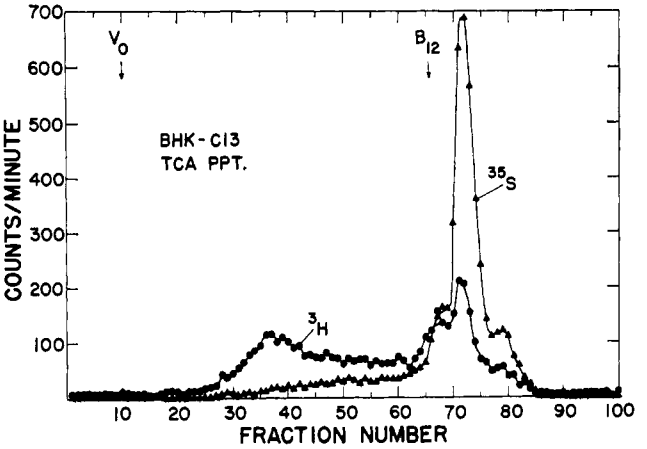

FIGURE 7: Bio-Gel P-10 chromatography profile of the nitrous acid generated fragments of one-half of the material harvested as indicated in Figure 6.

acid-soluble fraction and very little retained radioactivity of the material derived from the trichloroacetic acid precipitate. All of the Bio-Gel P-2 runs from both cellular fractions contained excluded radioactivity of both ^{3}H and ^{35}S types. All of the DEAE-cellulose runs from both cellular fractions of each cell line studied contained doubly labeled material that eluted in a fashion characteristic of heparan sulfate. In every case studied, the putative heparan sulfate material from the trichloracetic acid precipitates eluted later than the heparan sulfate from the direct acid-soluble fraction.

Results for the Don C line were indistinguishable from those of the CHO cell material previously reported (Kraemer, 1971). Moreover, preliminary studies (unpublished) of the major Don C glycopeptide classes were also similar to those previously reported for CHO cells (Kraemer, 1969). Thus, both of the Chinese hamster fibroblast lines appear to share a large number of species-specific characteristics.

Figure 6 illustrates the DEAE-cellulose elution profile for the macromolecular material derived from the acid precipitate of BHK-C13 Syrian hamster cells. As in the case of comparable material from CHO cells, these fibroblastic cells also synthesized other highly charged complex carbohydrates (peaks A and C), provisionally identified as hyaluronic acid and chondroitin sulfate on the basis of molecular size, charge, susceptibility to testicular hyaluronidase, and failure to degrade by direct nitrous acid treatment. The behavior of the heparan sulfates from BHK-C13 (*e.g.*, peak B, Figure 6) on Bio-Gel P-10 was similar to CHO and L5178Y cell material, and when this material was treated with nitrous acid, the cleavage products on Bio-Gel P-10 chromatographed in the usual way (Figure 7). The major difference found between CHO and BHK-C13 cells was that the direct acid-soluble fraction of the latter cells contained only a small amount of heparan sulfate, rather than about two-thirds of the total as found for CHO cells.

Chromatography runs comparable to those illustrated in Figures 5 and 6 are given for murine L-929 cell material in Figures 8 and 9. These murine fibroblasts apparently cannot synthesize hyaluronic acid and chondroitin sulfate under conditions where both CHO and BHK-C13 fibroblasts do so. Thus, the bulk of the $^{35}SO_4$ label of the acid precipitate derived material appears as heparan sulfate and the other $^{35}SO_4$-containing material elutes toward the end of the glycopeptide peaks. The identity of this latter material, which has been detected in all cell lines studied so far, is not known.

The Bio-Gel P-10 runs (Figure 9) for heparan sulfates of L-929 cells are significantly different from others studied. The untreated material (Figure 9A) behaves as if it were somewhat larger in molecular size and also as if two major size classes were present. When treated with nitrous acid (Figure 9B), the smaller cleavage products are similar to those previously illustrated; however, two sizes of larger fragments, with distinctly different $^{35}S/^3H$ ratios, are also present. The interpretation of these differences will require further experiments. Nevertheless, the L-929 cell material satisfies the basic operational definition of heparan sulfate in that doubly labeled macromolecular material degradable by direct nitrous acid treatment is present.

Experiments with HeLa cells have shown that, like the Chinese hamster lines, over half of the radioactivity of the total cellular heparan sulfate is isolable as directly trichloroacetic acid soluble material. These cells consistently appear to incorporate somewhat less radioactivity into heparan sulfate than the other cell lines studied. Another difference is that the size profiles of the nitrous acid generated fragments show almost all smaller fragments rather than significant radioactivity eluting before the vitamin B_{12} marker during Bio-Gel P-10 chromatography. Results for MEL diploid cells were similar to BHK-C13 cells in that all three types of glycosaminoglycans were synthesized.

