lecithin as substrate, the presence of a lecithinase (presumably a phospholipase A) has been demonstrated in homogenates of the GL, S, and W strains of *Tetrahymena*.

These results suggest that the *Tetrahymena* agent(s) which inhibits electron transport, uncouples phosphorylation, and stimulates latent ATPase of rat-liver mitochondria is a fatty acid(s) (and possibly a lysophosphatide) originating from the breakdown of particulate phospholipid(s) by the protozoan phospholipase. A comparison of the patterns of inhibition of various liver and *Tetrahymena* respiratory enzymes produced by oleic acid, lysolecithin, and the protozoan preparation, as well as other studies—all of which will be detailed elsewhere—supports this view. Thus it appears that the inhibitor(s) from *Tetrahymena* is similar to the uncoupling agents extracted by organic solvents from the mitochondria of blow-fly thoraces[4], wax-moth larvae[5], and rat liver[6], and from mitochrome[7].

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The enzymic degradation of heparitin sulfate

Extracts of flavobacteria had been found to degrade various mucopolysaccharides[1]. When the organisms were adapted to either heparin or heparitin sulfate, the "induced enzyme" degraded both substrates[2]. Heparitin sulfate resembles heparin in optical rotation and composition but has only one sulfate group per disaccharide unit. Approximately one-half of the hexosamine units are N-sulfated, the other half are N-acetylated[2].

It has been observed in all cases where "induced enzyme" was used that a loss of carbazole reaction for uronic acid accompanied the hydrolysis. In the case of hyaluronic acid and the chondroitin sulfates, this was shown to be due to the formation of an α-keto acid[3].

When heparitin sulfate was degraded by "induced enzyme", paper chromatography showed the presence of four major products (Fig. 1). The fastest moving component appeared to be N-acetylglucosamine. The three other components, Compounds I, II and III (Fig. 1) were isolated from a cellulose column[4] using butanol–acetic acid–water (50:12:25) as eluant. From 650 mg of a digest, 100 mg N-acetylglucosamine, 200 mg Compound I, 50 mg Compound II and 120 mg Compound III were

obtained. None of these compounds gave a significant carbazole reaction for uronic acid. On paper chromatograms, they could be detected by $AgNO_3$[5] but not by Ehrlich's[6] or ninhydrin[7] reagents.

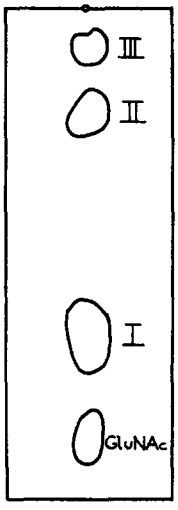

Fig. 1. Reproduction of a chromatogram of a heparitin sulfate digest. Solvent: butanol–acetic acid–water (50:12:25). GluNAc corresponds to N-acetylglucosamine.

Compound I: This material gave the semicarbazide and O-phenylenediamine reactions[3] for α-keto acids. The semicarbazone had the same ultraviolet spectrum as the semicarbazone of the α-keto acid derived from hyaluronate[3]. On paper chromatography in 2 solvent systems, the compound of Spot I had the same R_F value as the α-keto acid derived from hyaluronate.

Compound II: This compound migrated at the same rate as authentic[1] 2-deoxy-2-sulfoamino-D-glucose[8] in 2 solvent systems and on paper electrophoresis in borate buffer. The compound isolated as the barium salt gave the following analyses: hexosamine[9], 47 %; sulfate[3], 23 %, molar ratio sulfate to hexosamine, 1.1. After this compound had been heated to 100° for 1.5 h in 0.04 *N* HCl, paper chromatography showed that it was largely converted to glucosamine.

Compound III: This is also the slowest moving component in a butanol–ethanol–water solvent. However, when cetylpyridinium chloride was added to this solvent, a reversal of rates resulted with Compound III migrating more rapidly than Compound II indicating a higher negative charge[10]. The compound isolated as the barium salt gave the following analyses: heoxsamine, 29 %; sulfate, 33 %, molar ratio of sulfate to hexosamine 2.1. When this compound was heated at 100° for 1.5 h in 0.04 *N* HCl and chromatographed, a new spot, migrating slightly faster than Compound II, appeared. This spot could be detected by $AgNO_3$, ninhydrin or Ehrlich's reagents. A small amount of material corresponding to this new spot was isolated from a cellulose column and gave the following analyses: hexosamine (by ninhydrin[11]), 40 %; sulfate, 21 %, molar ratio sulfate to hexosamine 1.0. These data indicate that this compound is a glucosamine O-sulfate. Compound III was incubated with a glycosulfatase from marine snails (presumably Littorina). When the digest was

chromatographed, a spot with the same R_F value in 2 solvents as Compound II (*i.e.*, glucosamine N-sulfate) was detected.

These data indicate that Compound III is a glucosamine disulfate with one of the sulfates on the amino group and the other esterified with one of the available hydroxyls.

Some interesting conclusions about the structure and degradation of heparitin sulfate can be drawn from these data. The breakdown mechanism appears to be similar to that of other mucopolysaccharides degraded by "induced" flavobacterium enzyme, proceeding via unsaturated uronides[3]. Uronic acid color reaction is lost concomitant with hydrolysis, and acetylglucosamine, sulfated hexosamines and α-keto acid are the major degradation products. The presence of a disulfated glucosamine as a major product shows that the structure of heparitin sulfate is closely related to heparin. Since heparitin sulfate contains about 16 % sulfate and most of the N-sulfated glucosamine is also O-sulfated, the N-acetylated glucosamine moiety must be mainly sulfate-free.

A tentative structure of heparitin sulfate would be a polymer with a section containing repeating units of D-glucosamine disulfate and D-glucuronic acid. To this a sulfate-free portion, containing repeating units of N-acetylglucosamine and D-glucuronic acid, would be attached. This structure is also supported by studies on N-desulfated heparitin sulfate[2] which are in progress. When the N-desulfated compound (containing about 7 % sulfate) was incubated with the flavobacterium enzyme, the increase in reducing value was about one-half of that obtained with heparitin sulfate itself. When this digest was dialyzed, the non-dialyzable portion (about 40 % of the total) had a sulfate content of 15 %. A chromatogram of the dialyzable portion showed the presence of Compound I and acetylglucosamine only. The enzyme, apparently unable to hydrolyze the bonds at the free amino positions, had split the N-acetylated moieties, leaving a core containing repeating units of uronic acid and glucosamine O-sulfate. This finding, suggests the possibility of a branched structure of heparitin sulfate.

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