that after 2 weeks some mechanism (which in his case appeared to be regeneration of thyroid tissue) had compensated for glandular deficiency. Our results show clearly that 9 to 10 weeks after thyroidectomy an approximately 50% reduction in pancreatic amylase is evident.

Since the effects of hypophysectomy and thyroidectomy on pancreatic weight and amylase are of comparable magnitude it may be inferred that a large proportion of pituitary control over pancreatic acini is exerted through the thyroid gland. However, no aspect of altered pancreatic function in the hypophysectomized rat has yet been fully restored by therapy with thyroid hormones.

Summary. After surgical thyroparathyroidectomy, with or without additional treatment with I^{131}, the pancreatic weight and amylolytic activity/unit weight and /total weight of the pancreas were reduced significantly, the fall in concentration of enzyme being greater with the latter experimental procedure.

1. Barrett, J. L., Nishikawara, M. T., Haist, R. E., *Am. J. Physiol.*, 1955, v182, 35.
2. Baker, B. L., *Am. J. Clin. Nutr.*, 1957, v5, 445.
3. Baker, B. L., Pliske, E. C., *Symp. Soc. Exp. Biol.*, 1957, No. XI, p329.
4. Sesso, A., Tramezzani, J. H., Valeri, V., Migliorini, R. H., *C. rend. Acad. Sci.*, 1955, v241, 775.
5. Baker, B. L., Reid, N. L., Thoms, N., *Am. J. Physiol.*, 1956, v186, 57.
6. Kojima, M., *Quart. J. Exp. Physiol.*, 1917, v11, 255.
7. Herring, P. T., *ibid.*, 1917, v11, 231.
8. Cameron, G. R., *J. Path. Bact.*, 1926, v26, 177.
9. Leblond, C. P., Sergeyeva, M. A., *Anat. Rec.*, 1944, v90, 235.
10. Kinash, B., Haist, R. E., *Canad. J. Biochem.*, 1955, v33, 380.
11. Hashimoto, H., *Am. J. Physiol.*, 1922, v60, 365.

Received October 20, 1958. P.S.E.B.M., 1959, v100.

Toxic Protein Degradation Products. (24515)

HEINRICH RINDERKNECHT AND CARL NIEMANN (Introduced by Dan H. Campbell)
*Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena**

Vaughan(1) first noted that toxic polypeptides may be prepared from a variety of bacterial, plant and animal proteins by relatively simple chemical procedures. As more recent investigations(2-10) suggest that a number of products of endogenous proteolysis may possess substantial physiological activity it was decided to reinvestigate several of Vaughan's easily accessible and toxic "protein split products" with the aid of more modern procedures than those used previously(1) with the expectation that the results of such a study would be useful for detection and examination of similar products of *in vivo* origin. Before presenting our own observations it is necessary to refer to some results obtained by Vaughan and his associates(1). When bacterial and other proteins were treated with boiling 2% ethanolic sodium hydroxide these workers obtained, after neutralization of the reaction mixture with hydrochloric acid, ethanol-soluble fractions containing "protein split products" which, when injected into guinea pigs, progressively elicited all symptoms associated with anaphylactic shock, i.e., peripheral irritation, muscular incoordination, severe cyanosis, convulsions and death by respiratory failure. Such fractions invariably gave positive protein tests but a negative Molisch test. The preparations were hydroscopic golden-yellow powders which were readily soluble in water. Because of the slightly acid reaction of aqueous solutions of the preparations, Vaughan(1) concluded that the products were acidic in nature and that their physiological action was associated with blocking of some vital basic groups of tissue and serum proteins. Fractionation of the above materials failed to give crystalline products but did lead to preparations with con-

* Contribution No. 2410 from Gates and Crellin Labs. of Chemistry.

