All these findings demonstrate the toxicity of acetoacetate injections upon the structural integrity of the tissues. They also confirm and explain the biochemical findings as regards the aggravation of disturbances in scurvy by acetoacetate(10). The accumulative disturbances in carbohydrate metabolism thus produced finally tend to affect the architecture of the pancreatic islets accompanied with the degeneration of the nuclei of the β-cells, commonly observed in clinical diabetes(17), thus confirming the deleterious effect of acetoacetate in inducing diabetes in experimental animals and disclosing a possibility of excess accumulation of acetoacetate serving as a prominent factor for gradual development of clinical diabetes.

Summary. The islets of Langerhans of scorbutic guinea pigs and those injected with acetoacetate were hypertrophied. Injection of acetoacetate into scorbutic animals caused further degranulation and degenerative changes. Acetoacetate brought about hypertrophy of renal cortex and scurvy caused atrophy of glomeruli. Combined treatment aggravated these degenerative changes. Both scurvy and acetoacetate caused central degeneration of liver and this is enhanced by the combined treatment. In agreement with histologic changes, biochemical findings indicate the toxicity of acetoacetate. Acetoacetate may be a prime factor in clinical development of diabetes.

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Purification of Pancreatic and Urinary Callicrein. (22701)

MARION E. WEBSTER, WILLIAM R. CLARK, AND PEARL ANDERSON
Department of Biochemistry, Walter Reed Army Institute of Research, Washington, D.C.

The hypotensive substance callicrein (kallikrein or padutin) was first discovered by Abelous and Bardier(1) and systematically studied by Frey, Kraut Werle and co-workers for the next 3 decades(2-3). Procedures for the purification of both urinary and pancreatic callicrein(4-10), with the exception of that developed by Reisser(10), depend to some extent on the adsorption of impurities and/or callicrein on various colloidal salts. Since adsorption conditions are difficult to reproduce and frequently require the running of test samples before each step in the preparation,* the present method was devised based on an ethanolic fractionation scheme such as carried out by Cohn *et al.* upon plasma proteins(11) and utilized successfully by this laboratory for purification of hyaluronidases(12-13).

Methods. Callicrein content of the various fractions was determined by the method of

* Werle, E., personal communication.

Frey *et al.*(2). Dogs were anesthetized with 26 mg nembutal per kilo and the blood pressure recorded by a mercury manometer attached to the carotid artery. Injections of test solutions were made through a polyethylene catheter inserted in the femoral vein. Heparinized saline (50 mg per liter) was used to prevent clotting in the catheter. Samples of pancreatic callicrein containing ¼ to 2 Frey units† gave a response which was complete within one minute and solutions could readily be assayed at the rate of one every 2 minutes particularly with more highly purified preparations. However, with crude urinary fractions the blood pressure frequently did not return to its original level making the quantitation of the results difficult. *Total nitrogen* was determined by micro-kjeldahl digestion and direct nesslerization. For determinations where ammonium sulfate was present, aliquots were made alkaline with NaOH and the ammonia removed by aeration prior to digestion. Samples similarly treated, but not digested, served as controls. *Electrophoretic examination* and separation were conducted at 0-1° by moving boundary methods in the Perkin-Elmer equipment and the Aminco-Stern apparatus. Samples were equilibrated by dialysis with 0.1 ionic strength phosphate buffer pH 7.7.

Results. Purification of pancreatic callicrein: Fresh frozen bovine pancreas was thawed by standing overnight at 4°, then minced by machine. Three methods of extraction (autolysis(9), 0.1 M acetic acid and 0.25 M trichloroacetic acid) showed that 0.1 M acetic acid extracted the greatest number of callicrein units although autolysis gave the most highly purified product. Initial crude extracts were therefore prepared by addition of an equal weight of chilled 0.1 M acetic acid to cold mince. The preparation was stirred for 1 hour at 4° and then centrifuged at 2000 rpm and 26°. The extract was further clarified by filtration on Buchner funnels through filter paper pulp. The filtrate was measured and 1/10 its volume of concentrated buffer added to make 0.02 M phosphate at pH 5.5 containing 0.45% NaCl and 1:10,000 merthiolate. From 5 kg of pancreas a final volume of around 4,500 ml was obtained, containing 2.5-3 Frey units and 30-40 mg protein per ml as calculated from nitrogen assay.

