out mydriatic action whereas the esters of the corresponding tropic and benzilic acids are strongly mydriatic. The importance of the structure of the acid portion of the molecule is similarly emphasized in our series. The mydriatic and spasmolytic potencies of the β-diethylaminoethyl glycolates described here were much greater than those found for the corresponding acetates. Although two of the drugs described by us (Nos. 600 and 623) have spasmolytic action exceeding that of atropine, none is as strongly mydriatic as atropine. The reasons for this discrepancy are not readily apparent.

Summary. The β-diethylaminoethyl esters of di-α-thienyl-, diphenyl-, phenyl-α-thienyl- and cyclohexyl-α-thienylglycolic acids are anticholinergic drugs with significant mydriatic action. Of these, the cyclohexyl-α-thienylglycolic acid ester (No. 623) is the most mydriatic substance. The nitrate salt of this ester is very soluble in water and causes only small damage to sensitive tissues when used daily for several days at a concentration of 1.0 or 2.0%. It is suggested that this substance may have clinical applications as a mydriatic and cycloplegic agent.

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Enzyme Studies on Human Blood. I. Proteolytic Activity Associated with a Fraction of Plasma.*

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The demonstration of the proteolytic enzyme activity of the blood serum and plasma has attracted the attention of many workers since Dastre[1] originally inferred such an activity in serum. Delezenne and Pozerski[2] reported the proteolytic activity of serum which had been shaken with chloroform. Tagnon[3,4,5] studying this problem more thoroughly found that this enzyme is an euglobulin. Tillett and Garner[6] extracted from cultures of hemolytic streptococci a substance capable of dissolving fibrin clots. Milstone[7] later showed that this material did not act on highly purified fibrinogen and that a plasma euglobulin fraction was necessary for the "fibrinolysis." Christensen[8,10] and Christensen and MacLeod[9] found that the substance isolated from the streptococcic culture acted as an activator ("streptokinase") transforming the inactive plasma enzyme ("plasminogen") into "plasmin." They believe that this "plasmin" differs from trypsin but is identical with the chloroform-activated enzyme. Ferguson *et al.*[11] employing "fibrinogenolysis" as an assay technic of

* The protein fractions and the dried whole plasma were obtained through the courtesy of Dr. E. J. Cohn. They were prepared in collaboration between the Department of Physical Chemistry, Harvard Medical School, Boston, Mass., and the Division of Biologic Laboratories of the Massachusetts Department of Public Health from blood collected in collaboration with the American Red Cross.

1 Dastre, 1893, cited by Tagnon,[3] 1942.

2 Delezenne, C., and Pozerski, E., *C. R. Soc. biol.*, 1903, **55**, 690.

3 Tagnon, H. J., *Science*, 1942, **95**, 334.

4 Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 525.

5 Kaplan, M. H., Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 533.

6 Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

7 Milstone, H., *J. Immunol.*, 1941, **42**, 109.

8 Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

9 Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.

10 Christensen, L. R., *J. Gen. Physiol.*, 1946, **30**, 149.

11 Ferguson, J. H., Travis, B. L., and Gerheim, E. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 285.

"tryptic" activity demonstrated such after "streptokinase" activation in Fraction I of human plasma.

Much less conclusive data are available for the demonstration of the active enzyme without previous treatment with "streptokinase," chloroform, or other agents. Schmitz[12-14] isolated an enzyme by adsorption on calcium-fibrin and subsequent fractionation. It produced a slight increase in non-protein nitrogen when added to casein or gelatin. He postulated the existence in plasma of a kinase-trypsin-trypsin inhibitor system. However, recent evidence shows certain dissimilarities between trypsin and the chloroform—or the "streptokinase" activated-enzyme.[9,15] Taylor *et al.*[16] made observations on the proteolytic activity of plasma protein fractions prepared by low temperature-ethanol separation[17] and without previous chloroform treatment. Qualitatively, the enzyme activity was tested by observing the lytic action of the fractions on a fibrin clot. "Plus" proteolysis is reported in one out of 4 whole plasma samples, 2 out of 6 Fraction I, and in both of Fraction III-2 studied. Edsall[18] states that in Richert's method Fraction III-2 (or III-2,3) is treated with thrombin and the clot which forms adsorbs "plasminogen." This clot lysed spontaneously and was designated Fraction III-3. In 2 or 3 weeks at 0°C a slowly progressive proteolytic activity was demonstrated without the addition of any activator. Glazko[19] also reports the proteolytic activity of Fraction III-3. Christensen[10] observed that fractions of serum containing "plasminogen" will activate spontaneously in the absence of chloroform or "streptokinase" in the refrigerator after several weeks, when the inhibitor content is low.

