[CONTRIBUTION FROM THE DEPARTMENT OF ZOÖLOGY, UNIVERSITY OF WISCONSIN]

HORMONES OF THE CORPUS LUTEUM. THE SEPARATION AND PURIFICATION OF THREE ACTIVE SUBSTANCES[1]

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We have previously reported the extraction and separation of two active principles from the corpora lutea of the sow.[2] One of these was called relaxin due to its ability to cause relaxation of the pelvic ligaments. The second fraction, from which the relaxin had been eliminated, was physiologically active in producing several reactions ordinarily attributed to the corpora lutea, such as mucification of the vaginal mucosa of rats, production of a premenstrual endometrium in monkeys, development of the pseudopregnancy picture in the uterus of rabbits and growth of deciduomata in the uterus of castrate rats and guinea pigs.

We were at that time of the opinion that all these reactions, other than relaxation of the pelvic ligaments, were caused by the same active principle. At the present time, however, we have been able to separate from this fraction two substances, one of which produces the mucification reaction, while the second produces the premenstrual endometrium in monkeys, the pseudopregnancy picture in the rabbit's uterus, and deciduomata. We have, therefore, three hormones elaborated by the corpora lutea of the sow: *relaxin*, which has a specific action on the pelvic ligaments, *corporin*, which is concerned with the physiology of the uterus, while the mucifying principle acts on the vaginal mucosa.[3]

Methods of Testing.—Early workers almost invariably used inhibition of ovulation as a criterion for the potency of corpus luteum preparations. This end-point has been shown to be unreliable and this fact led to the confusion which existed for some time. However, in recent years several reliable physiological tests have been worked out by investigators in the field of endocrinology. One of these is the relaxation reaction used by Hisaw for the relaxative hormone.[4] The basis for other tests is the production of definite histological pictures in the uterus and vagina of various laboratory animals characteristic of the changes which normally occur during pregnancy or in the oestrous cycle when the corpora lutea of the ovary

[1] This work has been assisted by grants from the Committee for Research of Sex of the National Research Council and by the University of Wisconsin Research Funds.

[2] H. L. Fevold, F. L. Hisaw and R. K. Meyer, *Proc. Soc. Exptl. Biol. Med.*, **27**, 604, 606 (1930).

[3] The non-identity of the mucifying principle and the substance responsible for progestational development, etc., has also been recognized by Dr. R. K. Meyer and W. M. Allen, Rochester Medical School, Rochester, N. Y. They have also separated the two hormones from each other using the same general principles discussed here.

[4] F. L. Hisaw, *Anat. Rec.*, **37**, 126 (1927); *Phys. Zoölogy*, **2**, 59 (1929).

are functional. Four of these histological changes which may be used as physiological end-points in a chemical study are as follows: the production of placentomata;[5] the mucification of the vaginal mucosa of rats;[6] the production of the pseudopregnancy picture in the uterus of rabbits;[7] and the production of a premenstrual endometrium in the uterus of monkeys.[8]

All of these tests have been used in our laboratory and it has been definitely shown that these reactions are not all due to the same chemical substance. Relaxation of the pelvic ligaments is produced by relaxin, which does not produce any of the other four reactions named above. The pseudopregnancy reaction in the rabbit and the premenstrual picture in the monkey is produced by a second hormone which we named *corporin* and which Corner calls *progestin*,[7] while the mucification of the vaginal mucosa is due to a third separate principle which may be called the mucifying hormone. These three hormones have been separated from one another after being obtained in extract form by means of acid alcohol.

The activities of the corpus luteum hormones are measured in guinea pig units for relaxin, rat units for the mucifying hormone and rabbit units for corporin. The guinea pig unit for the relaxative hormone has already been described.[9] A rabbit unit of corporin is that amount of the active substance which brings about a very good pseudopregnancy picture in the rabbit's uterus when injected over a period of four days following castration of the rabbit in heat. The picture thus produced corresponds to that designated as + + + + by Corner. A rat unit of the mucifying hormone is that amount of the hormone which brings about a mucified condition of rats' vaginas similar to that of a six or seven day pseudopregnancy when injected over a period of four days following castration in full oestrum and killed on the fifth day.

