Incorporation of Sulphate by Chick-Embryo Corium

NATURE AND PRODUCTS OF THE PROCESS *IN VITRO*

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1. The incorporation of sulphate into the trichloroacetic acid-precipitable fraction of 9-day chick-embryo corium, incubated in Krebs-Ringer phosphate buffer, pH 7, is dependent on the sulphate concentration of the medium. Uptake of sulphate is linear with time for 3.5–4 hr. and is maximal at 37.5° in the presence of air or oxygen. D-Glucose stimulates the incorporation of sulphate but L-glutamine has no effect. 2. Incorporation of sulphate by the chick corium is enzymic and apparently involves the synthesis of active sulphate (adenosine 3'-phosphate 5'-sulphatophosphate) and the transfer of sulphate from adenosine 3'-phosphate 5'-sulphatophosphate to acceptor glycosaminoglycuronoglycan. This proposal on the nature of the process is suggested by the similarity between the energy of activation calculated for sulphate-incorporation in the chick-corium preparation and the energy requirement reported for sulphate-activation with purified yeast enzymes. 3. The 9-day chick-embryo corium is composed principally of fibroblasts; there are no histologically demonstrable mast cells. The young fibroblast is apparently responsible for the incorporation of sulphate into glycosaminoglycuronoglycans tentatively identified as chondroitin sulphate(s), heparan sulphate and heparin-like material.

Sulphated glycosaminoglycuronoglycans [the nomenclature of Jeanloz (1960) for the glycosaminoglycuronoglycans, the acidic polysaccharides that contain amino sugars, is used in this paper] of connective tissue are synthesized by fibroblasts and mast cells. Fibroblasts in tissue culture produce chondroitin sulphates (Grossfield, Meyer, Godman & Linker, 1957; Berenson, Lumpkin & Shipp, 1958), and the mouse mast-cell tumour actively synthesizes heparin *in vivo* and *in vitro* (Korn, 1959*a,b*). To study the synthesis of sulphated glycosaminoglycuronoglycans by fibroblasts alone the corium of the 9-day chick embryo was selected, since at this time the corium is composed principally of fibroblasts and there is no morphological evidence of mast cells. It was the purpose of this investigation to study the uptake of sulphate by fibroblasts *in vitro* and to determine the conditions for sulphate incorporation, the probable nature of the process and some characteristics of the newly sulphated glycosaminoglycuronoglycans.

The incorporation of [³⁵S]sulphate by connective tissue structures has been shown to reflect the synthesis of chondroitin sulphate-type glycosaminoglycuronoglycans (Boström & Aqvist, 1952; Boström & Mansson, 1952; D'Abramo & Lipmann,

1957; Dorfman & Schiller, 1958) and heparin (Korn, 1958). In several radioautographic and histochemical studies of tissues synthesizing sulphated glycosaminoglycuronoglycans, the presence of [³⁵S]sulphate was first observed in the fibroblasts or chondrocytes and subsequently in intercellular ground substance (Glücksman, Howard & Pelc, 1956; Mancini, Vilar, Stein & Fiorini, 1961; Dziewiatkowski, 1962; Thorp & Dorfman, 1963).

METHODS

Isolation of corium. Embryonated eggs from Kimber 137 White Leghorn chickens were incubated under conditions of controlled temperature and humidity for 9 days. To obtain the thin strips of corium, the intact embryo was removed from the egg, placed ventral side down and cut lightly on the dorsum with a razor blade across the nape of the neck, down the sides and above the tail. A narrow strip of dry filter paper was placed in contact with the dorsum and light pressure applied so that the paper adhered. The paper was teased away from the embryo and the corium strip removed into ice-cold 0.15 M-KCl. The epithelium conveniently adhered to the paper. The thin translucent strips were pooled in cold KCl and washed by brief low-speed centrifugation at 4°. Less than a minute was required to attain a speed of 1000 rev./min. and to stop the no. 269

horizontal head of the International model PR-2 refrigerated centrifuge. Small pieces of tissue and free epithelial cells in the supernatant were removed by aspiration, leaving a loose pellet of corium strips. For the morphological description of the corium preparation, samples of isolated corium strips and intact chick embryos were fixed in Carnoy solution, mounted in paraffin, sectioned and stained with haematoxylin-eosin and Azure A.

The isolation of the corium from one embryo required approximately 2 min., and thus preparation for an experiment involved a period of 1-2 hr. However, this interval between isolation and incubation did not alter the ability of the tissue to incorporate sulphate since no significant difference was observed in the extent of uptake of SO_4^{2-} by tissues stored in cold 0.15M-KCl for 1, 2, 2.5 or 3 hr. before incubation.