First ethanol fractionation: The crude extract was adjusted to pH 5.5 and fractionated with ethanol at —5°. The ethanol added was precooled at —20° and introduced in a thin stream with concurrent stirring of the cooled buffered solution. After addition of each increment of alcohol, the fraction was allowed to equilibrate for at least 18 hours before removal of precipitate by centrifugation at 4000 rpm and —5°. The precipitate was dissolved in the minimum amount of buffer at 0°. Preliminary fractionation using 0.05 *mole fraction* increments of ethanol demonstrated that 50% of the callicrein activity could be recovered in the precipitate between 0.15 and 0.25 mole fraction with a 6-fold increase in purity based on nitrogen determinations. However, occasional losses of callicrein, due to adsorption on the large precipitate obtained with 0.15 mole fraction, led to eventual adoption of a one step precipitation at 0.25 mole fraction. As illustrated in Table I, this one step procedure effected a 3-4 fold increase in purification and retained all callicrein activity of original extract.

Second ethanolic fractionation: Since callicrein was known to be stable from pH 5.0 to 8.0(14) a pH of 7.5 was chosen for the next ethanolic fractionation. Experimentation revealed that callicrein was now precipitated between 0.25 and 0.45 mole fractions. Precipitate obtained from the first fractionation was dissolved in 0.02 M phosphate buffer and diluted to 10-12 mg protein per ml. The pH was adjusted to 7.5 and precipitates removed following addition of 0.25, 0.3 and 0.45 mole fractions. Excessive adsorption of callicrein on relatively large precipitates obtained with 0.25 mole fraction was not noted at this concentration of protein (Table I). Precipitates obtained from 0.3 and 0.45 mole fraction were

† The authors are deeply indebted to Dr. E. Werle, University of Munich, Germany, for supplying vials of standard callicrein containing 10 Frey units. These preparations contained 5.5-6 Frey units/mg and 45-55 Frey units/mg N.

TABLE I. Ethanolic Fractionation of Pancreatic Callicrein.*

Purification procedure	Conc. ethanol mole fraction	Total callicrein units	% recovered	Purity index, units per mg N
0.1 M acetic acid extract		12,800	100	0.5
1st ethanol fract. pH 5.5	.25	15,000	117	1.6
2nd ethanol fract. pH 7.5	.25	6,400	43	2.5
	.3	2,280	15	25
	.45	12,000	80	37
3rd ethanol fract. pH 6.5	.3	500	3.5	23.5
	.35	650	4.5	45
	.5	5,100	42.5	104
	.55	550	4.2	25

* Above data are based on avg figures obtained from five 5 kg batches of pancreas.

usually sufficiently small to warrant collecting in two centrifuge tubes before dissolving in buffer. This procedure was followed so that residual ethanol present in the cups would not destroy callicrein. The resulting solutions were about 20 times as pure as that obtained in first ethanol precipitation.

Third ethanolic fractionation: This fractionation was conducted on combined precipitates from 0.3 and 0.45 mole fractions from above. They were diluted in buffer to 0.5 to 0.75 mg protein per ml with the pH adjusted to 6.5. Even though the pH was nearer the isoelectric point (pH 4.0 to 4.3(2)), the decrease in concentration of protein being fractionated caused the bulk of activity to precipitate betwen 0.35 and 0.5 mole fractions (Table I). Following the three ethanolic fractionations approximately 5,000 Frey units could be obtained from 5 kg of bovine pancreas. The final preparation was approximately 200 times purer than initial crude extracts. Average yield of material calculated as protein was 400 mg. Callicrein of this purity was reasonably stable when stored at 4° and at 0.1% protein concentration. It is possible that variation in activity obtained from one preparation to another at this stage may in part reflect a certain instability. Crude acetic acid extracts could be dialyzed against running tap water without inactivation. However, material from the third ethanolic fractionation lost 50% of its activity in 24 hours dialysis. It could be dialyzed against buffer at 4° without loss.