General Methods of Extraction.—The corpus luteum hormones are extracted from fresh sow corpora lutea by means of acidified 95% alcohol (20 cc. concd. HCl per liter). This solvent is more efficient in extracting the active principles than any other which we have used and the hormones may later be separated from one another quite readily. Corporin and the vacuolating hormone may be extracted from the tissue by means of neutral alcohol, but in this case little or none of the relaxin is obtained and the yield of the vacuolating hormone is reduced. This procedure is, therefore, not advantageous and complicates the process for the removal of the hormones. It is, however, possible to obtain a fairly complete separation

[5] C. K. Weichert, *Proc. Soc. Exptl. Biol. Med.*, **25**, 490 (1928); L. A. Goldstein and A. J. Tatelbaum, *Am. J. Phys.*, **91**, 14 (1929).

[6] R. K. Meyer, unpublished.

[7] G. W. Corner and W. M. Allen, *ibid.*, **88**, 326 (1929).

[8] F. L. Hisaw, R. K. Meyer and H. L. Fevold, *Proc. Soc. Exptl. Biol. Med.*, **27**, 400 (1930).

[9] H. L. Fevold, F. L. Hisaw and R. K. Meyer, THIS JOURNAL, **52**, 3340 (1930).

of relaxin from the other two hormones by this method. After extraction with neutral alcohol, the tissue is extracted for relaxin and purified as previously described.

The yield of corporin can be increased by extracting the tissue by means of hot acid alcohol. The presence of acid aids materially in this extraction due to the fact that during the process the tissue is disintegrated to a fine pulp, allowing a very efficient extraction of the active material. The yield of the vacuolating hormone is, however, not increased by this method and the relaxin is destroyed completely due to its lability toward heat. This method, therefore, can be used only when corporin and the vacuolating hormone are the active principles desired or a hot acid–alcohol extraction may be carried out on the residue after the cold extractions.

The separation of the three hormones has been made possible by their different solubilities in alcohol and ether. Relaxin is first separated from corporin and the mucifying principle due to the fact that it is insoluble in absolute (99%) alcohol while the other two are soluble in this medium. Corporin is also soluble in ether, acetone or petroleum ether whereas the mucifying hormone is insoluble in ether and petroleum ether and only slightly soluble in acetone. The character of these three hormones varies, therefore, from a substance which is probably peptide-like in nature to a substance which has the solubilities of a fat-like substance.

General Characteristics of Corporin and the Mucifying Hormone.— Corporin is extracted from the corpus luteum tissue by methods already described and is purified by the elimination of proteins and their degradation products, phospholipins, fatty acids, neutral fats and cholesterol with the result that a preparation is obtained which is active in very small amounts in producing the characteristic reaction. The active material is sparingly soluble in water, quite soluble in ether and petroleum ether, but more soluble in methyl and ethyl alcohol. It is soluble in benzene, acetone, pyridine and other organic solvents. It is stable toward dilute acids even at boiling temperatures but very unstable toward alkalies above a P_H of 9. It is also readily destroyed by oxidation, even by air at room temperature.

The mucifying hormone differs from corporin in many of its characteristics, its main difference being that it is not appreciably soluble in fat solvents such as ethyl ether, petroleum ether or benzene. This difference of solubility has enabled us to separate the two hormones from each other. It is, however, readily soluble in methyl or ethyl alcohol, pyridine and acetic acid, but more difficultly soluble in acetone. It is also readily soluble in distilled water. It is especially soluble in aqueous media above a P_H of 7, while in acidified solution a clear, stable colloidal solution is obtained.

The hormone is relatively stable as compared to relaxin and corporin. However, it is similar to these two in that it is unstable toward alkalies.

In alkaline solution above P_H 9.5 its activity is slowly destroyed while in more concentrated alkaline solution decrease of activity takes place rapidly. Acidified alcoholic or aqueous solutions are quite stable. Boiling such solutions for short periods seems to have no effect on their activity. Oxidation in aqueous solution with hydrogen peroxide or dilute nitric acid does not destroy the active principle. Formaldehyde, however, inactivates the preparation completely, as does also acetyl or benzoyl chloride and nitrous acid. It is not injured by tryptic or peptic digestion. It is not precipitated by picric, trichloroacetic or tannic acid nor have we been able to precipitate the active material by isoelectric precipitation. These points differentiate the mucifying factor from relaxin and indicate that it is not peptide-like in structure.