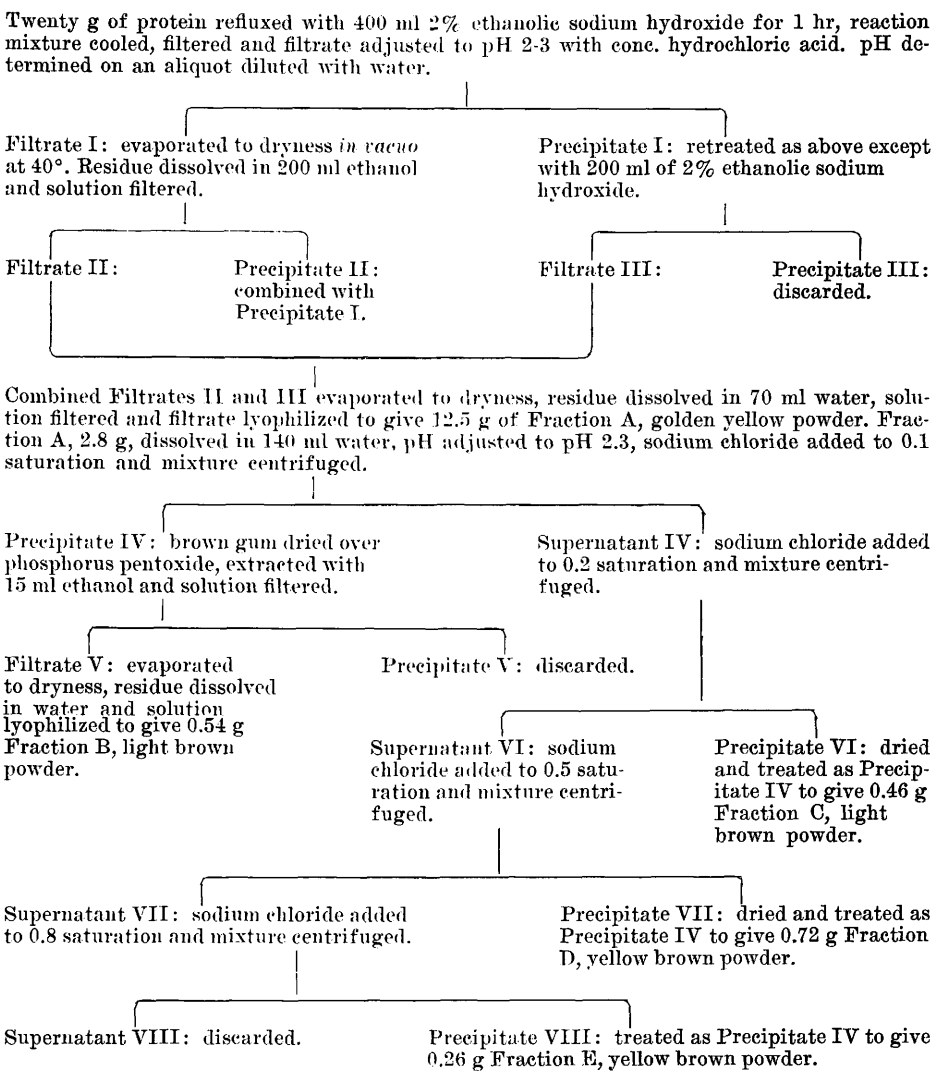

FIG. 1. Treatment of bovine serum albumin and fractionation of toxic polypeptides.

siderably enhanced toxicity. The most toxic preparations were lethal to guinea pigs when administered intravenously or intracardially in doses of approximately 1 mg/kg. However, they did not possess antigenic properties and were non-toxic when given orally.

Materials and methods. The materials employed in our study were Armour crystalline bovine serum albumin, Merck ovalbumin and edible gelatin, treated with 2% ethanolic sodium hydroxide, as described for bovine serum albumin in the flow sheet given in Fig. 1. Homogeneous reaction mixtures were obtained with bovine serum albumin and gelatin while with ovalbumin a small amount of precipitate failed to dissolve. In the toxicity tests, guinea pigs weighing approximately 500 g were employed whenever possible. However, animals deviating considerably from this weight occasionally had to be used. The animals were observed continuously for 90 minutes after injection, then checked after a lapse of 16 to 20 hours. A lethal dose usually killed in 2 to 20 minutes; death after survival for one hour was extremely rare and animals receiving a sub-lethal dose recovered within 24 hours. Toxicity tests on many intermediate fractions were performed on groups of 2

to 4 animals simply as a guide for subsequent operations. Groups of 4 to 8 animals were used for evaluation of toxicity of the final products.

