Table 1. W chromosome arm ratios (long arm length to short arm length) and sex chromosome ratios (W/Z) expressed as means with standard deviations for ZZZW triploidy and ZW diploidy.

Case	W chromosome arm ratio	Sex chromosome ratio (W/Z)	Reference
Triploid (ZZZW)	1.19 ± 0.09	0.46 ± 0.06	Present study
Diploid (ZW)	1.08 ± 0.13	0.42 ± 0.06	*6*
Diploid (ZW)	1.10		*4*
Diploid (ZW)		0.48	*5*

-4, diploid nucleolar distributions were also found. Thus, chromosomes 1, 2, 3, 4, and 5(Z) can be eliminated as the sole nucleolar-organizing chromosomes. This lends additional strength to the argument that a group of microchromosomes organizes the nucleolus (*8*).

Mechanisms for the origin of the 3A-ZZZW triploid were considered. Nondisjunction of the sex chromosomes in meiosis of the female could produce an A-ZW egg nucleus. If fertilized by a diploid (2A-ZZ) spermatozoon, or two haploid (A-Z) spermatozoa, a 3A-ZZZW zygote would result. The ZZZW sex chromosomal complement reported here is analogous to the XXXY modified Klinefelter's syndrome in man (*9*).

Note added in proof: After this paper was submitted, a case of double trisomy (trisomy-2,5Z) was found. The occurrence of trisomy-5Z lends further support to the hypothesis of meiotic nondisjunction of sex chromosomes. Further, simultaneous nondisjunction (in the male or female) of an autosome and a sex chromosome is suggested.

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References and Notes

1. S. E. Bloom and E. G. Buss, *Science* **153**, 759 (1966).
2. S. E. Bloom, *Chromosoma* **28**, 357 (1969).
3. W. F. Lamoreux, F. B. Hutt, G. O. Hall, *Poultry Sci.* **22**, 161 (1943).
4. A. Krishan and R. N. Shoffner, *Cytogenetics* **5**, 53 (1966).
5. N. Takagi and S. Makino, *Caryologia* **19**, 443 (1966).
6. S. E. Bloom, unpublished data.
7. N. S. Fechheimer, D. L. Zartman, R. G. Jaap, *J. Reprod. Fert.* **17**, 215 (1968).
8. S. Ohno, L. C. Christian, C. Stenius, *Exp. Cell Res.* **27**, 612 (1962).
9. J. J. Yunis, *Human Chromosome Methodology* (Academic Press, New York, 1965).
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Rapid Axonal Transport of Sulfated Mucopolysaccharide Proteins

Abstract. *When sulfur-35–labeled sodium sulfate is injected intraocularly in the goldfish, labeled sulfated mucopolysaccharides rapidly appear in the contralateral optic tectum of the brain, demonstrating the axonal flow of sulfated mucopolysaccharides. The transport rate is the same as that observed for proteins labeled after intraocular injection of tritiated proline. Treatment of the sulfur-35–labeled material with precipitants and enzymes reveals the presence of substances with properties similar to those of heparan sulfate (the major component) and chondroitin sulfate. Dermatan sulfate was not detected.*

Recent evidence has shown that chondroitin-4-sulfate and heparan sulfate are produced by rat glial cells in tissue culture (*1*). While histochemical (*2*) and autoradiographic (*3*) studies have suggested the presence of sulfated mucopolysaccharides (SM) in neurons as well and roles for SM have been postulated in nerve function (*2*), little is known about their origin and distribution.

In view of the possible functional significance of SM in neurons, it was of interest to determine whether they are among the rapidly transported axonal proteins. The optic tract of the goldfish provides a suitable system for approaching this question (*4, 5*). The eye is a convenient site for reproducible introduction of labeled precursors; the ganglion cells of the retina terminate in the contralateral optic tectum, which is easily removed; and the ipsilateral tectum can be used as an internal control for systemic labeling of the brain arising from precursor that escaped from the eye.