Experimental

Method of Extraction.—The details of the preparation and properties of relaxin have been previously reported[9] so the following paper deals chiefly with corporin and the mucifying hormones. The separations of all three hormones must necessarily be given, however.

The corpora lutea used in these investigations have been obtained from the slaughter house as soon after the animals have been killed as possible. They are dissected from the ovaries, ground and covered with alcohol until they are to be extracted. The initial steps in the process are carried out in the same manner as given for relaxin. However, the first steps will be briefly given here. The procedure given below is typical for the extraction of one kilogram of corpus luteum tissue.

The corpora lutea are extracted twice at room temperature with two-liter portions of acidulated alcohol. The extracts are removed by pressing and the residual tissue is refluxed with one and one-half liters of acidulated alcohol for one hour. The extract is removed from the pasty residue and united to the first extracts. The residual tissue is discarded.

The united extracts are neutralized to P_H of 6.8–7, filtered and the precipitate redissolved, reprecipitated and discarded. The neutralized extracts are evaporated to a thick paste under reduced pressure. During the course of this evaporation the solution foams strongly and it is practically impossible to accomplish the evaporation in the ordinary vacuum distillation set-up. We have made use of a special type of distillation flask designed by the Eastman Kodak Laboratories for frothing liquids.[10] The distillation from this type of flask presents no difficulties and very little attention is required during the course of the distillation. The temperature must not rise above 45–50°, since relaxin is decomposed quite rapidly above this temperature.

The thick paste which remains in the flask is then extracted with about 500 cc. of 95% alcohol. The residue is discarded while the alcoholic solution is again evaporated to dryness, emulsified in water and phospholipins eliminated by addition of one volume of acetone. The phospholipins are filtered off, the solution evaporated and again taken up in 95% alcohol.

The crude preparation which is thus obtained contains all three of the hormones of the corpus luteum. It contains about 1000 guinea pig units of relaxin, at least 125 rat units of the mucifying hormone and about 30 rabbit units of corporin. The next

[10] "Synthetic Organic Chemicals," Vol. IV, No. 2, 1930.

step in the procedure consists of the separation of relaxin from the other two hormones by means of absolute alcohol, relaxin being insoluble while corporin and the vacuolating hormone are readily soluble.

The alcoholic solution is evaporated to dryness and extracted with 250 cc. of 99% alcohol. The solution is allowed to stand for a few hours to permit the flocculent precipitate to settle out. The precipitate, which contains practically all of the relaxin, is centrifuged off, leaving the other hormones in solution. The purification of the relaxative hormone is then carried out as formerly described.[9]

Corporin and the mucifying hormone may then be separated from each other. This separation does not take place as smoothly and cleanly as does that of relaxin since in the presence of fats the mucifying hormone has a tendency to be somewhat soluble in ether. However, after the separation has been accomplished the mucifying hormone is insoluble or very slightly soluble in ethyl ether. Corporin on the other hand is readily soluble in this solvent and a separation can be effected.

The alcoholic solution is evaporated to small volume and 100 cc. of ethyl ether is added. A precipitate settles out which contains about 60 to 70% of the vacuolating hormone and very little or none of the corporin. The precipitate is centrifuged off and preserved in a little alcohol.

The ether solution is next evaporated to dryness and extracted with 100 cc. of dry ethyl ether. A second smaller precipitate is formed which is added to the first. This process is repeated until no more ether-insoluble material can be obtained. Approximately 90% of the mucifying hormone is obtained in the ether-insoluble material while the ether-soluble gives no reaction for this hormone. The separation is therefore practically complete. If any precipitate comes out when the ether-insoluble fraction is dissolved in 99% alcohol, it is centrifuged off and combined with the fraction containing the relaxin.

We have therefore at this point three preparations each containing one of three hormones elaborated by the corpora lutea of the sow, an alcohol-insoluble preparation containing relaxin, an alcohol-soluble but ether-insoluble fraction containing the mucifying hormone, and an alcohol-soluble and ether-soluble fraction containing corporin. The separation in each case is as complete as can be expected in work of this kind.

