In the meantime, whiskers are proving to be useful tools in many areas of research. As the methods of their growth become more widely known and experience is gained in their handling, their use will become increasingly more widespread.

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Biological Sulfate Activation and Transfer

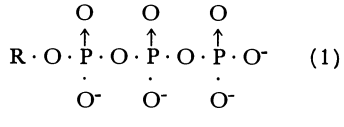
Studies on a mechanism of group activation and its role in biosynthesis are described.

Fritz Lipmann

Sulfate is bound, mostly in ester linkage, in a fairly large variety of compounds present rather commonly in living organisms. Of most importance among these compounds are the sulfated mucopolysaccharides, such as chondroitin sulfuric acid, the ground substance of cartilage, and the similar mucoitin-sulfuric acid in mucosous tissues. Heparin belongs in this group; it is outstanding for its high sulfate, partially bound here to the amino group of the glucosamine moiety. Furthermore, a sulfurylated cerebroside is present in the brain and other tissues. On the other hand, conjugation with sulfate is a means of phenol detoxication in the animal body. This sulfate conjugation of the phenols, mainly in liver and the intestine, has been used for many years for the study of the mechanism of sulfate transfer.

With such a large number of metabolically-formed sulfurylated substances, it appeared likely that there was a common metabolic carrier for activated sulfate which would serve as general sulfate donor in the enzymatic set-up of cells. This was all the more indicated when DeMeio (*1*), who pioneered in the field of sulfate activation, demonstrated that, in cell-free systems, ATP (*2*) could serve as the source of energy for sulfate activation. The kind of mechanism that occurs in sulfate activation was further clarified by Bernstein and McGilvery (*3*). All this work with the liver system indicated strongly that conjugation with phenol was a two-phasic process, the activation of sulfate being the primary, the transfer to phenol being a secondary and separate, step.

Since, therefore, in the process of activation the energy of a phosphoanhydride link of ATP



(1)

apparently was transmitted to the sulfate, it seemed likely that the formation

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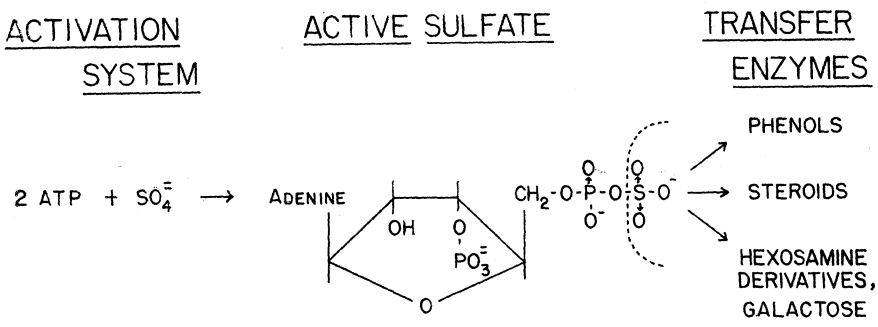
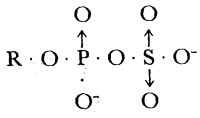


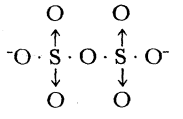
Fig. 1. Sulfate activation.

of a *mixed* anhydride between sulfate and phosphate,



(2)

might represent this process of activation. However, no information about the chemistry of such a mixed anhydride was found in the literature. Pyrosulfates are well-known compounds,



(3)

but they are very sensitive to water and are apparently about as unstable as organic acid anhydrides. For all these reasons, sulfate activation presented a rather special problem in group activation which seemed most attractive to me, and my co-workers and I decided, some three years ago, to try our hand at clearing up the chemistry of this intriguing reaction.

It will be helpful to start with the scheme in Fig. 1, which gives the essentials of the activation-transfer process as we were able to develop it. What I want to stress particularly is the strict separation between the activation process and the transfer reactions. The active sulfate is formed, as we now know and will explain in detail later, by a reaction between sulfate and two ATP's (*4, 5*), as a result of which adenosine-3′-phosphate-5′-phosphosulfate is formed. This appears to be the general sulfate donor in biological reactions, which, as suspected, carries sulfate in the form of a mixed anhydride. We have found this to be a sulfate donor in all cases studied so far —that is, in chondroitin sulfate and sulfatide synthesis, in steroid, and in phenol sulfurylation. Sulfate transfer from the activated sulfate to acceptors such as phenols, hydroxy steroids, and the hydroxy group of various compounds, and also probably amino groups, is catalyzed by enzymes which we call sulfokinases and which are more or less specific for the acceptor molecule.