Electrophoretic examination and separation. In order to obtain sufficient protein concentration for electrophoretic examination, 6,500 callicrein units which had been obtained by 3 ethanolic fractionations and which gave an average purity of 75 units per mg N were recombined in 350 ml, pH was adjusted to 5.5, and 0.3 mole fraction of ethanol added. The precipitate was extracted with 3 successive 12 ml portions of 0.1 ionic strength phosphate buffer pH 7.7. Approximately 75% of the enzyme was recovered in 3 solutions with 2-fold increase in purity for the first extract. The 3 solutions were separately dialyzed against buffer at 4°. No loss in total enzymatic activity was noted. The first extract showed another 2-fold increase in purity, the second extract went from 35 units per mg N to 150 units per mg N, while the third extract remained unchanged at 60 units per mg N. Each of the 3 solutions were analyzed electrophoretically by moving

TABLE II. Electrophoretic Separation of Partially Purified Callicrein.

	Total callicrein units	Purity index, units/mg N	Relative % of electrophoretic components 1	2	3	4	5	6
Extract No. 1	100	300	47	18	11	24	—	—
Ascending	75	430	69	19	6	6	—	—
Descending	7.5	85	18	18	18	45	—	—
Extract No. 2	850	150	27	22	6	13	8	23
Ascending	540, 540*	330, 297	42	29	6	11	4	8
Descending	61, 46	35, 37	14	16	6	15	11	38
Extract No. 3	100	60	14	9	2	5	5	65
Ascending	35, 75	320, 400	42	23	4	9	5	17
Descending	15, 30	38, 52	5	4	1	4	5	81

* Values represent duplicate determinations on same extract.

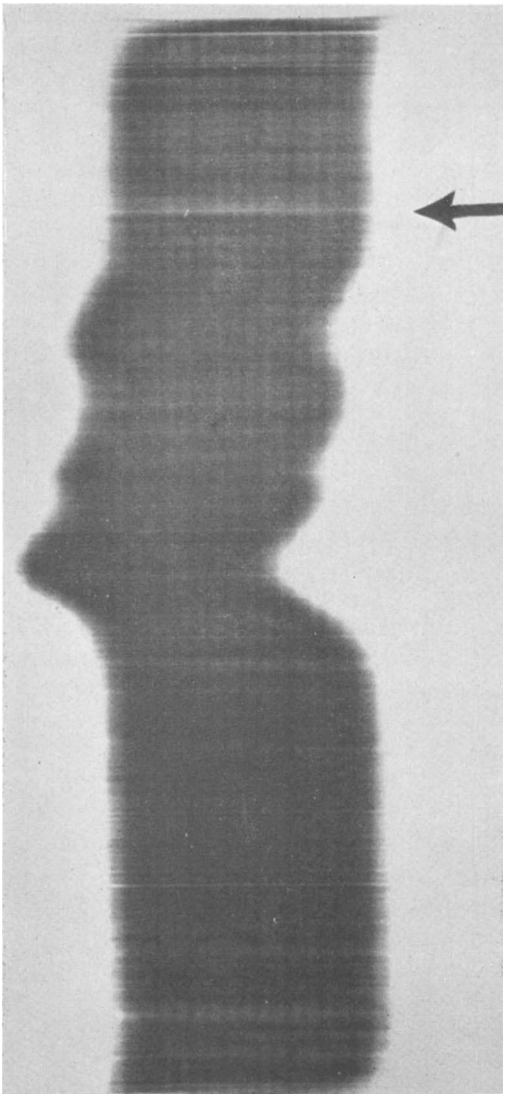

FIG. 1. Ascending electrophoretic pattern of partially purified callicrein (Extract No. 1). Time: 1920 sec. Volts: 5.0 volts/cm. Arrow indicates starting point.

