STUDIES ON A PROLACTIN-INACTIVATING ENZYME SYSTEM IN BLOOD SERA

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An Abstract

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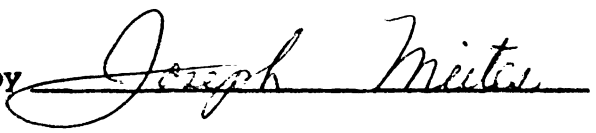
Approved by 

ABSTRACT

It has been demonstrated by several investigators that various protein hormones can be inactivated by whole blood and by certain blood constituents from a variety of species. The system(s) involved in protein hormone inactivation by blood has not been clearly defined. However, some authors attribute the action to that of fibrinolysin. The present experiments were designed to determine whether blood serum would inactivate prolactin and to attempt to establish some identity to the system.

Ovine prolactin was incubated two hours at 37.5°C in human and guinea pig serum, or euglobulins, human plasmin, plasminogen and streptokinase-activated plasminogen. Prolactin activity was assayed in white Carneau pigeons employing the method of Reece and Turner.

1. Incubation of prolactin in human blood serum of 10% and 45% concentration resulted in mean losses of 36% and 54% respectively. Incubation in human serum euglobulins of 50% concentration caused a loss of 41% prolactin activity.
2. The mean loss of prolactin activity resulting from incubation with 5.7 caseinolytic units plasmin was 94%. Plasminogen at the 5.0 mg level was not appreciably effective (prolactin loss, 19%), however activation by 2000 U streptokinase caused a loss of 74%.
3. Incubation in guinea pig serum or with serum euglobulins

produced losses of 50% and 45% respectively. The albumin-containing supernatant did not contain any demonstrable protease activity, nor could it be produced by addition of streptokinase.

4. Certain blood serum collections were devoid of protease and fibrinolytic activities. This was found to be attributable to the presence in serum of factors which inhibited the plasmin system. Euglobulins prepared from such inactive sera were contaminated with the inhibitors, but the concentration was less than that of the serum.

5. The prolactin-inactivating capacity of streptokinase-activated plasminogen was completely inhibited by antifibrinolysin, but was enhanced by e-amino-N-caproic acid (Grade C). An AR Grade of e-amino-caproic acid, reported to competitively inhibit fibrinolytic activity of streptokinase-activated plasminogen did not at the levels employed inhibit protease activity. Below 0.06 M concentration e-amino-caproic acid enhanced fibrinolytic activity of streptokinase-activated plasminogen. Above 0.06 M concentration protease but not fibrinolytic activity was enhanced.

INTRODUCTION

It is well known that a deficiency as well as an excess of hormonal activity can cause undesirable physiological effects; thus a hormonal balance or equilibrium must be maintained for normal body functions. Varied approaches have been applied in attempts to ascertain an understanding of some of the mechanisms involved in maintaining a homeostatic condition. As a result, considerable knowledge is available concerning the metabolism of steroid hormones, whereas knowledge of the metabolism of protein hormones is quite scant.

It has been shown that protein hormones can be inactivated by various biological tissues and tissue fluids, and that the rate at which they disappear from the circulating blood can be influenced by chemical poisoning of or by surgical removal of specific organs or glands. However, an understanding of the mechanisms involved remains to be determined. Results of some of the studies reported suggest the involvement of some yet unidentified enzyme system(s). Although the data are inconclusive, some authors support the involvement of the blood fibrinolysin system in mammals.

To effectively study and elucidate such systems active in protein hormone catabolism, it is necessary that the systems be isolated and studied outside the animal body. However, when conducting such <u>in vitro</u> studies as hormone inactivation by tissues or enzymes, it must be assumed

that the effects observed under *in vitro* conditions are similar to those produced *in vivo*. Since hormones are generally secreted directly into and transported throughout the body by the blood, it should be of first importance to investigate the fate of hormones while in the blood.

In the studies reported here, the protein hormone prolactin was chosen for investigation. The studies were designed to determine:

a. the effects on prolactin activity of incubation in blood serum
b. whether prolactin activity would be influenced by the fibrinolysin system
c. the blood serum fraction containing the prolactin-inactivating system
d. the relationship of the protease (prolactin-inactivating system) activity to the fibrinolytic system, and
e. whether the fibrinolytic activity of streptokinase-activated plasminogen (profibrinolysin) could be influenced by specific inhibitors without influencing the protease activity.

The results of these studies should give some information concerning the blood enzyme involved in prolactin inactivation, and whether one or more enzyme(s) are involved.

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DEFINITIONS

Antifibrinolysin - antiplasmin.

Fibrinolytic activity - lysis of fibrin.

Plasmin - fibrinolysin (FL).

Plasminogen - profibrinolysin.

Proteolytic (protease) activity - inactivation of prolactin.

SURVEY OF LITERATURE

In vitro and *in vivo* methods have been employed in studying the possible manner by which hormones are normally inactivated. Inactivation of protein and peptide hormones by various biological tissues has been reported.

The rate at which adrenocorticotropin (ACTH) disappears from the circulating blood has been studied by Richards and Sayers (1951). Two hours after administration only 2% of porcine ACTH activity could be detected in the blood of men. After three hours ACTH activity was not detectable in the blood. Similar results were obtained in the rat after injections of porcine ACTH and sheep ACTH. Rat ACTH disappeared rapidly after being injected intravenously into intact and adrenalectomized rats. Five minutes after injection into intact rats 40% of the ACTH activity was found in the extracellular fluid and 20% in the kidneys. After 15 minutes a negligible amount was detectable in the extracellular fluid and 15% in the kidneys. Less than 1/500th of the injected dose was estimated to be in the adrenals after five minutes. There was no detectable activity in the liver or urine. Reiss, et al (1951) reported that ACTH was rapidly inactivated by heparinized plasma from rats, rabbits, and humans.

Pincus, Hopkins and Hechter (1952) reported the presence of an ACTH inactivating system in bovine, rat and human blood, which would inactivate ACTH derived from pigs, sheep and whale. This system consisted of a

thermo-labile component and a thermo-stable component, both of which were present in plasma. The inactivation was heat-dependent and could be reduced or completely inhibited by heating the blood to 50°C for one-half hour. This suggested that an enzymatic process was involved. The inactivating system was later found to be concentrated in Cohn fraction III.

Geschwind and Li (1952) reported that incubation of sheep ACTH with rat plasma did not result in loss of activity, whereas incubation with liver and kidney slices, diaphragm and homogenates of liver, kidney and adrenal tissues inactivated ACTH. White and Gross (1957) compared the effects of bovine fibrinolysin with liver cathepsin, on the biological activity of corticotropin-A. Since the structure of corticotropin-A was known it was possible to determine the positions and types of esters attacked by the enzymes studied. These investigators found that bovine fibrinolysin at pH 6.0-7.5 splits corticotropin-A after arginine in position 8 and after lysine in position 15, causing a complete loss of biological activity. Bovine liver cathepsin split corticotropin-A after leucine in position 31 and after phenylalanine in position 35 without a resulting loss of ACTH activity. Their observations on the action of fibrinolysin were in agreement with those of Troll and Sherry (1954) who found that fibrinolysin attacks arginine and lysine esters.