Twenty goldfish (*Carassius auratus*, 6 to 7 cm in length and maintained at 20°C) were injected in the right eye with 40 μc (5 μl) of carrier-free $Na_2{}^{35}SO_4$ and killed 12 hours later. Left and right optic tectal hemispheres were collected separately with an equal amount of unlabeled tectum and fractionated (Table 1). There was no significant difference between the left and right optic tectal hemispheres in radioactivity present in either chloroform–methanol or methanol–water fractions, an indication that there had been no transport of free or lipid-bound labeled sulfate. The large difference in the protease digests from left and right tectal hemispheres was suggestive of rapid transport of labeled SM proteins. This view was substantiated by the fact that about half of the radioactivity in the digest had the solubility characteristics of SM—that is, precipitability in cetylpyridinium bromide (CPB) and ethanol and solubility in 10 percent trichloroacetic acid (TCA). In other experiments, a comparable distribution of radioactivity was seen. The yield of hexosamine in the isolated SM from tectum and whole brain was 50 μg/g (wet weight). This is similar to the values found previously in mammalian brain (*6*). The ratio of hexosamine to uronic acid was 1.1, a value expected for SM.

Further evidence for the SM nature of the material precipitable in CPB was obtained by electrophoresis (Fig. 1). The fastest moving band migrated with standard chondroitin-6-sulfate and contained 29 percent of the recovered radioactivity. The middle band migrated somewhat slower than standard heparan sulfate and contained 68 percent of the radioactivity. The slowest band migrated with hyaluronic acid and accounted for less than 5 percent of the radioactivity. The distribution of radioactivity in the two sulfate bands corresponded approximately with the intensities of staining, suggesting that the specific activities are similar.

The bands were further characterized by treatment with hyaluronidase, which is known to degrade hyaluronic acid and chondroitin sulfate but not heparan sulfate. Fraction 8 (25 μl) was hydrolyzed with 5 μl of a solution (1 mg/ml) of bovine testicular hyaluronidase (Worthington, 3600 unit/mg) in a mixture of 1*M* sodium acetate (*p*H 5.2) and 1.5*M* NaCl at 37°C for 4 hours. Only the center band stained after electrophoresis; it retained more than 95 percent of its original radioactivity. Paper electrophoresis of the hyaluronidase digest at high voltage (80 volt/cm in pyridine acetate, *p*H 4.3, for 20 minutes) yielded three radioactive regions. One remained at

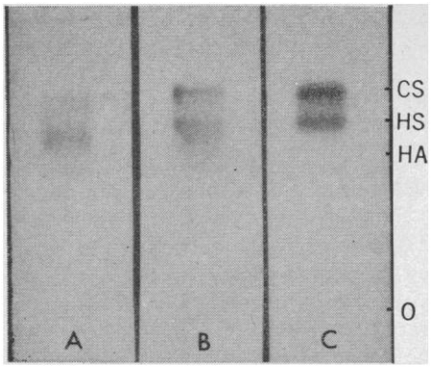

Fig. 1. Cellulose acetate electrophoresis of sulfated mucopolysaccharides. Fraction 8 (Table 1), containing 3.5 μg of uronic acid, was run on 16-cm strips of Sepraphore III cellulose acetate (Gelman Instruments) in cupric acetate buffer (containing 2 g of cupric acetate, 10 ml of acetic acid, and 90 ml of water) for 45 minutes at 16 volt/cm. The wet strips were immersed for 10 minutes in 1 percent Alcian blue in 5 percent acetic acid, then destained with 5 percent acetic acid. (A) Tectal SM; (B) mixture of tectal SM and standards; (C) standard hyaluronic acid (*HA*), heparan sulfate (*HS*), and chondroitin-6-sulfate (*CS*). This system did not separate authentic mammalian chondroitin-4-sulfate from shark chondroitin-6-sulfate. *O*, origin.

