Conclusions. The method of Zlatkis, Zak, and Boyle for the direct determination of serum cholesterol has been adapted to the determination of total adrenal cholesterol. The values thus obtained do not differ significantly from those obtained by the method of Sperry and Webb in normal, hypophysectomized and cold stressed animals. The time required to analyze 10 samples and 5 standards by the Zlatkis, Zak, and Boyle procedure requires less than one hour compared to the 2 days usually necessary when methods based on the Liebermann-Burchard reaction are utilized.

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Hyaluronic Acid Formation by *Streptococcus pyogenes.** (21282)

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Previous work(1) with 4 encapsulated strains of *S. pyogenes* demonstrated that these microorganisms form from 30 to 50 mg of hyaluronic acid per 100 ml of culture when grown for 12 hours in 1% glucose infusion broth. When galactose is substituted for glucose as the carbon source only 5 to 10 mg of the capsular polysaccharide can be detected per 100 ml of culture in the same period of time. That the substance found in such cultures is hyaluronic acid is indicated by our observation that it is susceptible to hydrolysis by both streptococcal and testicular hyaluronidases and that it precipitates under the same conditions as does the material from the glucose-containing cultures.

It has been reported(2,3) that the intact carbon skeleton of glucose is incorporated into the structure of hyaluronic acid by *S. pyogenes* in glucose-containing media. No data are available on the mechanism of capsule synthesis in the presence of galactose but certain factors which influence the production of hyaluronic acid in both glucose and galactose broth cultures of *S. pyogenes,* strain S23, will be reported here.

Methods. Organism S. pyogenes, strain S23, a type 14 mucoid organism, was used throughout. *Culture Media* Two and one-half per cent heart infusion broth (Difco) supplemented with 0.5% neopeptone (Difco) and 1.0% sugar was employed. The medium was adjusted to pH 7.5, diluted to 98% of final volume and autoclaved. Sufficient sterile 10% $NaHCO_3$ was then added to bring the medium to volume. The final pH, before inoculation, was approximately 7.8. *Cultivation* Culture stocks were maintained on coagulated whole egg slants with transfers at monthly intervals. Blood agar plates were streaked periodically to detect dissociation. This strain displayed a uniform capacity to synthesize hyaluronic acid when maintained under these conditions. Inocula, consisting of the centrifuged cells from 17 hour cultures in the standard medium modified to contain 0.2% glucose, were used in the hyaluronic acid formation studies. The volume of each inoculum culture was one-tenth that of the medium to be inoculated. Incubation of all cultures was at 37° C. *Quantitative Determinations* For the estimation of bacterial nitrogen and hyaluronic acid, two 5 ml aliquots of each culture were removed and centrifuged. The supernates were carefully withdrawn, pooled, and the individual cell sediments washed once with 5 ml of saline. Nitrogen determinations were performed as previously reported(1). The pooled supernates were heated in a water bath at 60° C for 45 minutes to remove trace amounts of

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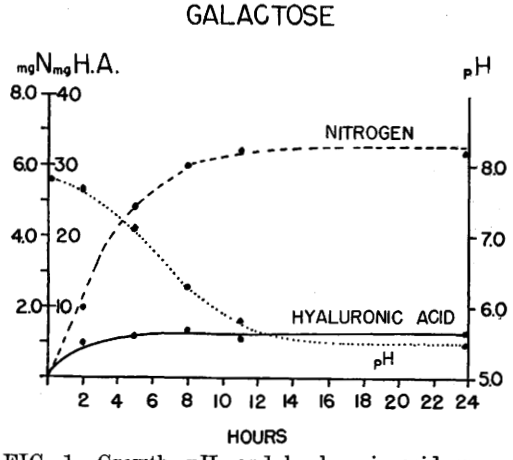

FIG. 1. Growth, pH, and hyaluronic acid curves of *S. pyogenes* strain S23 in 1% galactose infusion broth. Values per 100 ml of medium.

hyaluronidase activity. The pH was then adjusted to 6.0 and 0.1 ml of 1% merthiolate added as a preservative. Such supernates, when stored at 4° C, gave reproducible hyaluronic acid values for at least 6 weeks. Hyaluronic acid was measured by the procedure previously described(4).