Incubation. Small beakers containing 10.5 μC of carrier-free $\text{Na}_2^{35}\text{SO}_4$ (approx. 7.5×10^6 counts/min.) in 3 ml. of medium were used for incubation at 37.5° in a Dubnoff metabolic shaker (90 cyc./min.) in an atmosphere of air. The medium employed was Krebs-Ringer phosphate buffer (Umbreit, Burris & Stauffer, 1951), pH 7, in which MgSO_4 was replaced by MgCl_2 . Carrier-free [^{35}S]sulphate in weak HCl was obtained from the Union Carbon and Carbide Co., Oak Ridge, Tenn., U.S.A. D-Glucose, L-glutamine and carrier Na_2SO_4 were dissolved in the medium in concentrations indicated in the text. The amounts of these compounds added did not alter the pH of the reaction medium. For the study of the effect of pH on incorporation of SO_4^{2-} , the relative proportions of the two salts of the 0.1M- NaH_2PO_4 - Na_2HPO_4 solution used in the preparation of the Krebs-Ringer phosphate buffer were varied so that the incubation medium ranged from pH 6.45 to 7.25.

The pooled sample of corium strips was distributed into the small beakers containing the medium. Generally, tissue from approximately two embryos (180 μg . of N) was incubated per beaker.

Analyses and calculations. At the conclusion of the incubation, the vessel contents were transferred to a small tube with an equal volume of cold 20% (w/v) trichloroacetic acid, which precipitated the protein and stopped the reaction. The tissues were exhaustively washed at 4° with 10% (w/v) trichloroacetic acid and finally with 0.15M-NaCl so that the last wash contained only 0.1% of the protein-bound radioactivity. A portion (0.5 ml.) of 6N-HCl was added to the tissue, and the tube was sealed and heated for 16 hr. at 110°.

For the determination of radioactivity, a portion of the acid hydrolysate was neutralized and diluted. Samples were dried on aluminium planchets, which were rimmed with an anti-wetting agent (Mold Release 225; Ram Chemicals Inc., Gardena, Calif., U.S.A.) to produce a confined area of NaCl and were counted (minimum of 1000 counts) at least twice in a gas-flow counter with a thin window Geiger-Müller tube (model D47; Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.). Corrections were made for background and radioactive decay. Since the samples were neither infinitely thick nor thin, the NaCl formed in the neutralization of the hydrolysate contributed to self-absorption of the radioactivity. To obviate the need for self-absorption corrections, conditions of hydrolysis, neutralization and sampling were standardized to give a uniform quantity and thickness of NaCl. In addition, planchets were prepared and counted in quadruplicate. The

standard error for the number of counts accumulated was 0.35-3.2%.

In preliminary experiments, only carrier-free [^{35}S]sulphate was present in the medium. Although this resulted in the incorporation of a greater amount of label than in the presence of added carrier, tissues were subsequently incubated in medium containing small amounts of Na_2SO_4 . From the specific activity of the $^{35}\text{SO}_4^{2-}$ medium (counts/min./ μg . of SO_4^{2-}) and the radioactivity of the hydrolysate of the tissue, the amount of sulphate incorporated could be calculated. For the experiments with 'carrier-free' [^{35}S]sulphate, the concentration of sulphate given by the manufacturer on the description and assay sheet of the labelled material was used for this calculation. Radioactivity was expressed as μg . or μmole of SO_4^{2-} /mg. of N.

To check for adsorption of radioactivity, 26 beakers of corium strips were maintained at -20° for 3 hr. in carrier-free $^{35}\text{SO}_4^{2-}$ medium and analysed in the same manner as the incubated tissues. Since only 1-2% of the total radioactivity incorporated in 54 parallel incubations was fixed to the tissue (0.005 ± 0.003 and $0.36 \pm 0.09 \mu\text{g}$. of SO_4^{2-} /mg. of N respectively), it was not deemed necessary to correct results for this 'zero value'.

For the estimation of nitrogen, the extinction of a diluted portion of hydrolysate was measured in cells of 1 cm. light-path length at 260 and 280 $\text{m}\mu$. To relate these readings to nitrogen, six samples of corium strips, each from 20-40 9-day chick embryos, were isolated and hydrolysed as described. Nitrogen content of each hydrolysate, determined by nesslerization after digestion with H_2SO_4 and H_2O_2 , was correlated with its extinction at 280 and 260 $\text{m}\mu$. The equation describing this relationship for 1 ml. of solution analysed spectrophotometrically was: μg . of N/ml. = $61.34 \times E_{280} \times (E_{280}/E_{260})$.

Characterization of $^{35}\text{SO}_4^{2-}$ -labelled glycosaminoglycuroglycans. To characterize the $^{35}\text{SO}_4^{2-}$ -labelled product of the chick corium, sulphated glycosaminoglycuroglycans were isolated from each of five 3-hr. incubations of tissues from approximately 100 embryos and fractionated by the method of Schiller, Slover & Dorfman (1961). The tissues were defatted with acetone and digested with papain and trypsin. After treatment with trichloroacetic acid to remove remaining protein, the glycosaminoglycuroglycans were precipitated with cetylpyridinium chloride in the presence of 8 mg. each of heparin (General Biochemicals, Chagrin Falls, Ohio, U.S.A.), hyaluronic acid and chondroitin sulphate (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.). The precipitate was successively extracted with 0.4M-NaCl-0.1% (w/v) cetylpyridinium chloride (extract I), 1.2M-NaCl-0.1% (w/v) cetylpyridinium chloride (extract II) and 2.1M-NaCl (extract III). After the removal of cetylpyridinium chloride (Korn, 1959b) and dialysis, the non-diffusible material of the 1.2M and 2.1M extracts was adsorbed on 0.9 cm. $\times$ 44 cm. columns of Dowex 1(X2; Cl- form) and eluted stepwise with increasing concentrations of NaCl starting from 1.2M for extract II and from 1.4M for extract III; 100 ml. of each concentration of NaCl was used for elution. Each 10 ml. fraction of eluent was assayed directly for uronic acid (Bitter & Muir, 1962). A portion of each fraction was dialysed free of NaCl and dried (infinite thinness) for radioactivity determination.