Birmingham and Kurlents (1958) found that five-minute exposure of rat adrenals to ACTH, in Krebs-Ringer bicarbonate buffer, stimulated the formation of corticoids in an ACTH-free medium and decreased the ACTH activity

in the medium in which the adrenals had been for a short time. Exposure of one to one and one-half hours resulted in a complete loss of ACTH activity in the medium and there was no corticoid production by the adrenals subsequently placed in an ACTH-free medium. The interpretation was that the ACTH had become bound or absorbed by the adrenals and thus was inactivated.

Mirsky, Perisutti and Davis (1959) observed that somatotropin and ACTH were hydrolyzed by streptokinase-activated human plasma or plasminogen. Meakin, Tingey and Nelson (1960) did not feel that the loss of activity of exogenously administered ACTH was due to "binding" by adrenals of the dog. However, inactivation of porcine ACTH did occur when incubated with heparinized blood, plasma and serum of the dog. Meakin and Nelson (1960) reported that the ACTH-inactivating system of canine blood was heat dependent and was inhibited by L-cysteine.

Hopkins, Terner and Pincus (1961) isolated and concentrated from human plasma a factor which when incubated with plasminogen and porcine ACTH caused a marked loss of ACTH-activity, whereas the plasminogen alone was ineffective. The purified "co-factor" was found to be a heat stable, dialyzable substance of low molecular weight (less than 2000), itself devoid of activity.

Mersky, Perisutti and Davis (1959) reported that glucagon was hydrolyzed <u>in vitro</u> by blood plasma from various species and found that the

highest rates of hydrolysis occurred with plasma from rat and monkey. Lower rates were produced by plasma from chicken, man, guinea pig, rabbit, cat, dog and cow. It was possible by addition of streptokinase (SK) to the incubation mixture, to increase the glucagonolytic activity of the plasma from man, monkey and cat. It was proposed that the inactive precursor, plasminogen, was converted by SK to the active enzyme plasmin, which was responsible for the glucagonolytic activity of plasma. Mirsky et al (1949) (1955) described a specific hydrolytic enzyme system termed "insulinase" present in slices, homogenates and extracts of liver. A rapid degradation of insulin resulted when incubated with this system. Exogenously administered insulin was similarly rapidly inactivated by intact mice.

Sonenberg, et al (1957) found that S^{35} labelled or acetylated thyrotropin (TSH) did not cause enlargement of the thyroid of the rat or chick, but these same preparations did inhibit endogenous TSH activity. Sonenberg and Money (1957) prepared pituitary preparations having TSH and gonadotropic activities. Acetylation of these preparations destroyed the TSH and gonadotropic activities and also inhibited the activity of exogenously administered TSH.

Loeser (1934) observed that injected TSH disappeared from the blood of intact rabbits in about one hour; whereas it was detectable in the blood of thyroidectomized rabbits for as long as four hours. Seidelin (1940)

assayed TSH activity in the urine from intact and thyroidectomized guinea pigs which had been given injections of TSH. TSH activity was found only in the urine from thyroidectomized animals. Seidelin (1940) also observed that the ovaries might similarly reduce the amount of activity in the urine of rats following injections of gonadotropins.

Hall (1950) implanted pituitary tissue in various locations in immature female rats. He observed that ovaries of rats bearing subcutaneous grafts were larger than those of the rats bearing intrasplenic or intraperitoneal implants. It was thought that the absence of ovarian stimulation in those rats with intrasplenic or intraperitoneal pituitary grafts, was due to gonadotropin inactivation by the liver. Hall (1950) also reported that incubation for one hour at 37°C of human chorionic gonadotropin with homogenates of rat liver caused a marked reduction in gonadotropic activity.

Eser and Tuzunkam (1952) studied the effects of liver from rats, pigeons and frogs on melanocyte stimulating hormone (MSH). Rat liver was highly effective in reducing MSH activity. The MSH-inactivating capacity was reduced by about 50% in pregnancy, after adrenalectomy, and after production of toxic lesions of the liver. The inactivation of MSH by liver from frog or pigeon was considerably less than that caused by rat liver.

Shizume and Irie (1957) reported that exogenously administered MSH rapidly disappeared from the blood of normal dogs. The rate of disappearance from the blood of hepatectomized dogs was much less than that in intact

animals. Carbon tetrachloride poisoning of the liver also caused a reduced rate of removal from the blood. Homogenates of liver inactivated MSH; whereas kidney and muscle were much less effective. Long et al (1961) reported that liver and muscle tissue did not inactivate a highly purified preparation of MSH, but that brain tissue of cats greatly reduced MSH activity.

Meites (1940) followed the rates at which known amounts of subcutaneously administered prolactin were detected in the urine of rabbits. Approximately 1 % was detectable during the first 24 hours, only a trace during the second 24 hour period, and none was detectable in the urine collected during the third 24 hours. Intravenously administered prolactin could not be detected in the urine collected during the following 72 hour period. About half of the administered prolactin activity could be detected in the blood plasma five minutes after intravenous injection; none was assayable thirty minutes after injection.

Sonenberg et al (1951) administered I^{131} labelled prolactin to normal female rats. In studying the distribution of radioactivity in various organs of the rats it was found that the greatest concentration was in the corpora lutea. Essentially no activity was demonstrable in the mammary glands of normal adult, immature, pregnant or lactating females. Cox (1951) injected iodinated prolactin into mice and found that 20 minutes after the injection the greatest concentration of activity was in the liver and kidneys. Lesser amounts were found in the mammary glands, milk, ovaries and adrenals.

Sgouris and Meites (1952) incubated prolactin with various tissues including liver, kidney, brain and muscle of female rats; mammary glands from lactating and nonlactating guinea pigs and rabbits; pigeon crop glands; corpora lutea and the remaining ovarian tissue from pseudopregnant rabbits. All tissues employed were effective inactivators of prolactin with the exception of muscle and brain. Lactating mammary tissue, pigeon crop and ovarian tissues were the most effective. The inactivating system was shown to be completely inhibited by boiling the tissue preparations for ten minutes. Sgouris and Meites (1953) observed also that prolactin inactivation occurred more rapidly when incubated with lactating mammary tissue than when incubated with mammary tissue from pregnant (non-lactating) rats.

MATERIALS AND METHODS

Prolactin:

The ovine preparation used was generously supplied by the Endocrinology Study Section, N. I. H. and had a reported activity of 15 International Units (I. U.) per mg.