This may serve as a general orientation. Now I want to discuss in detail, first, the isolation and identification of active sulfate and the enzymatic mechanism of its formation, carried out by Phillips W. Robbins (*4–6*). Then, second, I will discuss a number of sulfate acceptor reactions which have been and are being studied at the Rockefeller Institute for Medical Research by John D. Gregory, Yoshitsugu Nose, Furio D'Abramo, and Irving H. Goldberg.

Table 1. Active sulfate fraction, analytical data. Adenosine was determined by absorption at 260 mμ, ribose by the Orcinol procedure, and phosphate, by the method of Fiske and Subbarow. Total phosphate was determined by hydrolysis in 1*N* HCl acid. The 12- and 30-minute phosphate was determined by hydrolysis in 1*N* HCl at 100°C. Phosphate hydrolyzable by the 3′-nucleotidase was determined by the method of Kaplan (*21*).

Component	Amount
Adenosine	1
Ribose	0.95
Phosphate, total	1.98
Phosphate, 12-minute	0.53
Phosphate, 30-minute	1.04
Phosphate, 3′-nucleotidase	0.85
Sulfate, enzymatic	0.2–0.85

Isolation and Identification of Active Sulfate

Helmuth Hilz, with whom this venture was started, found (*7*) that the reaction between ATP and sulfate appeared to yield pyrophosphate, and, using paper electrophoresis, he could show that the compound formed contained adenylic acid. This preliminary identification was carried out by the use of radioactive sulfate. In analogy to other activation reactions, in particular to the recent studies on acetate activation, we tentatively thought of sulfate activation as a reaction between ATP and sulfate which, through a substitution of the terminal pyrophosphoryl group in ATP, led to the suspected anhydride between sulfate and substituted phosphate. However, some of the data did not fit with this interpretation, and at that stage we cautiously reported that our observations had yielded evidence for the formation of an adenyl sulfate derivative, of the exact structure of which we were not sure.

As is often the case in studies of an unknown "active" compound, especially a substance of which no chemical analog has been known before, we expected the compound to be very unstable. It had appeared, indeed, in these preliminary experiments that "active" sulfate was quite unstable to strong acid. To get a step further, it was necessary to clean up the enzymes and concentrate the sulfate activation system for use in medium-scale preparative runs (*6*); thus, Robbins succeeded in preparing 50-μmole samples of active sulfate from ATP and sulfate, and tried Dowex chromatography. Fortunately, it appeared that the phosphosulfate link was more stable to acid than had been expected; it is perfectly stable at neutral, or higher, *p*H, and it tolerated chromatography with rather concentrated formic acid in the cold. Active sulfate is strongly bound by Dowex-1, and it was possible to remove from the column all other adenine derivatives by treatment with 4*N* formic acid–0.3*M* ammonium formate. Then 5*N* formic acid–1*M* ammonium formate eluted the remaining active sulfate, and in this manner a rather homogenous fraction of active sulfate was obtained. During concentration of this fraction by lyophilization, however, part of the sul-

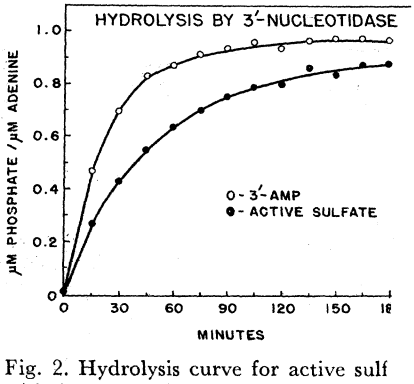


Fig. 2. Hydrolysis curve for active sulf with 3′-nucleotidase compared to that 3′-adenylic acid.

