arranged through a mercury seal, and a thermometer the bulb of which was immersed in the liquid when the material melted. The mixture was heated with stirring; carbon dioxide evolution was appreciable at 200°, and it was allowed to escape through the side tube of the flask and to bubble through water, which gave an indication of the progress of the reaction. The temperature was allowed to rise and was maintained at 260° until carbon dioxide evolution ceased (about four hours). It is necessary to continue until the carbon dioxide evolution has completely stopped. If a distillation is attempted before this point, a considerable quantity of anthraquinone is formed, which contaminates the product. The stirrer was taken from the flask, a short air condenser attached to the side tube and the thermometer raised out of the liquid as for distillation. The crude benzophenone was distilled over until the drops of distillate became dark in color; the weight of this crude product was 209 g., 86.6% of the theoretical. One crystallization from 95% alcohol gave pure benzophenone, m. p. 47–48°, in an amount corresponding to 82–84% of the theoretical based on the *o*-benzoylbenzoic acid. The crystallization may be replaced by distillation in a vacuum.

Summary

A method for the preparation of benzophenone has been described.

PRINCETON, NEW JERSEY

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF PARKE, DAVIS AND CO.]

THE ACTIVE PRINCIPLES OF THE POSTERIOR LOBE OF THE PITUITARY GLAND.[1] I. THE DEMONSTRATION OF THE PRESENCE OF TWO ACTIVE PRINCIPLES. II. THE SEPARATION OF THE TWO PRINCIPLES AND THEIR CONCENTRATION IN THE FORM OF POTENT SOLID PREPARATIONS

BY OLIVER KAMM, T. B. ALDRICH, I. W. GROTE, L. W. ROWE AND E. P. BUGBEE

RECEIVED DECEMBER 31, 1927 · PUBLISHED FEBRUARY 4, 1928

Introduction

The manifold physiological activities of extracts of the posterior lobe of the pituitary gland are now well known; namely, their effect in stimulating uterine contractions (oxytocic activity), their ability to raise the blood pressure (pressor activity), and their diuretic-antidiuretic effects (renal activity). These three types of activity have led the way to three definite and important medical applications; these are illustrated respectively by the use of pituitary solutions in obstetrics, in the treatment of surgical shock, and in the control of diabetes insipidus.

[1] Presented before the Detroit meeting of the American Chemical Society, September, 1927.

On the other hand, chemical information concerning the physiologically active constituents of pituitary extracts is rather meager; in fact it has not been shown definitely whether the above enumerated pharmacological activities are due to a single chemical substance (hormone) or to the presence of several different compounds. The American investigators, and this is true especially of the Johns Hopkins and the U. S. Hygienic Laboratory groups, have defended the evidence favoring a single active principle, whereas the English workers have argued for two or three principles, while the Germans at one time claimed the separation of even a larger number.

Our own work has been directed towards the isolation of the active principle (or principles) in pure form in the hope that something might be learned concerning its chemical nature. Assuming the presence of a single hormone, it is obvious that the same result will be attained irrespective of the particular method chosen for analysis. In our own experience the pressor assay method is capable of an accuracy of 10%, the oxytocic assay method of 10 to 20%, while the diuretic method is scarcely adapted to quantitative work. Because of its greater accuracy we adopted the pressor assay method and proceeded with the concentration of the active principle.

As a result of a new method of manipulation to be described presently, we succeeded in preparing a very potent product—in fact when this fraction was tested by the pressor assay method it appeared to be many times as potent as the very active product reported by Abel, Rouiller and Geiling,[2] although an accurate comparison could not be made. However, when this potent product was subjected to an accurate assay by the oxytocic method it was found that most of the smooth-muscle-stimulating principle had disappeared—either it had been eliminated in the fractionation process or it had been destroyed. Accordingly the work was repeated and all fractions, residues and solutions normally discarded were carefully reëxamined. As a result an efficient method was developed for the separation of the pressor from the oxytocic activity.

This illustrates the necessity of applying more than one quantitative assay method in any attempt at fractionation and concentration of extracts of the posterior lobe—a requirement that has been disregarded by most workers in this field. However, in demonstrating the presence of more than one active principle it is necessary not merely to obtain fractions that differ from each other when subjected to two or more methods of assay (since this result might be due to the partial injury of a single active principle), but it is necessary to obtain complete quantitative evidence to demonstrate that a separation can be effected, and by recombining the fractions in the original proportions it should be possible to obtain again a pituitary solution indistinguishable from the original, thus proving that

[2] Abel, Rouiller and Geiling, *J. Pharmacol.*, **22**, 289 (1923).

no injury of any active principle has taken place. This is exactly what we have done and, in addition, by continuing our fractionation process, we have effected the first substantially complete separation of two principles. Our diuresis experiments on rabbits seem to indicate that the diuretic principle is the same as the pressor principle, thus eliminating one of the complicating possibilities. In addition, we have obtained both principles (oxytocic and pressor) in highly potent form as solid preparations although not yet in crystalline condition. No definite chemical information is available at the present time concerning the chemical nature of these principles but they appear to be basic in character. For this reason we have designated them as alpha- and beta-hypophamines[3] (amines derived from the hypophysis).

Historical Part

The pressor action of extracts of the pituitary gland was discovered by Oliver and Schafer[4]; and later Howell[5] and Schafer and Vincent[6] were able to demonstrate that the pressor action is confined to the posterior lobe.

The Epinephrine Theory.—Since epinephrine (adrenaline) was the most active pressor compound known to occur in the animal organism, it was logical to suspect that pituitary extracts owe their blood-pressure-raising effect to an action on the suprarenal glands, whereby an increased amount of epinephrine is discharged into the circulation, or that a compound similar to epinephrine or that epinephrine itself occurs in the pituitary gland. In spite of the fact that Dale[7] had concluded in 1909 that epinephrine and the active principle of the posterior lobe are two different substances, the epinephrine theory was not readily abandoned; in fact, as recently as 1916 Watanabe and Crawford[8] concluded that "pituitary extracts when prepared by certain methods, yield color reactions which would suggest the presence of epinephrine or an epinephrine-like compound."

The Histamine Theory.—In 1919 Abel and Kubota[9] isolated histamine from a quantity of *whole* pituitary gland and on the basis of their chemical and pharmacological studies concluded that "histamine is the plain-muscle-stimulating and depressor constituent of the posterior lobe of the pituitary gland. The physiological and chemical evidence in favor of identity of

[3] These products are already being manufactured for experimental clinical use. α-Hypophamine is being supplied under the trade name of Oxytocin (designating quick birth) and β-Hypophamine under the trade name Vasopressin (designating elevation of blood pressure). They were first supplied for clinical use in August, 1927.

[4] Oliver and Schafer, *J. Physiol.*, **18**, 277 (1895).

[5] Howell, *J. Exp. Med.*, **3**, 245 (1898).

[6] Schafer and Vincent, *J. Physiol.*, **25**, 87 (1899).

[7] Dale, *Biochem. J.*, **4**, 427 (1909).

[8] Watanabe and Crawford, *J. Pharmacol.*, **8**, 75 (1916).

[9] Abel and Kubota, *ibid.*, **13**, 243–298 (1919).

the two principles coincide at every point." A separate pressor principle of minor importance was also recognized.

The claims of Abel and Kubota were promptly and effectively disproved by Dudley[10] in a masterly piece of experimental work. Histamine is stable towards alkali; it is unacted upon by trypsin; it is soluble in boiling chloroform but not readily extracted from acid solutions by butyl alcohol. The pituitary uterine stimulant exhibited exactly the opposite behavior in each one of these tests. In a subsequent paper by Abel and Nagayama[11] the Histamine Theory was withdrawn.

The Single versus the Multiple Hormone Theories.—In addition to the three primary physiological responses that have been obtained by the administration of pituitary extracts (oxytocic, pressor and renal activities) a number of additional effects have been reported, as for example the melanophore action, the galactagog action, the effect on coagulability of the blood, the effect on intestinal peristalsis, inhibition of gastric secretion, mydriasis, etc. It was natural therefore to consider the possibility of the presence of a variety of active principles and such a possibility has not been overlooked by the earlier workers.

Schafer and Vincent[12] showed that pituitary extracts contain a depressor as well as a pressor substance, and as early as 1900 Osborne and Vincent[13] called attention to the fact that the central portion of the posterior lobe appeared to contain more activity than the periphery. Herring[14] and more recently, Hogben and De Beer,[15] have sought to trace the active principles to physiologically distinct parts of the posterior lobe.

Fühner[16] in 1913 claimed to have isolated four crystalline principles from the posterior lobe but this work has never been verified by others.[17] It is likely that these crystalline fractions consisted of inorganic salts containing in admixture appreciable and variable amounts of physiologically active material.

