**Absorption Spectra of *d*-2-Nitro-octane and the Sodium Salt of *d*-2-Nitro-octane.**—The absorption spectra (Plate I) were determined in a K and E color analyzer which permitted measurements in the visible range only. A solution of 0.5191 g. of *d*-2-nitro-octane in 20 cc. of absolute alcohol was used for obtaining curve (A) and a sample of 0.5041 g. of 2-nitro-octane which was treated with one equivalent of sodium ethylate solution and diluted to 20 cc. was used to obtain curve (B).

## Summary

1. The preparation of (*d*) and (*l*) 2-nitro-octanes is described.

2. The sodium and potassium salts of *d*-2-nitro-octane were found to be optically active to the same extent.

3. The *d*-2-nitro-octane regenerated from the sodium salt at $-10°$ was optically active to the extent of 24.12% of the original *d*-2-nitro-octane.

4. By use of very low temperature for the reaction, the regenerated *l*-2-nitro-octane was optically active to the extent of 71.2% of the original *l*-2-nitro-octane.

5. Optically active *d*-2-bromo-2-nitro-octane was prepared from the active sodium salt of *d*-2-nitro-octane.

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[CONTRIBUTION FROM THE ZOÖLOGY DEPARTMENT, UNIVERSITY OF WISCONSIN]

# THE RELAXATIVE HORMONE OF THE CORPUS LUTEUM. ITS PURIFICATION AND CONCENTRATION[1]

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It is a matter of common knowledge, at least among those versed in the physiology of reproduction, that the pelvis of several species of mammals is modified during pregnancy to facilitate the birth of young. These pelvic modifications commonly involve the ligaments of the symphysis pubis and ilio-sacral unions in a fashion which enables these bones to move apart and thus increase the diameter of the pelvic canal. In some animals, only the ilio-sacral unions are changed, while in others both these and the symphysis pubis are affected. One of the most striking examples of this phenomenon is found in pregnant guinea pigs. The pelvic ligaments of these animals begin to show signs of loosening, both at the ilio-sacral and pubic regions, about the middle of pregnancy and they become more and more pronounced with the approach of parturition. At the termination of pregnancy the ilia can be freely moved, and a finger can be placed between the pubic bones at the symphysis. This remarkable modification of the guinea pig pelvis has, for lack of a better term, been designated as ligamentus relaxation.

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It was not until quite recently that these pregnancy changes were found to be under hormonal control.[2] It was first discovered that a substance was present in the blood of pregnant rabbits which, when injected into virgin guinea pigs during oestrum, would produce ligamentus relaxation, characteristic of that which normally occurs during pregnancy. This substance appears in the blood of pregnant rabbits, at about the seventh day of the gestation period, in sufficient amount to give a positive test when 2 cc. of serum is injected and disappears within twelve hours after the birth of young. So constant is the presence of this relaxative substance and so definite is its reaction that it may be used as a test for pregnancy in rabbits.

Although the chemical and physiological properties of the relaxative substance were first studied as it exists in the blood, it was later demonstrated that the substance had its origin in the corpora lutea and placenta. It is a hormone typical of the corpus luteum but, like the follicular hormone "oestrin," may also have other sources, particularly the placenta. The hormone is not species specific, but is found in the blood and tissues of certain mammals in greater amounts than in others.[2] However, no matter where it is found it can be extracted and purified by essentially the same chemical procedures.

The only physiological property thus far discovered for this hormone is its action on the pelvic ligaments and for this reason we propose the name "Relaxin." This designates its physiological activity and also adheres to the generally accepted nomenclature for hormones.

The factors governing the relaxation reaction can best be demonstrated in castrate virgin guinea pigs. When such animals are injected with relaxin the pelvic ligaments do not respond, but if they are first brought into artificial oestrum through the use of follicular hormone and are then given relaxin, positive results follow. The follicular hormone must first produce its effect before relaxin can act. If relaxin is given first, followed by follicular hormone, no relaxation occurs and when both hormones are given simultaneously over an extended period, the results remain negative until enough time has elapsed for the follicular hormone to put the animal in the proper physiological condition, so that the pelvic ligaments will respond to relaxin. Relaxation of the pelvic ligaments of the guinea pig depends upon the action of two hormones, namely, oestrin and relaxin, neither of which can produce the reaction when given alone, but the two must work in definite relationship to each other.

It also seems that oestrin and relaxin work with each other in a qualitative fashion in achieving and prolonging the ligamentus relaxation reaction. This idea is supported by the fact that a castrate female need not be brought into full oestrum before the relaxative hormone can function,

[2] Frederick L. Hisaw, *Phys. Zoölogy*, **2**, 59 (1929).

nor can the action of this hormone be hindered by large doses of oestrin. It is also true that once the relaxed condition is attained, relaxin alone cannot maintain it over an extended period, but can do so only when the animal is kept tuned up, so to speak, by frequent doses of oestrin.

