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THE PREPARATION AND PURIFICATION OF A PLASMA
CALCIUM RAISING PRINCIPLE FROM
BOVINE PARATHYROID GLANDS

By

James J. Smullen

A thesis submitted in partial fulfilment of the requirements
for the degree of Master of Science in Medicine in Loyola
University.

1931.

VITA

James Joseph Smullen

I was born November 10, 1908 in Johnstown, New York. I attended the Johnstown Public Schools and graduated from the grammar school in June, 1922. High school training was received at the Johnstown High School 1922-24, at the Robert A. Waller High School, Chicago, and graduation followed at Lakeview High School, Chicago, February, 1926. Pre-medical work was obtained at Crane Junior College, 1926-28. I entered Loyola University Medical School, 1928 and received the Bachelor of Science degree from Loyola University, 1930. I was student fellow in the Department of Physiological Chemistry during the year of 1930 to 1931.

I wish to extend to Dr. W. R. Tweedy my appreciation of his efforts to give me something more than an introduction to the field of research in this particular branch of chemistry, and my gratitude for the unsparing time and benefit of his experience he has given me in guiding and supervising my work on this problem. I also wish to thank him for his permission to incorporate verbatim from one of his previous publications Part I of the Method of Preparation, which was adhered to more or less strictly in preparing our material.

I also wish to thank Dr. W. C. Austin for the many courtesies he extended to me during this year.

Introduction.

During the past year, the author has sought to further purify the calcium raising principle contained in a fraction separated from parathyroid glands by a procedure developed by Tweedy (9). An additional procedure is herein given, which yields a uniformly active product, qualitatively and quantitatively different in several important respects from any thus far described.

Berkeley and Beebe (1) separated a nucleoprotein from bovine glands by extraction with slightly alkalinized 0.9 per cent sodium chloride solution which was claimed to completely relieve acute parathyroid tetany. Tryptic digestion, or the action of pepsin-hydrochloric acid for 48 hours severely injured, but did not completely destroy the activity of the nucleoprotein.

MacCallum and Vogel (2) prepared extracts by grinding ox parathyroids and, in two instances, dog parathyroids with Ringer's solution. In some cases glycerol was added to the Ringer's solution used in the extraction. They found the tetany-relieving principle present in the solution from which the nucleoprotein has been precipitated by acidification as well as in the precipitate.

Hanson (3) prepared extracts by boiling bovine parathyroid

glands with 0.2 per cent hydrochloric acid. In 1925 Hanson showed that the substance isolated by his method would not only relieve tetany, but would increase the blood calcium as well.

Berman (4) utilized acidified alcohol in extracting a crystalline substance which he describes as the parathyroid hormone. In a subsequent paper he does not refer to his product as crystalline, but presents proof that it will cause an increase in blood calcium.

Collip (5) was first to offer convincing evidence that an active serum calcium increasing principle might be obtained by acid extraction from bovine parathyroid glands. The active product is described by Collip and Clark (5) as a substance of the nature of a complex protein derivative, or else a simple substance associated with such a type of compound. It is described as soluble on either side of its isoelectric point which was found to be at pH 4.8 to 4.9. In the absence of salt it is precipitated at pH 4.6 to 5.2 with maximum flocculation at pH 4.8 to 4.9. However, with stronger acids such as 4 per cent hydrochloric acid it was again reprecipitated from solution. It was soluble in dilute alkali, and could be reprecipitated by acidification to the isoelectric point. The active substance was completely removed from acid solution by half saturation with ammonium sulphate, or by complete saturation with sodium chloride.

Collip described his product as giving the common protein reactions. The Molisch and orcin hydrochloric acid tests were negative. Iron and sulphur were present, but phosphorus was absent. The air dried substance assayed 14.6 per cent nitrogen, but when the product was dried over potassium hydroxide and concentrated sulphuric acid it analyzed 15.5 per cent nitrogen. It gave an amorphous precipitate with picric and picrolonic acid. It was slightly soluble in absolute alcohol, quite soluble in 80 per cent alcohol, and insoluble in ether, pyridine, and anhydrous acetone.

