STUDIES ON THE BIOSYNTHESIS OF THE M-PROTEIN OF GROUP A HEMOLYTIC STREPTOCOCCI[1]

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A preliminary report of the biosynthesis of the M-protein by nonproliferating group A hemolytic streptococci was presented by Fox and Krampitz (1954). The M-protein is the somatic antigen which confers type specificity upon group A streptococci (Lancefield, 1940). The experiments presented now represent an attempt to define some of the conditions which are required for synthesis of M-protein.

The technique employed was to remove the M-protein from the surface of the streptococcal cells with trypsin and to study the factors responsible for the *de novo* synthesis of the protein. This approach was suggested by the experiments of Lancefield (1943) which showed that all traces of the M-protein were removed by trypsin or pepsin and that such streptococci remained viable and virulent for mice. Injection of these treated streptococci into mice gave rise to cells with a normal amount of the specific M-protein. The M-protein could also be restored by directly subculturing trypsin-treated streptococci into fresh broth.

One might infer from Lancefield's experiments that the resynthesis of the M-protein *in vivo* or *in vitro* was associated with cellular multiplication. In the experiments to be discussed, however, M-protein synthesis was initiated in nutritionally incomplete media in the absence of apparent multiplication; furthermore, bacteriostatic concentrations of penicillin did not inhibit the synthesis.

MATERIALS AND METHODS

Streptococci. Group A β-hemolytic streptococci, types 14 and 24, isolated from patients with respiratory infections at the Streptococcal Disease Laboratory, Warren Air Force Base, Wyoming, were used in these studies.

A stock supply of frozen streptococci was maintained in vials containing brain-heart infusion (henceforth referred to as BHI) and 2 per cent defibrinated sheep blood. The contents of one of these vials containing 1 ml were added to 12 ml of BHI and incubated at 37 C for 12 hr. The latter 12-ml culture was used as an inoculum for larger volumes of BHI varying from 200 to 400 ml incubated at 37 C for 14 hr.

Antisera. Type-specific anti-M sera were prepared in rabbits with heat-killed streptococci according to the procedure of Lancefield (1947).

Trypsin. Initially, experiments were performed with a crude trypsin preparation called, "Trypsin, 1–300" obtained from Nutritional Biochemical Corp., Cleveland, Ohio. In later experiments twice crystallized trypsin containing 50 per cent $MgSO_4$ (obtained from the same company) was employed.

Trypsin inhibitor. Five times recrystallized soybean trypsin inhibitor obtained from the Worthington Biochemical Corp., Freehold, New Jersey, was used to inhibit the residual trypsin.

Amino acids. Acid hydrolyzed casein was used as a source of amino acids. This material, obtained from the Nutritional Biochemical Corp. as a 10-per cent solution of "vitamin-free" acid hydrolyzed casein, was evaporated to a syrup on a steam bath under a stream of air to remove some of the hydrochloric acid. The syrup was dissolved in water, neutralized with 20 per cent KOH, and treated with a small amount of activated charcoal to remove colored material. The concentrated solution was diluted to the original volume and stored in the refrigerator. Before use, aliquots were heated on a steam bath to redissolve any precipitated amino acids.

Polypeptides. A 100-ml solution of 10 per cent Casitone (a pancreatic digest of casein prepared by Difco) was dialyzed against running tap water for 48 hr. A small amount of toluene was added as

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a preservative. The nondialyzable material was further dialyzed against 4 or 5 L of distilled water for another 48 hr. The residue was concentrated to a small volume on a steam bath under a stream of air and dialyzed once more against distilled water for 24 hr. Insoluble material was removed by centrifugation and the soluble fraction was lyophilized. Ten grams of Casitone yielded 400 to 500 mg of "polypeptide" material. Longer dialysis resulted in only slightly smaller yields.

Preparation of streptococci for M-protein synthesis. In a typical experiment the bacterial cells from 300 ml of a 14-hr culture were removed by centrifugation and suspended in 100 ml of 0.01 M $NaHCO_3$ containing 2.5 mg of crystalline trypsin and incubated in a 37 C water bath with occasional stirring for 30 min. After digestion with trypsin, the cells were centrifuged and washed twice with 150 ml of 0.05 M sodium phosphate buffer, pH 7.0, to free them of trypsin. In spite of these two washings, traces of trypsin were carried along either as insoluble material or adsorbed to the cells. Additional washings did not remove the trypsin. To prevent the residual trypsin from interfering with subsequent protein synthesis, soybean trypsin inhibitor (2.5 mg per 35 ml) was added to the various media in which the streptococci were resuspended. After trypsinization and washing, the suspension was divided into 6 equal portions and each was used for a separate experiment.