The potency of different preparations of relaxin is standardized in terms of their ability to produce relaxation of the pelvic ligaments of virgin female guinea pigs which are in full oestrum. These animals are injected subcutaneously with a definite amount of the preparation to be tested and after ten to twelve hours the pelvic ligaments are palpated gently to see if they have become loose. Loosening can best be determined by alternately moving each half of the pelvis up and down vertically while at the same time keeping a finger over the symphysis pubis. The minimum amount of hormone which causes a definite loosening of the ligaments within ten to tweve hours after a single injection is taken as a guinea pig unit.

This paper deals only with the relaxative hormone of the corpus luteum, but it should be mentioned in passing that relaxin is only one of two hormones elaborated by the corpora lutea of the sow. This second hormone differs from relaxin in both its physiological and chemical properties. It does not produce relaxation but is responsible for various other reactions such as the growth of placentomata,[3] inhibition of ovulation and vaginal changes typical of pregnancy,[4] progestational development in the uterus of rabbits,[5,6] and formation of a premenstrual endometrium in the uterus of castrate monkeys.[7] This second hormone also depends upon the coöperation of oestrin to produce its effect in a manner similar to that described for relaxin, with the exception that the relationship here is a quantitative one.[8] The two corpus luteum hormones have been separated from each other quantitatively and the chemical nature of the second hormone is being studied at the present time. We wish only to state that it has been obtained in a highly purified, water-soluble condition.[9]

**General Characteristics of Relaxin.**—The relaxative hormone is extracted from corpora lutea, which is its chief source, or from other tissues, by means of acid alcohol. The extract is subsequently purified by elimination of proteins, phospholipins and other fatty substances by various methods with the result that a water-soluble extract, containing the active principle in concentrated form, is obtained. The hormone may then be obtained from this extract by either of two different methods. By crystallization of the evaporated aqueous extract from glacial acetic acid, the

[3] C. K. Weichert, *Proc. Soc. Exptl. Biol. Med.*, **25**, 490 (1928).

[4] Frederick L. Hisaw, R. K. Meyer and C. K. Weichert, *ibid.*, **25**, 754 (1928).

[5] G. W. Corner and W. M. Allen, *Am. J. Physiol.*, **88**, 326 (1929).

[6] W. M. Allen and G. W. Corner, *Proc. Soc. Exptl. Biol. Med.*, **27**, 403 (1930).

[7] Hisaw, Meyer and Fevold, *ibid.*, **27**, 400 (1930).

[8] Hisaw and Leonard, *Am. J. Physiol.*, **92**, 574 (1930).

[9] Fevold, Hisaw and Meyer, *Proc. Soc. Exptl. Biol. Med.*, **27**, 606 (1930).

hormone is obtained in a form in which it is associated with sodium chloride. The product is crystalline in nature and consists largely of sodium chloride. The active material is present in this crystalline preparation but whether it is merely adsorbed on the crystals or crystallized with the sodium chloride is open to question. Approximately one milligram of this crystalline material is sufficient to give the physiological reaction described above. On the other hand, the hormone may be precipitated from solution by means of picric acid and subsequently purified, with the result that a product is obtained which is effective in about 0.03-mg. doses. One gram of the purified material contains about 28,000 guinea pig units.

The purified hormone is soluble in glacial acetic acid, only slightly soluble in distilled water and 95% alcohol, insoluble in 99% alcohol and other organic solvents such as ethyl ether, acetone, petroleum ether and benzol. In its impure form it is sufficiently soluble in 95 or 97% alcohol so that these solvents may be used to purify the crude extract. The hormone dissolves in aqueous alkaline or acid solutions. In alkaline solution, however, above a $P_H$ of 9.0, the activity of the hormone is impaired quite rapidly, while in 2% sodium hydroxide the hormone is destroyed almost immediately. In acid (non-oxidizing) and neutral solutions the hormone is stable. Solutions at $P_H$ of 7.0 and 3.2 have been kept in the ice box for a year with no detectable decrease in activity.

The hormone is also affected by oxidation, by drying in air and by heating. Solutions of the hormone decolorize potassium permanganate with consequent loss of activity. One cc. of a $N/10$ solution of potassium permanganate will inactivate completely 25 G. P. units of the hormone. Dilute nitric acid renders it inactive, as do also bromine, chlorine and iodine. If the hormone is permitted to stand at room temperatures in dry form the activity decreases slowly but definitely. If the dry hormone is heated under atmospheric conditions over 50° the activity is completely lost in twenty-four to forty-eight hours. Heating the aqueous solutions at 70–80° for five or six hours destroys all activity, while boiling for ten or fifteen minutes brings about the same result.