Not only did Dudley[10,18] overthrow the "histamine theory," but he went farther and offered evidence suggesting the presence of more than one active principle. By means of butyl alcohol extraction of dilute aqueous pituitary solutions, Dudley was able to secure what appeared to be a partial separation of the oxytocic and pressor activities, although considerable decomposition took place.

[10] Dudley, *J. Pharmacol.*, **14**, 295–312 (1919).
[11] Abel and Nagayama, *ibid.*, **15**, 347–399 (1920).
[12] Schafer and Vincent, *J. Physiol.*, **25**, 87 (1899).
[13] Osborne and Vincent, *Brit. Med. J.*, 1, 502 (1900).
[14] Herring, *Quart. J. Exp. Physiol.*, 1, 149 (1908); **6**, 107 (1913).
[15] Hogben and De Beer, *ibid.*, **15**, 163 (1925).
[16] Fühner, *Z. ges. exp. Med.*, 1, 397 (1913).
[17] Abel and Pincoffs, *Proc. Nat. Acad. Sci.*, **3**, 507 (1917).
[18] Dudley, *J. Pharmacol.*, **21**, 103 (1923).

In contrast to the claims of Fühner, Guggenheim[19] offered evidence in favor of the unitarian theory. He found that the various physiological activities of pituitary extracts (the vasomotor, respiratory and oxytocic) are all destroyed equally readily upon exposure to alkali.

Since 1920 Abel and his co-workers have been the enthusiastic advocates of the unitarian theory. Although they refer to three products, A, B and C derivable from the posterior lobe, the latter two, a histamine-like substance and histamine itself, appear in animal extracts of all kinds. However, in respect to Compound A they concluded[20] that "the infundibulum contains but one active specific substance, or hormone, and that this in its uninjured state is not only a blood-pressure-raising but also a plain-muscle-stimulating substance." Their most recent publication[2] states that "we therefore conclude that all the evidence at hand is greatly in favor of our belief that the oxytocic, pressor, diuretic and respiratory activities referred to above are properties of one and the same substance."

The wealth of evidence presented in favor of the unitarian nature of Compound A is almost incontrovertible. Subjected to four different agencies, namely, heat, tryptic digestion, hydrochloric acid decomposition and alkaline hydrolysis, both the pressor and oxytocic activities disappeared as in the decomposition of a single compound. Finally, even more convincing evidence is claimed in the statement that in their highly purified products the pressor, oxytocic and diuretic activities stand in the same relationship as in an ordinary pituitary extract.

Recently Smith and McClosky[21] demonstrated the parallel destruction of the oxytocic and pressor activities by heat and thus claimed to have affirmed the evidence advanced by Abel in favor of chemical unity. Finally, in a study "On the Dialysis of the Physiologically Active Constituents of the Infundibulum" the same workers[22] summarized the situation thus: "The identical diffusion rate of the oxytocic, pressor and renal activities present in infundibular extracts argues in favor of their chemical identity, and confirms the view held by Abel and his collaborators on this matter."

The controversy between Dudley and Abel has been submitted to analysis by Hogben, Schlapp and MacDonald,[23] and although these investigators were not disposed to regard Abel's preparation as a pure homogenous substance, they likewise stated that "we cannot regard Dudley's conclusions as fully established until his fractions have been standardized by a method of comparison such as we here propose."

[19] Guggenheim, *Biochem. Z.*, **65**, 189 (1914); **81**, 277 (1917).
[20] Abel and Rouiller, *J. Pharmacol.*, **20**, 65–84 (1922).
[21] Smith and McClosky, *Hyg. Lab. Bull.*, No. 138, April, 1924.
[22] Smith and McClosky, *J. Pharmacol.*, **24**, 391 (1924).
[23] Hogben, Schlapp and MacDonald, *Quart. J. Exp. Physiol.*, **14**, 315 (1924).

In subsequent publications by Schlapp[24] and more recently by Draper[25] an account is given of a successful repetition of Dudley's experiment and an additional new method is presented by Schlapp in support of the multiple hormone theory. "By producing precipitates of lead sulfide in extracts by the passage of sulphuretted hydrogen gas through suitable concentrations of lead acetate all the active principles are to some extent absorbed. But the quantity of pressor and melanophore substance absorbed exceeds that of oxytocic absorbed by a significant amount."

Dreyer and Clark[26] as well as Fenn[27] have considered the melanophore stimulant as a separate principle but according to Schlapp[24] there is insufficient quantitative evidence that the pressor and melanophore responses are due to distinct substances.

It has been suggested that several pressor principles are present in posterior lobe pituitary extracts. Crawford[28] was primarily interested in the concentration of this type of activity but even in 1916 when he was still investigating the possibility of the presence of epinephrine he suggested that "in pituitary extracts there must be one or two depressor compounds, and one or more pressor compounds." Dudley[18] has also presented evidence suggesting the presence of a second pressor principle.

The present status of the work on the isolation of the active principles of the posterior lobe is thus expressed by Dudley:[29] "I am driven, therefore, to the conclusion that Abel and Rouiller's preparation, like my own, contained at least three, and possibly more, active substances mixed with an unknown quantity of inert material."

"My conclusion, I am aware, shows the prospect of isolating the pituitary active principles in a far less encouraging light than theirs; but the experience of the past few years has given me so strong an impression of the difficulties entailed in the search for this group of extremely active, unstable substances, present in relatively minute proportions in the scarce and costly material of the posterior lobe of the pituitary gland, that I feel it a duty to make clear, for the benefit of other workers on the subject, my own reading of the facts at present available."

General Discussion

In reading over the extensive experimental work that has been reported in connection with the search for the active principle (or principles) of the posterior lobe of the pituitary gland one is impressed by the great number of uncorrelated experiments. For this reason we shall attempt to present

[24] Schlapp, *Quart. J. Exp. Physiol.*, **15**, 327 (1925).
[25] Draper, *Am. J. Physiol.*, **80**, 90 (1927).
[26] Dreyer and Clark, *J. Physiol.*, **58**, xviii (1924).
[27] Fenn, *ibid.*, **59**, xxxv (1924).
[28] Crawford, *J. Pharmacol.*, **15**, 81 (1920); **8**, 75 (1916).
[29] Ref. 18, p. 121.

only the essential parts of our work but we shall endeavor to describe our experiments in sufficient detail so that the results can readily be duplicated.

Obviously it will be impossible to present here the hundreds of kymograph charts upon which our work is based even if it were desirable to do so. We can assure the reader, however, that none of our assays are based upon one or two contractions of a muscle in comparison with a standard solution or upon one or two blood-pressure readings. Samples submitted for assay were treated as unknowns and often checks were required from two workers. A brief account of the assay methods is given below.

Fortunately there is now available from the U. S. Bureau of Chemistry a Standard Powdered Pituitary Product and all oxytocic results can be expressed in terms of this standard. The official Solution of Pituitary of the Pharmacopeia of the United States represents in each cc. the activity derived from 5 mg. of this standard powder. The potency of this solution may be expressed in terms of International Units,[30] each cc. being equivalent to 10 International Oxytocic Units. It follows, therefore, that one milligram of the U.S.P. Standard Powdered Pituitary is equal to 2 International Units of oxytocic activity.

In all of our work we refer to the U.S.P. Standard Powdered Pituitary as possessing a potency of 100%. A product, therefore, that is reported as 1000% is 10 times as potent as the U.S.P. standard and contains 20 Units per milligram.

A Pressor Standard

The Pharmacopeia fixes only an oxytocic standard and obviously this would be sufficient if all of the activity of pituitary extracts were due to a single principle, as has been supposed by several of the leading investigators. In view of the fact that we have definitely separated two principles, the need of a Pressor Standard becomes apparent.

In working with fairly fresh pituitary glands we find that there is a surprising constancy in the relative amounts of pressor and oxytocic activities present in the gland, and indeed it is this constancy that has aided in promulgating the ideas of a single principle.

For the present we propose that the pressor standard be expressed also in terms of the U.S.P. Standard Powdered Pituitary Product, one milligram of this powder being considered as containing 2 Pressor Units. The official solution of pituitary would therefore be designated as containing 10 International Units of Oxytocic activity and also 10 Pressor Units.

In the subsequent work we shall refer to the U.S.P. Standard Powdered Pituitary as possessing a pressor potency of 100%. A fraction, therefore, that is reported by us as 5000% is fifty times as potent as the U.S.P. standard and contains 100 Pressor Units per milligram.

[30] League of Nations, p. 14 of report by Second International Conference on the Biological Standardization of Certain Remedies, Geneva, Aug. 31—Sept. 3, 1925.