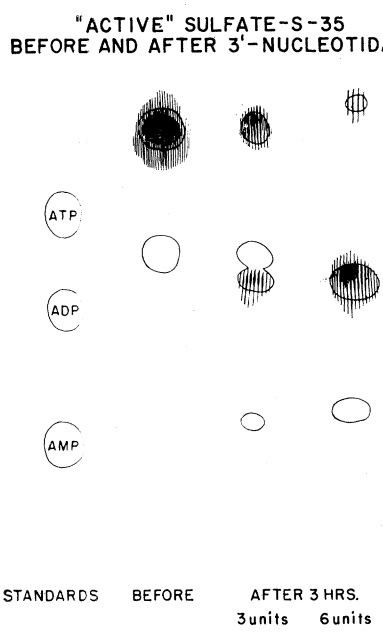


Fig. 3. Radioautogram of paper electrogram in citrate buffer of *p*H 5.9. Encircled areas are ultraviolet quenching; shaded and black areas are radioautogram tracings (see Fig. 6 in *6*).

fate was split off. The analysis of such a fraction is shown in Table 1.

It appeared from these data that the compound, to our surprise, contained two phosphates per adenine, and pentose. One phosphate was acid-stable, and the other one was unstable to heating with 1*N* HCl, but not as much so as would have been expected for a pyrophosphate link. The hydrolysis with acid rather suggested a 2′- or 3′-adenylic acid, particularly since the compound did not give the periodate reaction commonly obtained with adenosine-5′-phosphates. For further identification, Kaplan's 3′-nucleotidase was used. This enzyme liberated practically a full equivalent of phosphate from our "active" sulfate.

The active sulfate in all these cases was conveniently determined by enzymatic transfer to nitrophenol, the anion color of which disappears on conjugation.

As has been mentioned, during lyophilization part of the sulfate was split off, and this explains why the figures for sulfate in Table 1 vary between rather wide limits. Smaller batches, which could be lyophilized quickly, showed near equivalence between sulfate and adenosine. On the other hand, the sulfate-free residue gave ratios with regard to phosphate identical with those of active sulfate. In Fig. 2 the hydrolysis curve for active sulfate with 3′-nucleotidase is compared with that for 3′-adenylic acid. Hydrolysis is slower for the active sulfate than for the monophosphate; in general, the 3′-adenylic acid is the substrate most rapidly hydrolyzed by this enzyme, while additional substitutes slow down reactivity.

The position of the sulfate in the molecule was largely identified by the use of radioactive sulfate. If a mixture of active sulfate and sulfate-free residue is exposed to the 3′-nucleotidase, the sulfate remains with adenylic acid, as is shown in the paper electrophoresis pattern of Fig. 3 by the overlap of ultraviolet quenching and radiotracing. If incubated with a sufficient amount of nucleotidase, the upper spot of PAPS disappears almost completely and a new spot appears instead, which could be identified as adenosine-5′-phosphosulfate (APS). On the other hand, a lower spot appears which is adenosine-5′-phosphate (AMP), derived by hydrolysis of PAP, the sulfate-free residue. The adenosine-5′-phosphosulfate could be identified by comparison with synthetic compound, prepared according to the method of Baddiley *et al.* (*8*). The structure and some of the properties of the active sulfate are explained in Fig. 4, where it may be seen that the phosphosulfate link is rather sensitive to hydrochloric acid; 0.1*N* HCl split the sulfate completely off in about half an hour at 37°C. These compounds have an ultraviolet absorption indistinguishable from that of adenylic acid. This excludes any possibility of sulfate being linked to the amino group of adenine, since blocking of this amino group in all cases causes a shift of the ultraviolet absorption toward the visible.

For further identification, we argued that hydrolysis of the phosphosulfate link should liberate a secondary phosphate. This was shown to be the case by means of electrotitration between *p*H 5 and 8 before and after hydrolysis. All this evidence makes us feel sure that we are dealing with a compound of the constitution shown in the figures. This constitution has now been confirmed through synthesis by Baddiley *et al.* (*9*).