The prolactin was dissolved in distilled water to a concentration of 5. 0 mg prolactin per ml solution. A volume of 0. 1 ml (0. 5 mg) was used in each experiment.

Serum: Human

Collections of human blood were arranged and supervised by Mrs. Margaret Shick at Olin Health Center, Michigan State University. The blood was brought to the laboratory and after clotting had occurred, the blood was centrifuged and the serum collected and pooled. Each pool consisted of equal portions of serum from each of six to ten different blood samples. Each pool was aliquoted into volumes of 5-10 ml, stored in a freezer and thawed just prior to use. Serum concentrations for incubations were prepared by diluting with Krebs-Ringer phosphate buffer of pH 7. 2.

Serum: Guinea Pig

The guinea pig blood was given essentially the same treatment as the human blood. A 10 ml volume was collected from each guinea pig. The serum pool consisted of equal volumes of serum from each of three female guinea pigs.

Enzymes: Plasmin

Human Plasmin (Fibrinolysin): The fibrinolysin solution was supplied by The American Red Cross and Dr. J. T. Sgouris, Michigan State Health Laboratories. Each ml of fibrinolysin solution contained 57 caseinolytic units (C. U.) activity. Fibrinolysin solutions for incubation were prepared by adding 0. 1 ml (5. 7 C. U.) fibrinolysin solution to 0. 8 ml Krebs-Ringer phosphate buffer of pH 7. 2.

Plasminogen (Profibrinolysin):

Human plasminogen was supplied by Dr. J. T. Sgouris, Michigan State Health Laboratories. Plasminogen was not readily soluble at neutral pH. Therefore, a fine suspension was prepared in Krebs-Ringer phosphate buffer.

Enzyme Inhibitors: Antifibrinolysin

The author is indebted to Dr. Walter Seegers, Wayne State University, Detroit, for the antifibrinolysin which was a product of Parke-Davis and Co., and was prepared from human blood. The antifibrinolysin was dissolved in Krebs-Ringer phosphate buffer to a concentration of 500 Units per ml solution. A volume of 0. 2 ml (100 Units) was used for inhibiting plasmin activity.

E-Amino-N-Caproic Acid (Grade C) was purchased from California Biochemicals, Los Angeles, California.

E-Amino-Caproic Acid (Analytical Reagent) was purchased from Nutritional Biochemical Co., Cleveland, Ohio.

Enzyme Activators: Streptokinase

Streptokinase (SK) was a gift from Mr. Albert Ratner, Department of Physiology and Pharmacology, Michigan State University, and was a product of Lederle Laboratories.

Euglobulin Fractions:

The method of Milstone (1941) was employed in preparing the euglobulin fractions. Blood serum was diluted 20-fold; dilute acetic acid was added and by aid of a Beckman Zeromatic pH meter was adjusted to pH 5.3. After storage overnight at 4°C the euglobulins were collected by centrifugation. The euglobulin fractions were reconstituted in Krebs-Ringer phosphate buffer.

Incubation of Prolactin:

A volume of 0.1 ml (0.5 mg) prolactin solution was added to each flask containing the incubation medium. The total incubation volume was 1 ml. As a control, standard prolactin was added to Krebs-Ringer phosphate buffer and incubated simultaneously. The incubation flasks were placed in a Dubnoff Metabolic Shaking Incubator and after 2 hours' incubation at 37.5°C, each sample was diluted 10-fold with 0.9% saline solution to give an initial prolactin concentration of 0.05 mg/ml.

Assay of Prolactin Activity:

Prolactin activity was assayed in mature White Carneau pigeons employing the method of Reece and Turner (1937). Each pigeon was given 0.1 ml of the assay solution daily for four days. On the fifth day the pigeons were

sacrificed, and the crop sacs were removed and rated. The highly sensitive crop sac of the pigeon exhibits glandular proliferation in the presence of small amounts of prolactin. The degree of response represents the amount of prolactin activity present. By injecting incubated standard prolactin over one crop sac, and comparing the response with that of an equivalent amount of experimental sample injected over the other crop sac, each bird served as its own control. To minimize subjectivity, ratings were made by two independent co-workers who were unfamiliar with the assay materials, but experienced in the assay technic.

Assay of Fibrinolytic Activity:

Materials:

0. 1M phosphate saline buffer of pH 7. 4.

Fibrinogen: Human fibrinogen was supplied by Dr. J. T. Sgouris, Michigan State Health Laboratories.

Thrombin: Bovine thrombin was supplied by Dr. Clyde Cairy, Department of Physiology and Pharmacology, Michigan State University, and was a product of Parke, Davis & Co.

Preparation of Fibrin Plates and Assay of Fibrinolysin:

Petri dishes (9. 0 cm or 14. 0 cm diameter) were sterilized before being used. Fibrinolytic activity was assayed on fibrin plates employing a modified method of Astrup and Mullertz (1952) and the heated plate method of

Lassen (1952).

A 10.0 ml volume of 0.2% solution of human fibrinogen, which had previously been dialyzed for approximately 18 hours against 0.1M phosphate saline buffer, was poured into the 9.0 cm Petri dishes and was clotted by addition of 0.1 ml (10 units) bovine thrombin. When using the larger Petri dishes, 15.0 ml fibrinogen solution was clotted by 0.15 ml (15 units) thrombin. As many as four solutions could be assayed on the smaller dishes, and as many as six on the larger dishes.

When heated plates were desired, they were heated, after clotting, for 15 minutes at 80°C in a Dubnoff shaking incubator. After cooling, single drops (0.03 ml or 10.0 λ) of each sample were placed on the plates and incubated for 18 hours at 34°C. The area of the digested fibrin served as an index of enzyme activity. The product of the two perpendicular diameters was recorded in mm^2.

RESULTS AND DISCUSSION

A. Incubation of Prolactin in Human Blood Serum:

Human blood serum was diluted with Krebs-Ringer phosphate buffer. A volume of 0.1 ml (0.5 mg) prolactin solution was pipetted into the incubation flask which contained 0.9 ml of the dilute serum and the preparations were incubated for two hours at 37.5°C. The effects on prolactin activity of incubation with blood serum can be seen in Table 1. Incubation in human serum of 10% concentration resulted in moderate but significant losses (33, 36, and 40%) of prolactin activity. A somewhat greater loss (41, 52, and 70%) of activity occurred when a similar amount of prolactin was incubated in 45% serum.

These data show the presence, in human blood serum, of a system capable of inactivating ovine prolactin. The activity of the system is temperature dependent, since heating the blood for 30 minutes at 60° inhibited the inactivating system. This would tend to eliminate the possibility of inactivation due to surface reaction such as absorption or binding of prolactin with serum proteins.

Table 1. Mean Loss of Prolactin Activity Following Incubation in Human Blood Serum. (0.5 mg prolactin incubated in each experiment.)