The Oxytocic Assay Method

The oxytocic method of assaying pituitary solution depends upon the measurement of the contraction caused in a strip of uterine muscle when it is immersed in Locke's solution to which has been added some of the solution to be tested. This method was first suggested by Dale and Laidlaw.[31] The apparatus consists of a water-bath in which a temperature of 37.5° is maintained by an electric heating unit controlled by a thermostatic regulator. Partly immersed in the water-bath stands a vertical tube of about 150 cc. capacity marked at the 100cc. level. Locke's solution, previously heated to 37.5°, is run into the vertical tube up to the 100cc. level through a small tube near the lower end. The Locke's solution may be drained off conveniently through a second small tube in the lower end of the vertical tube. Air is bubbled slowly through the Locke's solution from a small glass tube which enters the upper and open end of the vertical tube and extends down almost to the bottom of the solution. The extreme tip of the air tube is bent into a hook shape. This hook furnishes a convenient place for the attachment of one end of a strip of muscle. A thread is attached to the other end of the muscle and in turn is attached to a light lever which bears against the smoked paper of a kymograph. In this way the contractions and relaxations of the muscle are recorded on the smoked paper. The most satisfactory uterine muscles are taken from virgin guinea pigs weighing from 200 to 250 g. Two strips are obtained from each guinea pig.

In making an oxytocic test a measured amount of a pituitary solution is added to the 100 cc. of Locke's solution in which the uterine muscle is immersed. The muscle contracts very quickly and relaxes again in a few minutes. The Locke's solution is then drained away and fresh Locke's solution is admitted up to the 100cc. mark. After a few minutes' rest the muscle may be used for another test. Various quantities of a standard pituitary solution of known strength are tried on the muscle until the dosage has been determined which is required to cause a contraction slightly less than the maximum contraction of that muscle. Then various quantities of the unknown solution are tried until the quantity has been found which will give a sub-maximum contraction equal to that produced by the standard solution. Then tests are made alternately with quantities of the standard and unknown solutions until the relationship between their activities has been determined.

In this Laboratory so many oxytocic tests have to be made that the original one- or two-tube apparatus proved inadequate. The apparatus has been increased in capacity by making the water-bath large enough to hold twelve vertical tubes so that twelve muscles can be used at the same time. Accurate assays are secured by testing a single extract on from three to six muscles.

[31] Dale and Laidlaw, *J. Pharmacol.*, **4**, 75 (1912).

The Pressor Assay Method

The pressor method of assay for posterior pituitary extracts depends upon the fact that consecutive injections of the same quantity of an active extract when given intravenously to a dog—a very definite technique being followed—cause the same amount of increase in blood pressure. Some of the details of the technique are similar to those necessary for the proper standardization of adrenaline.

The procedure is briefly as follows.

A medium-sized, healthy, normal dog is deeply anesthetized with chloretone[32] by intraperitoneal administration of a dose of a 40% solution of chloretone in 40% alcohol, amounting to 0.4 g. per kg. body weight of the dog (1 cc. of the 40% solution per kg.). Cannulas are inserted into one femoral vein and one carotid artery, the former for receiving the intravenous doses of diluted extract and the latter for connection to a mercury manometer for recording arterial blood pressure. Under this deep anesthesia—all reflexes destroyed except a slight conjunctival reflex—0.04 cc. (1 cc. of a 1–25 dilution) of a standard U.S.P. Pituitary Extract is injected. Every 15 minutes, not oftener, alternately there are injected intravenously the small doses of sample and standard until comparable doses of the two are found which will produce the same rise in blood pressure on the same dog in at least two series. The result should be checked on another dog if there is any doubt about the first test.

Pituitary extracts of the posterior lobe cause a rise in arterial pressure due to constriction of peripheral blood vessels. If the above technique is rigidly followed, there will be no preliminary fall in blood pressure or development of tolerance such as occurs if larger doses are given too frequently. Special emphasis is placed upon the requirement of using only 0.4 unit for the intravenous dose.

Experimental Part

The activity (both pressor and oxytocic) of the posterior lobe of the pituitary gland may be removed almost quantitatively by extracting the gland with water containing a small amount (0.1 to 1%) of acetic acid.[33] This method, originally proposed by one of the present writers (Aldrich),[34] is now a part of the official method in the Pharmacopeia and is in quite general use by manufacturers. Nevertheless, some of the scientific workers in the field have employed ordinary aqueous extracts and apparently have failed to recognize the importance of using acidified water. This is

[32] Rowe, *J. Pharmacol.*, **9**, 107 (1916); Hamilton, *J. Am. Pharm. Assocn.*, 1, 1119 (1912).

[33] The use of a highly ionized acid such as hydrochloric acid even in as low a concentration as 0.5% results in destruction of the active principles. Recent work on this point has been reported by Stasiak, *J. Pharmacol.*, **28**, 1 (1926).

[34] Aldrich, *Am. J. Physiol.*, 21, Proc. XXIII (1908).

true, unfortunately, of the important work of Schlapp,[24] of Draper[25] and possibly of Dudley.[10]

The Importance of P_H Control in Making Aqueous Extracts

Adams[35] has shown that considerable loss of oxytocic activity takes place when pituitary solutions are heated at a P_H of 5. We find that the reaction of extracts prepared without the use of acetic acid is near a P_H of 6, and that the loss of pressor activity is even more pronounced than the loss of oxytocic activity. Extracts prepared with the use of 0.25% acetic acid possess a P_H of about 3.8 to 4.4 and are comparatively stable.

The following summary gives the results of extraction of 1-g. samples of acetone-desiccated posterior lobe both with 0.25% acetic acid and with distilled water, the extracts being heated during only one-half hour as in the method described below.

TABLE I

ASSAYS BASED ON 1-G. SAMPLES OF DESICCATED POSTERIOR LOBE

(a)	Extracted with 0.25% acetic acid	= 1000 units (Oxytocic)
(b)	Extracted with 0.25% acetic acid	= 800–1000 units (Pressor)
(c)	Extracted without acetic acid	= 800 units (Oxytocic)
(d)	Extracted without acetic acid	= 250 units (Pressor)
(e)	Reëxtraction of residue from (d) with 0.25% acetic acid	= 30 units (Pressor)

It is evident that more than one-half of the pressor activity was lost and, although the oxytocic activity is more stable, a 20% loss was detected here also. A significant point is that the loss of pressor activity is not recoverable by reëxtracting the gland residue—it has actually been destroyed.

The above experiment was duplicated in work on a manufacturing scale although unintentionally. In Table II, Experiments (a), (b), (c) and (d) illustrate the uniformity with which the activity is extracted under

TABLE II

YIELDS OF ACTIVITY ON A MANUFACTURING SCALE

	1 No. units pressor according to assay	2 No. units pressor extracted	3 No. units in salted-out product
(a)	1,650,000	1,900,000[a]	1,300,000
(b)	1,650,000	1,650,000	1,630,000
(c)	1,650,000	1,460,000	1,600,000
(d)	1,650,000	1,800,000[a]	1,530,000
(e)	1,650,000	800,000	600,000
(f)	.....	40,000	.....

[a] These two values indicate yields of more than 100%, due no doubt to errors in the assay. This is verified by the values recorded in Col. 3, which represent the yields in the next step in the process.

[35] Adams, *J. Biol. Chem.*, **30**, 235 (1917).

specified conditions. In Expt. (e) the acetic acid was accidentally omitted and, although the error was detected a half-hour later after the solution had been heated, and the acid was then promptly added, a considerable loss of pressor activity had taken place. Expt. (f) shows that reëxtraction of the gland residue failed to yield the activity that had been lost.

Loss of Activity Due to Acetone Desiccation

The acetone liquors recovered from the desiccation process contain a negligible amount of pressor activity but a considerably larger amount of oxytocic activity.

Fifty posterior lobes were dropped in 200 cc. of acetone, which is the volume prescribed in preparing the standard powder. Several hours later the acetone was poured off and replaced with fresh acetone, as prescribed. The acetone liquors were evaporated at a low temperature and the residue extracted with dilute acetic acid. The acid solution was then assayed by both oxytocic and pressor methods; 160 oxytocic units, but only 40 pressor units, were found. The desiccated gland material which was not completely defatted and not separated from coarse material by sifting assayed 50% of U.S.P. standard and contained a total of 2000 International Units.

In the above experiment the pituitary glands were collected during the afternoon, kept in the refrigerator overnight without freezing them, and dissected on the following morning.

Smith and McClosky[36] have not recorded a study of the acetone liquors obtained in connection with the preparation of their standard powder but we are informed in a private communication from Dr. M. I. Smith that only 0.4% of the oxytocic activity was lost.

It appears, therefore, that there is very little loss if the glands are desiccated within a few minutes after the death of the animal but a number of hours later sufficient acidity is developed to permit the acetone to extract 2% of the pressor activity and 8% of the oxytocic activity.