Most of the M-regeneration experiments with trypsinized cells were carried out for 3 hr at 37 C. At the end of this time the cells were removed by centrifugation and suspended in 0.7 ml of 0.2 N HCl containing 0.9 per cent NaCl and heated in a boiling water bath for 10 min to solubilize the regenerated M-protein. The M-protein content of neutralized acid extracts with specific antisera was measured by the capillary precipitin method described by Swift, Wilson and Lancefield (1943). The precipitin reactions were graded from negative to plus four; doubtful reactions were given the notation, "±."

Manometric measurements. Large Warburg vessels (135 ml) were employed for manometric experiments because it was observed that fluid volumes less than 15 or 20 ml had an inhibitory effect on M-protein synthesis although respiration and fermentation were not so affected. The cells harvested from 50-ml aliquots of BHI were treated with crystalline trypsin and washed as previously described. These cells were suspended in 2 ml of distilled water in the side arm of the cups and tipped into 33 ml of fluid in the main compartment. At the end of a manometric experiment the cells were removed and the M-protein extracted. Rapid shaking also had an inhibitory effect on M-protein synthesis. This inhibition was overcome by decreasing the amplitude of the shaking apparatus to about one-third of the normal distance. Under these conditions the equilibration of gases for manometric measurements was only slightly retarded. The inhibitory effect of rapid shaking could not be explained.

Lactic acid was measured by the method of Barker and Summerson (1941).

Optical density determinations on cell suspensions were performed with a Klett-Summerson photoelectric colorimeter with a 660 mμ filter.

RESULTS

The effects of trypsin on the M-protein, growth, and viability. Two-thirds of a 200-ml culture of type 14 streptococcus were treated with crude trypsin and washed as described. The other one-third was retained on ice as an untreated control. One-half of the treated cells was stored on ice as a treated control. The other half was suspended in 75 ml of BHI containing 5 per cent defibrinated sheep blood and incubated at 37 C for 90 min. At the end of the incubation period extracts were prepared from each of the three portions of streptococci and assayed for their M-protein content. The results of such an experiment are shown in table 1. It can be seen that during the 90-min incubation the M-protein was almost restored to the concentration associated with untreated cells.

TABLE 1

Synthesis of the M-protein in blood broth

Extracted Antigen Diluted	Precipitin Reaction, Original 1/3 Culture Untreated	Precipitin Reaction, Trypsin-treated Cells Retained on Ice	Precipitin Reaction, Trypsin-treated Cells Incubated in Blood Broth
Undiluted	++++	−	++++
1:10	++	−	++
1:100	+	−	−
1:1000	−	−	−

Type 14 streptococci were treated with 0.05% crude trypsin in 0.02 M phosphate, pH 7.5, and washed. No trypsin inhibitor added to resuspending media.

TABLE 2

Cell growth and M-protein synthesis in BHI

Incubation Time	Optical Density (Klett Units)		Precipitin Reaction, Undiluted Extract	
	Trypsin treated cells	Cells treated with boiled trypsin*	Trypsin treated cells	Cells treated with boiled trypsin
Min				
0	46	30	−	++++
30	63			
60	79	50	+++	++++
90	81			
120	79	59	++	++++
180	75	57	+	++++

Type 14 streptococci treated with trypsin, etc., as in table 1.

* Trypsin solution was boiled 5 min just prior to use.

The same type of experiment was repeated without blood in the BHI broth so that density changes could be measured during the course of the experiment. Cells incubated in a boiled trypsin solution were used as a control. The results are given in table 2. The optical density figures show an approximate 2-fold increase after 60 min. The net change was about the same whether trypsin or boiled trypsin was used. In the test where untreated trypsin was used, the greatest amount of M-protein was detected at the end of 1 hr. After that time, the amount of M-protein gradually decreased, probably due to the presence of residual trypsin which gradually destroyed the M-protein as it was synthesized. In later experiments this effect was eliminated by the use of trypsin inhibitor.

Influence of amino acids on M-protein synthesis. The preceding experiments demonstrated that the M-protein could be resynthesized on the streptococcal cells in a relatively short time when the organisms were growing under optimal conditions.