Some of the properties of the active material would point to a peptide-like structure while other characteristics seem to eliminate that possibility. It has both basic and acidic properties, since it is soluble in both acid and alkaline solutions. It has a very definite isoelectric point, being precipitated from a 0.1% solution at a $P_H$ of 5.4–5.5. Proteolytic enzymes break down the material to a stage where it is no longer active. Trypsin and pepsin both destroy the hormone. It contains nitrogen but the amount is rather low to correspond to a peptide-like structure. A 0.5% solution of the active material is negative to Millon's reaction and to the ninhydrin reaction, but gives a faint violet coloration with Poser's ring biuret test. It is also negative to Molisch's reaction.

## Experimental Details

**Extraction of Corpus Luteum Tissue.**—The following describes the preparation of an extract of one kilogram of corpus luteum tissue from sows' ovaries.

Corpora lutea from sows' ovaries are finely ground and extracted twice, for forty-eight hours, with two liter portions of acidulated alcohol (2 cc. of concd. hydrochloric acid to 98 cc. of 95% alcohol) at room temperature, with frequent shaking and stirring. After each extraction the residue is pressed dry by means of a press. The two extracts are united and one-third of their volume of water is added. The fatty material which separates is filtered off and discarded.

The filtrate is adjusted to a $P_H$ of 6.8 by the careful addition of 10% sodium hydroxide. When the end-point is being approached, the extract becomes turbid and at a $P_H$ of 6.8 a flocculent precipitate separates out. After standing for a few hours (six to eight) the precipitate is filtered off, reëxtracted with 200 cc. of 70% alcohol, which has been acidified as before, reprecipitated by neutralization and discarded.

The united extracts are evaporated to a semi-dryness under reduced pressure at 40 to 45° or preferably in an air dryer at 40° and extracted with 450 cc. of 95% alcohol, using 150-cc. portions in three successive extractions, allowing one-half hour for each extraction. The insoluble residue is centrifuged off and discarded while the alcoholic solution is evaporated and the residue emulsified in 200 cc. of water. Phospholipins are precipitated by adding an equal volume of acetone, removed and discarded.

The aqueous acetone solution is again evaporated and the residue extracted with 200 cc. of 97% alcohol. The material must be triturated with the alcohol until it is in a finely divided condition. A large volume of alcohol must not be used as this results in a partial precipitation of the hormone. After removal of the alcohol as before, the solute is repeatedly extracted with ether or acetone to remove any remaining fats. The ether-insoluble residue is then dissolved in water at a $P_H$ of 3 to 4. The water solution is clear and light brownish in color. It is usually prepared so that 1 cc. is equal to 15 g. of the fresh tissue, although it can be concentrated to more than twice that strength. As a rule, enough of this extract to represent one gram of the fresh tissue is sufficient to give a good positive result in the relaxation reaction. This extract has been used for a long time in our laboratory and has proved very satisfactory. We shall designate this preparation as preparation "A."

If the residue after the ether extraction is dissolved in a small amount of 95% alcohol and then diluted with 99% alcohol until no more precipitate forms, the relaxative hormone is completely precipitated. The precipitate is somewhat soluble in distilled water but much more readily soluble in water at a $P_H$ of 3 to 4 (prepn. "B"). The potency of this preparation is the same as that of the original extract. Table I shows the number of units contained in preparation "A" from one kilogram of tissue and in preparation "B" after precipitation.

TABLE I

RELATIVE POTENCY OF PREPN. "A" AND PREPN. "B"

| Sample | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Prepn. "A," units per kilo | 1100 | 975 | 1000 | 800 | 850 |
| Prepn. "B," units per kilo | 1100 | 950 | 1000 | 750 | 850 |

**The Relaxative Hormone in the Blood of Pregnant Rabbits.**—The presence of the relaxative hormone in the blood of various pregnant animals has been previously reported.[2] The amount increases with the duration of pregnancy, being greatest during the last half of the gestation period.

At this time 2 cc. of pregnant rabbit serum is sufficient to produce very pronounced relaxation, while 0.25 cc. ordinarily gives a positive reaction.

The hormone can be obtained from rabbit serum by various methods. If the serum is evaporated to semi-dryness, the active material can be extracted by means of saline solution. Ninety-five per cent. alcohol will not extract the hormone from the dried residue, but if the residue is treated with 95% alcohol which has been acidified as previously described, the hormone is taken into solution. After neutralization and evaporation, the residue is then soluble in neutral 95% alcohol.