The Depressor Activity of Pituitary Extracts

Schafer and Vincent[12] not only demonstrated the presence of a depressor substance but showed that it is extractable from the glands by means of alcohol. Recent workers[37] have attached considerable significance to this depressor product, some referring to it as histamine.

When acetone-desiccated glands are extracted with absolute ethyl alcohol in a Soxhlet extractor, this depressor product is readily removed. We have tested it by three methods, as is shown in the following table.

[36] Ref. 21, p. 16.

[37] For references see the excellent paper by Sharpey-Schafer and Macdonald, *Quart. J. Exp. Physiol.*, 16, 251–280 (1926).

TABLE III

ASSAYS BASED ON EXTRACT FROM 1 G. OF DESICCATED POSTERIOR LOBE

(a)	Colorimetric assay for histamine	<1 mg.
(b)	Blood pressure assay for histamine	<1 mg.
(c)	Oxytocic assay	80 Oxytocic units = 100 mg. histamine

According to the oxytocic assay, approximately 100 mg. of histamine is indicated as being extractable from 1 g. of gland material. The blood pressure assay, however, indicates the presence of even less than 1% of this large quantity of histamine and the colorimetric test agrees in demonstrating that histamine is present only in small amounts. Two conclusions might be drawn: first, the depressor principle is not histamine but rather a substance much more potent than histamine in its oxytocic action; second, a small amount of histamine is present in the gland but in the process of alcohol extraction there is extracted simultaneously some of the true oxytocic principle.

We believe that the second conclusion is correct. Since the oxytocic principle is soluble in alcohol, there is no reason why it should not be extracted and our results show that in six hours' extraction approximately 10% of the oxytocic principle is removed.

Freshly collected pituitary glands contain very little histamine, but if this impurity is present it may be removed advantageously by salting-out methods. This is illustrated by the following experiment, in which a known quantity of histamine was intentionally added.

A pituitary extract was prepared so as to contain 20 mg. of pituitary proteins per cc. To this solution, which according to colorimetric assay contained material equivalent to less than 0.02 mg. of histamine per cc., there was added 0.5 mg. per cc. of histamine in the form of hydrochloride. Sufficient solid sodium chloride was then added to salt out the pituitary proteins and the precipitate was removed by centrifuging and purified merely by washing with a very small volume of saturated salt solution. The filtrate as well as the precipitate was then assayed colorimetrically for histamine with the following results:

Precipitate dissolved in water to original volume.....0.02 mg. histamine per cc.
Filtrate (calcd. to original volume) 0.45–0.50 mg. histamine per cc.

It is apparent that histamine is readily removed by the salting-out method, the variation from the theory being within the limits of accuracy of the colorimetric method of assay when conducted in the presence of salts.

The Concentration of the Active Principles

In our choice of methods for the concentration of the active principles of the posterior lobe, we have been guided by the results obtained from a study of the dialysis of pituitary extracts. The active principles dialyze more slowly than does the known substance, adrenaline, and the suggestion has been advanced that the pituitary principles may possess a molecular weight in the neighborhood of 600.[38] Although this conclusion is specula-

[38] Kamm, *Science*, Feb., 1928.

tive, it has proved a reliable guide. The active principles are relatively simple when compared with the protein-like material present in pituitary extracts. On the other hand, they are relatively complex when compared with most of the simpler glandular extractives, such as the inorganic salts, creatinine,[39] histamine, amino acids, etc.

The process may be illustrated by the separation of the active principles *B* from the mixture *A B C*, in which *A* represents low molecular weight substances and *C* represents the high molecular weight substances, such as proteins. In the absence of a specific precipitant for *B*, two methods of separation suggest themselves: first, *A B* can be separated from *C* and subsequently *B* can be fractionated from *A;* or, second, *A* can be separated from *B C* and subsequently *B* can be fractionated from *C*.

The method that we attempted to use for the separation of *A B* from *C* was that of fractional dialysis. Another method was based upon the use of uranium acetate[34] for the precipitation of *C*. Although most protein precipitates appear to adsorb the active principles, the use of uranium acetate appears to be the least objectionable. However, since these methods of attack are not used in our final method, we refrain from recording the hundreds of experiments that have been conducted in these directions.

Our final method involves the separation of *B C* from *A*. Although this first step may be partly accomplished by the use of effective protein precipitants such as tannic acid and phosphotungstic acid, the subsequent recovery, particularly from the phosphotungstate, requires rather heroic methods that might injure the active principles[40] and consequently we have used the more gentle process of salting out the proteins, together with the active principles, by means of salts such as sodium chloride or ammonium sulfate.

Salting-out Methods

Although Osborne and Vincent[41] had observed in 1900 that a part of the pressor activity of pituitary solutions is present in the salted-out fraction obtained by the addition of ammonium sulfate, no practical development resulted from this observation until 1913, when Clover[42] attempted to isolate the active principle by fractional precipitation by salting-out methods. Although mistaken in his conclusion that his potent salted-out fraction was an approximation of the active principle itself, Clover's work led to the first water-soluble pituitary preparations in solid, stable form.

[39] Dudley, *J. Pharmacol.*, 21, 111 (1923), isolated a considerable amount of creatinine from his extracts.

[40] Ref. 39, p. 104.

[41] Osborne and Vincent, *Brit. Med. J.*, 1, 502 (1900).

[42] Clover, U. S. Patent Application of March 3, 1913; see No. 1,373,551.

Recently we have standardized by both pressor and oxytocic methods one of the best specimens of this "Proto-pituitrin," prepared by Clover in 1913, with the following results.

TABLE IV

Assay in 1913	0.5 mg. per cc. yielded a pituitary extract equal to the 1913 standard
Assay in 1927	0.5 mg. = 8.5 Oxytocic units
Assay in 1927	0.5 mg. = 7.5 Pressor units

Two results are apparent: (1) this solid preparation remained stable for a period of fourteen years, and (2) the oxytocic and pressor activities are present approximately in the proportion in which they occur in the pituitary gland.

All of our work on products obtained by salting-out methods from relatively *concentrated* solutions has led to the conclusion that both principles are carried down into the precipitate in substantially the proportions in which they occur in the gland. Quite a different result is obtained in working with dilute solutions.

Comparison of the figures in Col. 1 of Table II with those in Col. 3 illustrates the completeness with which the activity is recovered by salting out in a fairly concentrated solution containing 50 to 100 units per cc. When relatively dilute solutions are treated with salt, a considerable loss of activity occurs, due to incomplete precipitation, as is illustrated by the following experiment.

One hundred cc. of a posterior lobe extract assaying 10 oxytocic units and 10 pressor units per cc. and containing 0.5% chloretone was treated with 30 g. of solid sodium chloride. The protein fraction together with the precipitated chloretone was removed with the centrifuge. The solution as well as the salted-out precipitate was then subjected to assay by both methods with the following result.

TABLE V

	No. of pressor units	No. of oxytocic units
Original solution	1000	1000
Salted-out fraction	500	700
Filtrate	400	250

The above results show not merely that dilute solutions are incompletely precipitated by salting-out methods, but also that the two activities are carried down with the protein fraction in unequal amounts. We have here another bit of evidence which disagrees with the theory that both types of activity are due to a single hormone.

The Separation of the Pituitary Hormones from Proteins: Separation of the Fraction B C into B and C

The hormones of the posterior lobe of the pituitary gland are not readily separated from foreign gland tissue and are carried along with the protein fractions. In this respect they resemble insulin and the parathyroid hor-

mone rather than the simpler hormone, adrenaline. However, the method of manipulation of these protein extracts that has proven successful with insulin, namely, iso-electric precipitation, and which can be applied also to the purification of the parathyroid hormone, fails when applied to pituitary extracts. A new method of manipulation was required and has been devised.

After partial purification by salting out, the pituitary protein fraction is much more susceptible to fractionation than is the crude gland extract. However, the usual organic solvents used for such fractionation purposes, as for example alcohol, do not yield very satisfactory results.

An essential part of our method consists in the choice of an organic solvent which acts chemically with the active principles, due possibly to salt formation, and which acts simultaneously as a solvent for the salts of the active principles but is a relatively poor solvent for foreign protein material. An important prerequisite for such a solvent is that it must not injure the active principles.

The ideal solvents have been found in the aliphatic monocarboxylic acids of low molecular weight. Our work has been chiefly with acetic, propionic, and butyric acids, all of them being used in relatively high concentration and in the case of acetic acid in practically anhydrous form. The solvent action of propionic acid containing 5% of water and butyric acid containing about 10% of water is practically equivalent to 98–100% acetic acid. Since the results with acetic acid are highly satisfactory and since this solvent is also the cheapest one, the experiments to be described will deal with this particular acid.

The solution of the active principles in glacial acetic acid at room temperature (25°) is stable for days. After a month a perceptible loss of activity is observed, but in the following experiments the material need be exposed to the acid during only an hour. The advantages of such a method are in obvious contrast to methods involving reactive agents such as mercuric chloride and barium hydroxide.