The influence of amino acids on the M-protein synthesizing system was next studied. Acid hydrolyzed casein with glucose as an energy source, with or without a supplement of purine and pyrimidine bases, did not support M-protein synthesis. Therefore, a semisynthetic medium was prepared from components suggested by a number of investigators who have studied the nutritional requirements of the hemolytic streptococci (McIlwain, 1940; Bernheimer *et al.*, 1942; Slade *et al.*, 1951). The composition of this medium is given in table 3. The organisms grew rather slowly in this medium although large inocula were used. The effect of the various components of this medium was studied. The results are summarized in table 4. The resynthesis of the M-protein occurred only in a complete medium.

TABLE 3

Components of the semisynthetic medium

Components	Amount per Liter	
Solution I		
Glucose (anhydrous)	25.0	gm
Solution II (Salts A)		
NH_4Cl	2.0	gm
KH_2PO_4	1.2	gm
K_2HPO_4	1.2	gm
Solution III (Salts B)		
$MgSO_4 \cdot 7H_2O$	0.4	gm
$FeSO_4 \cdot 7H_2O$	0.02	gm
$MnSO_4 \cdot 4H_2O$	0.04	gm
NaCl	0.02	gm
Solution IV ("AGUX")		
Adenine	20.0	mg
Guanine	20.0	mg
Uracil	20.0	mg
Xanthine hydrochloride	20.0	mg
Solution V (Vitamins)		
Thiamin hydrochloride	1.0	mg
Pyridoxine hydrochloride	2.0	mg
Pyridoxamine hydrochloride	0.6	mg
Pyridoxal hydrochloride	0.6	mg
Calcium pantothenate	1.0	mg
Riboflavin	1.0	mg
Nicotinic acid	2.0	mg
Para-aminobenzoic acid	0.2	mg
Biotin	0.002	mg
Folic acid	0.02	mg
B_{12}	0.75	μg
Solution VI		
Glutathione	2.0	mg
l-Glutamine	100.0	mg
Solution VII		
Amino acids (acid hydrolyzed casein; see Materials and Methods)	10.0	gm

The medium was prepared in double strength, steamed for 15 min, filtered to remove a slight precipitate which formed upon heating, and made up to full strength. The pH was adjusted to 7.3 before autoclaving for 15 min for final sterilization.

Under these conditions there was a small increase in optical density during a 4-hr incubation period.

The specificity of some amino acids in M-protein synthesis. The question then arose whether all the amino acids essential for the growth of streptococci or only those amino acids known to be present in the M-protein were required for synthesis of M-protein. It was previously shown by Fox and Krampitz (1953) that in various types of streptococci the M-protein contained little or no aromatic amino acids. The M-protein preparations did contain relatively large amounts of lysine and isoleucine. Both of these amino acids as well as tryptophan and phenylalanine were among 15-essential for the growth of hemolytic streptococci in a synthetic medium (Slade *et al.*, 1951). The resynthesis of the M-protein of streptococci should be contingent upon only those amino acids present in that protein and omission of aromatic amino acids from the resuspending medium should have little or no effect upon the synthesis.

TABLE 4

Components required for M-protein synthesis from free amino acids

Experiment Number	Medium	Incubation Time	Initial Density	Final Density	Precipitin Reaction, Undiluted Extract
		Min			
1	Complete	240	33	40	+++
2	Lacking solution VII (amino acids)	240	33	35	−
3	Lacking solution I (glucose)	240	31	31	−
4	Lacking solution IV (AGUX)	180	34	35	+
5	Lacking solution V (vitamins)	180	29	32	+
6	Lacking solutions IV and V	200			±
7	Lacking solutions IV and VI (AGUX and GSH)	200			±
8	Lacking solutions IV, II, and III (AGUX and salts A and B)	200			±

For each experiment, type 14 streptococci from 75 ml brain-heart infusion treated with crude trypsin as in table 1 and suspended in 100 ml of each medium. See table 3 for description of solutions.

TABLE 5

The specificity of four amino acids in M-protein synthesis

Medium and Supplement	Incubation Time	Initial Density	Final Density	Precipitin Reaction, Undiluted Extract
	Min			
Tryptophan assay medium	150	69	75	+++
Phenylalanine assay medium	180			+++
Lysine assay medium				
No lysine	150	75	75	+
10 mg l-lysine	150	72	84	+++
Isoleucine assay medium				
No isoleucine	180			+
10 mg l-isoleucine	180			+++

Type 14 streptococci treated with crude trypsin as in table 1 and suspended in 100 ml of each medium.