Experimental. Rate of hyaluronic acid formation in glucose and galactose broths. To determine the approximate rate of formation of the capsular material successive samples, during a 24 hour period, were analyzed for hyaluronic acid and bacterial nitrogen. The hydrogen ion concentration of the cultures was also measured at each sampling period. The curves thus obtained are plotted in Fig. 1 and 2. As shown in the figures, growth is more vigorous in glucose broth and is accompanied by an earlier fall in pH. In the glucose cultures an inhibitory hydrogen ion concentration is reached after about 5 hours, whereas in galactose broth some growth is still detectable at 8 to 10 hours. The rate of hyaluronic acid synthesis in glucose broth is maximal as the pH falls from about 7.0 to below 6.0, and continues at a slower rate until growth ceases due to the accumulation of acid. In galactose broth, polysaccharide formation is rapid only during the first 2 hours, before the pH has fallen to 7.0. After 2 hours, when the pH is below 7.0, there is little or no synthesis of capsular material.

Hyaluronidase formation. Pike(5) has reported that some streptococcal strains which synthesize capsular material during the early hours of growth in broth media, later destroy soluble capsular substance as a consequence of hyaluronidase formation. Likewise Sallman (6) has shown that many *S. pyogenes* strains may produce at least small amounts of the enzyme, if grown under suitable conditions. Thus, the low concentrations of hyaluronic acid encountered in galactose broth cultures could result from the activity of hyaluronidase elaborated by the organisms during growth. Hyaluronidase activity was measured as follows: At various intervals during the first 24 hours of growth, 10 ml samples were withdrawn, centrifuged, and the supernates adjusted to pH 6.0. Merthiolate was added to give a final concentration of 0.01%. This concentration has been shown not to inhibit hyaluronidase activity(7). The sample was then divided into 2 equal parts. One was heated at 60° C in a water bath for one hour to inactivate the enzyme. The heated and unheated samples were then incubated in rubber-stoppered tubes at 37° C for 5 days, after which time hyaluronic acid determinations were made. The results are presented in Table I. It appears unlikely, on the basis of these results, that the amount of enzyme produced by this strain in galactose broth is sufficient to account for the reduced capsular polysaccharide levels found in such cultures after 24 hours of incubation.

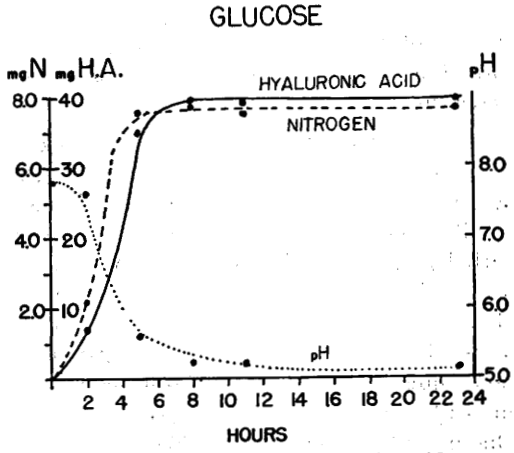

FIG. 2. Growth, pH, and hyaluronic acid curves of *S. pyogenes* strain S23 in 1% glucose infusion broth. Values per 100 ml of medium.

TABLE I. Hyaluronidase Production in Glucose and Galactose Broths.

Time of sampling, hr	Hyaluronic acid (mg/100 ml)			
	Glucose		Galactose	
	Heated supernate	Unheated supernate	Heated supernate	Unheated supernate
2	6	6	5	5
5	32	32	6	2
8	37	36	7	2
11	37	37	6	1
23	37	36	6	1

Effect of pH on hyaluronic acid formation in galactose broth. Inspection of Fig. 1 and 2 shows that hyaluronic acid synthesis in galactose broth occurs almost exclusively before the reaction of the medium has dropped below pH 7.0. The utilization of galactose for hyaluronic acid production might therefore involve at least one pH dependent metabolic step which occurs readily only at an alkaline reaction. To examine this hypothesis heavily inoculated cultures of strain S23 were grown for 6 to 8 hours in mechanically stirred galactose broth at either pH 7.8-8.0 or pH 6.6-6.8. The hydrogen ion concentration was determined with a glass electrode at 15 to 30 minute intervals and pH changes in the alkaline cultures controlled by the addition of sterile 6 N NaOH. Large amounts (35 mg/100 ml) of polysaccharide were formed in control glucose broth cultures at pH 7.8-8.0. No quantitative differences in the production of polysaccharide on galactose were found at the two pH ranges tested although growth was satisfactory. It appears therefore that hydrogen ion concentration is not an important factor controlling the formation of polysaccharide in galactose broth under our conditions.