The pituitary proteins are relatively insoluble in glacial acetic acid, whereas the active principles are readily extracted by this solvent. The amount of foreign protein extracted, however, is dependent upon the moisture content of the acid—for example, 98% acetic acid will extract appreciable quantities of protein, whereas 100% acetic acid will dissolve very little protein but it will extract the physiologically active compounds although with more difficulty than when the 98% acid is used. More dilute acid than 98% may be used as, for example, a solvent containing 10 to 20% (or even more) of water, but in this case the proteins and contaminating salt will dissolve completely and the subsequent fractionation by the addition of organic precipitants will be more tedious.

We find that the acetic acid extract may be subjected profitably to

fractional precipitation by the gradual addition of acetone, ether, and petroleum ether in the order specified. When this is done, the first few fractions are found to be relatively inert, but successive fractions show an increasing potency. Acetone is the best precipitant for the inert proteins; subsequently the pressor principle, together with varying quantities of the oxytocic principle, is precipitated in a series of fractions by the addition of sulfuric ether, and finally complete precipitation is secured by the addition of a hydrocarbon precipitant such as petroleum ether.

By several repetitions of the fractionation process, it is possible to obtain the active principles in very potent form.

The steps involved in this concentration of the active principles and their separation are as follows:

(1) Extraction of the gland material according to the known method, using dilute aqueous acetic acid.

(2) Concentration of the dilute extract at a low temperature.

(3) Separation of the activity, together with proteins, by the addition of salt.

(4) Extraction of the salted-out fraction with glacial acetic acid.

(5) Fractionation of the acetic acid extract by the addition of organic liquids such as acetone, ether and petroleum ether.

The U. S. P. pituitary powder is prepared under special conditions[43,21] from pituitary glands collected within thirty minutes after the death of the animal. We have already designated the potency of this powder as 100%. In four preparations we were able twice to duplicate material possessing this potency, but two other lots, for some unexplained reason, possessed a potency of only 70%.

Commercial desiccated posterior lobe obviously is less potent than the standard powder and in actual practice a good technical product will test 40 to 60% of standard. A satisfactory method of separating the active principles, however, should be independent of the potency of the starting material and, indeed, we have found this to be true of the method to be described.

Experimental Procedure

(1) An aqueous extract of the posterior lobe of the pituitary gland is prepared by extracting 100 g. of the commercial acetone-desiccated gland material testing 50% of U. S. P. standard with 10 liters of 0.25% acetic acid. The mixture is gradually heated to a temperature of about 95° during a half-hour period, cooled quickly and then filtered to remove the insoluble gland residue, which is reëxtracted with one liter of the acidified water in order to secure a fairly complete extraction. The combined filtrates should contain 100,000 units of oxytocic activity and an equal number of units of pressor activity.

(2) The filtrate is then concentrated at a low temperature to a volume

[43] *U. S. P.*, X, page 220.

of one liter. Since the active principles in acid solution are stable toward oxidation by air, evaporation with the aid of a current of warm air is permissible.

(3) The concentrated extract is treated with 550 g. of C. P. ammonium sulfate. The precipitate is filtered on a hardened filter paper, pressed, dried and powdered. The weight of salted-out product will be 20 to 40 g. according to the amount of salt present, an excess of which is not objectionable.

(4) The salted-out product is extracted with successive portions of glacial acetic acid (99–100%) a total volume of 500 cc. of acetic acid being used.[44] The amount of glacial acetic extracted material will be approximately 5 to 8 g., provided care is taken to prevent undue exposure to the moisture of the air during the extraction process.

(5) The acetic acid extract is now treated with 1250 cc. of sulfuric ether and then immediately with 2500 cc. of petroleum ether, which treatment precipitates the active material completely. The precipitate is filtered off by suction, washed with ether and dried. Its weight should be 5 to 10 g. varying with the water content of the acetic acid, and a potency test by both the pressor method and the oxytocic method should demonstrate the presence of 80 to 90% of the activity contained in the original 100 g. of desiccated gland material. Ninety to ninety-five per cent. of the inert material has thus been removed by our process up to this stage. Assay shows the product to possess a potency of 450 to 900% of standard according to the weight, the oxytocic and pressor activities being practically balanced.

The uniformity of this extraction and purification method is illustrated by the following four practical experiments conducted on a manufacturing scale.

TABLE VI

SEPARATION OF ACTIVE PRINCIPLES FROM INERT MATERIAL ON A MANUFACTURING SCALE

Expt. no.	No. of pressor units in desiccated gland	No. of oxytocic units in desiccated gland	No. of pressor units extracted	Yield of final product, g.	Potency by pressor test, %	Potency by oxytocic test, %
1	3,500,000	3,620,000	3,180,500	203	625	625
2	3,500,000	3,620,000	3,470,000	237	500	600
3	3,500,000	3,620,000	3,450,000	258	500	600
4	3,500,000	3,620,000	3,180,500	250	500	625

[44] In order to secure an efficient extraction the following method may be applied. The salted-out product is divided into four equal part lots A, B, C and D. Part Lot A is extracted with seven times its weight of acid and this first extract set aside. A is then reëxtracted with a second portion of acid but this second extract is used for extraction of Part Lot B after which the second extract is combined with the first. In making the third extract Lots A, B and C are extracted in this respective order and the procedure is continued in this manner until each fraction has been extracted four times before being discarded and a total of seven portions of acetic acid have been used.

Partial Separation of the Two Active Principles

As a next step it is advantageous to remove a part of the oxytocic activity by the following simple process. A complete separation at this stage is not feasible because of the tendency of the protein fractions to adsorb the oxytocic fraction.

Five g. of the material assaying 500–900% of the U. S. P. powder is dissolved in 250 cc. of 98% acetic acid at a temperature of 40° and precipitated immediately by the addition of 2.5 volumes of sulfuric ether previously warmed to 30°. The flocculent precipitate is allowed to settle rapidly (not over five minutes) and is filtered by suction, washed with ether and dried.

The precipitate is immediately redissolved in 250 cc. of 98% acetic acid and precipitated as before. The recovered material will weigh 4.5 to 4.7 g.

The ether filtrate contains oxytocic activity together with traces of salts such as ammonium acetate. The active fraction may be precipitated by the addition of petroleum ether but the resultant solution is usually so difficult to filter that the following procedure is preferred.

The two ether-acetic filtrates (above) are combined and refiltered through hardened filter paper and the *clear* filtrate is treated with 10 cc. of water (which must dissolve completely) and two volumes (about 3 liters) of petroleum ether. A fine mist is thrown out which carries down practically all the oxytocic activity and usually collects as a varnish on the sides and bottom of the container after standing for several hours or overnight. The *clear* liquid is decanted and the gummy precipitate dissolved in 50 cc. of water and filtered free from fat. Based on solid content, this petroleum ether precipitate contains oxytocic material of a potency of 5000 to 12,000% and not more than 3–4% of pressor activity per 100% oxytocic activity.

The following practical result has been duplicated many times. Ten g. of a partially purified solid assaying 600% oxytocic and 500% pressor activity yielded by this process 9.6 g. of a fraction assaying 500% pressor but only 325–375% oxytocic activity. However, the petroleum ether precipitate, taken up in 100 cc. of water, had a solid content of 350 mg. and contained 50,000 to 60,000 units of oxytocic activity, thus accounting for the oxytocic activity removed from the precipitate. Such a solution is suitable for subsequent use in the purification of the oxytocic principle and also, after suitable dilution, for practical clinical use, since it contains only small amounts of pressor activity.

Fractionation of the Pressor Principle

(a) Five g. of partially separated material testing 500–900% by the pressor test but only approximately one-half this potency according to the oxytocic test, is dissolved in 100 cc. of 98% acetic acid at room tem-

perature and the solution is filtered by suction from a small amount of salt and other insoluble impurities.

(b) To the filtrate is added 40 cc. of acetone. The precipitate, consisting mainly of protein containing only a small amount of active material, is filtered off, washed with ether and dried.

(c) The filtrate from (b) is treated with an additional 40 cc. of acetone and a second protein fraction is obtained as before, although this fraction contains a slightly more active material.

(d) The filtrate from (c) is treated with about 25 cc. of sulfuric ether and the precipitate filtered off and dried as before. This fraction is more potent than (c) by both the pressor and oxytocic tests.

(e) The filtrate from (d) is treated with 125 cc. of ether and the precipitate filtered off and dried. This fraction is very active by the pressor test but less active by the oxytocic test, thus plainly showing a separation of the active principles.

(f) The filtrate from (e) is treated with an excess (about 500 cc.) of sulfuric ether in order to precipitate the remainder of the pressor activity.