Experiment	No. pigeons used per assay	Average rating of pigeon crops		Loss of prolactin activity	
		Std. prolactin	Incubated in 10% serum	mg	%
1	4	2.56	1.50	.20	40
2	4	1.56	1.00	.18	36
3	4	1.40	.95	.17	33
			Average	.18	36 ± 2.0*
			Incubated in 45% serum		
4	3	1.91	.58	.35	70
5	5	1.70	1.00	.21	41
6	3	2.42	1.42	.26	52
			Average	.27	54 ± 8.9*

* Standard error

B. The Effects of Plasmin on Prolactin Activity:

Plasmin (fibrinolysin, FL) is a naturally occurring fibrinolytic enzyme present in some mammalian blood (Sherry, Fletcher, and Alkjaersig, 1959). Sherry and his collaborators showed that plasmin is a proteolytic enzyme with a more general action than fibrinolysis alone. Plasmin has been shown to hydrolyze fibrinogen, casein, gelatin, plasma accelerator globulins and other proteins, and attack arginine and lysine esters (Troll, Sherry, and Wachman, 1954). The proteolytic activity of plasmin is further demonstrated by its effect on the biological activity of prolactin. In Experiment B, 0.1 ml (0.5 mg) prolactin was pipetted into each incubation flask containing 5.7 caseinolytic units (CU) (14.1 Loomis Units) fibrinolysin. The results are presented in Table 2, where it can be seen that essentially a complete loss of prolactin activity resulted after two hours' incubation with fibrinolysin.

White and Gross (1957) and Hopkins, Terner and Pincus (1961) reported that adrenocorticotropin (ACTH) was readily inactivated by fibrinolysin. The former authors (1957) reported that bovine fibrinolysin splits ACTH after arginine in position 8, (Arginine-tryptophane) and after lysine in position 15, (lysine-lysine), resulting in a complete loss of the biological activity (adrenal ascorbic acid depletion) of ACTH. White and Gross (1957) expressed belief that the heat-labile component of the ACTH-inactivating factor reported by Pincus, Hopkins and Hechter (1952) might be fibrinolysin since the latter authors had traced the heat-labile component to fraction III which contains

Table 2. Mean Loss of Prolactin Activity Following Incubation with Fibrinolysin (5.7 CU). (0.5 mg prolactin incubated in each experiment.)

Experiment	No. pigeons used per assay	Average rating of pigeon crops		Loss of prolactin activity	
		Std. prolactin	Incubated with fibrinolysin	mg	%
1	7	1.39	.21	.43	85
2	7	1.57	.07	.48	96
3	10	1.37	.00	.50	100
			Average	.47	94 ± 5.2*

* Standard error

plasminogen, (Kline, 1953).

C. Inactivation of Prolactin by Streptokinase-Activated Plasminogen:

The presence of plasmin in the blood depends on the conversion of the enzymatically inactive precursor plasminogen into the active enzyme plasmin (Astrup, 1958). The manner by which this is done is complicated and has been defined by Alkjaersig, Fletcher and Sherry (1957) and by Astrup (1958).

A variety of agents known to activate plasminogen has been reported by Tillet (1935), Astrup and Permin (1947), Astrup and Sterndorf (1952, 1953), Sobel et al (1952) and Mullertz (1953). However, streptokinase is the most widely used activator of plasminogen for <u>in vitro</u> studies on fibrinolytic activity. Since prolactin was inactivated by plasmin, it was important to know what effect streptokinase-activated plasminogen would have on prolactin activity.

Prolactin (0.5 mg) was incubated with (a) plasminogen and (b) streptokinase-activated plasminogen. The results are presented in Table 3. At the 2.0 mg level, plasminogen with or without 2000 U streptokinase did not inactivate prolactin. Prolactin was not appreciably inactivated (19%) by 5.0 mg plasminogen, whereas 5.0 mg plasminogen activated by streptokinase produced a marked increase in proteolytic activity, causing a 74% loss of prolactin activity.

Table 3. Mean Loss of Prolactin Activity Following Incubation with Plasminogen and Streptokinase-Activated Plasminogen. (0.5 mg prolactin incubated in each experiment.)

Experiment	No. pigeons used per assay	Average rating of pigeon crops		Loss of prolactin activity	
		Std. prolactin	Experimental prolactin	mg	%
(1) 2.0 mg plasminogen	5	1.42	1.32	.04	7.0
(2) 2.0 mg plasminogen 2000 U Streptokinase	8	1.05	1.10	.00	0.0
(3) 5.0 mg plasminogen	10	1.50	1.20	.095	19.0
(4) 5.0 mg plasminogen 2000 U Streptokinase	10	1.58	0.46	.37	74.0

D. (1) Incubation of Prolactin in Guinea Pig Serum and Euglobulins, and in Human Euglobulins. (2) Demonstration of Anti-fibrinolysin in Some Sera.

The proteolytic activity (prolactin inactivation) of blood is not limited to human serum. In Table 4 it is shown that guinea pig serum or euglobulins at 50% concentration have essentially the same degree of proteolytic activity, and that precipitation of the euglobulin fraction removes all demonstrable proteolytic activity. Human euglobulins prepared from serum pool # 1 (Experiment A) did not exhibit the same degree of activity as shown by the whole serum. However, streptokinase activation increased the activity.

The proteolytic activity of human and animal serum shows a wide variation. This variation in activity may well be due to increased enzyme activators or inhibitors which have been shown to vary in different physiological conditions (Macfarlane and Biggs, 1946; Fantl and Simon, 1958; Sherry, Fletcher and Alkjaersig, 1959). Results in Table 5 show that guinea pig serum from pool # 2 and human serum pool # 4 were ineffective as inactivators of prolactin.

Fibrinolysin assays were done on unheated fibrin plates to determine whether the proteolytically inactive human and guinea pig sera contained any fibrinolytic activity, and also whether the absence of proteolytic activity could be due to the presence of high levels of anti-fibrinolysin. In each experiment (Table 6) the sera or enzyme was prepared in 1.0 ml Krebs-Ringer

Table 4. Mean Loss of Prolactin Following Incubation in Human Euglobulins and in Guinea Pig Serum and Euglobulins (0.5 mg prolactin incubated in each experiment.)

Experiment	No. pigeons used per assay	Average rating of pigeon crops		Loss of prolactin activity	
		Std. prolactin	Experimental prolactin	mg	%
1. 50% Guinea pig serum	5	2.30	1.15	.25	50
2. 50% Guinea pig euglobulins	5	2.00	1.10	.23	45
3. 50% Guinea pig serum (euglobulin-free)	5	2.17	2.20	.00	0
4. 50% Guinea pig serum (euglobulin-free) 2000 U Streptokinase	5	2.10	2.00	.00	0
5. 50% Human euglobulins	5	2.40	1.90	.10	20
6. 50% Human euglobulins 2000 U Streptokinase	5	2.20	1.30	.21	41

Table 5. Failure of Human or Guinea Pig Euglobulins to Inactivate Prolactin. (Guinea Pig Serum Pool # 2, Human Serum Pool # 4). (0.5 mg prolactin incubated in each experiment).

