Stability tests[10] on the penicillin in oil and in beeswax-peanut oil mixture show no deterioration in various batches kept at refrigerator, room and 37 degrees C. temperature for 30 to 62 days.

As initial experiments rabbits were injected intramuscularly with 5,000 to 10,000 Oxford units of penicillin contained in 1 cc of beeswax-peanut oil mixture and blood assays[11] were made. Whereas penicillin in saline maintained a level for only two hours, penicillin in beeswax-peanut oil mixture maintained a level for 6 to 12 hours.

Human subjects were then given single injections of 41,500 to 66,400 Oxford units of penicillin intramuscularly. These doses were contained in 2 to 2.4 cc of beeswax-peanut oil mixtures. The figure shows the maintenance and duration of penicillin levels in the blood obtained by the use of penicillin in saline, peanut oil and the various percentages of beeswax-peanut oil mixtures. The figure also compares the duration of excretion of penicillin in the urine after the injection of penicillin in saline and in beeswax-peanut oil mixture. The beeswax-peanut oil mixture delayed penicillin absorption and maintained a level in the blood for 6 to 7 hours. The presence of penicillin in the urine for 20 to 32 hours indicated a persisting level in the blood for that period of time, though not assayable by present methods.

None of the patients complained of local pain or irritation in the region where the penicillin beeswax-peanut oil mixture had been injected. Nothing suggestive of an allergic reaction occurred in any of the subjects.

Gross and microscopic studies of tissues from hamsters which have been injected with the penicillin beeswax-peanut oil mixture are in process of study.

Eleven of twelve patients[13] with gonococcic urethritis have been cured after receiving a single injection of penicillin beeswax-peanut oil mixture.

The detailed accounts of these investigations will be published in the October, 1944, issue of the *Bulletin* of the U. S. Army Medical Department.

SUMMARY

(1) Single injections of penicillin in beeswax-peanut oil mixture will produce and maintain levels of penicillin in the blood for 7 or more hours.

(2) These mixtures have maintained their potency at room, incubator and refrigerator temperatures for 30 to 62 days and show no signs of deterioration to date.

(3) Eleven of twelve patients with gonorrhoea have been cured by a single injection of penicillin beeswax-peanut oil mixture.[14]

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INHIBITION OF β HEMOLYTIC STREPTOCOCCI FIBRINOLYSIN BY TRYPSIN INHIBITOR (ANTIPROTEASE)*

TILLETT and Garner[1] in 1933 discovered that β hemolytic streptococci produce an extracellular substance which is fibrinolytic in that it can liquefy human fibrin. They also found that patients with β hemolytic streptococcal diseases develop a factor which is capable of inhibiting the *in vitro* action of the fibrinolysin. These observations gave impetus to numerous studies on the role of the fibrinolysin in diseases attributable to β hemolytic streptococci and on the development of antifibrinolysin in such patients. Consequently, evidence has been accumulated which suggests that the fibrinolytic potency of the organism plays a role in the pathogenesis of some aspects of hemolytic streptococcic infections.[2]

The mechanism whereby the streptococcal fibrinolysin liquefies solid human fibrin is unknown, although it is stated by some that it is not a proteolytic enzyme since the products of proteolysis are not apparent when liquefaction has occurred.[2] However, it is possible that the liquefaction of fibrin consequent to the action of fibrinolysin is due to some hydrolysis of the protein, the rate of proteolysis being such as to make the measurement of the products a difficult one.

During the course of studies on the significance of antifibrinolysin formation in streptococcal diseases, we found it necessary to evaluate also the significance of serum antiproteases. It soon became apparent that a serum which has a high antifibrinolysin titer also has a high antiprotease (antitrypsin) titer. This lent emphasis to the possibility that the two phenomena are related and consequently, that the streptococcal fibrinolysin is more closely related to proteases than hitherto believed. It is probable that the proper application of Bergmann's method[3] would

[10] Assays were made by the methods of Rammelkamp[11] and Rake.[12] Penicillin assays of the urine were also done by these methods.

[11] C. H. Rammelkamp, *Proc. Soc. Exp. Biol. and Med.*, 51: 95, October, 1942.

[12] G. Rake and H. Jones, *Proc. Soc. Exp. Biol. and Med.*, 54: 189, 1943.

[13] The cooperation of Captain Robert J. Murphy of the V.D. Ward is appreciated.

[14] An additional fifty-three cases have been cured by this method. Data on these cases will be published later with Captain Robert J. Murphy.

* From the AAF Rheumatic Fever Control Program.

[1] W. S. Tillett and R. L. Garner, *Jour. Expt. Med.*, 58: 485, 1933.

[2] W. D. Tillett, *Bact. Rev.*, 2: 161, 1938.