Purification and Chemical Characteristics of the Mucifying Hormone.—The absolute alcohol solution of the vacuolating hormone is treated with three volumes of ethyl ether. A precipitate is thrown down which is centrifuged off, redissolved in alcohol and reprecipitated with ether. The precipitate is inactive so is discarded. The ether–alcohol solution contains the active material. The solution is evaporated to dryness and dissolved in glacial acetic acid. To this solution are added three volumes of ethyl ether and a volume of petroleum ether equal to the combined volume of ethyl ether and glacial acetic acid. Brown drops of glacial acetic acid containing the active material gradually settle out. After standing for some time the concentrated glacial acetic acid is removed and the acetic acid evaporated in an air dryer.

The resulting preparation containing the active material is a golden brown substance which is very difficult to dry completely as it retains water and remains in a kind of gel-like condition. When dried down completely it is a brown substance of a rather waxy consistency. It is active in two-milligram doses injected daily for four days to a rat. It is best preserved in absolute alcohol. In such solution it has been kept for a year with no detectable decrease in activity.

The reaction of a few chemical agents on the active substance was investigated in an effort to obtain some knowledge about the possible nature of the active material itself. The preparation which we have obtained is undoubtedly still far from a pure substance and an elementary analysis of the material would show nothing as to the nature of the

hormone. However, some of the results reported below throw some light on the possible nature of the active molecule.

Action of Alkalies.—Three cc. of an active preparation containing fifteen rat units of hormone was adjusted to a P_H of 9.5 by means of sodium hydroxide. This solution was allowed to stand for fifteen hours at room temperature. The solution was then neutralized, extracted with absolute alcohol and tested on a series of rats. Only about five rat units were recovered, showing a decided decrease in activity due to the presence of alkali.

The same experiment was repeated using alcoholic potash solution with similar results. The decrease in activity seemed to be slower in this case, however, as about eight to ten rat units were recovered at the end of twenty hours.

Action of Formaldehyde.—Ten cc. of the same solution as above containing 50 rat units was adjusted to P_H of 4.5 and 5 cc. of formaldehyde solution was added. The solution was permitted to stand at room temperature for twenty hours. The formaldehyde was removed *in vacuo*, the residue extracted with ether and then taken up in alcohol and prepared for injection. No activity was present in the extract, showing a complete destruction due to the action of formaldehyde. In this respect this hormone is similar to relaxin since formaldehyde also destroys that hormone.

Action of Nitrous Acid.—Nitrous acid acts as a deaminizing agent on primary and secondary amines yielding alcohols and nitrosamines. If the active molecule of the hormone contains a primary or secondary amino group which is functional, nitrous acid would destroy the group and render the hormone inactive. The action of nitrous acid on the active preparation was therefore tried.

Ten cc. containing 50 rat units was acidified with glacial acetic acid (0.8 cc.) and treated with 2 cc. of 10% sodium nitrite solution. The solution was warmed to 35° for three hours, with occasional shaking. At the end of that period the solution was neutralized, concentrated, taken up in absolute alcohol and prepared for injection. Only about ten rat units was recovered from the fifty showing a marked decrease in activity.

Action of Benzoyl Chloride.—The presence of the primary or secondary amino group as well as OH groups might also be indicated by the action of benzoyl chloride. Experiments which demonstrate the effect of this reagent were carried out as follows.

Twenty cc. containing 100 rat units was dissolved in water and made alkaline (P_H 9) by addition of sodium carbonate. Two cc. of benzoyl chloride was added and the mixture shaken in a tube for fifteen or twenty minutes. An appreciable amount of heat was liberated accompanied by evolution of carbon dioxide. The mixture was acidified and the benzoic acid removed by shaking with ether. The remaining aqueous solution was tested for activity but none was present.

Attempts to restore the activity by acid hydrolysis were made. In several instances small amounts of the activity seem to have been regenerated while in others the results were negative. However, the instances of apparent success would lead one to believe that success along this line might be accomplished by carefully regulating the conditions under which the hydrolysis is carried out. At the present time, however, the results must be considered as being negative.

Action of Pepsin and Trypsin.—Many of the hormones, such as insulin, relaxin, active principles of the posterior lobe of the hypophysis and the hormones of the anterior lobe of the hypophysis are destroyed by the action of proteolytic enzymes, indicating that they are probably of a type similar to peptides or polypeptides. The action of these enzymes on the mucifying hormone was investigated to determine if it can also be assigned to this general class.