Experiment	No. pigeons used per assay	Average rating of pigeon crops		Loss of prolactin activity	
		Std. prolactin	Experimental prolactin	mg	%
1. 50% Guinea pig euglobulins	5	1.55	1.50	.015	3.0
2. 50% Human euglobulins	5	1.65	1.55	.03	6.0

Table 6. Inhibition of Fibrinolysin (FL) Activity by Human and Guinea Pig Blood Sera and Antifibrinolysin (AFL). (0.03 ml each solution put on fibrin plates 2 minutes after preparation).

Experiment	Lysis of fibrin, mm^2
1. 5.7 CU FL	361
2. 5.7 CU FL 100 U AFL	16
3. 50% guinea pig serum	0
4. 50% human serum	0
5. 50% guinea pig serum 5.7 CU FL	0
6. 50% human serum 5.7 CU FL	0

CU = Caseinolytic Units

phosphate buffer. One drop (0.03 ml) of each sample was put on fibrin plates two minutes after preparation. The fibrin plates were incubated for 18 hours at 34°C, after which the product of the two perpendicular lysed zones was recorded. The area of fibrinolysis caused by fibrinolysin was 361 mm^2. This activity was more or less completely inhibited by antifibrinolysin. It can be seen that the inactive human and guinea pig sera not only failed to cause fibrinolysis, but also exerted an inhibitory action on plasmin. Antiplasmin activity of serum has been reported by Milstone (1941); Macfarlane and Pilling (1946); Grob (1949); and Sherry, Fletcher and Alkjaersig (1959). Fibrinogen is contaminated with plasminogen, therefore, lysis of fibrin occurs if plasminogen activators are applied to the plate even in the absence of fibrinolysin.

Table 7 shows results of experiments designed to determine whether the antifibrinolytic action of human and guinea pig sera and euglobulins could be overcome by streptokinase and fibrinolysin. The sera and euglobulin fractions were prepared in 0.8 ml Krebs-Ringer phosphate buffer to which was added 0.2 ml (2000 U) streptokinase. One drop (0.03 ml) of each sample was put on the fibrin plate before addition of the streptokinase to the solution, one minute after adding streptokinase, 10 minutes after incubation with streptokinase, and finally after 10 minutes' incubation with 5.7 CU fibrinolysin. The results are presented in Table 7. Fibrinolytic activity was produced one minute after addition of streptokinase to the solution. The greatest amount of activity was in the euglobulin fractions. It is not known if this activity was

due to streptokinase-activation of plasminogen present in the serum and serum fractions, or to activation of plasminogen in the fibrin plate. Nevertheless, it is obvious that the sera contain greater concentrations of inhibitors of fibrinolysin; and that the guinea pig euglobulins contain the least amount. The fibrinolytic activity is shown to have increased following a ten-minute incubation period. It is believed that this increase was due to a production of plasmin during incubation. The relative increase in activity was approximately the same for human (75 mm^2) and guinea pig (84 mm^2) sera. The smallest increase in activity, 18 mm^2, was in the guinea pig euglobulin fractions, whereas the increased lysis caused by human euglobulins was 47 mm^2. Lysis caused by a similar amount of streptokinase which had been incubated for ten minutes before application to the fibrin plate was 256 mm^2.

Addition of 5.7 CU fibrinolysin to the incubation mixture did cause a moderate increase in the lysis of fibrin, but the sum of the activities of fibrinolysin, streptokinase-activated plasminogen of serum, or euglobulins and plasminogen present in the fibrin, were not sufficient to overcome the inhibitory actions of the antiplasmins. The area of lysis caused by control fibrinolysin which had been incubated for 10 minutes was 484 mm^2; lysis caused by fibrinolysin added to the streptokinase-activated human or guinea pig serum was 196 mm^2 each; for fibrinolysin added to streptokinase-activated human and guinea pig euglobulins, 400 and 408 mm^2 respectively. It is conceivable that the greatest portion of lysis at this time was due to the action of the

added fibrinolysin; particularly in the euglobulin preparations, since the activities were essentially the same for both species.

The data presented in Table 7 give a possible explanation for the failure of these serum fractions to inactivate prolactin. (Table 5). It is still uncertain whether any appreciable degree of plasmin was produced in the serum samples. Lassen (1952) has shown that heating the fibrin plate denatures the plasminogen, but does not appreciably reduce the sensitivity of fibrin to lysis by plasmin. Therefore, any lysis of fibrin is due to fibrinolysin added to the plate and not due to activators of plasminogen as in the unheated plate. By use of the heated fibrin plates, it was possible to determine if any fibrinolytic activity was produced in the serum or euglobulins.

One drop (0.03 ml) of serum or euglobulin solution was put on the plate before addition of, and after 10 minutes' incubation with streptokinase. It can be seen from the data presented in Table 8 that the euglobulins caused some lysis of fibrin. This experiment was run seven days after preparation of the euglobulins and three days after the preceding experiment (Table 7). Plasminogen is known to undergo spontaneous activation on storage (Sherry, 1958). Such a process could have occurred during storage of the euglobulins, and would explain the presence of fibrinolytic activity without addition of streptokinase to the mixture. The expected increase in fibrinolytic activity was produced by streptokinase-activation of the euglobulins, however, the sera were not activated by streptokinase. It must be assumed, therefore, that

Table 7. Inhibition of Fibrinolysin by Human and Guinea Pig Sera and Euglobulins Devoid of Proteolytic Activity

	Lysis of Fibrin, mm^2			
	Without SK	1 min. after adding 2000 U SK	10 min. inc. with SK	10 min. inc. with 5.7 CU FL
1. 50% Human serum	0	25.0	100.0	196.0
2. 50% Human euglobulins	0	259.0	306.0	400.0
3. 50% Guinea Pig serum	0	36.0	120.0	196.0
4. 50% Guinea Pig euglobulins	0	306.0	324.0	408.0
5. 5.7 CU Fibrinolysin	Incubated 10 minutes			484.0
6. 2000 U SK	Incubated 10 minutes		256.0	

SK = Streptokinase

Table 8. SK-Activation of Serum Euglobulins
(Heated Fibrin Plates)

	Lysis of Fibrin, mm^2	
	Without SK	10 min. inc. with 2000 U SK
50% Human serum	0	0
50% Human euglobulin	44.9	108.2
50% Guinea pig serum	0	0
50% Guinea pig euglobulin	42.3	122.0
1000 U Streptokinase	0	0

these sera from human and guinea pig blood contained inhibitors that inhibited plasmin and probably activation of plasminogen.