[3] M. Bergmann, "Advances in Enzymology," 1: 63, 1941.

elucidate the relationship of the fibrinolysin to some specific protease. However, our facilities prevented the application of such procedures and therefore other experiments were designed.

Since we observed a close relationship between the serum antiprotease and antifibrinolysin, we decided to test the effect of substances with known anti-tryptic properties on the action of the fibrinolysin secreted by β hemolytic streptococci. Toward that end two antiproteases of different sources were employed, *viz.*, the crystalline "trypsin-inhibitor" isolated from beef pancreas by Northrop and Kunitz[4] and the soybean trypsin-inhibitor described by Ham and Sandstedt.[5] We are indebted to Dr. M. Kunitz for a sample of crystalline trypsin-inhibitor and to Dr. Ham for samples of soybean extracts containing potent anti-tryptic activity.

Human fibrin, prepared from relatively purified fibrinogen and thrombin, was employed as the substrate. To a solution of fibrinogen was added a definite quantity of a fibrinolysin preparation and after mixing and making up to a definite volume with saline, thrombin was added. A solid clot formed within one minute. In the experiments with the antiproteases, the fibrinolysin preparation was mixed with a definite concentration of the antiprotease before it was added to the fibrinogen solution. Table 1,

TABLE 1

INHIBITION OF β HEMOLYTIC STREPTOCOCCUS FIBRINOLYSIN BY TRYPSIN-INHIBITORS

	Fibrinogen solution (1 per cent.) ml	Saline ml	Fibrinolysin* ml	Trypsin inhibitor ml	Thrombin ml	Time for liquefaction
A	0.25	0.5	0.5	0	0.1	39 min.
	0.25	1.0	0	0	0.1	40.5 hrs.
	0.25	0	0.5	0.5†	0.1	63 hrs.
	0.25	0.4	0.5	0.1†	0.1	3.5 hrs.
B	0.25	0.5	0.5	0	0.1	43 min.
	0.25	1.0	0	0	0.1	+24 hrs.
	0.25	0	0.5	0.5‡	0.1	+24 hrs.

* Filtrate from an 18-hour broth culture of β hemolytic streptococci.
† 5 mgm crystalline trypsin inhibitor per ml (Northrop and Kunitz).
‡ Soybean extract (Ham and Sandstedt).

illustrative of many similar experiments, reveals that crystalline trypsin-inhibitor of pancreatic origin (A) and crude soybean extract with potent anti-trypsin activity (B) completely inhibit the action of a β hemolytic streptococcal fibrinolysin. The potency of the antiproteases was tested by noting their ability to inhibit pancreatic trypsin. Of interest is the fact that the trypsin inhibitors not only inhibited the streptococcal fibrinolysin but, in sufficiently high concentrations, also inhibited the spontaneous lysis of the fibrin which in turn is due presumably to a non-bacterial fibrinolysin.

It made little difference as to how the fibrinolysin was prepared. Thus, similar results were obtained with the whole 18-hour streptococcal cultures, filtrates of such cultures and the alcoholic precipitates of such filtrates, all of which were of proven fibrinolytic potency.

These preliminary data reveal that substances of animal and plant origin which have the property of inhibiting the action of trypsin can also inhibit the fibrinolysins produced by streptococci. Whether the antiproteases can influence other exotoxins produced by streptococci must await further studies, though Grob[6] did observe a retardation of bacterial growth in media containing pancreatic trypsin-inhibitor.

It is of interest in this connection to note Netter's demonstration that tyrothricin and actinomycin A are not only antibiotic but can also inhibit the fibrinolysin present in the filtrates of hemolytic streptococci.[7] Whereas tyrothricin is very toxic, preliminary studies indicate that both pancreatic and soybean trypsin-inhibitors are not toxic when injected in relatively large dosage into the peritoneal cavity of mice and guinea pigs.

The observations reported herein lend support to the hypothesis that the streptococcal fibrinolysins are proteolytic enzymes which are related to trypsin. They suggest also that the antiprotease concentrations of the blood may have much clinical significance. Serial assays of the serum antiprotease concentration in various diseases lends emphasis to this possibility and will be reported in greater detail in another communication.

With the ready availability of non-toxic preparations of antiproteases either from pancreas or from soybeans it may be possible to influence the course of β hemolytic streptococcal diseases by preventing the *in vivo* effects of the fibrinolysin secreted by the bacteria. This and other clinical applications of trypsin-inhibitors (antiprotease) in various disease states are now under investigation.

SUMMARY

Crystalline "trypsin-inhibitor" of pancreatic origin and the antitrypsin of soybean origin completely inhibit the fibrinolytic activity of cultures of β hemolytic streptococci, of filtrates of such cultures and of concentrates of such filtrates. It is suggested that the streptococcal fibrinolysin is a protease and that it may be related to trypsin.