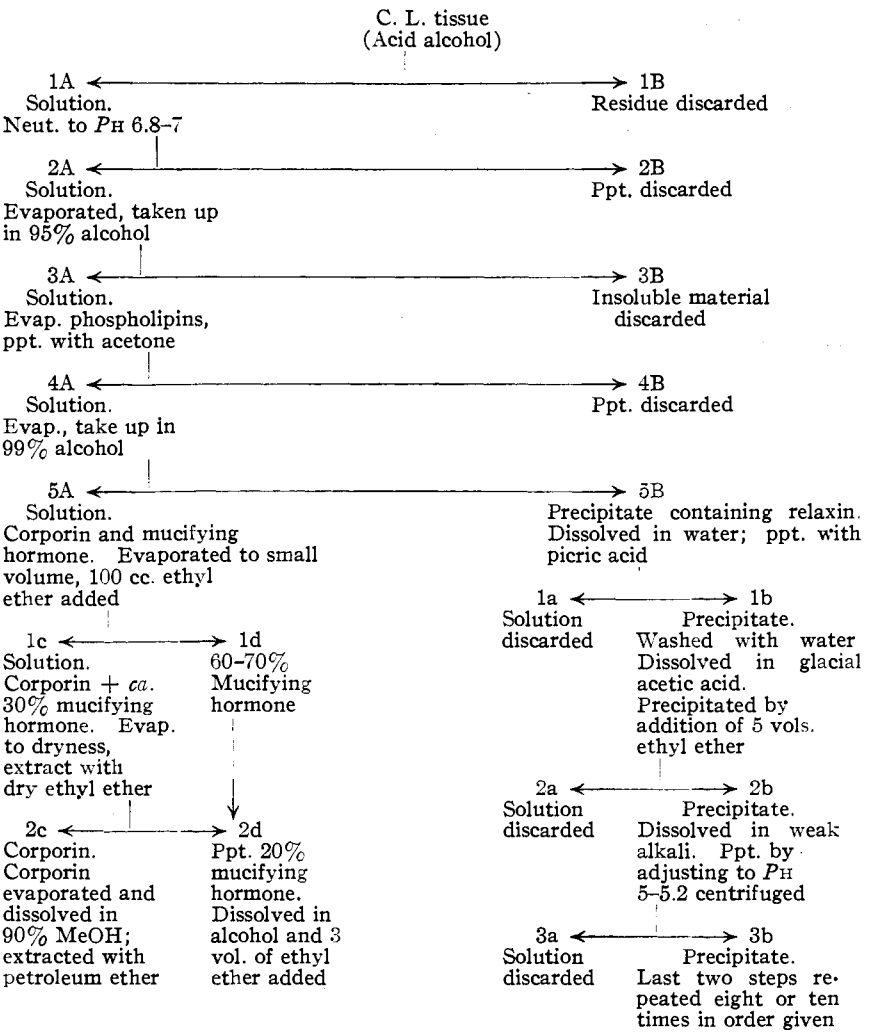
Twenty rat units was dissolved in an aqueous medium at a P_H of 2.5 and subjected to peptic digestion for forty-eight hours. The solution was then concentrated almost to dryness, extracted with 99% alcohol and filtered. The filtrate was evaporated to dry-

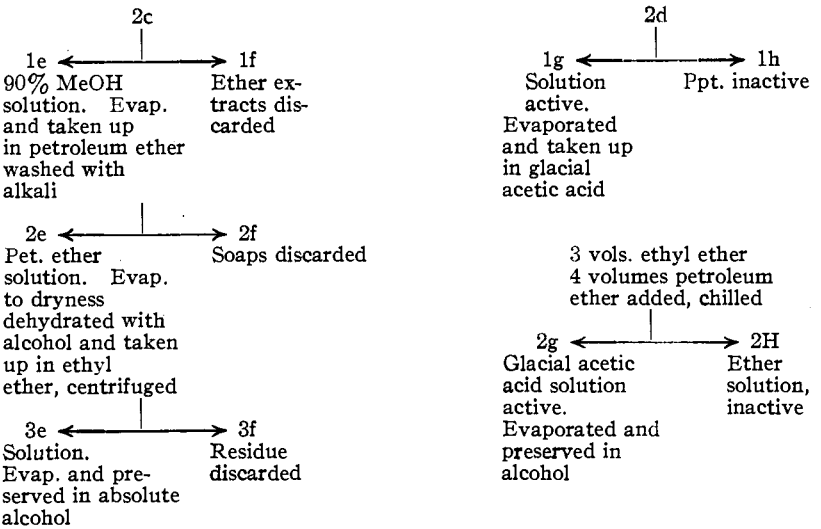
ness and the solute prepared for injection in saline solution. No decrease in activity could be detected since the twenty rat units was still present.