Difco media, prepared for the microbiological assay of lysine, isoleucine, phenylalanine, and tryptophan, were employed in this study. The results obtained when trypsinized streptococci were incubated in these media are shown in table 5. From these data it may be seen that media devoid of either tryptophan or phenylalanine had little or no effect on the resynthesis of the M-protein, whereas media devoid of lysine or isoleucine supported only a slight synthesis of M-protein. When lysine and isoleucine were added to the respective media, the yield of M-antigen was increased. These media when reconstituted with the missing amino acids supported only minimal growth of Group A streptococci over a long incubation period even when large inocula were used. These media did not support as much synthesis of M-protein as did a complete medium (table 1). Nevertheless these experiments demonstrated the expected fact that amino acids, especially those present in the M-protein, were required for regeneration of this protein.

The stimulatory effect of polypeptides. The work of Woolley (1941) suggested that a polypeptide or mixture of polypeptides such as strepogenin might be a stimulatory factor in protein synthesis.

TABLE 6

The requirements for polypeptides, amino acids, and glucose for M-protein synthesis

Experiment Number	Medium Containing Only:	Gm/100 Ml	Precipitin Reaction, Undiluted Extract
1	Amino acids Glucose Polypeptides	1.0 2.0 0.03	+++
2	Amino acids Glucose	3.0 2.0	±
3	Amino acids Polypeptides	1.0 0.03	+
4	Glucose Polypeptides	2.0 0.03	±

Type 14 streptococci from 100 ml BHI treated with crude trypsin, washed and suspended in 100 ml of each of the above media containing 0.05 M phosphate, pH 7.5, and incubated for 180 min at 37 C.

Therefore, the possible role of peptide intermediates in M-protein synthesis was explored.

A sample of polypeptides was prepared by prolonged dialysis of a pancreatic digest of casein (see Materials and Methods). These polypeptides were probably similar to the material briefly described by Slade (1951) as a growth factor of group A hemolytic streptococci. When added to the semisynthetic medium described in table 3, these polypeptides had a marked stimulatory effect on M-protein synthesis and streptococcal growth. On the assumption that the polypeptides exert their effect by by-passing some of the synthetic processes required for the synthesis of the M-protein in the semisynthetic medium, various components of the medium were omitted in successive experiments. Representative experiments of this type are shown in table 6. It is clear from these data that polypeptides, amino acids and glucose were the only substances required for M-protein synthesis. With these components no increase in optical density was observed during 3-hr incubation period. Experiment 2 in table 6 shows that three times the normal concentrations of amino acids did not replace the requirement for polypeptides under the given conditions.

In order to determine the specific amino acids required for M-protein synthesis, a mixture of 17 amino acids simulating those present in the Difco phenylalanine assay medium were prepared. By elimination it was found that only 8 amino acids (in the presence of glucose and polypeptides) gave near-optimum yields of M-protein with trypsinized streptococci. These 8 amino acids were alanine, arginine, glutamic acid, histidine, isoleucine, proline, threonine, and valine. All the amino acids essential for M-protein synthesis were, therefore, present either as free amino acids or within the polypeptides; the omission of one or more of the amino acids had a progressively diminishing effect on the yield of M-protein.

Properties of the polypeptides. A 1 per cent solution of the polypeptide mixture was stable to autoclaving at a neutral pH, gave a strong biuret color (80 per cent of the color of an equal amount of crystalline bovine albumin), was very weakly ninhydrin positive, and was not precipitated at 0 C in a final concentration of 10 per cent perchloric acid. The polypeptide material was very soluble in water but insoluble in 95-per cent alcohol. The ratio of the ultraviolet light absorption extinctions at 260 and 280 mμ in a

TABLE 7

The effects of inhibitors on M-protein synthesis and glycolysis

Experiment Number	Inhibitor	Precipitin Reaction Undiluted Extract	Micromoles Lactate Formed
1	No inhibitor	++++	560
2	2,4-Dinitrophenol (10^{-4} M)	+++	
3	Sodium arsenate (10^{-3} M)	++	540
4	Sodium fluoride (5×10^{-3} M)	+	178
5	Iodoacetic acid (5×10^{-4} M)	+	160
6	Penicillin (100 units/ml)	++++	
7	No inhibitor and no glucose	+	52