The peaks of radioactive material from each of three chromatographic analyses were freeze-dried. Glucosamine and galactosamine analyses (Neuhaus & Letzring, 1957;

Pogell & Koenig, 1958) were made on a portion of the freeze-dried material which had been heated in 3N-HCl for 8 hr. at 110° in a sealed tube.

To check for the presence of keratan sulphate, anthrone-positive material was determined on a water-solution of the freeze-dried material and on the carriers (Dreywood, 1946; Morris, 1948).

RESULTS

Morphology of the isolated corium. Histological examination of the corium preparation of the 9-day chick embryo indicated that most of the cells were fibroblasts, the epithelium, approximately two cells in thickness, having been removed during the isolation of the corium (Fig. 1). No mast cells were seen. Only a few epithelial cells from capillaries and a few feather bud cells contaminated an otherwise pure sample of fibroblasts.

Conditions for maximum incorporation. Incorporation of SO_4^{2-} increased linearly at a rate of $0.12 \text{ m}\mu\text{g. of SO}_4^{2-}/\text{mg. of N/hr.}$ (Fig. 2). Beyond 3.5–4 hr. no further incorporation occurred. The extent of uptake of SO_4^{2-} after 3.5 hr. was $0.50 \pm 0.09 \text{ m}\mu\text{g. of SO}_4^{2-}/\text{mg. of N}$ for 18 incubations.

Fig. 1. (a) Section of dermis from intact 9-day chick embryo. E, epithelium; C, corium; Cap, capillary. (b) Chick-embryo preparation used in sulphate-incorporating system *in vitro*. Haematoxylin-eosin ($\times 300$).

With an increase of the sulphate concentration of the medium (5.1 mM-glucose; 2.9 mM-glutamine) from that of the carrier-free ^{35}S sulphate to $5 \mu\text{M}$ (approximately 100-fold), the 3-hr. incorporation value rose from 0.38 ± 0.04 to $38 \pm 9 \text{ m}\mu\text{g. of SO}_4^{2-}/\text{mg. of N}$. Maximum incorporation of SO_4^{2-} occurred in medium which was approximately $50 \mu\text{M-SO}_4^{2-}$ (Fig. 3).

Carrier sulphate did not affect the time-course of the incorporation of SO_4^{2-} described in Fig. 2; a maximum value at the higher amount of incorporation was attained after 3.5 hr. incubation. Incorporation was approximately doubled between 1.5 and 3 hr. at any of the concentrations of sulphate studied, e.g. 0.21 to 0.39 (carrier-free $\text{Na}_2^{35}\text{SO}_4$).

Fig. 2. Time-course of incorporation of sulphate by chick-embryo corium. Tissue was incubated in pH 7.0 carrier-free medium containing $^{35}\text{SO}_4^{2-}$ (5.5 mM-glucose and 1.26 mM-glutamine). The equation for incorporation between 0.5 and 3.5 hr. ($y = 0.028 + 0.12x$) was calculated by the method of least squares. Each point is an average of three or more incubations.

Fig. 3. Effect of sulphate concentration on rate of incorporation of sulphate by chick-embryo corium. Tissue was incubated for 1.5 or 3 hr. in pH 7.0 medium containing $^{35}\text{SO}_4^{2-}$ (5.5 mM-glucose and 1.24 or 2.9 mM-glutamine). Each point is an average of three or more incubations.

Table 1. Effect of D-glucose on incorporation of sulphate by chick-embryo corium

Corium strips were incubated for 3 hr. with indicated glucose concentration in pH 7.0 medium containing $^{35}\text{SO}_4^{2-}$ ($37\text{ }\mu\text{M-SO}_4^{2-}$ and 0, 1.24 or 2.48 mM-glutamine). Values are given $\pm$ s.d. with the numbers of incubations in parentheses.

	Concn. of D-glucose (mM)	Uptake of SO_4^{2-} ($\mu\text{mg. of SO}_4^{2-}$ / mg. of N)	Increase in uptake of SO_4^{2-} (%)	P*
a	0	81 ± 16 (21)		
b	1.39	99 ± 14 (6)	22	(a:b 0.02)
c	2.76	123 ± 20 (17)	52	(b:c < 0.01)
d	5.50	120 ± 21 (41)	48	

* Probability of a chance occurrence of the difference between the two values.

65 to 119 ($35\text{ }\mu\text{M-SO}_4^{2-}$) and 91 to 231 $\mu\text{g. of SO}_4^{2-}$ /mg. of N ($50\text{--}90\text{ }\mu\text{M-SO}_4^{2-}$).