boundary technic (Fig. 1) and then separated into 2 fractions, one containing the major portion of the fast component and the other the slow material with the intermediate substance being divided between the 2 fractions. As is shown in Table II, the sample with highest purity index, extract 1, showed 4 components with mobilities ranging from about —6 to —3 x 10^{-5} $cm^2sec^{-1}volt^{-1}$. The fastest component (as estimated from distribution pattern areas) accounted for 47% of the original material and the slowest 24%. Electrophoretic separation increased the concentration of fast material to 69%. The other two extracts contained 6 or more components varying in mobility from —6 to —0.5 x 10^{-5} $cm^2sec^{-1}volt^{-1}$. Their decrease in purity index paralleled the decrease in content of fast material and the corresponding increase in slower moving components. In addition, the activity of preparations following separation was invariably associated with the fast component. Callicrein, therefore, is tentatively identified as the most rapidly migrating substance present in these solutions. Activity of these purer preparations of callicrein, rapidly lost at room temperature, could be maintained only by storage of samples in frozen state. Further purification by moving-boundary procedure did not appear feasible since mobilities of various components were similar and diffusion rates were fast. Paper electrophoresis at 4° and 25°, could also be used for further purification of this enzyme. However, considerable loss was incurred both from irreversible adsorption on paper and by inactivation during time period required for the experiment.

Ammonium sulfate fractionation. Ammonium sulfate fractionations were carried out at 4° and individual precipitates were allowed to equilibrate for 24 hours before being removed by centrifugation at 4000 rpm, 4°. As is shown in Table III levels of purity were obtained which compare favorably with those achieved by electrophoretic separation. However, callicrein of this purity again readily lost activity at room temperature. The technic of adding albumin to maintain high protein concentrations which had been successfully used in stabilization of hyaluronidase up to 100,000 T.R.U. per mg N(15), failed

TABLE III. Ammonium Sulfate Fractionation of Partially Purified Pancreatic Callicrein.

Saturation of $(NH_4)_2SO_4$	Total callicrein units	% recovered	Purity index, units/mg N
.3	750	7.5	10
.4	1,200	12.0	50
.5	4,950	49.5	450
.6	1,500	15.0	300
Supernatant	750	7.5	5

to prevent inactivation of the hypotensive enzyme.

Purification of urinary callicrein. Methods originally devised for purification of bovine pancreatic callicrein were subsequently applied to purification of urinary callicrein from human urine. Urine was collected and stored for short periods of time under xylene. Addition of suitable amounts of 0.02 M phosphate buffer was made and pH adjusted to 5.5. Preliminary fractionation indicated that despite the increased dilution of enzyme in solution (0.075 Frey unit per ml as compared to 2.5-3.0 per ml for crude pancreatic extract) 90-100% of the enzyme was precipitated by the 0.25 mole fraction of ethanol. However to insure complete precipitation of callicrein 0.3 mole fraction was utilized. In 100 liters of human urine used in present studies, the average number of callicrein units was 0.75 Frey unit per 10 ml. This number of units is within the range found by previous investigators(2).

Callicrein in 40 liters of normal urine was concentrated by precipitation at pH 5.5 with 0.3 mole fraction. The solution was then diluted to 5 mg protein per ml as calculated from nitrogen assay, the pH adjusted to 7.5 and ethanol added to 0.15 mole fraction. Although the bulk of activity failed to precipitate until 0.3 mole fraction of ethanol, this stepwise separation was utilized in order to minimize loss by adsorption. The ethanol concentration was then raised to 0.25 mole fraction and a second inactive precipitate was removed. Precipitates from 0.3, 0.35 and 0.4 mole fractions were also obtained. As is shown in Table IV, the 0.35 fraction contained half the starting activity with better than a 50-fold increase in purity. Urinary callicrein differed from pancreatic in that precipitation at pH 7.5 occurred at a lower mole fraction despite an increased dilution of enzyme in solution (0.7 Frey unit per ml as compared to 3 units for pancreatic preparation).