For each experiment, type 14 streptococci from 50 ml of BHI were treated with crystalline trypsin and washed. The cells were suspended in 35 ml 0.05 M phosphate, pH 7.0 containing 3.9 m M glucose, 1 per cent amino acids, 10 mg polypeptides, and 2.5 mg trypsin inhibitor. Incubation was for 180 min at 37 C.

neutral phosphate solution was 1.0, indicating that the material was relatively low in aromatic amino acids. The latter apparently were eliminated in the smaller dialyzable fragments produced by pancreatic digestion. Orcinol-positive material was less than 1 per cent as measured by the method of Morse and Carter (1949).

A number of chemical and physical techniques were applied in an attempt to purify the polypeptide material. Precipitation with alcohol, ammonium sulfate, tungstic acid, and calcium hydroxide, and chromatography with Dowex-50 and starch yielded fractions which were no more active than the original dialyzed material. Neither whole casein, crystalline bovine albumin, nor protamin could replace the casein polypeptides.

The action of inhibitors on M-protein synthesis and glycolysis. Since glucose was immediately and rapidly fermented by the trypsinized cells in the presence of amino acids and polypeptides, the effects of inhibitors upon glycolysis and M-protein synthesis were studied. These results are summarized in table 7. 2,4-dinitrophenol in a concentration of 10^{-4} M had only a slight inhibitory effect on the yield of synthesized M-protein. Sodium arsenate (10^{-3} M) inhibited M-protein synthesis although the lactic acid formed from glucose was the same as that formed in the absence of arsenate. When glucose was omitted (experiment 7), the amount of M-protein formed was small and approximately the same as in the presence of glucose plus arsenate. Since arsenate inhibits energy utilization from glucose its effect on M-protein synthesis may reflect the endergonic nature of the process. The common inhibitors of glycolysis, sodium fluoride and iodoacetic acid were both effective in inhibiting M-protein synthesis and decreasing the yield of lactic acid to about one-third of the normal amount.

Penicillin, in a concentration 1,000 times the amount required to inhibit the growth of a comparable amount of streptococci in BHI, was ineffective in inhibiting M-protein synthesis.

Rates of glycolysis measured as the displacement of CO_2 from bicarbonate buffer by lactic acid showed that the trypsinized streptococci decomposed glucose immediately and rapidly in the presence of polypeptides and amino acids. Only small amounts of M-protein were formed during the first hour; the maximum amount was synthesized between the second and third hour.

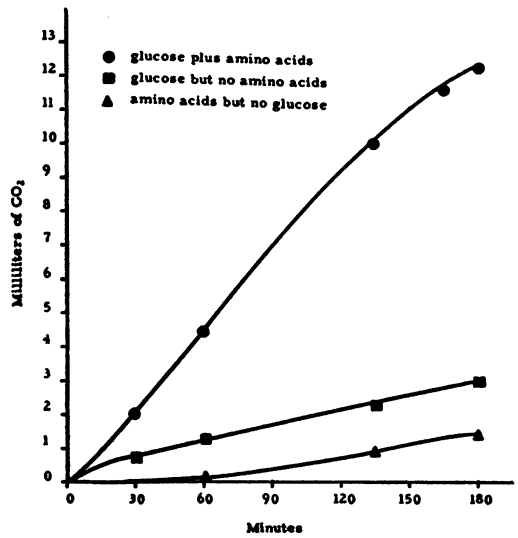

Figure 1. Trypsin-treated cells from 50 ml brain-heart infusion in 35 ml 0.05 M bicarbonate plus 0.025 M phosphate buffer, pH 7.8, containing 2.5 mg trypsin inhibitor and 10 mg polypeptides; supplemented with: ●, 3 mM glucose plus 1% amino acids; ■, 3 mM glucose but no amino acids; ▲, 1% amino acids but no glucose.

In the absence of amino acids, trypsinized streptococci would not ferment glucose much above the endogenous rate. Untreated streptococci fermented glucose at a rapid rate in buffer containing only glucose. This phenomenon is demonstrated by figure 1. The polypeptides would not substitute for or supplement the amino acids in reconstituting the glycolytic system. No evidence of fermentation was obtained when trypsin-treated or normal cells were suspended in amino acids alone.