the origin, one migrated with the hyaluronidase digestion product of standard chondroitin sulfate, and the third migrated with standard heparan sulfate. When fraction 8 was heated with 0.05*N* HCl at 100°C for 1 hour (conditions which hydrolyze *N*-sulfates but not *O*-sulfates) (*7*), 42 percent of the sulfate became dialyzable. This value is consistent with the conclusion that the major band was heparan sulfate. On the basis of the above results, it would appear that heparan sulfate and chondroitin sulfate are the major and minor components, respectively, of the labeled tectal SM. Dermatan sulfate, which might have been expected on the basis of work with mammalian brain (*8*), was not visible on staining after digestion. The nature of the fraction not precipitable in CPB awaits further study. It is 75 percent nondialyzable and has a mobility on high voltage electrophoresis which is less than that of marker SM, including keratan sulfate.

These results suggest that SM are labeled in the eye and are axonally transported. It is possible that radioactive precursor, such as sulfate, was rapidly transported down the axon, forming labeled SM in the tectal hemispheres, and that this precursor had left the tectum prior to the time when the fish were killed (12 hours). To test this possibility and to compare the kinetics of transport of the labeled SM and protein, fish were injected in the right eye with a mixture containing 10 μc of $Na_2{}^{35}SO_4$ and 1.2 μc of ^{3}H-proline (0.14 μmole); they were killed at intervals in groups of six and treated as described in Table 1. Proline was used because it is a particularly effective label for axonally transported proteins (*5*).

Large differences in the amount of ^{35}S in the left and right tectal fractions are absent in the combined washes at any time, precluding the possibility that SM was labeled locally by axonal transport of a precursor molecule (Fig. 2A). The time course of appearance of radioactivity in the protease digest was remarkably similar for the two isotopes (Fig. 2B) with large differences between left and right fractions developing between 3 and 6 hours. Similar results were obtained in an additional double-label experiment. In the case of proline, we calculated the rate of transport to be 70 to 100 mm/day at 20°C (*5*). The percentage of the ^{35}S radioactivity which was precipitable with CPB ranged from 40 to 50 percent at the various times. These results suggest that SM are components of rapidly transported proteins. While extra-axonal migration of labeled protein has generally been excluded (*9*), the possibility remains that ^{35}S-labeled material is transported extracellularly.

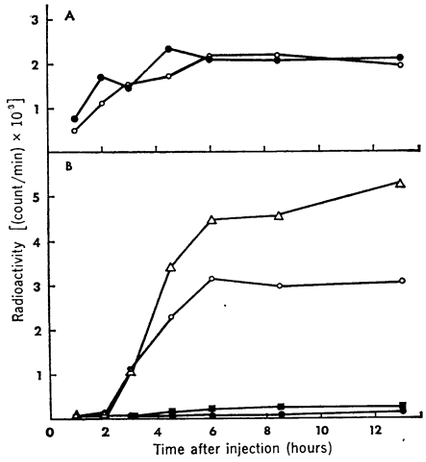

Fig. 2. Appearance of radioactivity in left and right tectal fractions following injection of ^{3}H-proline and $Na_2{}^{35}SO_4$ into the right eye. (A) Combined chloroform-methanol and methanol-water washes (fractions 1 plus 2). Open circles, ^{35}S in left tectum; and closed circles, ^{35}S in right tectum. (B) Supernatant after protease digestion (fraction 3). Open triangles, ^{3}H in the left tectum; closed squares, ^{3}H in the right tectum; open circles, ^{35}S in the left tectum; and closed circles, ^{35}S in the right tectum.

The axonal migration of sulfated mucopolysaccharide proteins has not to our knowledge been previously observed. This migration may furnish material for synaptic maintenance or growth. A possible special function of these highly charged anionic molecules may be to serve as carriers of specific cations to the synapse.