(g) The filtrate from (f) contains only a trace of pressor activity but contains the bulk of the oxytocic activity. The latter may be obtained by the addition of petroleum ether as already described. Due to the presence of moisture this fraction may precipitate as a gummy mass but can be obtained in the form of a white solid by dissolving in absolute alcohol and precipitating with an excess of ether. If it precipitates as a liquid it must be concentrated at a low temperature.

The results obtained by this fractionation method are shown in the following experiment selected at random from a large number of similar results.

Thirty g. of material assaying 750% by pressor and 375% by oxytocic test was treated with 600 cc. of 98% acetic acid and fractionated with the following results.

TABLE VII

FRACTIONATION OF PRESSOR PRINCIPLE AND SEPARATION OF A POTENT OXYTOCIC FRACTION

Fraction number	Amount and kind of solvent added	Assay of fraction, %	Weight of fraction, g.
1	Acetic acid	Practically inert	5.4
2	225 cc. Acetone	300 Pressor, 150 oxytocic	7.7
3	225 cc. Acetone	400 Pressor, 200 oxytocic	8.4
4	150 cc. Ether	750 Pressor, 250 oxytocic	1.9
5	750 cc. Ether	3125 Pressor, 700 oxytocic	4.2
6	4000 cc. Ether	1875 Pressor, 1625 oxytocic	1.0
7	Petroleum Ether	120 Pressor, 5000 oxytocic	0.8
		Total	29.4

In the above experiment 220,000 pressor units were recovered from a total of 225,000 and likewise 100,000 oxytocic units from a total of 122,000 shown by the initial assay.

Examination of the above table shows that fractions 2, 3 and 4 are relatively weak. They consist chiefly of protein material which naturally carries along an appreciable amount of activity. However, a separation and concentration of the two activities is taking place as is shown by the fact that fraction *four* is three times as potent by pressor as by oxytocic test, and the large fraction, *five*, is more than four times as potent by pressor as by oxytocic test. The oxytocic activity is accumulating in the residual solution and it is therefore to be expected that the last solid fraction (No. 6) will be comparatively potent in oxytocic activity also, since the oxytocic principle is not very soluble in ether.

Refractionation and Further Concentration of the Pressor Principle

The more potent of the separated fractions may be further concentrated by a repetition of the process already described, applying the same to the individual fractions.

The following laboratory experiment illustrates the results obtained in actual practice.

Seven g. of material assaying 3125% by pressor test and 700% by oxytocic test (equivalent to Fraction 5, Table VII) were dissolved in 210 cc. of 98% acetic acid and refractionated with the following results.

TABLE VIII

FRACTIONATION OF THE PRESSOR PRINCIPLE

Fraction number	Amount and kind of precipitant added	Assay results, %	Weights of fraction, g.
1	210 cc. Acetone	1000 Pressor, 150 oxytocic	1.35
2	105 cc. Acetone	1625 Pressor, 400 oxytocic	0.95
3	75 cc. Ether	3000 Pressor, 500 oxytocic	1.15
4	300 cc. Ether	4000 Pressor, 750 oxytocic	2.40
5	600 cc. Ether	2500 Pressor, 2000 oxytocic	0.52
6	Filtrate	150 Pressor, 4000 oxytocic	0.25
		Total	6.62

Repeated fractionation of the 4000% pressor fraction yields a fraction testing 6250% but it is difficult to increase the potency beyond this figure by this method. The subsequent use of other solvents, such as the alcohols, has raised its potency only to 8000%. This product is now being subjected to various purification methods in the hope of obtaining it in a condition of maximum potency.

The distribution of the pressor hormone among the various fractions in this process is shown graphically in Fig. 1, which is based upon the refractionation of 46.5 g. of a partially separated product testing 750% by pressor and 375% by oxytocic tests. The dark areas represent the amount of pressor principle present in the respective fractions, assuming a potency of 7500%, a value which we already know is somewhat below the actual potency of the pure principle. The white areas represent inert material.

Results analogous to those illustrated in Series 2 have already been recorded in Table VII. The best Fraction, 2E, testing 3125%, was refractionated and yielded Fractions 3A, 3B, 3C, 3D and 3E, testing 1000,

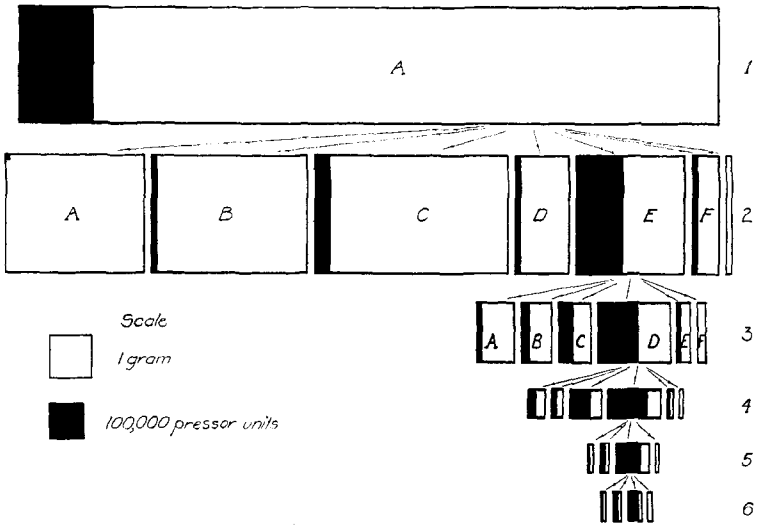

Fig. 1.—Separation of the pressor principle.

1625, 3000, 4000 and 2500%, respectively. The latter results are recorded in Table VIII.

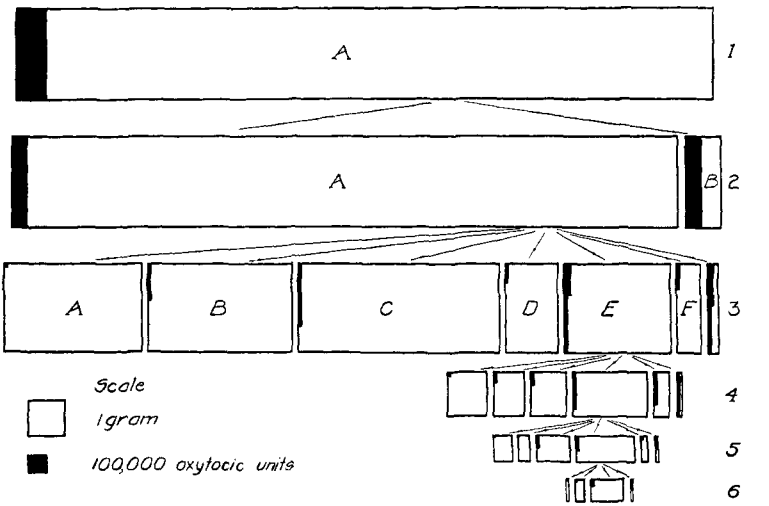

Fig. 2.—Separation of the oxytocic principle.

Fraction 3D, testing 4000%, was refractionated and yielded Fractions 4C and 4D, both testing 5000%. The latter fraction yielded a large

Fraction 5C, testing 5000%, and refractionation of this yielded the Fractions of the 6 series, the most potent of which tested 6250%.

The distribution of the oxytocic hormone among the various fractions in the process that has been described in detail is shown graphically in Fig. 2, which is partly based upon the oxytocic values recorded in Tables VII and VIII. The dark areas represent the amount of oxytocic principle present in the respective fractions, assuming a potency of 20,000% for the pure principle, an estimate which is, no doubt, considerably below the actual value. The white areas represent inert material.

The separation shown in Series 2, Fractions A and B, corresponds to the results obtainable by the procedure described above under the section headed "Partial Separation of the Two Active Principles." The fractions in Series 3 are identical with the fractions in Series 2 of Fig. 1; for example, Fraction 2E of Fig. 1 and 3E of Fig. 2 are based upon the same fraction, the former chart illustrating the proportion of pressor principle and the latter chart the amount of oxytocic principle in this sample.

It is to be noted that the oxytocic activity tends to accumulate in the end fractions shown at the right-hand end of the chart.