Addition of D-glucose to the medium significantly increased incorporation of SO_4^{2-} (Table 1). At 2.76 mM-glucose, incorporation was maximal.

It has been suggested that L-glutamine serves as nitrogen donor for the hexosamine moiety of the glycosaminoglycuronoglycan (Rodén, 1956). However, in the corium preparation *in vitro*, no significant change occurred in the 3-hr. value for incorporation of SO_4^{2-} when the concentration of glutamine was increased from zero to 3.45 mM.

The effect of the pH of the medium on incorporation is indicated in Fig. 4. Uptake of sulphate was maximal at pH 6.95 but fell only to an average of 76% of this value between pH 6.8 and 7.2 ($0.42 \pm 0.07\text{ }\mu\text{g. of SO}_4^{2-}$ /mg. of N for 33 incubations). However, when the pH was raised from 6.45 to 6.80, incorporation of SO_4^{2-} was doubled (0.19 to $0.40\text{ }\mu\text{g. of SO}_4^{2-}$ /mg. of N), and, at pH 7.25, only 56% of the maximum ($0.31\text{ }\mu\text{g. of SO}_4^{2-}$ /mg. of N) was incorporated.

Incubation of the corium strips in air or oxygen resulted in comparable incorporations of sulphate, whereas in nitrogen or oxygen-carbon dioxide ($95:5$, v/v) incorporation was reduced to 31 and 68% respectively of the value observed in air.

Nature of the process of incorporation of sulphate. The dependence of incorporation upon sulphate concentration suggested that sulphate was functioning as a substrate in the process. A double-reciprocal plot (Lineweaver & Burk, 1934) of the data of Fig. 3 from 5 to $90\text{ }\mu\text{M-SO}_4^{2-}$ gave an apparent K_m $24.8\text{ }\mu\text{M}$ and a V_{max} $750\text{ }\mu\text{moles of SO}_4^{2-}$ /mg. of N/hr.

An elevation of the incubation temperature from 20° to 37.5° resulted in an increase in the rate of incorporation of SO_4^{2-} . However, at 40° the rate

Fig. 4. Effect of pH on incorporation of sulphate by chick-embryo corium. Tissue was incubated for 3 hr. in carrier-free medium containing $^{35}\text{SO}_4^{2-}$ (5.5 mM-glucose and 1.24 mM-glutamine). Standard deviations are given for mean values of three or more incubations.

Fig. 5. Lineweaver-Burk plots for incorporation of sulphate: $\circ$, 37.5°; $\bullet$, 30°; $\square$, 27.5°; $\blacksquare$, 20°. v , $\mu\text{mole of SO}_4^{2-}$ /mg. of N/hr.; s , $[\text{SO}_4^{2-}]$ (mM). Tissue was incubated for 1.5 or 3 hr. at the indicated temperature in pH 7.0 medium containing $^{35}\text{SO}_4^{2-}$ (5.5 mM-glucose, 1.24 mM-glutamine and 5, 10 or $25\text{ }\mu\text{M-SO}_4^{2-}$). The best lines were calculated by the method of least squares.

was 40% less than that observed at 37.5°. Q_{10} for the process of incorporation of SO_4^{2-} was approximately 2 between 20 and 37.5°. These temperature effects were observed at three concentrations of sulphate (5, 10 and $25\text{ }\mu\text{M}$). The Lineweaver-Burk plots for the data at 20°, 27.5°, 30° and 37.5° are shown in Fig. 5; there was a progressive increase in the apparent V_{max} from 200 to $930\text{ }\mu\text{moles of$

Fig. 6. Arrhenius plot for incorporation of sulphate by chick-embryo corium. $V_{\max}$, $\mu\text{moles of SO}_4^{2-}/\text{mg. of N/hr.}$ Experimental details are given in Fig. 5. The best line was calculated by the method of least squares.

$\text{SO}_4^{2-}/\text{mg. of N/hr.}$ and in the apparent K_m from 12 to $32 \mu\text{M-SO}_4^{2-}$.

The effect of temperature on incorporation of SO_4^{2-} can be described by the Arrhenius equation (Fig. 6). A linear relationship was obtained from a plot of the logarithms of the values of $V_{\max}$ obtained from the Lineweaver-Burk plots of Fig. 5 against the reciprocals of the absolute temperature, and from the slope an activation energy of $15.78 \text{ kcal. mole}^{-1}$ was calculated.

The incorporating capacity of the tissue was destroyed either by a boiling period of 1 min. or repeated freeze-thawing (five times) before incubation.

Characteristics of labelled glycosaminoglycuronoglycans. The radioactivity in the washed tissue was present as bound sulphate; an average of only $2.1 \pm 1.1\%$ of the activity in hydrolysates of the trichloroacetic acid-precipitated tissue was not recovered as barium sulphate. For the five large-scale incubations, an average of 82% of the radioactivity of the cetylpyridinium-glycosaminoglycuronoglycans complex was extracted with 1.2M-sodium chloride whereas 14% was extracted with 2.1M-sodium chloride.