Use of endogenous cell substance; occurrence of dissociation. If the hyaluronic acid synthesis observed in galactose broth cultures results from utilization of internal reserves of carbohydrate then the exhaustion of such reserves should result in cessation of polysaccharide synthesis. To examine this possibility, strain S23 was transferred concurrently at 24 hour intervals through 7 consecutive cultures in 0.2% glucose and 0.2% galactose broths. One milliliter inocula were added to 49 ml of media at each transfer, and blood agar plates were streaked to detect dissociation. After the seventh transfer, the galactose- and glucose-propagated organisms were each inoculated into 1% glucose and 1% galactose broths for hyaluronic acid and bacterial nitrogen determinations. No quantitative differences were observed in the formation of hyaluronic acid in galactose broth between the first and seventh transfer. In addition it was found that cells kept in galactose broth continuously for one week were still able to form capsular substance in either medium. It appears unlikely on the basis of these observations, that the polysaccharide found in galactose culture supernates is produced from reserve cellular material. Appreciable dissociation to the glossy phase did not occur during transfer in either medium.

Utilization of an unidentified medium component. Use of E. coli-fermented broth. In galactose broth cultures hyaluronic acid may be synthesized from some unidentified component or components present in the medium in limited concentration. That the reducing sugar of the heart infusion is not involved was demonstrated by the use of infusion broth exhaustively fermented with *E. coli.* Strain S23 either does not grow or grows only to a very limited extent in such a medium unless sugar is added. When 1% of galactose is supplied, growth and hyaluronic acid production occur in the usual manner. In a typical experiment the medium, without added sugar, was fermented with *E. coli* for 48 hours at 37° C and residual reducing sugar determined as glucose by the method of Nelson(8). The reducing sugar of the medium was reduced from 20 mg/100 ml to 2.3 mg/100 ml by this procedure. The nature of the residual carbohydrate has not been determined. On a quantitative basis this small amount of carbohydrate could not account for the amount of capsule produced. Attempts to re-inoculate *S. pyogenes* into fermented galactose containing broth, even after neutralization and supplementation with galactose, always resulted in failure of the organism to grow.

Modification of the medium. The galactose medium was modified in a number of ways in an attempt to establish a correlation between hyaluronic acid production and one of the medium constituents. Protein hydrolysate was

TABLE II.
Effect of Modifications of Galactose Broth Medium upon Hyaluronic Acid Formation.

Heart infusion (%)	Neopeptone (%)	Hydrolysate* (%)	Galactose (%)	Hyaluronic acid (mg/100 ml)	Bacterial nitrogen (mg/100 ml)	H. acid / N ratio
			Experiment 1			
4.0		.5	1.0	8	6.7	1.2
2.0		.5	1.0	4	5.0	.8
.5		2.0	1.0	2	4.6	.4
.2		2.0	1.0	1	2.5	.4
.1		2.0	1.0	0	2.1	—
2.5	.5		.1	2	3.0	.7
2.5	.5		1.0	6	5.4	1.1
2.5	.5		3.0	10	7.4	1.4
2.5	.5		4.0	9	6.2	1.5
			Experiment 2			
.2		2.0	4.0	5	1.8	2.8
.8		2.0	4.0	11	3.1	3.5
1.6		2.0	4.0	13	5.3	2.5
4.0		2.0	1.6	11	3.1	3.5
4.0		2.0	.8	11	6.0	1.8
4.0		2.0	.2	5	3.9	1.3

* Tryptic digest of casein.

used because of its relative freedom from carbohydrate. As indicated in Table II, no clear-cut relationship between any of the medium constituents and hyaluronic acid synthesis could be found. One could infer from these data that for maximal growth and capsule formation, heart infusion and a utilizable source of energy such as galactose must both be present in fairly high concentration.

Discussion. The experimental data reported here favors, in general, the idea that galactose is not utilized for capsule synthesis. The fact that increased concentrations of heart infusion stimulate hyaluronic acid formation supports the possibility that a constituent of the infusion other than glucose may be the source of raw material for the synthesis of the polysaccharide. We have at present no indication of the nature of this constituent.