This report concerns experiments on human whole plasma and its primary protein fractions separated only by low temperature-ethanol procedure and the demonstration of considerable proteolytic activity in Fraction I without previous treatment with activators.

Methods and Material. The dried whole plasma and its fractions were stored in a domestic-type mechanical refrigerator. No special precautions as to temperature, humidity and air exposure were taken during weighing of samples. Unless otherwise specified, a test mixture consisted of equal volumes of 2% aqueous solutions of specified protein fractions and 2% suspension of "C.P." casein in 1/15 M Sorensen phosphate buffer of specified pH, both prepared on the day of incubation. With each test 2 controls were run simultaneously consisting of equal volumes of buffered casein and distilled water and equal volumes of protein solution and buffer. Each test and control mixture was overlayed with C.P. toluene, 1% of the total volume. As additional bacteriological control measure, frequent smears and cultures were taken. The pH was determined with a glass electrode electrometer. Incubation occurred at 37.5°C and the reactions were terminated by the addition of cold 20% trichloracetic acid equal in volume to the reaction mixture. After one hour in the refrigerator, the precipitated proteins were separated by filtration. Either acid soluble total N or "tyrosine" or both were determined in duplicate on one cc aliquots of the filtrate. The micro-Kjeldahl-Nesslerization and the Folin-Ciocalteu[20] procedures, as employed in this communication, have average approximate sensitivities of 0.004 mg N and 0.001 mg tyrosine respectively per 1% on the transmission scale of the Coleman Junior Spectrophotometer. Enzymatic activity is expressed as the mg of acid soluble total N or "tyrosine" liberated per 100 cc of the protein solution obtained by subtracting the amounts found in the enzyme and substrate controls from that found in the

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13 Schmitz, A., *Z. physiol. Chem.*, 1937, **250**, 37.
14 Schmitz, A., *Z. physiol. Chem.*, 1938, **255**, 234.
15 Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 331.
16 Taylor, F. H. L., Davidson, C. S., Tagnon, H. J., Adams, M. A., MacDonald, A. H., and Minot, G. R., *J. Clin. Invest.*, 1945, **24**, 698.
17 Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, **68**, 459.
18 Edsall, J. T., *Advances in Protein Chem.*, 1947, **3**, 383.
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20 Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, **73**, 627.

TABLE I.
Control Studies on Protein Solutions and Casein Substrate.

2% protein solution	Principal components*	Acid soluble: Total N mg/100 cc†	Acid soluble: "Tyrosine" mg/100 cc†
Fraction I	Fibrinogen	5.0	7.6
" II-III	Gamma and beta globulins	0.8	0.0
" IV-1	Lipids and alpha globulin	0.4	1.3
" IV-4	Alpha and beta globulins	0.0	0.0
" V	Albumin	0.0	0.0
Whole plasma		1.6	1.0
Casein		16.4	14.0

* Information directly from Dr. E. J. Cohn.
† After 48 hrs at 37.5°C, pH 7.1.

TABLE II.
Proteolytic Activity of Fraction I.