Table 1. Radioactivity in optic tectum 12 hours after intraocular injection of $Na_2{}^{35}SO_4$. Combined tectal hemispheres (300 mg wet weight) were homogenized in 20 volumes of a mixture of chloroform and methanol (1 : 1), centrifuged, and the pellet washed with an additional 20 volumes of solvent. The pellet was washed three times with 20 volumes of a mixture of methanol and water (90 : 10) 1 m*M* in sodium sulfate. The residue was treated overnight at 60°C with 1.2 mg of a protease from *Streptomyces griseus* (type IV, Sigma Chemical) in 4 ml of 0.14*M* tris(hydroxymethyl)aminomethane (*p*H 8) and centrifuged to remove a small amount of unsolubilized material. The supernatant was stirred 1 hour with 1 ml of 2 percent cetylpyridinium bromide (CPB). The resultant precipitate was washed in 1 ml of 0.05 percent CPB, dissolved in 1 ml of 60 percent *n*-propanol, and precipitated overnight with 4 ml of ethanol containing 5 percent potassium acetate. The precipitate was washed with 1 ml of the ethanol-acetate solution and dissolved in 0.8 ml of water to which 0.2 ml of 50 percent TCA was added. After 30 minutes at 0°C, the resulting precipitate (nucleic acids) was recovered; the SM were reprecipitated with 4 ml of ethanol-acetate and redissolved in 0.1 ml of water. Determination of radioactivity was achieved by liquid scintillation counting with the aid of an internal ^{35}S standard.

Fraction	Radioactivity (count/min)	
	Left tectum	Right tectum
1. Chloroform-methanol extracts	5,781	4,619
2. Methanol-water washes	23,470	24,270
3. Protease supernatant	86,000	5,320
4. Protease residue	773	49
5. CPB supernatant	33,900	2,800
6. CPB precipitate	30,100	2,010
7. TCA precipitate	314	5
8. Final ethanol precipitate	31,380	1,580

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References and Notes

1. A. Dorfman and Pei-Lee Ho, *Proc. Nat. Acad. Sci. U.S.* **66**, 495 (1970).
2. H. V. Castejón, *Acta Histochem.* **35**, 161 (1970).
3. J. D. Robinson and J. P. Green, *Yale J. Biol. Med.* **35**, 248 (1962).
4. B. S. McEwen and B. Grafstein, *J. Cell Biol.* **38**, 494 (1968).
5. J. S. Elam and B. W. Agranoff, *J. Neurochem.*, in press.
6. M. Singh, E. V. Chandrasekaran, R. Cherian, B. K. Bachhawat, *ibid.* **16**, 1157 (1969); K. Saigo and F. Egami, *ibid.* **17**, 633 (1970).
7. D. Lagunoff and G. Warren, *Arch. Biochem.* **99**, 396 (1962).
8. W. L. Cunningham and J. M. Goldberg, *Biochem. J.* **110**, 35P (1968).
9. S. Ochs and J. Johnson, *J. Neurochem.* **16**, 845 (1969).

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Plasticizers from Plastic Devices: Extraction, Metabolism, and Accumulation by Biological Systems

Abstract. *Phthalate ester plasticizers were found to be extracted by blood from plastic tubing and from plastic bags used for blood storage. One such plasticizer was metabolized by the isolated perfused rat liver while another was found to be accumulated in the liver unchanged. In addition, this latter plasticizer was identified in samples of human tissue taken from patients who had received transfusions of blood stored in plastic bags. The biological implications of these observations are considered.*

In experiments in which the technique of the isolated, perfused rat liver was used, three acidic ultraviolet-absorbing materials were observed to accumulate in the plasma of the perfusion medium. In Fig. 1 are shown the elution patterns from the chromatographic fractionation of acid-soluble extracts of perfusion plasma which had either perfused a liver or had circulated in the apparatus in the absence of a liver. The first peak (I) is common to both experimental conditions and on the basis of its ultraviolet spectrum and elution volume was identified as uric acid. Peak II, a compound formed only when a liver is present, has not yet been identified. Peak III, which also appears only when a functioning rat liver is present in the perfusion system, was selected for further identification.

The unknown material in peak III was extracted from acidified aqueous solutions with diethyl ether. Furthermore, treatment of the ether-extractable material with diazomethane, a methylating agent, led to a loss of the compound's water solubility. These results suggested the presence of an organic acid. The methylated derivative was subjected to nuclear magnetic resonance spectroscopy and mass spectrometry. Analysis of the spectrophotometric data led to the tentative conclusion that the unknown molecule was an ester of glycolic acid and phthalic acid, glycolyl phthalate (GP), as shown in Fig. 2.