Results. The unfractionated ethanol-soluble polypeptides, *i.e.*, fraction A, prepared from bovine serum albumin or ovalbumin were lethal when administered intraperitoneally at level of approximately 100 mg/kg. The same fraction prepared from gelatin was not toxic. When sodium chloride was added to aqueous solutions of fraction A and the precipitates collected successively at 0.1, 0.2, 0.5 and 0.8 saturation, the precipitates obtained at 0.5 saturation were more toxic than those obtained at lower or higher concentrations of sodium chloride (Fig. 1). Fraction D prepared from either bovine serum albumin or ovalbumin was lethal when administered at 75 mg/kg intraperitoneally or 1 mg/kg intracardially whereas the minimum lethal dose of fraction E was estimated to be approximately 90 mg/kg intraperitoneally or 5 mg/kg intracardially.

A solution of fraction D obtained from bovine serum albumin was placed in cellophane casing and dialyzed against distilled water. The dialysis was followed by determination of optical density at 280 mμ. After 24 hours it was estimated that approximately ⅔ of the original material within the casing had dialyzed whereupon both the dialysate and undialyzed portion were collected and lyophilized. The undialyzed fraction when administered either intravenously or intracardially was lethal at a dose of approximately 2 mg/kg whereas the fraction which had dialyzed was lethal at a level of 0.5 mg/kg. This latter material was approximately twice as toxic as Vaughan's most potent preparation(1).

Electrophoresis of fractions A and D (Fig. 1), on filter paper (Whatman No. 1) at pH 4.1 (acetic acid-sodium acetate buffer) and 8.53 (veronal buffer) did not lead to significant separation of the various components. However, it did establish the cationic nature of these fractions and indicated the presence of many closely related species of similar electrophoretic mobility in the material under investigation. For example, when a preparation of fraction D derived from bovine serum albumin was subjected to electrophoresis at 600 volts and pH 8.53 for 4 hours a diffuse wedge with a spread of approximately 20 cm from point of application towards the cathode was observed. However, more than 50% of the material had migrated less than 6 cm from point of application. While these data, and those obtained from dialysis experiments, can only suggest that fraction D contains cationic components whose molecular weights may vary from 5,000 to 20,000, they explain why Vaughan(1) was led to the erroneous conclusion that the products of the limited alkaline degradation of proteins are acids. It is now clear that the acidic reaction exhibited by aqueous solutions of preparations such as fraction A is due to the fact that these materials are hydrochlorides of basic substances.

With the recognition of the intrinsic basic nature of fraction A, preparations of this fraction, derived from bovine serum albumin or ovalbumin, were dissolved in water and introduced into columns charged with the weakly acidic ion exchange resin IRC-50 (Rohm and Haas) which had been prewashed with an acetic acid-sodium acetate buffer of pH 4.7. Initially the charged columns were washed with water and the "neutral" effluent collected in successive 25 ml portions. The optical densities of these fractions were determined at 280 mμ and when these values were plotted against the fraction number only a single broad maximum was observed. A material balance revealed that the bulk of the original material was retained on the resin. Therefore, it was eluted with 0.1 *N* hydrochloric acid and fractions of the eluate were collected as before. Again spectrophotometric examination of the various fractions revealed only a single diffuse peak indicative of the presence of numerous components of very similar properties. Paper electrophoresis of the desalted "neutral" effluent and the hydrochloric acid eluate showed both to be basic polypeptides although the former appeared to possess fewer basic groups as indicated by its lack of retention on the column and its less intense coloration with bromphenol blue when the latter substance was applied following paper electrophoresis. The hydrochloric acid eluate was lethal at a level of 0.5 mg/kg when given

intravenously. The "neutral" eluate was approximately one-half as toxic. There was no significant difference in toxicities of the fractions derived from bovine serum albumin or ovalbumin.