Protein solution			Exp.	pH	Incubation, hr	Net acid soluble: Total N mg/100 cc	Net acid soluble: "Tyrosine" mg/100 cc
2%	I	Run 25	A	6.7	48	150.4	
"	"	" "	B	6.3	"	113.6	120.9
"	"	" "	"	6.7	"	124.6	167.2
"	"	" "	"	7.1	"	9.4	24.4
"	"	" "	"	7.5	"	9.6	22.0
"	"	" "	"	7.9	"	7.6	21.8
0.25%	"	" "	C	6.7	"	4.4	2.4
1	"	" "	"	"	"	10.0	9.8
2	"	" "	"	"	"	23.0	27.6
"	"	" "	D	"	24		14.2
"	"	" "	"	"	33		21.2
"	"	" "	"	"	48		23.5
"	"	" "	"	7.5	24		17.0
"	"	" "	"	"	33		24.0
"	"	" "	"	"	48		27.4
"	I*	" "	"	6.7	33		—1.3
"	"*	" "	"	"	48		0.0
"	I	" "†	E	"	"		76.1
"	"	" "†	"	7.5	"		17.4
"	"	" AVL-20	"	6.7	"		18.0
"	"	" " "	"	7.5	"		108.8
"	"	" 186	"	6.7	"		92.0
"	"	" "	"	7.5	"		124.0
"	"	" 8/44	"	6.7	"		0.8
"	"	" "	"	7.5	"		1.0

* Inactivated for 3 min in 80°C water bath.
† Second sample of this preparation received from the Department of Physical Chemistry, Harvard Medical School.

enzyme-substrate mixture.

Results. Table I presents data which emphasize the necessity of both enzyme and substrate controls. It is shown that there were present in whole plasma and Fraction I, after 48 hours incubation alone, at pH 7.1, slight but significant amounts of acid soluble total nitrogen and "tyrosine." Similar studies were also made at pH 6.3, 6.7, 7.5, and 7.9 with almost identical results. It was also observed that this amount in Fraction I was constant whether there was spontaneous clot formation and subsequent clot disappearance.[21] There were traces of acid soluble N in Fractions II-III and IV-1 and none in Fractions IV-4 and V. These experiments could be duplicated. However, results with casein substrate alone were variable depend-

[21] Shinowara, G. Y., unpublished observations, 1947.

ing on the particular lot of casein used. The data in Table I represent the highest values obtained. In this laboratory, it has been found that the acid soluble total nitrogen or "tyrosine" of Fraction I alone and of casein alone increases progressively with time of incubation.

Table II contains data on the proteolytic activity of Fraction I upon casein substrate. Experiment A was done 10 days; B, 25 days; C, 36 days; and D, 39 days after receiving the material labeled Run 25 from the Department of Physical Chemistry, Harvard Medical School. Experiment E was done one day after receiving a second shipment composed of material from different runs. With each experiment one or more of the other fractions and whole plasma were run as controls. The summary of results in mg net acid soluble "tyrosine" released from 2% casein by 100 cc of 2% solution of the specified protein at pH 6.3 to 7.9 and 48 hours incubation follows: whole plasma, 5.5 to 13.8; II-III, 6.4 to 12.0; IV-1, 0.0 to 7.8; IV-4, -4.5 to 0.4; and V, -10.2 to 0.0.

The data in experiments A, B, and E, Table II, definitely demonstrate the high degree of proteolytic activity of Fraction I, Run 25, although of a lesser degree, and demonstrate that the activity is apparently dependent upon the period of incubation and the concentration of the enzyme. The one sample of Fraction I, Run 8/44, which did not show enzyme activity differed from the other preparations of this fraction in several respects: It was pure white instead of light amber or gray in appearance; it was more than 90% insoluble in water or saline; and did not clot upon addition of thrombin. Runs 25, AVL, and 186 of Fraction I had clotting times with thrombin between 11 and 14 seconds, whereas Run 8/44, Fraction I, and samples of Fractions II-III, IV-1, IV-4, and V did not clot in 60 seconds.

In Experiment B, Table II, the greatest activity of Fraction I occurred at pH 6.7 and 6.3, but in Experiment D, there was approximately the same amount of proteolysis at pH 6.7 and 7.5. Variable results in pH studies with Fraction I, Runs 25 (second sample received), AVL, and 186 were obtained in Experiment E. Therefore, from the data presented here, it can be stated that apparently the enzyme in Fraction I is active near neutrality. It is also evident from Experiment D that the enzyme activity was completely abolished by heating the 2% solution of Fraction I in an 80° bath for 3 minutes before mixing with the buffered casein substrate.