Collip found that his product was completely inactivated when it was boiled for one hour with 5 per cent sodium hydroxide, or 10 per cent hydrochloric acid. The enzymes, pepsin and trypsin, completely inactivated it. He also found that the dialysate from 1000 units was inert. Norit or Folin-Wu tungstic acid reagents were found effective in removing it from water solution.

Collip states that the product on which the above tests were made had a potency such that 30 milligrams were equivalent to 100 units, or in other words, the amount he found necessary to inject subcutaneously into a 20 kilogram dog to bring about an increase of 5 milligrams in blood serum calcium per 100 cubic centimeters after an interval of 15 to 16 hours.

Fisher and Larson (6) prepared active extracts by means of normal hydrochloric acid and also by 3 per cent acetic acid in 95 per cent alcohol at room temperature.

Hjort, Robison and Tendick (7) showed that 0.1 normal hydrochloric acid, and acid alcohol extraction yielded potent products, thus corroborating the claims of Hanson and Berman that very weak acid was an effective extracting reagent. They showed that at room temperature extraction was not nearly as effective as extraction at higher temperatures. They further demonstrated that the lipoid free portion of the gland yielded the active fraction.

Davies, Dickens and Dodds (8) obtained a potent preparation by acetone picric acid extraction without preliminary extraction with hydrochloric acid. The picrate is described as insoluble in water, but convertible to the hydrochloride which is easily soluble.

Tweedy (9) described a plasma calcium-increasing principle prepared by hydrochloric acid extraction of acetone-desiccated and defatted parathyroid glands. The active principle is described as adsorbable by kaolin at pH values greater than 5. Subsequent acidification to pH values of 1 to 3 failed to release the activity. In crude form the active material was found to be partially soluble and stable in liquid ammonia, and in ethyl lactate. 2 to 3 hours irradiation with ultra-

violet light did not destroy or increase the calcium-raising activity of the material. 8 to 20 milligrams were found effective in increasing the blood calcium of dogs weighing from 14 to 19 kilograms. In one instance 20 milligrams were found sufficient to increase the blood calcium of a 20 kilogram dog 5 milligrams above the control value within 16 hours. In a subsequent publication (9) a modified method of preparation is described, together with additional observations on the yield, potency and solubility of the product.

Experimental

In as much as I have found phenol a very useful reagent in the preparation of a uniformly potent plasma calcium increasing principle, it seems worth while to summarize the use which has heretofore been made of this reagent in connection with protein studies.

Kjeldahl (10) has shown that the vegetable protein gliadin is soluble in phenol and Mathewson (10) that it is soluble in paracresol. The latter author has also shown that gliadin is precipitated from solution in phenol by adding ether, acetone, pyridine, benzene, or chloroform. He has further found that when gliadin is dissolved in phenol, the solution can be heated to 140° C. without producing any notable effect on the specific rotation of the dissolved gliadin.

Moloney and Findley (11) have reported phenol to be a solvent for insulin. Abel and Geiling attempted to use either crystalline or 90 per cent phenol as a reagent for hydrolyzing or condensing impurities associated with active insulin. Although unsuccessful, they were able to use it as a solvent by means of which the active principle was freed of much of its associated impurities. Abel, Rouiller, and Geiling (12) have also found phenol to be a solvent for the active principles of the pituitary gland.

Recently Tweedy (9) reported warm 90 or 100 per cent phenol to be a solvent for the active principle of the parathyroid gland.

Method of Preparation of Glandular Extracts

The method of preparation is described in two parts. There follows first, the procedure as developed in Tweedy's previous work. The second portion of the method of preparation represents the additional procedure developed this year.