Two Enzymatic Steps in Biosynthesis of Active Sulfate

The unexpected appearance of two separately linked phosphates in the active sulfate, as now identified, indicated right away a two-phasic synthesis as likely. Initially, we had speculated (*7*) on the possibility of adenosine-5′-phosphosulfate being active sulfate, formed by pyrophosphate substitution on ATP. It now appeared that this reaction was the first step in the sequence shown in Fig. 5. This was completed by a second reaction, of phosphokinase type, whereby the terminal phosphate of a second ATP was transferred to the 3′-position of APS. The initial reaction is catalyzed by an enzyme which we call sulfurylase. It catalyses the attack of one of the oxygens of sulfate on the proximal phosphorus in adenosine-5′-triphosphate with

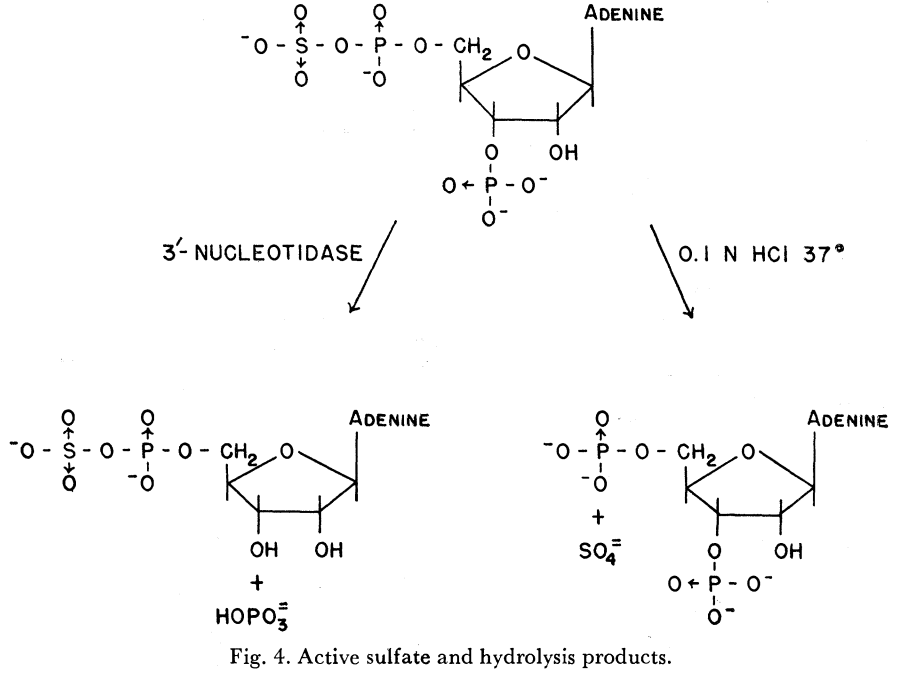


Fig. 4. Active sulfate and hydrolysis products.

the displacement of pyrophosphate by sulfate. The APS, thus formed initially, in *entirely inactive* as a sulfate donor in enzymatic reactions.

It is important to realize that the reaction as written is actually much more favored energetically in the backward direction; in other words, the sulfuryl potential in APS is considerably higher than the pyrophosphoryl potential in ATP. We believe that this energetic situation is the reason for the further phosphorylation. In this manner, the energy of a second energy-rich phosphate is used to force the reaction into the forward direction by a "masking" of the reaction product through 3′-phosphorylation. The over-all energy balance, even then, is not too favorable, and the rather energy-rich phosphosulfate bond becomes still further stabilized through removal of the initial reaction product, pyrophosphate, by the quite ubiquitous pyrophosphatase.

In proving this mechanism, the independent work of Bandurski *et al.* (*10*) has been rather important. These workers found that the sulfate activation system in yeast could be separated into two inactive fractions, active only after recombination. When Bandurski's work came to our attention, we turned to the yeast system for elaboration of the mechanism, as it appeared preferable to the liver system we had used so far. Confirming Bandurski, we were able to separate from yeast two fractions, which were identified with (i) sulfurylase, and (ii) APS-kinase.