E. Effects of Some Amino Acids on the Proteolytic and Fibrinolytic Activities of Streptokinase-Activated Plasminogen.

Alkjaersig, Fletcher and Sherry (1959) reported that e-amino-caproic acid competitively inhibited activation of human or bovine plasminogen by streptokinase; at low concentrations it enhanced the action of plasmin, whereas, at higher (above .06 M) levels it acted as a noncompetitive inhibitor of plasmin. Sakar (1958) reported that ovomucoid would inhibit the fibrinogenolytic activity of plasmin, but did not effectively inhibit the fibrinolytic activity. Conversely, e-amino-caproic acid inhibited fibrinolytic activity, but did not inhibit fibrinogenolytic activity. These observations suggested the possibility that the active enzyme, plasmin, has more than one active site and that one of these can be blocked by specific inhibitors, while not interfering with another enzymic property of the molecule. Streptokinase-conversion of plasminogen to plasmin was shown (Alkjaersig, Fletcher and Sherry, 1959) to be blocked by e-amino-caproic acid. Caseinolytic activity as well as fibrinolytic activity was inhibited, suggesting that these two proteins, casein and fibrin, are hydrolyzed by the same site(s) of the plasmin molecule. The activation of plasminogen by streptokinase involves an enzymic process causing a release of approximately 17% of the nitrogen from plasminogen (Sherry, Fletcher and Alkjaersig, 1959). It is not known if

e-amino-caproic acid alters the site at which plasminogen is split or just what is involved in inhibiting the process.

Hopkins, Terner, and Pincus (1961) reported that plasminogen could be activated by a factor prepared from human blood. The activated plasminogen had peptidase (inactivation of ACTH) activity, but did not cause lysis in the fibrin plate test. Streptokinase is a protein of relatively high molecular weight, which can convert plasminogen to an enzyme (plasmin) with diverse proteolytic activities. The "factor" employed by Hopkins, Terner and Pincus (1961) was considerably smaller (molecular weight less than 2000). Therefore, it differs from streptokinase as well as other known activators of plasminogen which with two exceptions are proteolytic enzymes (Sherry et al., 1959). It would appear in this case, that the type of activity produced would be determined by the plasminogen activator employed. Such observations, if applicable to prolactin inactivation should make it possible to show what relationship exists between the prolactin-inactivating system and the fibrinolysin system.

In the following studies the effects on the activities of streptokinase-activated plasminogen of two preparations of e-amino-caproic acid were studied. In the first of these experiments e-amino-N-caproic acid (Grade C) was incubated 10 minutes with plasminogen before addition of streptokinase. After the 10 minute incubation period, 2000 U streptokinase and 0.5 mg prolactin were added and the preparation was incubated for two hours. Following

the incubation period, one drop (10 λ) of each preparation was put on the heated fibrin plate to assay for fibrinolytic activity. A portion of the remaining solution was diluted and assayed for prolactin activity.

Table 9 shows that the protease activity (prolactin inactivation) and the fibrinolytic activity are markedly increased by e-amino-N-caproic acid. Since it has been demonstrated that e-amino-caproic acid of high purity inhibits the fibrinolytic activity of streptokinase-activated plasminogen (Alkjaersig et al, 1959), the next experiment was designed to see if fibrinolytic activity could be inhibited without interfering with the proteolytic activity.

Plasminogen (5.0 mg) was incubated for 10 minutes with 5.0, 10.0, or 50.0 mg e-amino-caproic acid in 0.7 ml Krebs-Ringer phosphate buffer. This incubation period was to allow the amino acid to react with plasminogen before adding streptokinase. At the end of the incubation period 0.2 ml (2000 U) streptokinase and 0.1 ml (0.5 mg) prolactin were added, and the mixture was incubated for two hours. One drop (10 λ) of each incubation mixture was put on a heated fibrin plate and incubated 18 hours at 34°C.

Table 10 shows that the initial protease and fibrinolytic activities of the plasminogen mixtures were increased by streptokinase-activation. As was shown previously (Table 9), antifibrinolysin inhibits both activities. Fibrinolytic activity was increased by 5.0 mg (0.026 M) e-amino-caproic acid, but the protease activity was not influenced. The increase in fibrino-

Table 9. Effects of Antifibrinolysin (AFL) and E-Amino-N-Caproic Acid on the Proteolytic and Fibrinolytic Activity of Streptokinase-Activated Plasminogen.

Experiment	No. pigeons used per assay	Average rating of pigeon crops		Loss of prolactin activity		Lysis of Fibrin on heated fibrin plate (mm^2) 10 λ incubation medium
		Std. prolactin	Experimental prolactin	mg.	%	
1. 5.0 mg plasminogen	5	1.80	1.37	0.12	24	27
2. 5.0 mg plasminogen 2000 U SK	5	2.25	1.05	0.27	53	45
3. 5.0 mg plasminogen 2000 U SK 100 U AFL	5	1.90	1.85	0.00	0	0
4. 5.0 mg plasminogen 2000 U SK 500 mg E-Amino-N-Caproic Acid	5	1.75	0.15	0.46	91	86

Table 10. Effects of Antifibrinolysin (AFL) and E-Amino-Caproic Acid on the Proteolytic and Fibrinolytic Activities of Streptokinase-Activated Plasminogen.

	Experiment	No. pigeons used per assay	Average rating of pigeon crops		Loss of prolactin activity		Lysis of fibrin on heated fibrin plate (mm2)
			Std. prolactin	Experimental prolactin	mg	%	10 incubation medium
1.	5.0 mg plasminogen	10	1.52	1.08	0.15	29	36
2.	5.0 mg plasminogen 2000 U SK	10	1.75	0.87	0.25	50	75
3.	5.0 mg plasminogen 2000 U SK 100 U AFL	5	1.75	1.66	0.02	3	0
4.	5.0 mg plasminogen 5.0 mg E-Aminocaproic acid 2000 U SK	5	1.60	0.85	0.24	47	158
5.	5.0 mg plasminogen 10.0 mg E-Aminocaproic acid 2000 U SK	5	1.30	0.35	0.37	73	132
6.	5.0 mg plasminogen 50.0 mg E-Aminocaproic acid 2000 U SK	5	1.50	0.75	0.25	50	132

lytic activity is in agreement with reports of Alkjaersig, Fletcher, and Sherry (1959) that e-amino-caproic acid below .06 M concentration increases the fibrinolytic activity of plasmin. Incubation with 10.0 mg e-amino-caproic acid (0.052 M) caused a significant increase in protease activity, but the degree of fibrinolytic activity was not correspondingly increased. At higher concentration (50.0 mg; 0.26 M) the protease activity was lower than that produced by 10.0 mg e-amino-caproic acid; whereas fibrinolytic activity was the same.

It can be concluded from the data in Table 10 that fibrinolytic and protease activities of streptokinase-activated plasminogen are not directly related. An increase in protease activity was not accompanied by a corresponding increase in fibrinolytic activity. In working with such an impure enzyme preparation (proenzyme) as plasminogen, it is impossible to state whether this represents more than one active site on the transformed product (enzyme), since it is possible that other enzymes (trace) present were influenced by streptokinase as well as by e-amino-caproic acid.