[4] J. H. Northrop and M. Kunitz, *Jour. Gen. Physiol.*, 6: 267, 1932–33.
[5] W. D. Ham and R. M. Sandstedt, *Jour. Biol. Chem.*, 154: 505, 1944.
[6] D. Grob, *Jour. Gen. Physiol.*, 26: 431, 1943.
[7] E. Netter, *Proc. Soc. Exp. Biol. and Med.*, 49: 163, 1942.

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EFFECT OF SPINAL FLUID FROM PATIENTS WITH MYASTHENIA GRAVIS ON THE SYNTHESIS OF ACETYLCHOLINE IN VITRO[1, 2]

IT was found that less acetylcholine was synthesized in the presence of serum from patients with myasthenia gravis than serum from healthy persons or patients with diseases other than myasthenia gravis.[3, 4] Some of the factors modifying the synthesis of acetylcholine seem to be of relatively small molecular size, since they pass through a semipermeable Cellophane membrane.[4] To ascertain whether these factors are able to pass into the spinal fluid the effect of spinal fluid of patients with myasthenia gravis and of control subjects on the synthesis of acetylcholine *in vitro* was investigated.

METHOD

The amount of synthesis of acetylcholine was ascertained using an adaptation[4] of the method of Quastel, Tennenbaum and Wheatley.[5] The spinal fluid was assayed immediately after collection. The pH of the spinal fluids was adjusted to 7.4. One cc of spinal fluid was added to a mixture containing minced frog brain (100 mg), Ringer's solution (2 cc) at pH 7.4, physostigmine salicylate (3 mg) and glucose (4.8 mg). The mixtures were shaken and incubated aerobically at 37° C for 4 hours and the amount of free and total acetylcholine synthesized was assayed biologically, using the sensitized rectus abdominis muscle of frog.

RESULTS

The effects of spinal fluid of 3 patients with myasthenia gravis and 25 control subjects were studied. The clinical states of the patients with myasthenia gravis are summarized in Table 1. The control subjects were patients with convulsions, fits, displaced intervertebral disks, headaches, brain tumors or were suspected of having brain tumor.

TABLE 1

SHORT SUMMARY OF THE CLINICAL STATE OF THE PATIENTS WITH MYASTHENIA GRAVIS

Name	Sex	Age	Severity of disease	Duration (yrs)	X-ray treatment	Thymectomy	Symptomatology	Neostigmine (Prostigmine Bromide Hoffmann-LaRoche)	
								Dose mg/day	Achievement after medication
R	F	23	3+	9	yes	no	moderate lid ptosis, occasional diplopia, occasional difficulty in chewing and swallowing, moderate muscular fatiguability	90	Walks 1–2 blocks
Sa	F	32	3+	7	no	no	moderate lid ptosis, occasional diplopia, occasional difficulty in chewing, moderate muscular fatiguability	90–150	housework
P	M	36	2+	2	no	no	difficulty in chewing, moderate muscular fatiguability	45–75	Walks, but unable to work

The amounts of acetylcholine synthesized in the various mixtures are summarized in Table 2. In the presence of spinal fluid an average of 50 per cent. more acetylcholine was synthesized than in the presence of serum from the same subject. This observation suggests that at least some of the factors increasing the synthesis of acetylcholine pass into the spinal fluid. Less acetylcholine was synthesized in the presence of spinal fluid from patients with myasthenia gravis than with spinal fluid from the control subjects. The percentage defect in the synthesis of acetylcholine in the presence of spinal fluid from the patients with

TABLE 2

AMOUNTS OF ACETYLCHOLINE SYNTHESIZED IN THE PRESENCE OF SPINAL FLUID FROM PATIENTS WITH MYASTHENIA GRAVIS AND CONTROL SUBJECTS

Subject	Average of acetylcholine synthesized			
	Free acetylcholine in μg per 100 mg frog brain	Per cent. of control	Total acetylcholine in μg per 100 mg frog brain	Per cent. of control
Controls	2.11 ± 0.053		3.16 ± 0.049	
Patients with myasthenia gravis				
R	1.21 ± 0.025	57	1.87 ± 0.032	59
Sa	1.27 ± 0.025	60	2.00 ± 0.037	62
P	1.41 ± 0.029	67	2.20 ± 0.040	69

[1] This study was aided by a grant from the Josiah Macy Jr. Foundation.

[2] From the New York Hospital and the Departments of Medicine (Neurology) and Psychiatry, Cornell University Medical College, New York, N. Y.

[3] C. Torda and H. G. Wolff, SCIENCE, 98: 224, 1943.

[4] C. Torda and H. G. Wolff, in press, *Jour. Clin. Invest.*, September, 1944.

[5] J. H. Quastel, M. Tennenbaum and A. H. M. Wheatley, *Bioch. Jour.*, 30: 1668, 1936.