There was considerably less or no proteolytic activity in the whole plasma and the other fractions tested. No kinases or other activators were added, nor was there a previous treatment with chloroform or fibrin (calcium or thrombin) formation in these and other experiments.

Discussion. The direct demonstration of considerable proteolytic activity of Fraction I was made under analytical conditions intended to give minimal values: These included the use of both enzyme and substrate controls and of cold 20% trichloracetic acid to precipitate the proteins at the end of the incubation period. By careful experiments, Hiller and Van Slyke[22] showed that increasing the concentration of trichloracetic acid increases the precipitation of non-protein nitrogen compounds.

The proteolytic activity of Fraction I can be explained on the basis that the low temperature-ethanol separation resulted in a preparation containing the zymogen and low, if any, concentration of inhibitor. This explanation would be based upon Christensen's[10] hypothesis that the spontaneous activation, without streptokinase, of "plasminogen" occurs when the inhibitor content is low or absent or when the inhibitor has been inactivated by chloroform treatment of serum, resulting in a process which "appears to be autocatalytic in nature." His hypothesis is in agreement with his earlier observation[8] that serum fractions prepared by dilution and acidification to pH 5.3 or by one-third saturation with ammonium sulfate may develop proteolytic activity without kinase or chloroform after a variable period of aging in the cold. The activity was

[22] Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, **53**, 253.

measured by the gelatin-viscosity method. Christensen and MacLeod[9] concluded that these solutions of inactive serum protease contained little or no inhibitor.

The higher activity of Fraction I at pH range 6.3-6.7 than at 7.1-7.9 in Experiment B, Table II requires some comment. In subsequent Experiments C and D, although significant degree of activity was still present, it had dropped appreciably. A possible explanation is that inadvertently the protein sample was left outside the refrigerator for several hours before Experiment C and this occurred when only a small amount remained in the sample bottle. Christensen[10] reports that in the presence of active enzyme both the "plasminogen" and "plasmin" are destroyed. Nevertheless, the pH studies in experiments B and E may have significance in view of Grob's[23] observation on the shifting of pH optima of leucoprotease and trypsin in the presence of serum from pH 7-8 to pH 6-6.5 as a result of the progressively weakened inhibitor effect of serum upon exposure to pH below 6.5 or above 9.7.

Other preparations exhibiting activity without kinase include Schmitz's[12] calcium-fibrin adsorbed enzyme and the thrombin-fibrin adsorbed Fraction III-3.[18] It appears from the data on these preparations and that reported here that the understanding of the significance of kinase and/or zymogen in the proteolytic system of human plasma *per se* requires further experiments with inhibitor-free kinase, and purer preparations of zymogen, active enzyme, and naturally occurring inhibitors of plasma. Moreover, it is suggested that the liquefaction of a fibrin clot ("fibrinolysis"), the alteration of fibrinogen as measured by clotting time with thrombin ("fibrinogenolysis"), the change in gelatin viscosity, and the increase in acid soluble nitrogen are not representative of an identical enzyme system. In many of the incubation experiments with Fraction I alone "fibrinolysis" and "fibrinogenolysis" were observed while the acid soluble nitrogen did not increase appreciably; *i.e.*, not more than 2% of the total available nitrogen. A comparative study of the various criteria of proteolytic activity will be reported separately. True proteolytic activity (*i.e.*, the cleavage of the peptide bond) of all preparations of Fraction I, which are water and saline soluble, is demonstrated in this report.

Summary. The action of whole dried plasma and its primary fractions separated by low temperature-ethanol procedure on casein was investigated. No kinase was added and there was no previous treatment of the protein preparations with chloroform or any other agents. Proteolytic activity was demonstrated by determinations of acid soluble total nitrogen and "tyrosine."

Human plasma Fraction I contains a high degree of proteolytic activity. The enzyme is inactivated at 80°C and functions at near neutrality.

[23] Grob, D., *J. Gen. Physiol.*, 1946, **29**, 219.