The failure to effectively inhibit streptokinase conversion of plasminogen to fibrinolysin may well be due to the excessive amount of activator employed in the system (2000 U streptokinase). This would be of particular importance if the following equation could be applied: $E + I \rightleftharpoons EI$; where the enzyme E (plasminogen) and inhibitor I complexing is reversible. The relative concentrations of e-amino-caproic acid and streptokinase would then determine the

rate at which plasmin would be formed.

In Table 10, Experiment 1, it can be seen that some plasmin was in the system before activation by streptokinase. The increase in protease activity caused by streptokinase was relatively small, but significant. Approximately two-thirds of the activity was present before adding streptokinase to the system. The fibrinolytic activity (initial) was approximately 50% of the activity present after streptokinase activation. E-amino-caproic acid, at all levels employed here, increased fibrinolytic activity, but only at the 10.0 mg level was the proteolytic activity influenced. If the fibrinolysin system was the only enzyme system involved, then it is quite possible that enzyme activity of activated plasminogen can be determined by the activator employed and the type of inhibitor present, or that the plasmin molecule possesses more than one active site. It is also shown that the purity of e-amino-caproic acid is of importance. E-amino-N-caproic acid at approximately ten times the molar concentration of the highest concentration of e-aminocaproic acid markedly enhanced the proteolytic activity of streptokinase-activated plasminogen (Table 9). The results suggest that e-amino-caproic acid at certain molar concentrations can enhance the proteolytic and fibrinolytic activity of streptokinase-activated plasminogen, but that enhancement of the two activities does not occur at the same molar concentration.

From the data presented the following conclusions can be drawn. The

prolactin-inactivating system of blood serum is heat dependent and probably enzymic in nature. All of the demonstrable protease (prolactin inactivating) activity was present in the euglobulin fraction and could be increased by addition of streptokinase. Since plasminogen, the precursor of the proteolytic enzyme plasmin, is concentrated in euglobulin fractions, it is possible that plasmin was involved in prolactin inactivation.

CONCLUDING REMARKS

The methods employed previously in studying the effects of blood on protein hormone activity have not been such that would elucidate the nature of any enzyme system involved in protein hormone inactivation. It has been shown that several of the protein hormones (ACTH, STH, and glucagon) can be inactivated by blood serum or plasma from various species, and that the degree of inactivation could in some instances be increased by addition of streptokinase, a product of bacterial origin. Streptokinase has been extensively studied and found to be a highly effective activator of the fibrinolysin system, but has not been found to activate other enzyme systems. Thus, some degree of specificity exists between streptokinase and fibrinolysin.

On this basis alone some investigators have assumed that the fibrinolysin system is responsible for the loss of protein hormone activity resulting from incubation in blood. As a result of studies presented here no strong dissension should result from such assumptions providing there be some clarification of terminology. The terms "fibrinolysin" and "plasmin" are at times used interchangeably. However, in the studies reported here reference to the action of "fibrinolysin" has been restricted to the dissolution or lysis of fibrin. Protease activity (inactivation of prolactin) has been attributed to the action of "plasmin," which in certain instances may or may not have fibrinolytic activity.

The present studies show that the two activities (fibrinolytic and protease) coexist to some degree, but that one may be increased without a corresponding increase in the other activity. Sakar (1958) reported that fibrinolytic activity of plasmin could be inhibited by e-amino-caproic acid without interfering with fibrinogenolytic activity; ovomucoid inhibited fibrinogenolytic activity but did not appreciably inhibit fibrinolytic activity. Hopkins, Terner and Pincus (1961), by use of an activator prepared from human blood, converted plasminogen to an enzyme possessing peptidase (ACTH-inactivating) activity, but without fibrinolytic activity. The above observations support the multifariousness of activated plasminogen.

The present observations are in agreement with reports of inactivation of protein hormones by blood, plasmin and by streptokinase-activated plasminogen. The failure of certain samples of sera to inactivate prolactin was shown here to be due to the presence of inhibitors which inhibited fibrinolytic and protease activities of the serum and also inhibited the fibrinolytic activity of plasmin. These inhibitors are therefore similar if not identical to antifibrinolysin, which in these studies should more aptly be termed antiplasmin. Additional studies employing specific inhibitors and varying amounts of streptokinase as well as other activators of plasminogen should be explored in acquiring more details on the nature of these enzyme systems.

BIBLIOGRAPHY

Alkjaersig, N., Fletcher, A. P. and Sherry, S. E-aminocaproic Acid, an Inhibitor of Plasminogen Activation. J. Biol. Chem. 234:832-837, 1959.

Astrup, T. Activators and Inhibitors of Fibrinolysis, in Proceedings of the Fourth International Congress of Biochemistry. 10:147-171, 1958. Pergamon Press, Inc., New York, N.Y.

Astrup, T. and Mullertz, S. The Fibrin Plate Method for Estimating Fibrinolytic Activity. Arch. Biochem. Biophys. 40:346-351, 1952.

Astrup, T. and Permin, P. M. Fibrinolysis in the Animal Organism. Nature 159:681-682, 1947.

Astrup, T. and Sterndorf, I. An Activator of Plasminogen in Normal Urine. Proc. Soc. Exper. Biol. and Med. 81:675-678, 1952.

Astrup, T. and Sterndorf, I. A Fibrinolytic System in Human Milk. Proc. Soc. Exper. Biol. and Med. 84:605-608, 1953.

Birmingham, M. K. and Kurlents, E. Inactivation of ACTH by Isolated Rat Adrenals and Inhibition of Corticoid Formation by Adrenocortical Hormones. Endocrinology 62:47-60, 1958.

Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Meline, M. and Taylor, N. L. Preparation and Properties of Serum and Plasma Proteins. I. Am. Chem. Soc. 68:459-475, 1946.

Cox, L. The Preparation and Distribution of Iodo-Prolactin Labelled with I^{131}. Anat. Rec. 109:285, 1951.

Eser, S. and Tuzunkam, P. Concerning the Inactivation and Augmentation in the Organism of the MSH at the Normal State and of Certain Pathological Conditions. Ann. d'Endocrin. 13:905-909, 1952.

Fanti, P. and Simon, S. E. Fibrinolysis Following Electrically Induced Convulsions. Australian J. Exper. Biol. and Med. Sci. 26:521-529, 1958.

Geschwind, I. E. and Li, C. H. Inactivation of Adrenocorticotropic Hormone in Vitro by Tissues. Endocrinology 50:226-233, 1952.

Grob, D. Further Studies on Protein, Polypeptide, and Other Inhibitors of

Serum Proteinase, Leucoproteinase, Trypsin and Papein. J. Gen. Physiol. 33:103-124, 1949.