After chromatography of each of these $^{35}\text{SO}_4^{2-}$ -labelled sodium chloride extracts, satisfactory recoveries of the radioactivity applied to the column (86% for 1.2M-sodium chloride and 96% for 2.1M-sodium chloride) and of uronic acid for the 1.2M-sodium chloride (106%) were realized. However, uronic acid recovery on the 2.1M-sodium chloride extract was poor and did not appear to be related to an effect of sodium chloride on uronic acid analysis. Only 56% of the uronic acid of a sample of the commercial heparin was recovered from the column.

Table 2. Chromatographic separation of $^{35}\text{SO}_4^{2-}$ -labelled glycosaminoglycuronoglycans of chick-embryo corium

Tissue was incubated for 3 hr. in pH 7.0 medium containing $^{35}\text{SO}_4^{2-}$ (5.5 mM-glucose, 1.24 mM-glutamine and $35 \mu\text{M-SO}_4^{2-}$). Details of isolation and separation of $^{35}\text{SO}_4^{2-}$ -labelled glycosaminoglycuronoglycans are given in the text.

Eluent (M-NaCl)	Radio- activity (% of that found in extract)	Uronic acid (% of hexosamine in eluent)	Galactosamine (% of hexosamine in eluent)
1.2M-NaCl extract (extract II)			
1.2	50	6	50
1.4	20	38	98
1.6	28	54	84
2.1M-NaCl extract (extract III)			
1.6	70	40	0
1.8	30	60	0

In chromatographic separation of the 1.2M-sodium chloride extract (extract II), radioactivity and uronic acid were eluted in three peaks, i.e. one each in 1.2M-, 1.4M- and 1.6M-sodium chloride (Table 2). A single peak was eluted with 1.6M-sodium chloride and one with 1.8M-sodium chloride from the 2.1M-sodium chloride extract (extract III) of the cetylpyridinium chloride complex. The activity (counts/min./ $\mu\text{g. of uronic acid}$) in each peak was relatively consistent even though there was some tailing of the peak. Higher concentrations of sodium chloride did not elute significant amounts of either measured quantity.

The glycosaminoglycuronoglycans eluted with 1.2M-sodium chloride contained 50% of the radioactivity and 6% of the uronic acid of extract II; 50% of the hexosamine of the material was galactosamine. The 1.4M-sodium chloride and 1.6M-sodium chloride eluents contained 20 and 28% of the radioactivity and 38 and 54% of the uronic acid respectively. An average of 98 and 84% of the hexosamine was galactosamine in the 1.4M and 1.6M eluents respectively. The carrier chondroitin sulphate contained only galactosamine.

In extract III, 70 and 30% of the radioactivity and 40 and 60% of the uronic acid were found in the 1.6M and 1.8M eluents respectively; only glucosamine was present.

The results from the hexose determinations were inconclusive. Each of the five peaks eluted from the two extracts and the carriers contained anthrone-positive material.

DISCUSSION

The corium of the 9-day chick embryo incorporates inorganic sulphate under conditions in

vitro into glycosaminoglycuronoglycans which are trichloroacetic acid-precipitable. The incorporation proceeds linearly for 3.5–4 hr., but after this little additional uptake of sulphate occurs. This loss of the sulphate-incorporating capacity of the fibroblast may be due to a general exhaustion of the cell under these conditions *in vitro* or to the inherent 'leaky' property of the fibroblast in aqueous suspension, permitting Krebs-cycle intermediates to pass into the medium (Paul, 1962). Loss of 70% of the incorporating capacity of the tissue in an atmosphere of nitrogen indicates the requirement of oxygen for the process. The reduction in incorporation observed with the oxygen-carbon dioxide mixture can be attributed to the decrease in pH of the medium to approximately 6.5 during the period of incubation.

Incorporation of sulphate into an organically bound form might suggest the new synthesis of glycosaminoglycuronoglycans. However, evidence indicates that it is preformed polysaccharide which is sulphated in the biosynthesis of sulphated glycosaminoglycuronoglycans (Korn, 1959c; Suzuki & Strominger, 1959, 1960b; Perlman, Telser & Dorfman, 1964). Since the report of the transfer of sulphate from adenosine 3'-phosphate 5'-sulphatophosphate* to chondroitin sulphate by extracts of embryo-chick cartilage (D'Abramo & Lipmann, 1957), several polysaccharides have been shown to serve as acceptors in this process (Adams, 1960; Davidson & Riley, 1960; Suzuki & Strominger, 1960a). Heparan sulphate, chondroitin and its three sulphated isomers accept sulphate when incubated with [³⁵S]PAPS and a soluble enzyme system from hen oviduct (Suzuki & Strominger, 1960a) or from embryo-calf cartilage (Hasegawa, Delbrück & Lipmann, 1961). However, heparin, keratan sulphate and hyaluronic acid were inactive in the former system.