Sulfurylase was measured, as is indicated in Table 2, in the reverse direction by means of synthetic APS. Sulfurylase from yeast could be rather highly purified by electrophoresis on Geon 426, as is shown in Fig. 6. With this purified enzyme, equilibrium studies were made which showed that, as seen in Fig. 7, a small but definite amount of APS was formed in the forward direction. The equilibrium constant for the reaction

$$ATP + S \rightleftharpoons APS + PP$$

at *p*H 8 is approximately 10^{-8}, and therefore $\Delta F°$ equals 11,000 cal. But APS was found to have a high affinity to APS-kinase, giving, indeed, the highest reaction rate at the lowest measurable concentration, $5.10^{-6}M$. The amounts formed enzymatically are, under "physiological" conditions, probably of similar magnitude, and therefore the little that is formed can thus immediately be phosphorylated by APS-phosphokinase and can thereby be eliminated from equilibrium. This drives the reaction in the direction of synthesis of PAPS, helped by pyrophosphatase, removing pyrophosphate, the other product of sulfurylase reaction. There are interesting general implications in this use of two or more energy-rich phosphates for the fixation of bonds of higher group potential, such as the phosphosulfate bond, the additional energy serving to pull an initially-formed, thermodynamically very unstable compound over the energy hump:

$$ATP + S \xleftarrow{\text{sulfurylase}} APS + PP \qquad \Delta F°, +11{,}000 \quad (4)$$

$$PP \xrightarrow{\text{PP-ase}} 2P \qquad \Delta F°, -5{,}000 \quad (5)$$

$$APS + ATP \xrightarrow{\text{APS-kinase}} PAPS + ADP + H^+ \qquad \Delta F°, -6{,}000 \quad (6)$$

$$2ATP + S \rightarrow PAPS + 2P + ADP \qquad \text{over-all} \quad \Delta F°, \quad 0 \quad (7)$$

The energy data are rough approximations, to give an impression of the over-all $\Delta F°$, which, taking into account the formation, at *p*H 8, of H^+ as calculated for hexokinase in (*11*), just about balances.

Table 2. Separation of yeast enzymes. National Bakers yeast was used, and extracts were prepared essentially according to the method of Jones *et al.* (*22*). The formation of ATP from APS was measured by following pyrophosphate disappearance or by measuring ATP formation with hexokinase and glucose-6-phosphate dehydrogenase. Formation of PAPS was followed by transfer to nitrophenol or by PAP assay. The PAP assay depends on the catalytic activity of PAP in the transfer of sulfate from *p*-nitrophenol to phenol. The rate of nitrophenol formation is measured at 400 mμ in the Beckman DU spectrophotometer. The reaction between APS and P was routinely followed by measuring the disappearance of P with chromatographic checks on ADP formation.

Fraction	ATP-sulfurylase (APS + PP → ATP) − PP, (μmole/mg hr)	APS-kinase (APS + ATP → PAPS) PAPS, (μmole/mg hr)
Dialyzed extract, I	2.1	
NaCl precipitate, II	10.1	0.55
17–23 percent EtOH, III	22.1	3.5
*p*H 5.4 precipitate, IVa	85.0	0.6
Supernatant + 10 percent EtOH, IVb	0.5	4.1
40–50 percent $(NH_4)_2SO_4$, V	0	12.5

Table 3. Sulfate transfer from $PAPS^{35}$ to chondroitin sulfate. The enzyme preparation, prepared from three chick embryos, as in the previous experiments, was extracted with 6.5 ml of saline-phosphate solution and centrifuged. The supernatant was mixed with 0.25 ml of $PAPS^{35}$, and 1 ml was immediately heated (zero time). Each tube contained 1 ml of enzyme in a 1.35-ml total and 15,000 counts. Precipitation was with sodium acetate and 3 vol of alcohol; there were 10 washings with 80 percent alcohol.

Incubation (37°C) (hr)	$PAPS^{35}$	ATP 1 μmole	UTP 1 μmole	Count/min	Δ	Incorporation (%)
0	+	−	−	80		
2	+	−	−	340	260	1.7
2	+	+	−	600	520	3.4
2	+	−	+	670	590	4
2	+	+	+	550	470	3

Transfer of Active Sulfate to Various Acceptors

With the solution of the problem of sulfate activation, the way had been opened to approach more intelligently the metabolic utilization of sulfate. This, in a sense, forced us to enter metabolic territory rather foreign to the experience of workers in our laboratory—some steroid metabolism but, more seriously, polysaccharide synthesis and lately some lipid chemistry. Generally, in these experiments, isolated PAPS was not used, but rather PAPS was fed in by way of an enriched enzymatic generating system from yeast (*5*) or liver (*6*). For confirmation, the various acceptor reactions were then checked with isolated PAPS.