To determine the type of material with which the hormone is associated in the blood, the following experiments were carried out. The globulins of the blood serum were precipitated, using sodium sulfate as the precipitant. The globulin-free serum was found to be inactive, while the globulin fraction produced relaxation. The euglobulins were then precipitated by the addition of 13 to 14% sodium sulfate. In this case the precipitated fraction was inactive while the remaining serum retained its activity. It appears, therefore, that the hormone was associated with the pseudoglobulin fraction of the serum proteins. To test this further, the entire globulin fraction was precipitated from a sample of pregnant rabbit serum by one-half saturation with sodium sulfate. They were then fractionated by fractional precipitation with the same precipitant. As before, the fraction designated as the euglobulins was inactive while the pseudoglobulins contained the hormone.

Preparation of the Hormone Associated with Sodium Chloride.—The ether-insoluble material from one kilogram of fresh tissue is dissolved in 200 cc. of glacial acetic acid. The solution is then permitted to evaporate slowly, in the air dryer, until all the acetic acid has been removed and crystals of a definite form appear. These crystals are insoluble in ethyl alcohol, so the brown sirupy material is removed by dissolving in 50 cc. of 99% alcohol. Here again the volume must be kept small. If a large volume of alcohol is used, a flocculent precipitate will appear. This precipitate dissolves if the volume of alcohol is small, while the crystals remain insoluble. To remove any traces of the flocculent precipitate, the following procedure is used. The 50 cc. of alcoholic extract is diluted with 99% alcohol until no more precipitate forms, the precipitate is centrifuged off, and the alcoholic solution again concentrated to 50 cc. The impure crystals are then reëxtracted, using the alcoholic solution as the solvent. Any of the flocculent material which remained will dissolve. After this has been repeated twice, the crystals are washed several times with absolute alcohol, leaving them in a "pure" condition. Microscopically nothing remains but the crystalline material. The crystalline fraction contains the relaxative hormone while the alcohol solution is relatively inactive. Approximately 85% of the hormone is obtained in the crystalline fraction while 15% remains in the alcoholic solution. This can be completely removed by the addition of a small amount of sodium chloride and recrystallization from acetic acid. The crystals in this case are, however, as a rule irregular and contain an excess of sodium chloride.

The crystalline product is composed of approximately four parts ash and one part volatile material. The ash is made up of sodium chloride, as is shown by the following facts. If the ash is dissolved in a little water and crystallized on a slide, characteristic

crystals of sodium chloride appear. If the chlorine in the ash is determined and calculated as sodium chloride the results agree with the weight of the ash. Also, the crystals give a strong sodium test while tests for other cations of non-volatile chlorides have been negative. The volatile fraction contains the active material which, as we shall see later, contains a definite amount of nitrogen.

The crystals dissolve readily in water. When this aqueous solution is injected, in the same proportion as that given for the tissue extract, it produces relaxation of the pelvic ligaments of guinea pigs in oestrum. The dose which gives a positive reaction represents approximately one milligram of crystalline material or two-tenths of a milligram of volatile material. The amount of crystalline material obtained, in different preparations, from the same amount of tissue, is relatively constant although not entirely so. One kilogram of corpus luteum tissue yields approximately 0.8 g. of crystalline material, varying in individual preparations by $\pm 0.2$ g.

**Purification of the Hormone by Precipitation with Picric Acid.**—The aqueous solution of the crystalline material is made acid ($P_H$ 4 to 5) with hydrochloric acid and an excess of picric acid is added. A precipitate is formed which is separated by centrifuging. The precipitate is washed several times with distilled water, dissolved by adding just enough sodium hydroxide to cause solution and reprecipitated by making acid with hydrochloric acid. The product is insoluble in water, alcohol and ether but dissolves in glacial acetic acid. It retains the activity of the original preparation quantitatively, as is shown by Table II.

TABLE II

RELATIVE POTENCY OF CRYSTALLINE PREPARATION AND PICRIC ACID PRECIPITATE

| Sample | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Crystalline prepn., G. P. units | 700 | 800 | 850 | 770 | 800 |
| Picric acid prepn., G. P. units | 700 | 800 | 835 | 740 | 775 |

While this insoluble picrate was first prepared from the crystalline preparations, it was later found that the hormone could be precipitated and purified more conveniently before crystallization from glacial acetic acid as follows. The hormone is precipitated by means of 99% alcohol as previously described and dissolved in water acidified with hydrochloric acid (Prepn. "B"). To this solution is added an excess of picric acid, which precipitates the active principle as described. The product is again just as active as the solution from which it was precipitated.