The failure of glutamine, shown in some systems to be the nitrogen donor in the synthesis of the hexosamine moiety of the polysaccharide (Rodén, 1956; Pogell & Koenig, 1959), to enhance incorporation of sulphate speaks against the synthesis of the hexosamine molecule in the preparation of chick-embryo corium *in vitro*. It has been reported that other sulphate-incorporating systems *in vitro*, rat cartilage (Salmon & Daughaday, 1957) and guinea-pig granulation tissue (Loewi & Kent, 1957), are unaffected by the addition of L-glutamine. Perhaps in these systems and in the chick corium an adequate supply of glutamine or perhaps ammonia existing in the tissue is used in the synthesis of the hexosamine molecule. The role of ammonia in hexosamine synthesis has been demon-

strated in hog kidney (Comb & Roseman, 1958) and in subcutaneous granulomas (Sekhara-Varma & Bachhawat, 1963). Incorporation of sulphate by the fibroblast of the 9-day chick corium appears to be enzymic since the process does not occur after the tissue has been boiled or repeatedly freeze-thawed. Furthermore, the rate of the reaction is dependent on the concentration of a substrate, an effect due neither to a change in pH nor to a significant increase in ionic strength of the medium by the addition of SO₄²⁻ to a concentration of 90 μM. The overall reaction of incorporation of sulphate by the cells of the corium strips can be described over a wide range of sulphate concentration by the Michaelis-Menten equation when the reciprocal of the velocity during the 3 hr. period of linear incorporation is plotted against the reciprocal of sulphate concentration of the medium. This relationship may be due to a rate-limiting step in the process of incorporation of sulphate or to the reduction of more complex mechanisms to Michaelis-Menten kinetics by the conditions *in vitro*.

Sulphate, two ATP molecules, magnesium and two enzymes, ATP sulphurylase and adenosine 5'-phosphosulphate (adenosine 5'-sulphatophosphate) phosphokinase, are required in the enzymic reactions leading to activation of sulphate (PAPS) (Lipmann, 1958). Sulphate is subsequently transferred from PAPS to an acceptor polysaccharide molecule in the presence of a sulphotransferase (sulphokinase). Thus there are three known enzymic reactions involved directly in the activation of sulphate and its transfer to acceptor, any of which would be rate-limiting.

The crucial step in activation is the creation of the reactive phosphosulphate link in adenosine 5'-phosphosulphate by sulphurolysis of ATP. Since incorporation of sulphate by the chick corium was enhanced by the addition of sulphate, it may be that this initial reaction involving the formation of adenosine 5'-phosphosulphate in the presence of ATP sulphurylase is the rate-limiting step.

From equilibria studies, the ΔF^0 for the formation of adenosine 5'-phosphosulphate was found to be 11 kcal. (Robbins & Lipmann, 1958b). However, the energetically unfavourable reaction is driven forward in the direction of PAPS synthesis because of the high affinity of adenosine 5'-phosphosulphate for adenosine 5'-phosphosulphate phosphokinase, the removal of pyrophosphate by pyrophosphatase and the energy supplied by the second energy-rich phosphate. Thus, for PAPS formation, 2 molecules of ATP are required. This energy is used for the formation of a bond of higher group potential than the pyrophosphoryl bond of ATP, i.e. the phosphosulphate bond, and for forcing the initially formed, thermodynamically unstable compound (adenosine 5'-phosphosulphate) to PAPS formation. At pH 7,

* Abbreviation: PAPS, adenosine 3'-phosphate 5'-sulphatophosphate.

the pyrophosphate group potential in ATP is 7.6–7.8 kcal.mole⁻¹ (Robbins & Boyer, 1957), and Robbins & Lipmann (1958b) have reported 18–19 kcal.mole⁻¹ for the sulphate group potential in adenosine 5'-phosphosulphate and presumably in PAPS.

The free energy of activation calculated for the overall process of incorporation of sulphate by the chick corium was 15.78 kcal.mole⁻¹, a value approximating to the energy which might be supplied by 2 molecules of ATP in the formation of PAPS. This similarity lends support to the proposal that the process *in vitro* involves the activation of sulphate. The enzyme system for the synthesis of PAPS has been demonstrated in several animal tissues, including mammalian liver and mast-cell tumour (Pasternak, 1960), chick-embryo cartilage (D'Abramo & Lipmann, 1957) and colonic mucosa (Kent & Pasternak, 1958). The sulphokinases active in transfer of sulphate from PAPS to the acceptor in sulphated glycosaminoglycuroglycan synthesis have been found in hen oviduct (Suzuki & Strominger, 1960c) and in ox-cornea epithelial extract (Wortman, 1963).

The pH optimum for the chick corium system for incorporation of sulphate is 6.95. Whereas the sulphate-activating enzymes isolated and purified from yeast have higher pH optima, i.e. 7.5–9.0 for ATP sulphurylase (Robbins & Lipmann, 1958a,b) and 8.5–9.0 for adenosine 5'-phosphosulphate phosphokinase (Robbins & Lipmann, 1958a), the pH optima for the transfer of sulphate from PAPS to acceptors are 6.4 and 6.5 in extracts of calf-embryo cartilage (Delbrück & Lipmann, 1960) and hen oviduct (Suzuki & Strominger, 1960a) respectively. Another enzyme essential to the synthesis of PAPS is pyrophosphatase. The pH optimum for hydrolysis of pyrophosphate to orthophosphate is 6.9–7.0 in yeast (Bailey & Webb, 1944) and 6.5–7.4 in brain (Gordon, 1950).