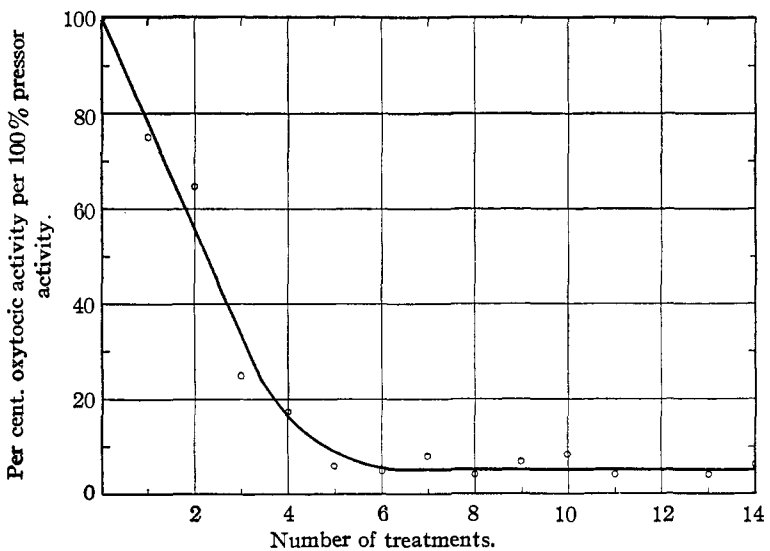

Fig. 3.—Separation of oxytocic principle from the pressor principle.

Limits of Separation of the Oxytocic Principle from the Pressor Principle

The most potent pressor material obtained by the acetic acid fractionation method, namely, a product testing 6250%, still contains approximately 10% of oxytocic activity per 100% pressor activity. It is difficult to reduce the oxytocic activity below 4% per 100% pressor. This is illustrated in Fig. 3, which represents fourteen treatments aiming at the removal of

the oxytocic activity by the method already described, namely, solution in glacial acetic acid and precipitation with ether.

The curve illustrates the fact that the oxytocic activity is rapidly removed in connection with the first four treatments with acetic acid. After the sixth treatment the amount of oxytocic activity remains almost constant.

These results suggest that a small amount of oxytocic activity may be inherent in the pressor principle itself and we have in progress a new series of experiments aiming at a solution of this interesting problem. The fact that the pressor principle, as such, may possess a slight stimulating effect on smooth muscle would not be an unique case, since some relatively simple substances (as for example tyramine) also show both types of activity.

Purification of the Oxytocic Principle

The oxytocic principle resembles the pressor principle in every way except that it is appreciably soluble in organic solvents and particularly in a mixture of ether and acetic acid. A practical method of obtaining a potent solution of the oxytocic principle has already been described in the above experimental procedure under the heading "Partial Separation of the Two Active Principles."

The ether-acetic acid solution of the oxytocic fraction may be evaporated to dryness at a low temperature and the residue taken up in 0.25% acetic acid and filtered. This acid extract is then assayed for total solids and for oxytocic activity and usually tests 5000% of U. S. P. standard. Precipitation of the ether solution by the addition of petroleum ether according to the methods already described yields a product testing up to 12,000%, which is relatively low in pressor activity, and this method is preferable. The gummy precipitate is then merely dissolved in 0.25% acetic acid.

The aqueous solution containing the oxytocic fraction as the acetate may be evaporated at a low temperature. The residue is treated with alcoholic tartaric acid, which dissolves the active substance but may leave a crystalline residue of ammonium acid tartrate which is filtered off. From the alcoholic solution the oxytocic tartrate may be fractionally precipitated by the addition of ether. This process, however, has not raised its potency beyond 15,000–20,000%, and a more satisfactory method of purification is being sought.

Assay of such a fraction by the pressor method indicates that 98 to 99% of the pressor activity of the gland has been removed. Apparently there is little difficulty in removing the pressor from the oxytocic principle, and even for practical clinical work we have been able to supply a product which assays only 4% pressor for each 100% by oxytocic assay.

Adrenaline, which is an amine, may be precipitated from aqueous solutions of its salts, when present in sufficient concentration, by neutraliza-

tion with ammonia. The pituitary hormones also appear to be amines but they are not precipitated from aqueous solutions of their salts upon neutralization, since the bases themselves are extremely soluble in water. Further work on the purification of these products will appear in subsequent articles.

Conclusive Evidence of the Presence of Two Principles

The evidence that we have presented in the quantitative results reported above should leave little doubt that at least two principles are present in pituitary extracts and that these two principles may be separated by practical methods. It seems advisable, however, to demonstrate that in our process none of the activity has been injured or one product converted into another. In other words, the sum of the parts should make a whole; and this should be demonstrated not merely by adding up the separate assay results but also by synthesis of the original by recombining the parts. This has been done in the following experiment.

A sample of a partially purified product was found to test 625% by both the oxytocic and pressor methods (see Table VI, Expt. 1). Ten g. of this material was then subjected to the acetic acid fractionation method with the following results.

TABLE IX

INITIAL FRACTION OF A PURIFIED PRODUCT CONTAINING ACTIVITIES IN ORIGINAL PROPORTIONS

Fraction	Wt., g.	Oxytocic assay, %	Pressor assay, %	Oxytocic units	Pressor units
1	3.8	175	125	13,300	9500
2	2.75	200	200	11,000	11,000
3	1.2	300	400	7200	9600
4	1.55	1875	2500	58,000	77,500
5	0.28	6000	500	33,600	2800
6	8 liters filtrate	..	...	2500	2500

Fractions 1 and 2 were obtained by acetone precipitation, Fractions 3 and 4 by ether precipitation and Fraction 5 with the aid of petroleum ether. Note that Fraction 1 contains more oxytocic than pressor activity, but in 3 and 4 the pressor activity is in excess, whereas in Fraction 5 the oxytocic activity is again in great excess. The large volume of filtrate contained only 2% of the total activity.

The initial material used in this experiment contained 125,000 oxytocic units and 125,000 pressor units. The assays of the separate fractions total up to 125,600 oxytocic units and 113,100 pressor units.

The fractions were then recombined and the resultant mixture was submitted to assay as an unknown. The resultant pituitary product on the basis of the physiological tests was indistinguishable from the original product and assay demonstrated it to test 575% by pressor test and 625% by oxytocic test. This represents 110,400 pressor units and 120,000 oxytocic units, results which are within the limits of experimental error.

The above experiment has been repeated using known quantities of highly purified oxytocic and pressor fractions. Such solutions when combined in theoretical proportions were indistinguishable pharmacologically from plain pituitary extracts. Incidentally, these tests show that the oxytocic assay is not influenced seriously by the

presence of the pressor principle and, *vice versa*, the pressor assay method is not adversely affected by the presence of moderate amounts of the oxytocic principle. The effect of the presence of excessive amounts of one principle upon the assay of the other requires further study.

The Diuretic-Antidiuretic Principle Appears to be Identical with the Pressor Principle

Extracts of the posterior lobe of the pituitary have a diuretic-antidiuretic action. Under some conditions injection of such extracts causes diminished excretion of urine but under different conditions the flow of urine is markedly increased.

We were interested in finding out which of the active principles, the oxytocic principle or the pressor principle, has the diuretic-antidiuretic action. In order to determine this question extracts containing the two activities were tested on rabbits.

The method of experiment was similar to that used by Magnus and Schafer,[45] Schäfer and Herrington,[46] and Houghton and Merrill[47] on dogs; and more recently on rabbits by Abel, Rouiller and Geiling,[2] Mackersie,[48] Smith and McClosky,[49] and others.

The rate of flow of urine was observed by counting the number of drops in five-minute intervals falling from a cannula tied into the urinary bladder. The rabbits were anesthetized with urethan (ethyl carbamate), 2 g. per kilogram of body weight, given by subcutaneous injection. The cannulas used were funnel-shaped at the end which was tied into the bladder and obliquely pointed at the other end. They were 1 cm. in diameter so as to prevent the possibility of capillary action interfering with the free flow of urine. In making a test the rabbit was laid on a board so that the cannula extended vertically downward through a hole in the board. Rabbit and cannula being in this position permitted the urine to drop as fast as it entered the bladder without the possibility of pocketing.

The solutions used for the diuresis experiments were Pituitrin which tested 100% pressor and 100% oxytocic, a solution of the pressor fraction which tested 100% pressor and 8% oxytocic; and a solution of the oxytocic fraction which tested 100% oxytocic and 4% pressor.

A typical experiment showing the effect of intravenous injections of Pituitrin, pressor fraction and oxytocic fraction is illustrated in Fig. 4. These three substances were used in rotation so as to compare their effects on the same rabbit. The dosage of Pituitrin is stated in International Units, the dosage of the oxytocic fraction is stated in units equal to the International Units, and the dosage of the pressor fraction is stated in units of the pressor activity which we have defined above. The rabbit weighed 2.1 kilograms and had been fed on oats and green food with water always accessible.

It will be noted that Pituitrin and the pressor fraction caused a marked increase in the flow of urine while the oxytocic fraction caused only a slight increase in the flow. This difference is even more noticeable in some of

[45] Magnus and Schafer, *J. Physiol.*, 27, Proc. ix (1901).

[46] Schäfer and Herrington, *Proc. Roy. Soc.* (*London*), 77B, 571 (1906); *Phil. Trans. Roy. Soc.*, 199B, 1 (1908).