Part I.* Fresh frozed parathyroid glands, from which extraneous fat is trimmed, are ground in a meat chopper. The finely ground material is then transferred to a conical flask of

*Quoted from Dr. Tweedy's article with his permission, J. Biol. Chem. 1930, 88, 649.

suitable size, covered with 200 cc. of 3 per cent hydrochloric acid per 100 gr., and heated in a water bath at 70° for 20 minutes. The flask is immediately cooled under a stream of cold water, while being rotated in order that liquefied fat may cool and solidify on its inner surface. The acid extract is further freed of fat particles, and of undissolved glandular material by pouring through several folds of cheese cloth. By means of weak sodium hydroxide the reaction of the solution is adjusted until near the neutral point, but slightly on the alkaline side of Congo red. An equal volume of redistilled anhydrous acetone is added, and the mixture set aside in the ice chest overnight.

The following day the aqueous acetone extract is filtered through a coarse filter paper, and evaporated in vacuo on a water bath at 50° C. until approximately one third of its volume is reached. It is then adjusted to the same volume as the neutralized acid extract by the addition of water and a sufficient volume of a freshly prepared solution of trichloroacetic acid to produce a concentration of 2½ per cent. After setting a few hours or overnight in the ice chest the finely divided precipitate has settled to the bottom of the beaker.

The trichloroacetic precipitate is separated from most of the supernatant solution by decantation, the remainder being removed by centrifugation. The tightly packed precipitate is stirred up with little alcohol, transferred to a desiccator,

and dried in vacuo at room temperature. After further drying for several hours over anhydrous calcium chloride, the material is ground to a fine powder. It is then tightly wrapped in an ashless filter paper, transferred to a Soxhlet apparatus and extracted with redistilled anhydrous chloroform as long as lipid material is being removed.

Part II. A typical experiment will illustrate the procedure developed for the further purification of the potent fraction prepared in Part I. of the method of purification.

Blend 4, consisting of 12.3981 grams was covered with 65 cc. of 90 per cent aqueous phenol, and heated at 70° C. in a water bath for five minutes. The undissolved material was stirred, and the heating continued a few more minutes until complete solution was attained. 10 cc. portions were then transferred to several 50 cc. centrifuge tubes and 30 cc. of absolute alcohol were gradually added while stirring. The tubes were covered and set aside in the ice box for a period of one hour.

The "alcohol" precipitate was collected by centrifugation, and the mother liquor set aside in the ice chest until further treated. The "alcohol" precipitate was washed several times with ether at the centrifuge to remove uncombined phenol and was finally dried of ether in vacuo. The weight of the fraction precipitated by alcohol was 9.5010 grams, and its potency

is shown in Table II.

20 cc. of the mother liquor were transferred to each of several centrifuge tubes, and an equal volume of anhydrous ether was added while stirring. The tubes were covered and set aside in the ice chest for one hour, after which the "ether" precipitate was separated by centrifugation. The "ether" precipitate was washed several times with anhydrous ether and dried in vacuo. The weight was found to be 2.3700 grams. Its potency is shown in Table III.

The total weight of the alcohol and ether precipitates was 11.8710 grams, or 95.7 per cent of the starting material. Several experiments have shown that when the directions outlined here are closely followed, there is very little variation in the yield of each active fraction, or in the amount of unrecovered material.

Method of Biological Assay

The method of assay has been essentially the same as that described by Tweedy (9). The analytical values which appear in the tables represent in most all cases average values of duplicate analyses.

TABLE I.
POTENCY OF ACTIVE FRACTIONS

Preparation Number	gms. of yield	Dog Kg.	Dose Mg.	Blood Plasma Calcium mg. per 100 cc.	
				Initial	After 15-16 hrs.
Blend 2					
P87 A	2.0000	14.0	100	normal	16.00
Blend 3					
P88 A	5.9320	17.3	100	12.68	17.04
P88 B	3.2432	14.5	100	normal	14.30
P87 A	2.3262	14.0	100	"	16.00
Blend 4					
P85 A	3.8814	17.5	100	11.03	12.94
P85 B	4.4334	12.7	100	normal	16.50
P87 B	4.8800	19.1	60	11.45	12.40
Average		15.6	93	11.72	15.02

TABLE II.