The increase in incorporation of sulphate on glucose addition is similar to that observed in cartilage slices (Boström & Jorpes, 1954; Boyd & Neuman, 1954) and in granulation tissue (Loewi & Kent, 1957), and may be due to the utilization of glucose as a general source of energy, particularly in the synthesis of ATP. Since the incorporation of labelled glucose *in vitro* into chondroitin sulphate has been demonstrated (Schiller, Mathews, Cifonelli & Dorfman, 1956; Pogell & Koenig, 1959), the possibility cannot be excluded that the glucose may participate in the synthesis of the polysaccharide.

From the sodium chloride-extraction pattern of the labelled material precipitated as a cetylpyridinium chloride complex, it would appear that 82% of the newly sulphated glycosaminoglycuroglycan is chondroitin sulphate(s) and heparan sulphate

(1.2M-sodium chloride: extract II) and the remainder is heparin-like (2.1M-sodium chloride: extract III).

Heparan sulphate is eluted with 1.25M-sodium chloride from extract II (Schiller *et al.* 1961), and 41% of the total radioactivity extracted from the cetylpyridinium chloride complex of the chick corium was present in this eluent. However, since 50% of the hexosamine was galactosamine, it is likely that chondroitin sulphate low in sulphate content was eluted with heparan sulphate. Of course, the extent of incorporation of [³⁵S]sulphate in these two sulphated glycosaminoglycuroglycans, heparan sulphate and chondroitin sulphate, cannot be determined from the data.

The three isomeric chondroitin sulphates, not resolved by this technique, are eluted with 1.6M-sodium chloride (Schiller *et al.* 1961), and 92% of the carrier chondroitin sulphate of extract II was eluted with 1.4M-sodium chloride and 1.6M-sodium chloride. Since the 1.4M-sodium chloride eluent contained only galactosamine, the radioactivity may be tentatively assigned to chondroitin sulphate (16% of the total radioactivity of the cetylpyridinium complex). Based upon the relative content of galactosamine, the 1.6M-sodium chloride eluent would also be principally chondroitin sulphate (84%). Schiller *et al.* (1961) reported that 5% of the heparin precipitated with cetylpyridinium chloride may be extracted with 1.2M-sodium chloride, and, if this were the case, the presence of glucosamine in the 1.6M-sodium chloride eluent of extract II would be reasonably explained. Again, the distribution of radioactivity (23% of the total) between the two types of polysaccharide in the 1.6M-sodium chloride cannot be specified.

In extract III, only glucosamine was found. The small amount of chondroitin sulphate which Schiller *et al.* (1961) observed in this extract was apparently not present. The carrier heparin recovered from the column was about equally distributed between the two peaks eluted from this extract, and, of the total radioactivity of cetylpyridinium chloride complex, 10% was present in the 1.6M-sodium chloride and 4% in the 1.8M-sodium chloride. Since it is known that the degree of sulphation varies in heparin, it might be suggested that both the ³⁵SO₄²⁻-labelled heparin-like material from the tissue and the carrier heparin were under-sulphated when compared with the heparin eluted at 2.0M-sodium chloride by Schiller *et al.* (1961).

The existence of chondroitin sulphate and heparin-like material with various degrees of sulphation in embryo-chick corium is suggested by these results. This proposal seems reasonable since the chondroitin sulphate of the epiphyseal cartilage of the 13-day chick embryo is incompletely sulphated (Perlman & Dorfman, 1963). Chondroitin

6-sulphate and chondroitin 4-sulphate with low degrees of sulphation have been found in tadpole cartilage; during metamorphosis, the degree of sulphation increases (Mathews & Hinds, 1962). Conclusive identification and characterization of the acidic glycosaminoglycans produced by the young fibroblast are currently under investigation by our laboratories.

Experimental evidence of others suggests that the synthesis of glycosaminoglycuronoglycans and the incorporation of sulphate into these compounds occur intracellularly (Glücksmann *et al.* 1956; Mancini *et al.* 1961; Dziewiatkowski, 1962; Thorp & Dorfman, 1963). Since fibroblasts are the principal cells in the chick-embryo corium, the incorporation of sulphate by this tissue indicates the participation of the fibroblast in this process. Another synthetic activity of the fibroblast, the production of collagen, is indicated by preliminary experiments with the chick-corium preparation. [¹⁴C]Proline was shown to be converted into a non-diffusible [¹⁴C]hydroxyproline. Thus the 9-day chick fibroblast in this system *in vitro* effects the sulphation of polysaccharides and the hydroxylation of proline, two classical properties of connective tissue cells. Since the 9-day chick-embryo corium contains no histologically demonstrable mast cells, our data suggest that the young fibroblast may also produce a heparin-like material.