Except in the case of phenol and steroid conjugation, we are still in the more exploratory phase, using almost exclusively S^{35} as a guide. We have made available cell-free preparations from embryonic cartilage for synthesis of chon-

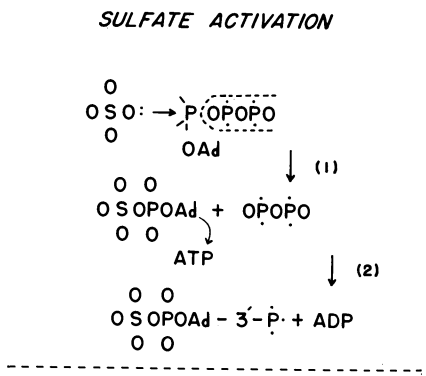


Fig. 5. Reaction 1: the displacement by sulfate of pyrophosphate in ATP, yielding APS, is catalyzed by sulfurylase. Reaction 2: the phosphorylation of APS by the terminal phosphate of ATP is catalyzed by APS-phosphokinase.

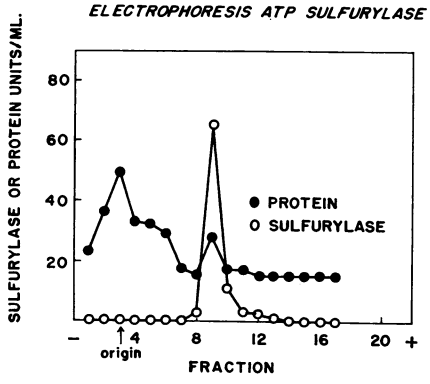


Fig. 6. Electrophoresis in a Geon 426 (Goodrich) bed (see Fig. 8).

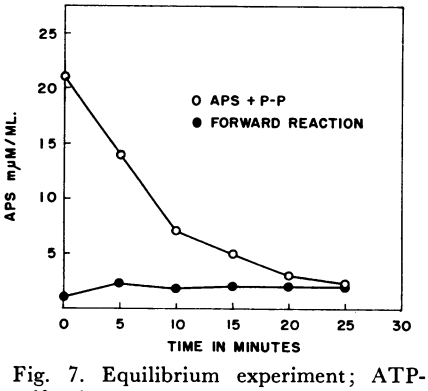


Fig. 7. Equilibrium experiment; ATP-sulfurylase.

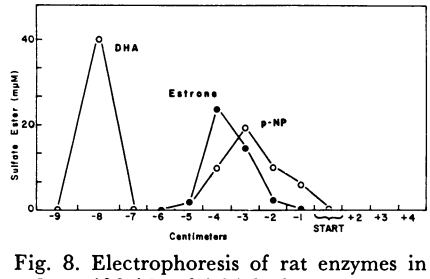


Fig. 8. Electrophoresis of rat enzymes in a Geon 426 (Goodrich) bed.

droitin sulfate, and from rat liver and brain for synthesis of sulfatide, but many needed details are still missing.

For orientation, I would like to refer once more to Fig. 1, in which it may be seen that, after resolving the left and middle part of the scheme, which concerns the generation of active sulfate, we now move into the nearly virgin territory of sulfate utilization outlined in the right-hand part of the figure.

Conjugation of Steroids and Phenols

This reaction has been studied by DeMeio (*12*), and by Roy in England (*13*), and we have used the latter's method for the estimation of sulfurylated steroids. Y. Nose, from the University of Kyoto, has worked in my laboratory on the separation of the enzymes responsible for the conjugation of phenols and polycyclic acceptors, using mainly electrophoresis of a prepurified liver preparation. It may be seen in Fig. 8 (*14*) that by this means three enzyme fractions were obtained, one for dihydroandrosterone and other 3′-β-OH steroids. Another enzyme conjugates with estrone and is different from ordinary phenol-sulfokinase. The last-named, however, seems to accept sulfate relatively unspecifically on a great variety of variously substituted phenols, as elaborated in greater detail by John D. Gregory (*15*). The dihydroandrosterone sulfokinase also reacts with isoandrosterone and with progesterone, all having a 3′-β-hydroxyl. It therefore appears to be a 3′-β-hydroxysteroid sulfokinase.