Crowley(9,10) has demonstrated amylase and "depolymerase" activity in broth cultures of certain serologic types of Group A streptococci. Various substrates were hydrolyzed, including starch, inulin, citrus pectin, bacterial dextrin, and glycogen. In general there was a direct correlation between these activities and hyaluronidase production. We have found slight hyaluronidase activity in supernates of galactose but not glucose broth cultures of strain S23. It is possible that some amylase and/or depolymerase activity may occur during growth in this medium, leading to degradation of infusion carbohydrate of high molecular weight with the release of smaller units utilizable for capsule synthesis.

Summary. 1. *S. pyogenes,* strain S23, grows rapidly in glucose broth, forms large amounts of hyaluronic acid throughout the growth period and induces a rapid fall in the pH of the medium. In galactose broth growth is slower, polysaccharide formation is diminished, occurring only during the first 2 or 3 hours of growth and the reaction of the medium remains above pH 7.0 for a much longer period of time. 2. While no hyaluronidase could be detected in glucose culture supernates, a small amount of activity was detected in galactose supernates. It was not of sufficient magnitude to account for the low levels of hyaluronic acid in galactose cultures. 3. Maintenance of galactose broth cultures at pH 7.8-8.0 did not increase hyaluronic acid formation. 4. It has been shown that neither endogenous cell substance nor infusion reducing sugar serve as raw materials for capsule synthesis in galactose broth. 5. A direct correlation between hyaluronic acid formation and galactose concentration could not be established. 6. From the data obtained, it seems likely that galactose is not directly involved in hyaluronic acid

formation, but that an unidentified component of the infusion is utilized for this purpose.

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Ethionine-Induced Depression of Plasma Antihemophilic Globulin in the Rat. (21283)

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To date there have been few studies relevant to the origin of antihemophilic globulin (AHF). Boldyreff(1) reported some work with dogs from which he concluded that the pancreas produced AHF. In these studies, either pancreatic fistula or complete pancreatectomy was followed by a prolongation of the whole blood coagulation time; however, no demonstration of the nature of the clotting defect was made. Graham and associates(2) produced acute liver injury in dogs by chloroform intoxication. In these animals AHF was well preserved despite a degree of liver damage which caused almost complete depletion of prothrombin and fibrinogen. Moreover, it was found that dicumarol was unable to depress AHF even at a dosage sufficient to produce severe hypoprothrombinemia. These studies would seem to exclude the liver as the source of AHF, in contrast to its role in the synthesis of most clotting factors. This same group of investigators showed that AHF was unaffected by heavy total body X-irradiation(3), indicating that the cells which produce AHF must be relatively radio-resistant.

Ethionine, the ethyl analog of methionine, is a metabolic blocking agent which interferes with the synthesis of certain proteins and produces striking histological changes in some tissues(4-6). In the studies to be reported, it was found that this drug produced a profound depression of plasma AHF levels in rats. The tissues which exhibited damage were interpreted as suggested sites for further and more definitive investigation of a possible role in AHF production.

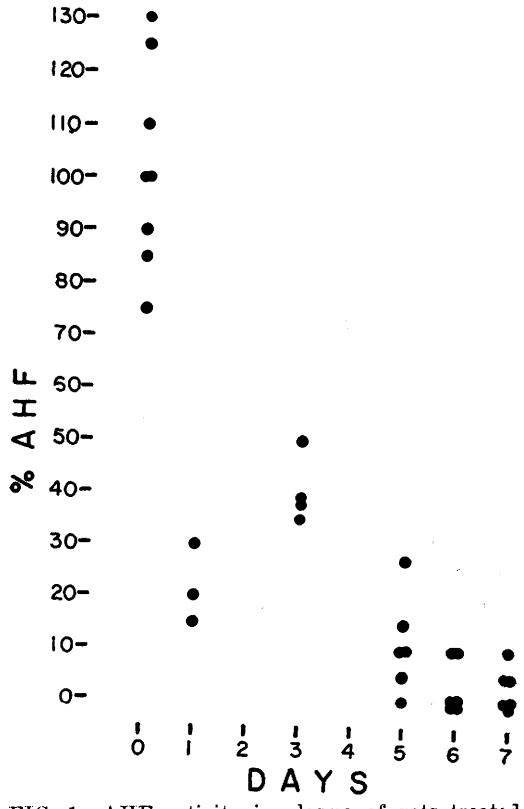

FIG. 1. AHF activity in plasma of rats treated with ethionine. Each dot represents one animal.

Methods and materials. Male albino rats