Discussion. Although originally reported to be present in relatively large amounts in the saliva, pancreas and urine, more recent evidence by Werle and Maier(3) suggests that pancreatic and urinary preparations are chemically and pharmacologically different from the submaxillary substance. Callicrein is reported to exert its hypotensive effect by enzymatic action on callodinogen, present in normal plasma, to release a smaller molecule, presumably polypeptide, called callidin. In this manner the callicrein-callidin system with respect to hypotension can be compared to the renin-hypertensin (angiotonin) mechanism which has been associated with hypertension(16).

TABLE IV. Purification of Urinary Callicrein.

Purification procedure	Conc. ethanol mole fraction	Total callicrein units	% recovered	Purity index, units per mg N
Normal human urine		3,000		.01
1st ethanol fract. pH 5.5	.3	2,880	96	1.0
2nd ethanol fract. pH 7.5	.15	47.3	1.6	1.0
	.25	100	3.5	1.6
	.3	280	9.7	14
	.35	1,440	50.0	57
	.4	160	5.5	10

Callicrein has previously been obtained at high levels of purity by two authors(5,7) who utilized a variety of technics. The purity achieved by Bischoff and Elliot(5) from human urine is probably similar to that obtained by us with pancreatic callicrein. However, Kraut and Schweitzer(7) have reported preparation of callicrein solutions containing 2 micrograms of organic material per callicrein unit. Calculation of these data based on the assumption that a pure protein had been isolated would give a purity index of 3000 Frey units per mg N. On the other hand, if the fast component in electrophoretic analysis at pH 7.7 can be taken as a measure of callicrein, then a solution containing 700 Frey units per mg N should represent a homogenous preparation. The discrepancy between these calculations is so great that further work would be necessary to elucidate the difference. Those authors using bovine pancreas(9-10) attempted only preliminary purification of the callicrein. While it is undoubtedly true that more units of callicrein can be obtained by autolysis than by extraction with dilute acetic acid, in our hands, as well as those of Reisser(10), autolysis at 37°

gave secondary reactions which were undesirable and caused inconsistent results. The autolysis devised by Reisser which takes place for 72 hours at 2° has not been attempted in this laboratory since it was desired to take all possible precautions against contamination of the extracts. However, this method of extraction should cause a 4-fold increase in the recoverable units.

The isolation of callicrein as a chemical entity would appear to depend on the finding of a method for stabilizing this hypotensive substance. The instability encountered in our preparation at around 400 Frey units per mg N and those reported by other authors(5,7) has hampered further attempts to characterize this enzyme.

Summary. A method has been devised for preparation of highly purified pancreatic callicrein. The fractionation procedure involves three ethanolic fractionations at pH 5.5, 7.5, and 6.5 followed by one ammonium sulfate precipitation. Solutions of hypotensive enzyme at this level of purity proved highly unstable and further purification was abandoned. Preliminary purification of urinary callicrein revealed that the method could be adapted to this source.

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Tolerance of the Rat for N,N'-Diphenyl-P-Phenylenediamine. (22702)

H. H. DRAPER, S. GOODYEAR, K. D. BARBEE, AND B. C. JOHNSON

Division of Animal Nutrition, University of Illinois, Urbana

The demonstration that N, N'-diphenyl-p-phenylenediamine (dppd) is effective in the prevention of encephalomalacia in chicks fed vit. E-low diets containing fish oils, and in the stabilization of carotene and vit. A in practical rations, has led to its widespread use in commercial mixed feeds. The concentration commonly employed is 0.025% of the diet. Matterson *et al.*(6) have reported that chicks which received a diet containing 1% dppd throughout a 12-week growth trial remained free of serious lesions and maintained a normal growth rate. Draper and Johnson (1) observed no adverse effects from administering dppd to lambs at a level of 0.1% for 7 weeks, and Jensen and coworkers* reported that 0.025% dppd improved reproductive performance in turkey hens fed a tocopherol-low ration.

The present experiments were designed originally to determine the degree of replace-

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