POTENCY OF ACTIVE FRACTION PRECIPITATED FROM 90 PER
CENT AQUEOUS PHENOL (PART II) BY ALCOHOL.

Dog No.	Wt. Kg.	Parathyroid Hormone		Blood Plasma Calcium	
		Blend No.	Alcohol Precipitate	mg. per 100 cc. Initial	After 15-16 hrs.
22	13.2	2	60 mg.	9.27	14.20
15	14.1	4	30 "	13.00	12.50
21	20.3	2-4 inc.	100 "	12.93	20.45
18	17.3	2-4 "	100 "	12.33	18.13
21	21.1	2-4 "	100 "	11.36	15.46
16	14.5	2-4 "	60 "	11.33	15.27
Av.	16.7		75 "	11.70	16.00

TABLE III.

POTENCY OF ACTIVE FRACTION PRECIPITATED FROM 90 PER
CENT AQUEOUS PHENOL (PART II) BY ETHER.

Dog No.	Wt. Kg.	Parathyroid Hormone		Blood Plasma Calcium	
		Blend No.	Ether Precipitate	mg. per 100 cc. Initial	After 15-16 hrs.
15	14.1	2	30 mg.	11.36	15.31
16	15.0	3	30 "	9.30	15.33
16	15.0	4	30 "	12.60	16.79
17	19.8	2-4 inc.	30 "	12.38	16.42
18	17.3	2-4 "	30 "	12.75	16.88
19	16.4	2-4 "	30 "	11.27	15.11
20	15.9	2-4 "	30 "	11.10	15.86
21	21.1	2-4 "	30 "	11.15	14.41
Av.	16.8		30 "	11.49	15.76

TABLE IV.

COMPARATIVE POTENCY OF ACTIVE FRACTIONS PRODUCED IN PART I
AND PART II.

Parathyroid Hormone Preparations	Number of tests	Dog Wt. Kg.	Dose Mg.	Average Values	
				Blood Plasma Calcium mg. per 100 cc. Initial	After 15-16 hrs.
Active Fractions (Part I)	7	15.6	93	11.72	15.02
"Alcohol" Precipitate (Part II)	6	16.7	75	11.70	16.00
Ether Precipitate (Part II)	8	16.8	30	11.49	15.76

Analyses of Active Fractions

The non-volatile material obtained on ashing the "ether" precipitate in platinum constituted 3.5 per cent. Iron and phosphorus were present, but sulphur was absent. On a 0.2 per cent solution, the Molisch test was negative, but was positive on a 1 per cent solution on standing. After hydrolysis for one hour with 5 per cent sulphuric acid, both the 0.2 per cent and 1 per cent solutions gave the Molisch test. The orcin hydrochloride test was negative on both the 0.2 per cent and 1 per cent solutions before and after hydrolysis with 5 per cent sulphuric acid for one hour.

The biuret test was positive on the "ether" precipitate. The ninhydrin test, however, was negative. Millon's test proved positive, but since the material probably contained a trace of phenol, and since a 0.05 per cent solution of phenol gave a color many times more intense when carried out under identical conditions, this may mean nothing. With Ehrich's p-dimethyl-amino-benzaldehyde test, a pink color appeared on addition of the aldehyde reagent to both the 0.2 per cent and 1 per cent solutions after heating with hydrochloric acid for one-half minute. Upon addition of the sodium nitrite solution, however, a yellow color appeared, and any color other than yellow could not be obtained even though the nitrite solution was varied from very weak to very strong. A 0.2 per cent solution

of the "ether" fraction did not yield a positive Hopkins-Cole test, but a 1 per cent solution developed a very slight reaction. Analysis of the nitrogen content by Pregl's (13) micro-Kjeldahl distillation method gave 13.09 per cent nitrogen when dried over concentrated sulphuric acid and solid potassium hydroxide, and when corrected for non-volatile ash.