An equal amount of the mucifying hormone was dissolved in aqueous solution at a P_H of 7.4 and subjected to tryptic digestion for forty-eight hours. At the end of that time the hormone was prepared for injection exactly as in the preceding experiment. As before the preparation retained its activity in full.

Purification and Chemical Characteristics of Corporin.—The alcoholic solution containing the corporin is evaporated to dryness and taken up in 50 cc. of methyl alcohol. The methyl alcohol solution may be packed in ice and the cholesterol partially chilled out by permitting it to stand for twenty-four to forty-eight hours, after which the crystallized cholesterol is centrifuged off, washed, reprecipitated and discarded.

SCHEMATIC DIAGRAM OF SEPARATION OF CORPUS LUTEUM HORMONES





To the methyl alcohol solution is added 5 cc. of water and the resulting 90% methyl alcohol is extracted three times with an equal volume of petroleum ether. The petroleum ether extracts are united and thoroughly washed with 90% methyl alcohol. The methyl alcohol solutions are then united. By this process none of the activity is lost to the petroleum ether and the methyl alcohol solution is free from all but traces of cholesterol and is active in 20-mg. doses. This step in purification may also be applied before the preliminary freezing out process.

The methyl alcohol solution is evaporated and taken up in petroleum ether. The petroleum ether solution is freed from any remaining fatty acids by the usual procedure of washing with dilute alkali until washings remain alkaline. The solution is freed from excess alkali by washing with acid and water. The soaps are washed with petroleum ether and discarded.

The resulting product is active in 15 to 20 mg. doses representing 25 to 50 g. of tissue. It is best preserved in absolute alcohol in a dark cool place. It must not be exposed to air for any length of time. For injection the material is dissolved in mazola oil so that it may be conveniently handled. It is not advisable, however, to keep it in this medium for more than four or five days.

The hormone is very unstable to alkalies since at a P_H of 9.5–10 the activity decreases very rapidly. On the other hand it is very stable to heat and acids (under non-oxidizing conditions). This is amply demonstrated by the fact that the corpus luteum tissue may be refluxed with two volumes of acidified alcohol for three hours and the resulting extract instead of showing a decrease in activity shows an increase, undoubtedly due to more complete extraction since the acid completely disintegrates the tissue.

Extraction of the tissue by means of hot acid alcohol affords a quick and convenient method to obtain a very active extract for physiological purposes. As stated before, the relaxin is completely destroyed in this procedure. It is carried out as follows.

To one kilogram of the tissue in a 5-liter round-bottomed flask is added two liters of acidified alcohol. This is refluxed for two hours on a sand-bath after which the extract is removed from the residual tissue. The residue is washed with a liter of 95% alcohol and discarded. The extracts are neutralized, filtered, evaporated to dryness *in vacuo*, taken up in 95% alcohol, discarding any insoluble material. More inactive

material may be eliminated by again evaporating to dryness and extracting with 99% alcohol. The alcoholic solution is evaporated to dryness and extracted thoroughly with 200-cc. portions of acetone until the last portions of acetone are practically colorless. The acetone solution is evaporated and extracted with ether removing any insoluble material. The ether is evaporated and the solute is fractionated between 90% methyl alcohol and petroleum ether as before. The preparation thus obtained is active in twenty-five gram equivalents of the fresh tissue, varying somewhat depending on the activity of different lots of corpus luteum tissue.

Discussion

In our preliminary report of the separation of relaxin from the active substance responsible for the other physiological reactions we stated that the preparation was water soluble. This was true but this preparation was far more active with respect to the mucifying factor than with respect to corporin. At that time we were using the mucifying reaction as our routine test in the laboratory and verifying our results with the pseudopregnancy reaction. The water-soluble preparation gave both reactions but as we later found much of the corporin was lost. Corporin may be prepared in aqueous medium but it is more soluble in organic solvents. The mucifying hormone, however, is readily soluble in water.