Elementary analysis revealed that preparations obtained by the various procedures possessed nitrogen contents which varied from 11.0 to 13.5% and sulfur contents from 0.6 to 1.3%. The fractions which had the highest nitrogen content and a sulfur content of approximately 1% were the most toxic.

All of the polypeptide fractions were characterized by considerable absorption at 270-280 mμ, a region in which the original proteins possessed a minimum. Acid hydrolysis failed to alter the characteristics of the absorption curves of the polypeptide fractions indicating that treatment of the original proteins with boiling 2% ethanolic sodium hydroxide produced changes at the amino acid as well as at the peptide level. Because the polypeptide fractions derived from gelatin, which is deficient in the aromatic amino acids, follow the same pattern the latter amino acids hardly can be implicated in the appearance of the new chromophore. It is also evident that absorption at 270-280 mμ is in no way related to toxicity of the degradation products.

The amino acid composition of representative toxic polypeptide preparations relative to that of the parent proteins was determined by 2-dimensional paper chromatography of the respective acid hydrolysates based upon use of t-butanol-water-formic acid and phenol-water-amonia as developing agents (11). The most striking result was the absence of cystine in polypeptide hydrolysates (Fig. 2). Since hydrogen sulfide was liberated when the original reaction mixtures were acidified (Fig. 1), it is probable that most of the cystine was lost through a process involving β-elimination of hydrogen sulfide during treatment with the 2% ethanolic sodium hydroxide(12). A similar reaction appears to have occurred with respect to serine and threonine since both of these amino acids were present only in trace amounts in the polypeptide hydrolysates. It is not known whether these structural changes were in any way associated with degradation of the original protein.

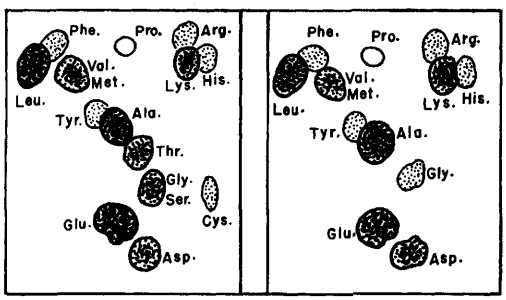

FIG. 2. Representation of paper chromatograms of acid hydrolysates of bovine serum albumin (left) and toxic polypeptide fraction derived from the same protein (right).

The remaining amino acid pattern of the polypeptide hydrolysates did not vary significantly from those of the parent proteins. It was surprising to find that arginine, which is known to give ornithine upon alkaline hydrolysis, had withstood degradation by the ethanolic sodium hydroxide reagent and there was no indication, at the amino acid level, of extensive decarboxylation of the acidic amino acids which might have been anticipated in view of the basic nature of the polypeptide preparations.

Although it is well known that the exposure of proteins to alkaline conditions may result in at least partial racemization no attempt was made to explore this area at an amino acid level largely because none of the polypeptide fractions were toxic when administered orally and those obtained from bovine serum albumin and ovalbumin were relatively non-toxic when given intraperitoneally. With this indication that the polypeptide preparations were capable of being degraded by *in vivo* proteinases and peptidases it did not appear worth while to examine their *in vitro* enzymatic degradation.