The general comparative results for most of the cell lines studied are presented in Table I, all of which have been, in a sense, normalized to similar cell numbers which have doubled in the presence of the same amount of exogenous radioactive precursors. It is apparent from these data that there was a surprising uniformity in incorporation of both glucosamine-6-*t* and $Na_2{}^{35}SO_4$ into acid-precipitable heparan sulfate of the various cell lines. Although no mass data have yet been achieved, one is tempted to suggest that all of these cell lines may synthesize similar amounts of acid-precipitable heparan sulfate despite their widely different synthetic capabilities in regard to other complex carbohydrate species. Directly acid-soluble heparan sulfate appears to be much more variable between cell lines, and only CHO, Don C, and HeLa cell lines possessed majority fractions in this cellular fraction. The degree of sulfation also appears to be quite variable between cell lines and, again only in the case of the Chinese hamster cell lines, there were clear differences in the $^{35}S/^3H$ ratio between acid-soluble and acid-precipitable cell fractions found. In every case studied, of the total incorporated ^{35}S ("total incorporation" refers to the sum of the macromolecular radio-

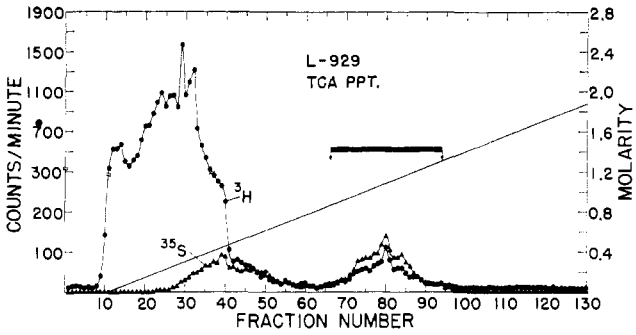

FIGURE 8: DEAE-cellulose chromatography profile for L-929 cells procedurally analogous to Figures 3B and 6.

activity harvested as excluded material from the primary Bio-Gel P-2 column runs), the fraction isolated as heparan sulfate was a large one, ranging from 37% for BHK-C13 cells to 82% for CHO cells. The remainder of incorporated ^{35}S appeared as the unidentified material eluting with glycopeptide species as well as, in some cases, material tentatively identified as chondroitin sulfate. The fraction of total incorporated ^{3}H appearing as heparan sulfate was much smaller, ranging from about 5% for the murine lymphoma cell line L5178Y to 18% for CHO cells.

Discussion

The foregoing results show that synthesis of heparan sulfate is a common, if not universal, capability of cultured mammalian cells. Cells tested in this study included a wide range of cell types whether classified by species of origin, by morphology, by retention of differentiated functions, or by ability to grow in suspension culture. For instance, BHK-C13 syrian hamster fibroblasts and CHO and Don C Chinese hamster fibroblasts, all three of relatively recent origin, retained the ability to synthesize hyaluronic acid and chondroitin sulfate, as did the diploid early-passage MEL cells. On the other hand, mouse L-929 fibroblasts, of much older origin, have apparently lost these differentiated functions. The murine L5178Y cell line retains the morphological and biochemical attributes of lymphoblasts, while human HeLa cells are morphologically considered to be epithelioid cells; neither of the latter synthesized detectable amounts of hyaluronic acid or chondroitin sulfate, while the L5178Y cells probably produced large amounts of immunoglobulins. Despite these differences, all seven cell types, under similar growth conditions, incorporated similar amounts of radioactivity from glucosamine-6-*t* and $Na_2{}^{35}SO_4$ into acid-precipitable heparan sulfate.

Heparan sulfate appears to be widely distributed in animal tissues (Brimacombe and Webber, 1964); many workers have apparently assumed that this represented the distribution in tissue of particular differentiated cell types in analogy to the correlations found between mast cell content and heparin content of various tissues (Brimacombe and Webber, 1964). Studies of cultured cells have the advantage of providing experimental material from cells of relatively homogeneous cell type and, thus, the studies reported here raise the possibility that many or all animal cells synthesize heparan sulfate *in vivo*.

In this connection, it is of interest to compare the results reported here with the studies of collagen synthesis of Green and Goldberg (1965) and Green *et al.* (1966). These workers reported that the rate of synthesis of this functionally differentiated protein varied over a range of 10^4 between various types of cultured cells. In their experience, established cell lines of fibroblastic origin synthesized collagen at rates 10–100 times greater than that of established lines of epithelial origin such as HeLa cells, while lymphocytes and lymphoma cells failed altogether to synthesize detectable amounts. They speculated that, in the latter class of cell types, the complete repression of collagen synthesis was in some way related to differentiation of the cells for other functions. Thus, comparison of the collagen-synthesis work with the results here encourages the belief that heparan sulfate synthesis is indifferent to the general phenomenon of cytodifferentiation.