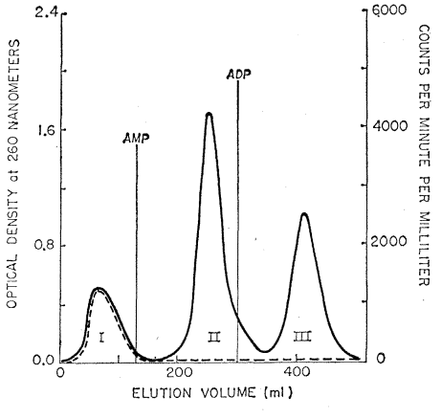

Fig. 1. Circulation of perfusion fluid in the presence (———) and the absence (- - - - -) of a liver. The perfusion fluid, a mixture of 70 ml of whole rat blood containing 70 units of heparin per milliliter and 35 ml of Krebs-Ringer-bicarbonate buffer containing 4 percent BSA and 80 mg of glucose per 100 ml, was circulated in the liver perfusion apparatus of Miller *et al.* (*17*). This system was used to perfuse an isolated rat liver for 4 hours or the perfusion fluid was allowed to circulate in the absence of a liver for the same length of time. At the end of the experiment, the total plasma was isolated by centrifugation and acidified with perchloric acid. After removal of the precipitate, the acid-soluble supernatant was neutralized with KOH and centrifuged, and the supernatant recovered. An amount of [^{14}C]adenosine diphosphate and [^{14}C]adenosine monophosphate was added to the neutralized extract to act as a marker during further chromatographic fractionation. The total extract was applied to a 0.7 by 10 cm column of Dowex-1 (formate form) anion exchange resin. Elution of the column was with a nonlinear gradient of ammonium formate (0 to 2*N*, *p*H 5.5), and the absorbance at 260 nm was monitored continuously in a Gilford spectrophotometer. Portions of each fraction were counted in a Packard TriCarb liquid scintillation counter. In order to simplify this figure, only the peak radioactive fractions are displayed.

As there is little information on the biological origin of phthalates and since it is known that esters of phthalic acid are used as plasticizers in the formulation of various plastics (*1*), the possibility was investigated that the GP arose as a metabolite of a plasticizer extracted from the polyvinyl chloride tubing used in the perfusion apparatus. The manufacturer of the tubing (*2*) identified the plasticizer as butyl glycolylbutyl phthalate (BGBP). Thin-layer chromatography of organic extracts of perfusion plasma, which had circulated in the apparatus in the absence of a liver, revealed appreciable quantities of BGBP.

In Fig. 3 is shown the time course for the accumulation of GP in the perfusate under three experimental conditions: (i) experiment A, in which a large quantity of BGBP was added exogenously to the perfusate at time zero; (ii) experiment B, in which polyvinyl chloride tubing was the only source of BGBP; and (iii) experiment C, in which ether-washed gum rubber tubing was used. It can be seen from experiment C that in the absence of exogenously added or endogenously available BGBP, no GP can be detected, while experiments B and A demonstrate that the polyvinyl chloride tubing or exogenously added BGBP leads to accumulation of GP. Thus it is concluded that BGBP is extracted from plastic tubing by blood, is metabolized by the liver, and its product GP is secreted into the perfusion medium.

A second phthalate ester plasticizer commonly encountered in plastics used in biological and medical practice is di(2-ethylhexyl)phthalate (DEHP) (*3*). Tubing formulated with this plasticizer (*4*) was also tested for extraction of the phthalate ester by the perfusion medium. Significant levels of DEHP (0.01 to 0.05 mg per milliliter of plasma) could be detected in perfusion fluid that had circulated through this type of tubing in the perfusion apparatus in the absence of a liver. As was the case with BGBP, no DEHP was seen in the per-