It should be mentioned that in nitrophenyl sulfate, the sulfate group was shown by John Gregory (*15*) to be of a group potential only less than 2000 calories below that of PAPS; a PAP-mediated sulfate transfer from nitrophenol to phenol has been studied with phenol-sulfokinase. Nitrophenyl sulfate was also shown, by Egami and his collaborators (*16*), to act as sulfate donor in a sulfatase-catalyzed, PAP-independent reaction, which reminds one of the rather outstanding activity of nitrophenyl phosphate for donating phosphate unspecifically with phosphatase (*17*).

Synthesis of Chondroitin Sulfate

The structure of chondroitin sulfate is presented in Fig. 9, for orientation. After unsuccessfully trying various cartilage preparations, which had been shown by other workers to incorporate radioactive sulfate in chondroitin sulfate in in vivo or in slice experiments, we turned to embryonic cartilage from 15-day-old chick embryos, which yielded rather active nonparticulate extracts. Figure 10 shows that active sulfate was formed in such an extract, together with small amounts of the precursor APS; in this experiment, the charcoal absorbate of the incubate was eluted with pyridine and then put on the paper for electrophoresis.

Chondroitin sulfuric acid was isolated by the usual methods of precipitating

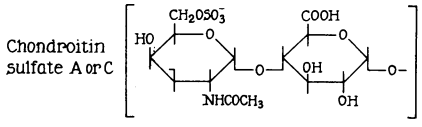


Fig. 9. Repeating unit of chondroitin sulfate.

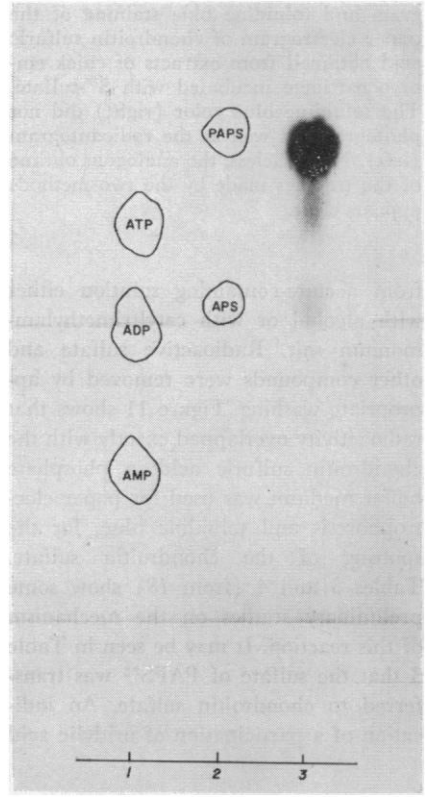


Fig. 10. Radioautogram of paper electrogram from $SO_4^{35=}$ incubated cartilage extract. Cartilages from two embryos were homogenized in 3 ml of saline phosphate to which 12 μmole of Mg^{++}, 10 μmole of Na-ATP, 1.5 μmole of UTP, 2 μmole of glutamine, 2.7 ml of enzyme, and 2 ml of $SO_4^{35=}$ (100 μc, carrier-free) were added; the total volume was 5.3. The mixture was incubated for 2 hours at 37°C. Markers electrographed on the same paper strip are identified in radioautograms Nos. 1 and 2 by ultraviolet quenching, as indicated by encircling lines. Number 3 represents the radioautogram of paper electrogram in citrate buffer (*p*H 5.9) of the pyridine eluate from a charcoal adsorbate of incubate.