Discussion. A detailed account of the pharmacology of toxic polypeptides has been given by Vaughan(1) and by Edmunds(13). Under their influence splanchnic or sciatic nerve of dogs did not respond to stimulation, indicating peripheral paralysis of the vasomotors. Paralysis of the ganglia by large doses of nicotine failed to prevent the characteristic fall in blood pressure following injection of

the toxic polypeptides proving their action to be peripheral to the ganglia. Furthermore, it was found that epinephrine was capable of counteracting the fall in blood pressure associated with moderate doses of the peptide material thus demonstrating that the peripheral nerve endings were the structures primarily affected. However, large doses of the polypeptide appeared to have a more generalized action affecting not only the nerve endings but also the receptive and contractile substances of the blood vessels. Our own observations tend to corroborate this latter finding as pretreatment with epinephrine at a level of 0.6 mg/kg subcutaneously failed to modify the toxic action of a normally lethal dose in the guinea pig. Because of the role assigned to histamine in anaphylactic reactions it was of interest to observe that antihistaminic agents had little or no effect upon the toxic action of the polypeptide preparations. Pretreatment of guinea pigs with 2-(2-dimethylaminoethyl) (p-methoxybenzyl) amino pyridine (Neo-Antergan) at a level of 45 mg/kg intraperitoneally, which should have protected the animals against 200 lethal doses of histamine, in no way altered the toxicity of the polypeptides.† It should be noted that antihistaminic agents also were unable to prevent the inflammatory response of tissue to the polypeptides which Spector(5) isolated from turpentine induced tissue exudates.

Danielopolu and Simionescu(14) have postulated that primary shock is not due to action of histamine but to liberation of acetylcholine. However, pretreatment of guinea pigs with atropine sulfate, at levels of 10 mg/kg subcutaneously or 5 mg/kg intracardially, failed to protect animals given a lethal dose of the polypeptide. As no symptoms of toxicity were observed in the mature control animals when either Neo-Antergan or atropine sulfate were administered at the levels indicated it may be concluded that the toxic action of the polypeptides is not mediated in a primary sense by liberation of histamine or acetylcholine nor is the former substance a component of the peptide preparation.

Addition of toxic polypeptides to an isolated strip of guinea pig small intestine in Tyrode solution, to give a concentration of approximately 1:20,000, caused moderate contraction similar to that observed by Armstrong *et al.*(15) upon exposure of guinea pig uterus and jejunum to a pain producing peptide from plasma, bradykinin, hypertensin, oxytocin, etc.

Although a direct analogy between the chemical degradation of serum proteins by ethanolic sodium hydroxide and enzymatic *in vivo* proteolysis is not permissible, it is tempting to speculate on the toxicity and physiological role of polypeptides arising from elevated *in vivo* protease activity in certain disease conditions(2). The similarity of symptoms elicited by lethal doses of toxic polypeptides to those observed in anaphylactic shock is a particularly striking example. If *in vivo* proteolysis is assumed to yield products of a toxictiy comparable to that of the polypeptide materials considered in this study, 50-100 γ intravenously would suffice to produce severe pathological symptoms in adult guinea pigs. The difficulties that would be encountered in isolating and characterizing such a small amount of material from the entire circulatory system of an animal are obvious and perhaps it is for this reason that such products have so far eluded detection.

Summary. Toxic polypeptides, similar to those obtained by Vaughan(1), have been prepared by treatment of certain proteins with boiling 2% ethanolic sodium hydroxide. These substances are soluble in water and in ethanol, are capable of dialyzing through a cellophane membrane and have been shown to be a mixture of essentially basic polypeptides of similar composition. Their characteristic absorption at 270-280 mμ does not appear to be related to their toxic properties. At an amino acid level the polypeptides differ from their parent proteins in that they contain little or no cystine, serine or threonine. The most toxic preparations, obtained by a salting out procedure, followed by dialysis or by ion exchange chromatography, while non-

† Neo-Antergan at this level was lethal to immature animals weighing approximately 250-300 g. However, intracardial injection of 20 mg/kg of histamine phosphate reversed the action and animals so treated survived.

toxic when given orally were lethal to guinea pigs at a level of 0.5 mg/kg intravenously. Pretreatment of the animals with atropine, epinephrine or Neo-Antergan failed to prevent or modify the effect of a lethal dose of the polypeptide thus excluding liberation of histamine or acetylcholine as the principal cause of toxicity.