The non-volatile material obtained on ashing the "alcohol" precipitate in platinum was found to represent 2 per cent of the whole material. Iron and phosphorus were present, but sulphur was absent. On hydrolysis for one hour with 5 per cent sulphuric acid, the Molisch test was positive, but the orcin hydrochloric acid test proved negative. Half saturation with ammonium sulphate precipitated the active fraction from solution. With Millon's reagent there was a slight reaction. However, as previously indicated, a trace of phenol may have been responsible for the reaction. The nitrogen content after correction for non-volatile ash was found to be 10.68 per cent.

Discussion

Upon finding the potency of the active fraction prepared by Part I of our method of preparation lower than usual, our first hope was to effect a separation of completely inert material from the active principle by fractionation from phenol

Several experiments have convinced us that while a uni-

formly active product can be separated from a less active product, neither fraction is rendered completely inert by repeated dissolving in 90 per cent phenol and precipitation therefrom with alcohol and ether.

A remarkable observation has been the very apparent gain in total activity obtained by fractionation of the active material from phenol. With due consideration for the small fraction of 5 to 10 per cent left behind in the phenol, and the factors concerned in estimating the potency of the material, we are still convinced that the phenol treatment results in a decided increase in total activity.

We cannot say definitely how the increase in potency is produced, but can only suggest some possibilities. It may be that further hydrolysis occurs, resulting in the separation of some of the hormone from substances which hold it in inactive form. Another possibility is that a calcium lowering substance is separated from the active fractions, and remains behind in the alcoholic phenol solution. The latter theory appears less likely to us than the first, although the first theory is also objectionable. The time of contact with the phenol is very brief, and the temperature is only allowed to remain at 70° C. a few minutes. Furthermore, as a hydrolyzing agent phenol is comparable to aqueous acid alcohol, which is without effect in increasing the total activity.

The qualitative and quantitative findings are also interesting since they differentiate our most active fraction from Collip's most active product. Our most active preparation is most decidedly different in containing no sulphur. This shows that none of the sulphur containing amino acids form a part of the hormone. Our preparation gives positive phosphorus and Molisch tests, while Collip's product does not. These findings are no so significant, since it may be that impurities are responsible for these positive tests.

Collips preparation gives a positive ninhydrin test, indicating some free carboxyl and amino groups, while our product does not give this test. We are at a loss to explain this difference as we know we are dealing with a protein-like substance. It may be that the phenol has produced changes which render either free carboxyl or amino groups unavailable for the reaction, or it may be that the colored product formed with ninhydrin is destroyed, or not allowed to develop.

Thus far in our work we have only made one quantitative measurement. Several micro-Kjeldahl determinations for total nitrogen, carried out by Pregl's procedure on both the "alcohol" and "ether" fractions give results too low for pure protein. The non-volatile material of each fraction is also very high. On carefully ashing each fraction we have also noticed a considerable quantity of white solid which is crystallizable and

distills off when the platinum crucible is brought to dull redness. Our most obvious conclusion would be that the material is inorganic as the melting point is well above 300° C.

A small amount of this material has been crystallized from absolute alcohol, and examined microscopically. While there were some small crystals resembling sodium chloride cubes, there was also another crystalline form present. While we would expect some sodium chloride to be converted to sodium carbonate by the heat treatment, we were not able to produce crystals similar to this unknown form by crystallization of sodium carbonate from alcohol. Potency tests made on ashed samples have been negative.

Conclusions

Phenol is a most useful solvent from which the plasma calcium raising principle of the parathyroid glands may be separated in a very potent form.

There appears to be a decided increase in total activity of active material dissolved in phenol and fractionated therefrom by means of alcohol and ether.

The most active preparations thus far prepared do not contain sulphur, and do not give the ninhydrin test differing in these respects from Collip's active preparations.

It appears that the active group is associated with par-

tially hydrolyzed protein like substances that have undergone denaturation.

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