Corner and Allen[7,11] have prepared a neutral alcohol extract of corpus luteum tissue which contains a hormone which they call progestin. This is apparently the same hormone which we call corporin since it is the same in its physiological action and also similar chemically. Their crude preparation would also contain the mucifying hormone which is, however, eliminated in the process of purification by means of ethyl and petroleum ether. Relaxin is not extracted from the tissue by means of neutral alcohol. The method used by these workers is, however, efficient for the extraction of corporin but is rather time-consuming.

With regard to the chemical natures of these three hormones not much can be said conclusively at the present time. Relaxin as far as it has been characterized is quite similar to peptides or polypeptides. The mucifying hormone is not of this type since neither pepsin nor trypsin has any effect on the substance. However, formaldehyde which presumably acts on the amino group completely destroys the active molecules as do other agents which would act on this grouping. Corporin on the other hand is a fat-soluble substance and as such is different in nature from the other two corpus luteum hormones. It is more similar to the follicular hormone of the ovary, its main difference being its instability to alkalies.

Summary

1. The corpora lutea of the sow secretes three active principles which have been extracted from the tissue and separated from one another.

2. The purification of the mucifying hormone and corporin is given.

[11] W. M. Allen, *Am. J. Phys.*, **92**, 174 (1930).

3. A few chemical characteristics of these two hormones are also given.

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[CONTRIBUTION FROM THE QUEEN'S COLLEGE, OXFORD]

THE CONDENSATION OF BUTYL CHLORAL HYDRATE WITH ARYL HYDRAZINES

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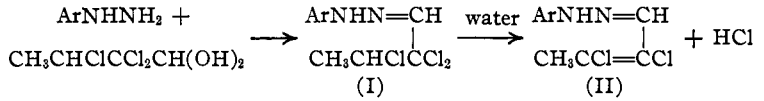
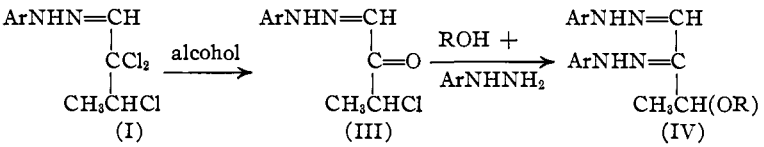
When butyl chloral hydrate is condensed with any aryl hydrazine, the hydrazone (I) first formed very readily undergoes a further reaction, the nature of which depends upon the solvent in which the change occurs.

In aqueous solution the initial hydrazone (I) loses hydrogen chloride and yields an *α,β-dichlorocrotonaldehyde-arylhydrazone* (II).[1]

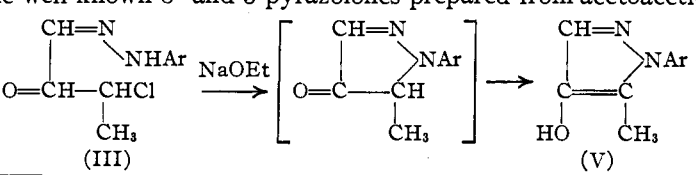


When, however, the condensation takes place in alcohol, the two α-chlorine atoms of the initial hydrazone (I) are replaced by an atom of oxygen and a *β-chloro-α-ketobutaldehyde-arylhydrazone* (III) is formed.[1]

These ketonic hydrazones (III) are very interesting substances for, when heated with a substituted arylhydrazine and any alcohol, many of them yield sparingly soluble *osazones* (IV) in which not only is the α-ketonic oxygen atom replaced by a hydrazine residue but also the remaining β-chlorine atom of the aldehyde chain is replaced by an alkoxy group.[1]



Again, when heated with an alcoholic solution of sodium ethoxide, these ketonic hydrazones (III) lose a molecule of hydrogen chloride, and ring closure takes place with the formation of the corresponding *4-hydroxy-1-aryl-5-methylpyrazoles* (V).[2] This reaction affords a general means of synthesis for these hitherto unknown 4-hydroxypyrazoles which are isomeric with the well-known 3- and 5-pyrazolones prepared from acetoacetic ester.



[1] Chattaway and Irving, *J. Chem. Soc.*, 87 (1930).

[2] Chattaway and Irving, *ibid.*, 786 (1931).