Cell-free extracts of trypsin-treated streptococci were prepared by triturating the bacteria in a paste with Alcoa "alumina" A-301. The extracts would ferment glucose only after a 30 or 40 min lag. This lag period could be shortened to 10 or 15 min by the addition of amino acids. Without amino acids catalytic amounts of adenosine triphosphate or hexose diphosphate initiated immediate and rapid glycolysis. Substrate quantities of hexose diphosphate in the absence of glucose or amino acids were fermented without a lag period. Cell-free extracts of untreated streptococci fermented glucose immediately in the absence of amino acids or any added organic phosphate. These data indicate that the inhibition of glycolysis by trypsin was probably

due to a depletion of cellular high energy phosphate required to initiate the glycolysis and not to a destruction of glycolytic enzymes. The stimulatory action of amino acids on glycolysis could be due to some utilization of the amino acids in energy-yielding reactions.

DISCUSSION

The data presented indicate that trypsin-treated hemolytic streptococci require three components for the *de novo* synthesis of M-protein. These components are amino acids, glucose, (presumably as an energy source), and polypeptides. When the latter are omitted, vitamins, purines, pyrimidines and other compounds shown in table 3 must be substituted for the polypeptides. Even under these conditions the compounds of the semisynthetic medium do not completely supplant the polypeptides.

Under the conditions employed it is difficult to determine whether or not the biosynthesis of M-protein is associated with cellular multiplication. In view of the exacting nutritional requirements of the group A streptococci it is doubtful that multiplication occurred in those media employed which are considered nutritionally inadequate for the organisms.

The casein polypeptides that stimulated the M-protein synthesis were similar to the strepogenin materials described by Sprince and Woolley (1944) and by Slade (1951) as growth factors for streptococci and lactobacilli. The observation by Woolley and Merrifield (1954) concerning the strepogenin activity of the synthetic polypeptide hormones, oxytocin and arginine-vasopressin (du Vigneaud *et al.*, 1953*a*, 1953*b*) demonstrated that the active material of strepogenin was a peptide. These experiments also demonstrated that biologically-active strepogenin peptides from diverse sources need not have the same composition or amino acid sequence.

Numerous instances are found in the literature where peptides have a marked stimulation upon the growth of bacteria and animals. These stimulations are over and above the amount provided by the individual amino acids in the peptides. Several authors have theorized on the reasons for the growth-promoting properties of peptides. Peters and Snell (1954) have shown that antagonisms between certain amino acids as growth factors for lactobacilli may be alleviated by the addition of a peptide containing one of these amino acids. They suggest that the stimulating effect of these peptides may be merely due to the relief of these antagonisms.

At the present time there is no direct evidence for the participation of peptides in animal or microbial protein synthesis. The indirect evidence that has been accumulated falls in two main categories, (1) the stimulatory effects (already cited) of peptides on bacterial and animal growth, and (2) the demonstration of a nonuniform incorporation of radioactive amino acids into enzymes synthesized by animal tissues (Steinberg and Anfinsen, 1952, and Vaughan and Anfinsen, 1954). The latter phenomenon implies that these labeled amino acids may be in equilibrium with pools of intermediate reactants, namely peptides. Halvorson and Spiegelman (1952, 1953*a*, 1953*b*), and Rotman and Spiegelman (1954) studied adaptive enzyme synthesis in *Saccharomyces cerevisiae* and *Escherichia coli* and conclude that the induced biosynthesis of enzymes evolved entirely from free amino acids derived from the medium without the participation of intracellular intermediates or enzyme precursors. The role which polypeptides have in M-protein synthesis remains to be established.

The inhibition of M-protein synthesis by the well-known inhibitors of glycolysis is in all probability related to the exergonic nature of glycolysis and the endergonic requirements for protein synthesis.

SUMMARY

Group A streptococci stripped of the M-protein antigen by treatment with trypsin reestablished the M-protein within an hour when incubated in a nutritionally rich medium. Under more defined conditions the utilization of amino acids for protein synthesis was dependent upon purine and pyrimidine bases, vitamins, and other accessory substances required for growth. Omission from the medium of certain amino acids known to be present in the M-protein, prevented the synthesis of the protein by washed, trypsin-treated streptococci. Polypeptides from a pancreatic digest of casein showed a marked stimulatory effect on M-protein synthesis in the absence of apparent cellular reproduction. Amino acids and glucose were required to supplement the peptides but no other constituents were necessary. The action of a number of inhibitors of glycolysis and

assimilation was observed with respect to M-protein synthesis.

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