Hall, B. V. Inactivation of Gonadotrophins by Liver and Liver Homogenates. Fed. Proc. 9:54-55, 1950.

Hopkins, T. F., Terner, C. and Pincus, G. Preparation and Properties of the Cofactor of an ACTH-inactivating Enzyme System from Human Blood. Endocrinology 69:728-734, 1961.

Kline, D. L. The Purification and Crystallization of Plasminogen (Profibrinolysin). J. Biol. Chem. 204:949-955, 1953.

Lasen, M. Heat Denaturation of Plasminogen in the Fibrin Plate Method. Acta Physiol. Scandinav. 27:371-376, 1952.

Loeser, A. Die Schilddrusenwirksame Substanz des Hypophysenvorderlappens. Arch. Exper. Pathol. u. Pharmakol. 176:697-728, 1934.

Long, J. M., Krivoy, W. A. and Guillemin, R. On a Possible Role of B-Melanocyte Stimulating Hormone (B-MSH) in the Central Nervous System of the Mammalia: Enzymatic Inactivation in Vitro of B-MSH by Brain Tissue. Endocrinology 69:176-181, 1961.

Macfarlane, R. G. and Biggs, R. Observations on Fibrinolysis: Spontaneous Activity Associated with Surgical Operations. Lancet 2:862-864, 1946.

Macfarlane, R. G. and Pilling, J. Observations on Fibrinolysis: Plasminogen, Plasmin and Antiplasmin Content of Human Blood. Lancet 2:562-565, 1946.

Meakin, J. W. and Nelson, D. H. The Catabolism of Adrenocorticotropic Hormone: Some Characteristics of an ACTH Inactivating System in Plasma. Endocrinology 66:73-79, 1960.

Meakin, J. W., Tingey, W. H. and Nelson, D. H. The Catabolism of Adrenocorticotropic Hormone: The Stability of Adrenocorticotropic Hormone in Blood, Plasma, Serum and Saline. Endocrinology 66:59-72, 1960.

Meites, J. Masters Thesis, University of Missouri, 1940.

Milstone, H. A Factor in Normal Human Blood Which Precipitates in Streptococcal Fibrinolysis. J. Immunol. 42:109-116, 1941.

Mirsky, I. A. and Broh-Kahn, R. H. The Inactivation of Insulin by Tissues. The Distribution and Properties of Insulin Inactivating Extracts (Insulinase). Arch. Biochem. and Biophys. 20:1-9, 1949.

Mirsky, I. A., Perisutti, G. and Davis, N. C. The Destruction of Glucagon, Adrenocorticotropin and Somatotropin by Human Blood Plasma. J. Clin. Invest. 38:14-20, 1959.

Mirsky, I. A., Perisutti, G. and Davis, N. C. The Destruction of Glucagon by the Blood Plasma from Various Species. Endocrinology 64:992-1001, 1959.

Mirsky, I. A., Perisutti, G., and Dixon, J. J. The Destruction of I^{131} Labelled Insulin by Rat Liver Extracts. J. Biol. Chem. 214:397-408, 1955.

Mirsky, I. A., Perisutti, G., and Jinks, R. Destruction of Insulin by Intact Mice. Endocrinology 56:484-488, 1955.

Mullertz, S. A Plasminogen Activator in Spontaneously Active Human Blood. Proc. Soc. Exper. Biol. and Med. 82:291-294, 1953.

Pincus, G., Hopkins, T. P. and Hechter, O. An ACTH-Inactivating Factor in Mammalian Blood. Arch. Biochem. Biophys. 37:408-418, 1952.

Reece, R. P. and Turner, C. W. The Lactogenic and Thyrotropic Hormone Content of the Anterior Lobe of the Pituitary Gland. Mo. Agr. Exper. Sta. Res. Bul. 266: 1937.

Reiss, M., Badrick, F. E., Halkerston, I. D. K., and Plaice, C. Rapid Inactivation of ACTH Incubated with Heparinized Plasma from Rats, Rabbits and Humans. Nature 168:206, 1951.

Richards, J. B. and Sayers, G. Fate and Excretion of Adrenocorticotrophic Hormone. Proc. Soc. Exper. Biol. and Med. 77:87-93, 1951.

Sakar, N. K. Activators and Inhibitors of Fibrinolysis, in Fourth International Congress of Biochemistry 10:145, 1958.

Seidelin, S. M. The Metabolism of the Thyrotrophic and Gonadotrophic Hormones. Endocrinology 26:696-702, 1940.

*Sgouris, J. T. and Meites, J. Inactivation of Prolactin by Body Tissues in Vitro. Am. J. Physiol. 169:301-304, 1952.

Sgouris, J. T. and Meites, J. Differential Inactivation of Prolactin by Mammary Tissue from Pregnant and Parturient Rats. Am. J. Physiol. 175:319-321, 1953.

Sherry, S. Biochemistry of Plasminogen and Plasmin, in Fourth International Congress of Biochemistry 10:142- , 1958. Pergamon Press, Inc., New York, N. Y.

Sherry, S., Fletcher, A. P. and Alkjaersig, N. Fibrinolysis and Fibrinolytic Activity in Man. Physiol. Rev. 39:343-382, 1959.

Shizume, K. and Irie, M. The Role of the Liver in the Inactivation of the Melanocyte Stimulating Hormone in the Body. Endocrinology 61:506-513, 1957.

Sobel, G. W., Mohler, S. R., Jones, N. W., Dowdy, A. B. C. and Guest, M. M. Urokinase an Activator of Plasma Profibrinolysis Extracted from Urine. Am. J. Physiol. 171:768, 1952.

Sonenberg, M. and Money, W. L. Inhibition of Pituitary Hormone Activity with Derivatives of Pituitary Preparations. Endocrinology 61:12-19, 1957.

Sonenberg, M., Money, W. L., Berman, M., Brener, J. and Rawson, R. W. Inhibition of Thyrotrophic Activity with Chemically Modified Thyrotrophin Preparations. Assoc. Am. Physicians 70:192-201, 1957.

Sonenberg, M., Money, W., Keston, A. S., Fitzgerald, P. and Godwin, J. T. Tracer Studies with Labelled Prolactin Preparations. J. Clin. Endocrinol. 11:747-748, 1951.

Tillet, W. S. Fibrinolytic Activity of Hemolytic Streptococci in Relation to Source of Strains and to Cultural Reactions. J. Bact. 29:111-130, 1935.

Troll, W., Sherry, S. and Wachman, J. Action of Plasmin on Synthetic Substrates. J. Biol. Chem. 208:85-93, 1954.

White, W. F. and Gross, A. M. Studies on Adrenocorticotropin. XIV. Action of Bovine Fibrinolysin and of Liver Cathepsin on Corticotropin-A. Effect on Biological Activity. J. Am. Chem. Soc. 79:1141-1145, 1957.