[47] Houghton and Merrill, *J. Am. Med. Assocn.*, 51, 1849 (1908).

[48] Mackersie, *J. Pharmacol.*, 24, 83 (1924).

[49] Smith and McClosky, *ibid.*, 24, 371 (1924).

our other experiments. In only one experiment has there been any great flow of urine following the injection of the oxytocic fraction. It seems probable in that exceptional experiment some source of experimental error changed the apparent outcome. These diuresis experiments are still in progress and will be reported more in detail later by Dr. E. P. Bugbee.

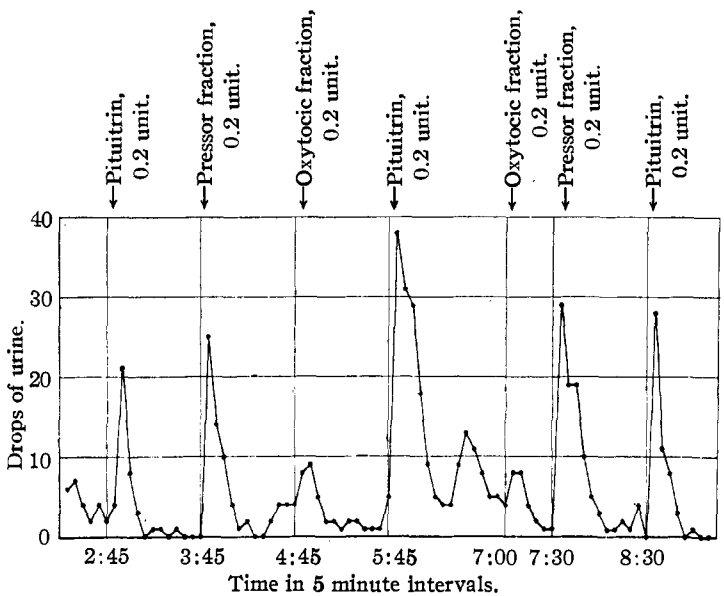

Fig. 4.—Showing the diuretic-antidiuretic effect of intravenous injections of pituitary extracts on a rabbit under urethan anesthesia.

The Pressor Effect of the Pressor Hormone

We have repeated the historical experiments of Howell[5] and of Schafer and Vincent[12] who have shown that when doses of pituitary extracts are administered intravenously in fairly rapid succession and in relatively large amounts a tolerance is quickly established so that after the second or third injection no further pressor effect is demonstrable.

When plain pituitary extracts are used the typical rise in pressure may actually be replaced by a depressor action due either to the presence of traces of histamine or to the acidity. Abel[50] has demonstrated this depressor action even with his purified fraction.

In repeating this early work we have used a purified pressor fraction and find that it acts exactly like an unmanipulated pituitary extract in respect to the development of tolerance, thus showing that the product has not been modified. However, the successive large doses fail to give

[50] Abel, Rouiller and Geiling, *J. Pharmacol.*, **22**, 303 (1923).

any depressor action, thus attesting to the degree of purification of the product. The details of this work will appear in a separate publication by Mr. L. W. Rowe.

Therapeutic Indications

In an article presented from the standpoint of the chemist it is naturally inadvisable to discuss in any great detail the medical aspects of this problem. However, in view of the fact that many of our readers may be interested in the possible applications of these hormones, the subject of therapeutic indication will be commented upon briefly.

The oxytocic principle seems indicated in those obstetrical cases where the physician wishes to avoid elevating a blood pressure that is already too high. Incidentally there is a possibility that this principle will yield more satisfactory and uniform oxytocic results when administered by itself than when mixed with the pressor hormone.

There is a strong probability that the pressor principle is the active principle which is of value in the treatment of diabetes insipidus, and preliminary clinical reports seem to confirm this opinion.

Pituitary extracts have not proved successful for the relief of asthmatic conditions. It has been suggested that the beneficial effects produced by one principle are neutralized by the unfavorable actions of the other. Obviously such practical questions can now be studied in a more scientific manner since the shotgun is replaced by the rifle.

In view of the fact that the two separated principles are available for study it is now possible for the physiologist and the pharmacologist to analyze more correctly the physiological responses caused by the administration of pituitary extracts.

General Discussion

We have no doubt that most of the previous investigators in this field have actually been working with partial separations of the active principles.

The simple operation of desiccating fairly fresh pituitary glands with acetone results in yielding a very crude extract which contains 8% of the total oxytocic activity and only 2% of the pressor activity. Investigators who have made aqueous or saline extracts without using acid have dealt with material deficient in pressor activity, but in general this fact was not realized because the deficient extracts were used as standards of comparison.

Abel[50] was convinced that his methods yielded all the various activities in the identical proportions present in the original gland material but upon repeating his work we were surprised to obtain in the first step involving the mercuric chloride treatment a yield of only 30% of pressor activity. This experiment was checked three times with almost identical results. In the light of our present work we repeated such an experiment

and found the yield of oxytocic activity to be 60% and that of pressor activity to be 30% of the total amount originally present. It is evident, therefore, that the very first step in Dr. Abel's process involves a partial separation of the two principles.

The products that we have obtained by the processes described in the experimental part of this paper, in sufficient detail so that the results may be readily duplicated, are certainly close approximations to the active principles themselves. Although we feel that additional purification may raise the potency values above those reported here and that ultimately we or others will be able to secure these two principles in crystalline form, such purification is not essential to the successful clinical application of these two new products.[51]

In contrast to all previous workers in this field we are able not merely to obtain fractions differing from each other when subjected to the two acceptable quantitative assay methods (which result might be due to partial injury of a single principle), but we have been able to secure the following results which we believe are new.

(1) Complete quantitative evidence has been obtained proving that a separation may be effected and by recombining the fractions in the original proportions a pituitary solution indistinguishable from the original is again obtained, thus proving that no injury of any active principle has taken place.

(2) By continuing our fractionation process we have effected a separation of the two principles which for all practical purposes may be considered as complete.

(3) Both principles have been obtained in the form of stable, highly potent, water-soluble powders.

(4) The separated principles have for the first time been made available to the medical profession for experimental clinical use and thousands of ampoules of the purified separated products have been distributed for this purpose.

Summary

1. The posterior lobe of the pituitary gland contains two important active principles: one which raises blood pressure and another which stimulates contraction of uterine muscle.

2. A substantially complete separation of these two active principles has been accomplished by the employment of salting-out methods and, subsequently, by the use of appropriate solvents and precipitants.

3. Solutions of these separated active principles have been recombined

[51] Note: This is analogous to the Insulin case. This product has proved to be a successful remedial agent and although recently it was obtained in somewhat more potent form and also in crystalline condition, it is not likely that the clinical use of Insulin will be greatly influenced.

to form a pituitary extract identical with the original from which they were prepared, thus proving that no decomposition has taken place.

4. The substantially pure pressor principle (β-hypophamine) has been obtained in the form of a white, stable, water-soluble powder 80 times as potent as the International Standard Powdered Pituitary.

5. The separated oxytocic principle (α-hypophamine) has been obtained in the form of a white, stable, water-soluble powder which is more than 150 times as potent as the International Standard Powdered Pituitary.

6. The pressor principle has been shown to be responsible for the diuretic-antidiuretic action of pituitary extracts.

7. The pressor principle when tested on animals for demonstration of pressor effects shows the development of tolerance which is characteristic of active pituitary extracts. It has been shown to possess no appreciable depressor action.

8. Both active principles are basic bodies, presumably amines.

9. Practical manufacturing methods have been developed for the separation of these two hormones and they have been made available to the medical profession for careful clinical trial.

10. As a result of this preliminary work the foundation is now laid for an investigation of the chemical nature of the separated hormones of the posterior lobe of the pituitary gland, together with a more exhaustive study of their pharmacological properties.

DETROIT, MICHIGAN

NEW BOOKS

General Chemistry. A Cultural Course Based upon the Texts of the Late Alexander Smith. By JAMES KENDALL, Professor of Chemistry, Washington Square College, New York University. The Century Company, 353 Fourth Avenue, New York City, 1927. xxix + 676 pp. 170 figs. and several plates. 13.5 × 20.5 cm. Price $3.50.

In this text Dr. Kendall has presented what he calls a cultural course in chemistry. It must be said, however, that if beginning students in chemistry master this book they will be entirely prepared to take advanced courses; hence it can be applied to any college or university freshman course with profit.

A number of additions to the usual content of the Smith texts have helped make the book interesting. The illustrations scattered liberally through it are very well chosen. This applies not only to those showing commercial processes but to the personal portraits of eminent chemists as well. The discussion of the personalities of these chemists is one of the ways in which human interest is aroused.

Familiar notes by Kendall add much to the ease of understanding of such topics as catalysis, ionization, etc. The citation of articles which may be