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REFERENCES

- Adams, J. B. (1960). *Biochem. J.* **76**, 520.
 Bailey, K. & Webb, E. C. (1944). *Biochem. J.* **38**, 394.
 Berenson, G. S., Lumpkin, W. M. & Shipp, V. G. (1958). *Anat. Rec.* **132**, 585.
 Bitter, T. & Muir, H. M. (1962). *Analyt. Biochem.* **4**, 330.
 Boström, H. & Aqvist, S. (1952). *Acta chem. scand.* **6**, 1557.
 Boström, H. & Jorpes, E. (1954). *Experientia*, **10**, 392.
 Boström, H. & Mansson, B. (1952). *J. biol. Chem.* **196**, 483.
 Boyd, E. S. & Neuman, W. F. (1954). *Arch. Biochem. Biophys.* **51**, 475.
 Comb, D. G. & Roseman, S. (1958). *J. biol. Chem.* **232**, 807.
 D'Abramo, F. & Lipmann, F. (1957). *Biochim. biophys. Acta*, **25**, 211.
 Davidson, E. A. & Riley, J. G. (1960). *J. biol. Chem.* **235**, 3367.
 Delbrück, A. & Lipmann, F. (1960). *Ber. ges. Physiol.* **215**, 21.
 Dorfman, A. & Schiller, S. (1958). *Recent Progr. Hormone Res.* **14**, 427.
 Dreywood, R. (1946). *Industr. Engng Chem. (Anal. Ed.)*, **18**, 499.
 Dziewiatkowski, D. D. (1962). *J. Cell Biol.* **13**, 359.
 Glücksmann, A., Howard, A. & Pelc, S. R. (1956). *Amer. J. Anat.* **90**, 478.
 Gordon, J. J. (1950). *Biochem. J.* **46**, 96.
 Grossfield, H., Meyer, K., Godman, G. & Linker, A. (1957). *J. biophys. biochem. Cytol.* **3**, 391.
 Hasegawa, E., Delbrück, A. & Lipmann, F. (1961). *Fed. Proc.* **20**, 86.
 Jeanloz, R. W. (1960). *Arthritis Rheumat.* **3**, 233.
 Kent, P. W. & Pasternak, C. A. (1958). *Biochem. J.* **69**, 453.
 Korn, E. D. (1958). *J. Amer. chem. Soc.* **80**, 1520.
 Korn, E. D. (1959a). *J. biol. Chem.* **234**, 1321.
 Korn, E. D. (1959b). *J. biol. Chem.* **234**, 1325.
 Korn, E. D. (1959c). *Biochim. biophys. Acta*, **32**, 554.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Lipmann, F. (1958). *Science*, **128**, 575.
 Loewi, G. & Kent, P. W. (1957). *Biochem. J.* **65**, 550.
 Mancini, R. E., Vilar, O., Stein, E. & Fiorini, H. (1961). *J. Histochem. Cytochem.* **9**, 278.
 Mathews, M. B. & Hinds, L. (1962). *Fed. Proc.* **21**, 167.
 Morris, D. L. (1948). *Science*, **107**, 254.
 Neuhaus, O. W. & Letzring, M. (1957). *Analyt. Chem.* **29**, 1230.
 Pasternak, C. A. (1960). *J. biol. Chem.* **235**, 438.
 Paul, J. (1962). In *Conf. Biology of Connective Tissue Cells*, pp. 55, 56 and 146. Ed. by Castor, C. W., Merchant, D. J. & Shulman, L. E. New York: The Arthritis and Rheumatism Foundation.
 Perlman, R. L. & Dorfman, A. (1963). *Fed. Proc.* **22**, 413.
 Perlman, R. L., Telser, A. & Dorfman, A. (1964). *J. biol. Chem.* **239**, 3623.
 Pogell, B. M. & Koenig, D. F. (1958). *Nature, Lond.*, **182**, 127.
 Pogell, B. M. & Koenig, D. F. (1959). *J. biol. Chem.* **234**, 2504.
 Robbins, E. A. & Boyer, P. D. (1957). *J. biol. Chem.* **224**, 121.
 Robbins, P. W. & Lipmann, F. (1958a). *J. biol. Chem.* **233**, 681.
 Robbins, P. W. & Lipmann, F. (1958b). *J. biol. Chem.* **233**, 686.
 Rodén, L. (1956). *Ark. Kemi*, **10**, 333.
 Salmon, W. D., jun. & Daughaday, W. H. (1957). *J. Lab. clin. Med.* **49**, 825.
 Sekhara-Varma, T. N. & Bachhawat, B. K. (1963). *Biochim. biophys. Acta*, **69**, 464.
 Schiller, S., Mathews, M. B., Cifonelli, J. A. & Dorfman, A. (1956). *J. biol. Chem.* **218**, 139.
 Schiller, S., Slover, G. A. & Dorfman, A. (1961). *J. biol. Chem.* **236**, 983.
 Suzuki, S. & Strominger, J. L. (1959). *Biochim. biophys. Acta*, **31**, 283.
 Suzuki, S. & Strominger, J. L. (1960a). *J. biol. Chem.* **235**, 257.
 Suzuki, S. & Strominger, J. L. (1960b). *J. biol. Chem.* **235**, 267.
 Suzuki, S. & Strominger, J. L. (1960c). *J. biol. Chem.* **235**, 274.
 Thorp, F. K. & Dorfman, A. (1963). *J. Cell Biol.* **18**, 13.
 Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1951). *Manometric Techniques and Tissue Metabolism*, p. 119. Minneapolis: Burgess Publishing Co.
 Wortman, B. (1963). *Fed. Proc.* **22**, 413.