The active substance, whether it is prepared from the crystalline fraction or from preparation "B," is freed from picric acid and obtained in an extremely pure form by the following procedure. The picrate is dissolved in a small amount of glacial acetic acid. To this solution is added five times its volume of ethyl ether. The active material is precipitated as a brownish-yellow flocculent precipitate. It is centrifuged off and dissolved in distilled water, in which it now dissolves readily. The aqueous solution is adjusted to a $P_H$ of 5.4, whereupon the active substance again precipitates and is centrifuged off. The successive precipitation from acetic acid with

ether and from aqueous solution at its isoelectric point is repeated several (4–5) times. The purified product finally comes down as a flesh colored flocculent precipitate which dries in clear, shiny amber plates with no definite structure. Table III gives the yield in milligrams from different kilogram samples and the guinea pig units contained in each sample.

TABLE III

ASSAY OF PURIFIED RELAXATIVE HORMONE

| Sample | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Yield per kg., C. L. tissue, mg | 35 | 30 | 17 | 21.5 | 43.5 | 32 |
| G. P. units | 1000 | 858 | 500 | 672 | 1230 | 914 |
| Mg. of dried material per G. P. unit | 0.035 | 0.035 | 0.034 | 0.032 | 0.034 | 0.035 |

The figures in Table III are selected to show the variation which may occur in different samples of tissue. The general yield which is ordinarily obtained varies from 850 to 1100 guinea pig units per kilogram of fresh tissue, but wider variations sometimes occur as shown in the table. Variation in the yield from a given amount of tissue is to be expected since no attempt is made to obtain corpora lutea only from pregnant or non-pregnant sows in the same stage of development. The more important fact, which is demonstrated in Table III, is that the amount of purified material representing a guinea pig unit is constant throughout.

The nitrogen content of the isolated material has been determined by Kemmerer's micro Kjeldahl method.[10,11] The samples were prepared by reprecipitation at the isoelectric point at least ten times, dried at 35° under atmospheric conditions, ground in an agate mortar and then dried at 60° *in vacuo*. The amount of nitrogen found in different preparations is constant as shown in Table IV.

TABLE IV

NITROGEN CONTENT OF PURIFIED MATERIAL

| Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Nitrogen, % | 11.52 | 11.78 | 11.55 | 11.24 | 11.55 | 11.49 | 10.78 | 11.38 |

## Discussion

As to the chemical nature of relaxin, not much can be said conclusively at the present time. We believe that the purified product which we have obtained is at least a close approach to the pure substance and while we have not as yet obtained it in a pure crystalline form we may hope to do so. The product seems to act as a chemical individual toward all solvents and reagents to which we have subjected it. Its composition seems to be constant, if we may judge from the constant amount of nitrogen in every sample. As mentioned before, the product is amphoteric in nature and

[10] G. Kemmerer and L. T. Hallett, *Ind. Eng. Chem.*, **19**, 1295 (1927).

[11] The authors are indebted to T. F. Setterquist for the analysis of the purified material.

has several other properties which would point to a peptide-like structure. However, with a nitrogen content of only 11% the chemical structure of the material appears to be different from typical peptides or polypeptides. The work which is reported in this paper lays the foundation for a more exact study of the chemical nature of the hormone.

Since insulin is generally considered to be peptide-like in nature, it is interesting to compare the two hormones, relaxin and insulin. Insulin is more definitely positive to protein reactions than is relaxin, but it can readily be seen that the two are very similar in a number of their properties.

TABLE V

COMPARISON OF THE PROPERTIES OF INSULIN AND RELAXIN

| | Insulin | Relaxin |
|---|---|---|
| Isoelectric point | About *P*H 5.0 | *P*H 5.4–5.5 |
| Acid solution | Stable | Stable |
| Alkaline solution | Unstable | Unstable |
| Millon's reaction | Questionable | Negative |
| Biuret reaction | Positive | Questionable |
| Molisch Reaction | Negative | Negative |
| Picric acid | Precipitated by | Precipitated by |
| Trypsin | Destroyed | Destroyed |
| Pepsin | Destroyed | Destroyed |

## Summary

1. Methods for the extraction and purification of the relaxative hormone (Relaxin) are given.

2. The hormone has been obtained in a form in which it is associated with sodium chloride in relatively pure condition.

3. The hormone has also been concentrated to a highly purified form in which 0.035 mg. constitutes a guinea pig unit.

4. The chemical properties of the purified product are given. These are compared with those of insulin.

5. The name "Relaxin" is proposed to designate the relaxative hormone.

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