1. Vaughan, V. C., Vaughan, V. C., Jr., Vaughan, J. W., *Protein Split Products,* Lea and Febiger, 1913.
2. Menkin, V., *Am. J. Med. Sci.,* 1936, v190, 583.
3. ———, *J. Exp. Med.,* 1936, v64, 485.
4. Duthie, E. S., Chain, E., *Brit. J. Exp. Pathol.,* 1939, v20, 417.
5. Spector, W. A., *J. Pathol. Bact.,* 1951, v63, 93.
6. Greuer, W., Hess, E., Rosteck, I., *Arznerimittelf.,* 1957, v6, 269, v7, 128.
7. Balls, A. K., Hale, W. S., *Cereal Chem.,* 1940, v17, 243.
8. Balls, A. K., Hale, W. S., Harris, T. H., *ibid.,* 1942, v19, 279, 841.
9. Stuart, L. S., Harris, T. H., *ibid.,* 1942, v19, 288.
10. Coulson, E. J., Harris, T. H., Axelrod, B., *ibid.,* 1942, v19, 301.
11. Rockland, L. B., Underwood, C., *Anal. Chem.,* 1954, v26, 1557.
12. Swan, J. M., *Nature,* 1957, v179, 965.
13. Edmunds, C. W., *Z. J. Immunitaets forsch.,* 1913, v17, 105.
14. Danielopolu, D., Simionescu, S., *Acad. Rep. Populare Romane, Bull. Stünt B. Stünte Med.,* 1949, v1, No. 4, 6.
15. Armstrong, D., Keele, C. A., Jepson, J. B., Stewart, J. W., *Nature,* 1954, v174, 791.

Received October 15, 1958. P.S.E.B.M., 1959, v100.

Humoral Regulation of Erythropoiesis V. Relationship of Plasma Erythropoietine Level to Bone Marrow Activity. (24516)

FREDERICK STOHLMAN, JR. AND GEORGE BRECHER
Natl. Inst. of Arthritis and Metabolic Diseases, N.I.H., P.H.S., U. S. Department of Health, Bethesda, Md.

Erythropoietine has been demonstrated repeatedly in plasma of experimental animals following exposure to acute hypoxia and also in certain clinical disorders with severe chronic hypoxia(1). Rate of release of erythropoietine, its disappearance following discontinuance of the stimulus, and maintenance of plasma levels have not been extensively studied. Prentice and Mirand(2) noted a decrease in plasma level of erythropoietine following prolonged exposure to low O_2 concentrations at ambient pressure. Erslev(3) was unable to confirm these observations in anemic rabbits or those exposed to low O_2 concentrations. Since the conditions which influence plasma level of erythropoietine are important in the role of erythropoietine in clinical anemias, a further evaluation of those factors is here reported.

Methods and materials. Erythropoietine was assayed in fasted Sprague-Dawley rats as suggested by Fried *et al.*(4) and described elsewhere(5). Release of erythropoietine was stimulated by bleeding rats 2% of body weight or by exposing them to simulated altitude of approximately 23,000 feet (310 mm Hg) in decompression chamber. In studies on effect of chronic anemia in dogs, plasma was acidified (pH 5.5), heated 10 minutes at 90°C (internal temperature). The material was lyophilized and final preparation concentrated 5-fold to compensate for loss of activity due to heating(6) and dialyzed against isotonic phosphate buffer at pH 7.0. The effect of bone marrow function on plasma erythropoietine levels was studied in rats in which erythroid hypoplasia was produced by ionizing radiations. A dose of 400 r was delivered using a 2.5 MEV Van de Graaff generator at average dose rate of 50 r/minute measured with Bendix ionization chamber known to be air equivalent at that energy.

Results. It was possible to demonstrate erythropoietine in plasma of rats exposed for 2-3 hours at a simulated altitude of 23,000 feet. Plasma concentration of erythropoietine