Fig. 11. Comparison between radioautogram and toluidine blue staining of the paper electrogram of chondroitin sulfuric acid obtained from extracts of chick embryo cartilage incubated with S^{32}-sulfate. The toluidine blue color (right) did not photograph as well as the radioautogram (left). Nevertheless, the analogous outline of the tracings made by the two methods appears clear.

from acetate-containing solution either with alcohol or with cetyltrimethylammonium salt. Radioactive sulfate and other compounds were removed by appropriate washing. Figure 11 shows that radioactivity overlapped exactly with the chondroitin sulfuric acid; a phosphate buffer medium was used for paper electrophoresis and toluidine blue, for the spotting of the chondroitin sulfate. Tables 3 and 4 (from *18*) show some preliminary studies on the mechanism of this reaction. It may be seen in Table 3 that the sulfate of $PAPS^{35}$ was transferred to chondroitin sulfate. An indication of a participation of uridylic acid in the synthesis may be seen in the slightly better activation, in this case, when UTP was used instead of ATP. In other experiments, however, UTP was often inhibitory. Table 4 shows that with these extracts, ATP and magnesium were necessary for chondroitin sulfate synthesis from inorganic sulfate. This was also true if isolated PAPS was used, confirming the *de novo* synthesis of polysaccharide. Further evidence for *de novo* synthesis of chondroitin sulfate in these extracts was obtained by showing incorporation of radioactive acetate.

A great deal remains to be done. The polysaccharide field is a vast area of chemistry, and I have to confess that it is not our intention to become polysaccharide chemists. However, we will try to stay with this type of reaction for a little while, since it has been forced on us through our progress with sulfate activation. For similar reasons, we ventured also into the equally complex field of lipid synthesis. Irving Goldberg is studying in vitro incorporation of sulfate into lipid fractions. He has obtained preliminary evidence of a formation in liver extracts and brain homogenates of a sulfatide similar to Blix's cerebroside sulfate (*19*), shown in Fig. 12. This problem seemed attractive to me mainly because we might, from present experience, anticipate here a direct transfer of a galactose sulfate derivative to ceramide. This would present a simpler metabolic sequence than chondroitin sulfate synthesis, which is complicated by the polymerization problem. That, however, makes the latter all the more challenging.

Finally, I would like once more to underline the fact that we are dealing, in the area of sulfate transfer, with a process in which a central activated

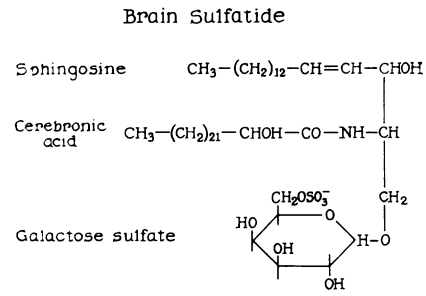


Fig. 12. The structure of cerebroside sulfate according to Blix (*19*).

molecule is first elaborated. This carries the sulfate in activated form, and from there, sulfate is picked up by quite a large number of separate acceptor enzymes which we call sulfokinases. This is a repetition of a rather general scheme in biosynthesis. It has been well defined in the case of acetyl activation and transfer (*20*) and, of course, with phosphate activation and transfer, and it seems to apply in many other cases of group transfer.

Table 4. Incorporation of $S^{35}O_4^=$ into chondroitin sulfate. Condyles from tibias and femurs of three chick embryos were broken up in deep-freeze-cooled mortar with quartz sand, with 6 ml of saline and 0.01*M* phosphate (*p*H 7.4), and centrifuged at 6000*g* (International centrifuge) for 10 minutes. The supernatant was mixed with 1.2 ml of carrier-free $S^{35}O_4^=$ (60 μc). There was 1 ml of enzyme-S^{35} mixture in each sample; final volume was 1.35 ml.

Sample No.	Incubation (37°C) (hr)	ATP 5 μmole	Mg 6 μmole	Glutamine 1 μmole	UTP 1 μmole	Counts/min	%
1	0	–	–	–	–	60	
2	2	–	–	–	–	60	
3	2	+	–	–	–	405	
4	2	+	+	–	–	4760	0.6
5	2	+	+	+	–	5390	0.7
6	2	+	+	+	+	2007	0.28

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2. The following abbreviations have been used in this article: ADP, adenosinediphosphate; AMP, adenosine monophosphate; APS, adenosine-5′-phosphosulfate; ATP, adenosine triphosphate; CoA, coenzyme A; P, inorganic phosphate; PAP, 3′,5′-diphosphate; PAPS, adenosine-3′-phosphate-5′-phosphosulfate (active sulfate); PP, pyrophosphate; S, sulfate; UTP, uridine triphosphate.
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