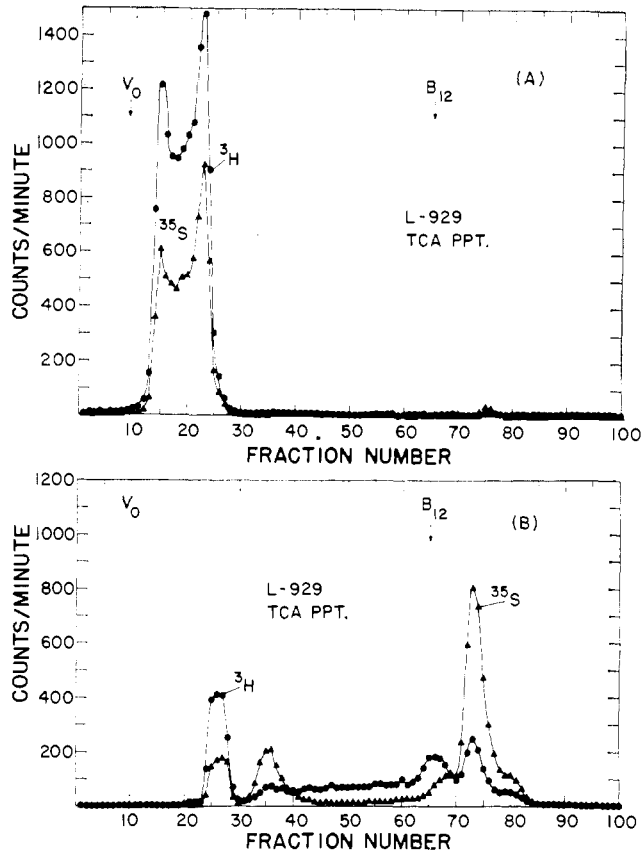

FIGURE 9: Bio-Gel P-10 chromatography profiles of untreated and nitrous acid treated heparan sulfate of L-929 cells. Material harvested as indicated in Figure 8 was divided into two equal portions, one run untreated (A) and one-half run after direct nitrous acid treatment (B). All of each 2.0-ml fraction was counted for each run.

If the capability of heparan sulfate synthesis is indifferent to cytodifferentiative processes yet regularly persists in established cell lines (even in cases like L-929 cells that have lost some of their differentiated functions), then one must consider the possibility that this capability is vital for some general life process. For instance, the hypothesis that "heparin-like" materials are involved in the process of cell division was advanced earlier by Heilbrunn (1956), and more recently Kinoshita (1969) has reported that changes in the intracellular compartmentation of a heparin-like polysaccharide coincides with the sea urchin egg-cleavage process. However, at the moment very little biochemically credible data exist concerning the cellular metabolism and physiology of heparan sulfate; hence, the function of this species and its physiological relation to heparin must be considered an open question.

Acknowledgments

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Added in Proof

Since preparation of this paper, Dietrich and Montes de Oca (1970), using somewhat different methods, have reported the biosynthesis of heparan sulfate by HeLa, L. mouse embryo, and rat embryo cells in culture.

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Identification of an Ommochrome in the Eyes and Nervous Systems of Saturniid Moths*

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ABSTRACT: A substituted phenothiazine pigment has been isolated and characterized from the optic lobes, larval ocelli, and nerve cords of saturniid moths. By comparative spectrophotometric, colorimetric, chromatographic, and chemical degradative techniques we have demonstrated it to be related to ommin—an ancillary pigment found in the compound eyes of insects. The optic lobe, ocellar, and ganglionic ommochrome display absorption maxima in the blue region of the spectrum when oxidized by molecular oxygen or hydrogen peroxide. A shift toward green wavelengths accompanies reduction by ascorbic acid, sodium dithionite, and similar reductants in a variety of solvents. Estimation of the oxidation potential with indicator dyes has placed the E_0' of the pigment at pH 7 between 0.123 and 0.217 mV.

The ommochromes are a group of natural organic pigments widely distributed in the animal kingdom (Fox and Vevers, 1960). They are biochemically defined as products of tryptophan metabolism and arise from the oxidative coupling of 3-hydroxykynurenine molecules. There are three classes of ommochrome pigments, all of which show a characteristic change of color upon oxidation or reduction (Butenandt and Schäfer, 1962; Linzen, 1966).

In arthropods ommochromes have been found mainly in the eyes and ocelli (Becker, 1942; Ziegler, 1961; Butenandt and Schäfer, 1962). In this paper we report the extraction and purification of ommochromes from the compound eyes of two saturniid moths. These pigments are then used as reference standards for the identification of the embryonic ocellar, pupal optic lobe, and ganglionic pigments of the same species.

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Materials and Methods

1. Experimental Animals and Preparation of Tissue Samples. *Hyalophora cecropia* and *Antheraea pernyi* were purchased from dealers or reared indoors on an artificial diet as described by Riddiford (1968) and chilled at 5°. Optic lobes and ganglia excised from diapausing pupae and heads from adults of both species were minced in distilled water, lyophilized, and stored at −20°. Embryos during the last third of embryonic development were removed from the eggs and decapitated. The heads were lyophilized from distilled water and stored at −20° until several grams of material had accumulated.

2. Extraction and Purification of Pigments. Each of the pigment sources was homogenized in 80% acetone (v/v), the homogenate was centrifuged at 10,000*g*, and the supernatant was discarded. The pellet was resuspended and washed twice again using the same procedure, and was then preextracted with 50% methanol (v/v) for 12 hr at 40°. The mixture was centrifuged as above, the supernatant was discarded, and the pellet was washed twice with methanol. Ommochromes were extracted from this pellet into 0.5% concentrated HCl–meth-