SCIENCE

VOL. 82 FRIDAY, AUGUST 2, 1935 No. 2118

SCIENCE: A Weekly Journal devoted to the Advancement of Science, edited by J. MCKEEN CATTELL and published every Friday by

THE SCIENCE PRESS

New York City: Grand Central Terminal

Lancaster, Pa. Garrison, N. Y.

Annual Subscription, $6.00 Single Copies, 15 Cts.

SCIENCE is the official organ of the American Association for the Advancement of Science. Information regarding membership in the Association may be secured from the office of the permanent secretary, in the Smithsonian Institution Building, Washington, D. C.

THE ISOLATION OF CRYSTALLINE PROGESTIN[1]

By Dr. W. M. ALLEN

SCHOOL OF MEDICINE AND DENTISTRY, UNIVERSITY OF ROCHESTER

THE actual isolation of a hormone in pure form, determination of its chemical formula or even its synthesis is oftentimes simpler than the basic researches necessary to show that a given physiological condition exists only when a certain hormone is acting. The hormonal rôle of the corpus luteum and the isolation and synthesis of its hormone are no exceptions to this dictum, and for this reason I must describe briefly some of the work, already classical, showing that this little structure is a gland of great importance in the propagation of the race.

In all the higher forms of animal life reproduction is brought about by union of specialized cells of the two sexes, but the development of the fertilized ovum into a new animal is carried out in a variety of ways, depending on the type of individual. In birds, for example, the fertilized egg is incubated outside the mother's body and hence there can be no direct effect of the mother on her offspring after the egg has been laid. With mammals, on the other hand, the early development of the embryo is quite different, for in them the embryo must first be nourished within the body of the mother for a considerable length of time before it reaches a stage of development such that it can be born, and even then the young are so premature as to require suckling for some time. It is not surprising, therefore, that we find highly specialized structures in the mammalian female designed exclusively for use in rearing the young.

The differences between the reproductive organs and the reproductive processes of the lower animals and

[1] Address at the meeting of the American Chemical Society, New York, April, 1935, on the occasion of the presentation of the first Eli Lilly and Company award in biological chemistry.

the mammals have been appreciated of course, for many years, but it was not until the latter part of the last century that any attempt was made to study these differences in much detail. In fact, the modern school dealing with the physiology of reproduction owes its origin in part to a German embryologist, Born, who first fully appreciated the significance of the fact that the ovary of all mammals contains a special structure, known as the corpus luteum, which is not present in the lower forms of animal life. Purely by analogy he reasoned that this gland should have some relation to the development of the placenta and the intra-uterine growth of the embryo because it occurs only in those animals in which intra-uterine development takes place and because it reached its greatest size during pregnancy. He postulated, therefore, that removal of the corpus luteum should interrupt pregnancy. Unfortunately, Born did not live to try out this beautiful hypothesis, which marked the first step in our understanding of the intricate phases of mammalian reproduction; but he passed the idea on to one of his students, Ludwig Fraenkel.

The experiments by Fraenkel, which showed for the first time that the mammalian ovary had a function other than egg-bearing, were carried out in 1903. He showed by studies in rabbits that removal of the corpora lutea or the ovaries during the first few days of pregnancy invariably prevented a continuation of the pregnancy. At that time, these results evoked so much criticism that Fraenkel repeated them in 1910, but the findings were exactly the same, *i.e.*, the corpora lutea were indispensable for the continuation of pregnancy and his master's hypothesis was fully substantiated.

At this point a little digression into the anatomy of the rabbit will serve to clarify the experiments of Fraenkel and at the same time refresh our memory regarding the details of the mammalian ovary. The ovary contains numerous cystic structures, known as graffian follicles, which are lined with specialized cells and in which the ovum reposes. These follicles gradually increase in size and eventually rupture, discharging the ovum into the abdominal cavity. From there they soon find their way, by procedures as yet unknown, into the fallopian tubes, where fertilization takes place. The fertilized ova then spend 4 days wandering down the tube and into the uterus where they grow and become attached by means of placentae. During this period of transportation the ovary has not been idle; the cells lining the collapsed follicle have increased in size, capillaries have grown in and a new structure, the corpus luteum, has been formed. This process always takes place following ovulation, so that the corpus luteum is invariably associated with the recently extruded ovum and the young embryo. These bodies gradually increase in size and last for two weeks, unless fertilization takes place, in which event they persist throughout gestation, regression starting shortly before parturition takes place. These are the structures which Fraenkel removed and which he found so important in the normal continuation of pregnancy.

This pioneering work of Fraenkel was soon followed in 1907 by the work of Leo Loeb, which showed that during the first few days after ovulation the uterus is in a special physiological state whereby implantation is made possible. He found, using guinea pigs for the experiments, that if the uterus were traumatized on the seventh day after ovulation, a tumor would develop in the endometrium which was identical with the maternal portion of the placenta. This result could not be obtained at other times in the cycle and could never be obtained if the corpora lutea were removed at the time the uterus was injured.

In 1910, Ancel and Bouin began the work which produced a morphological explanation of Fraenkel's results by showing that the rabbit's uterus, while under the influence of the corpus luteum, undergoes very remarkable changes. They found that in the resting state, there were a few small glands, but when the corpus luteum appeared there was associated with its growth and persistence a remarkable change in the uterus. It became enlarged and congested, and its epithelium underwent great mitotic proliferation, resulting in a highly complicated picture. This specialized state was interpreted as being necessary for implantation of the fertilized ova. At other times (1910, 1924) they have shown that excision of the corpora lutea always prevents development of this endometrial proliferation.

We had, therefore, ample evidence that the uterus undergoes very special changes when under the influence of the corpus luteum, but as yet there was no complete explanation of why embryos failed to implant when the corpus luteum was removed, as in Fraenkel's experiments, since nobody had actually investigated the fate of the embryos under these circumstances. This phase of the subject was completed in 1928 by my chief, Dr. Corner. Again using rabbits, he was able to show that, if the corpora lutea were removed 18 hours after mating when the young embryos are already in the tubes, the embryos developed normally for about 4 days, but after that they immediately stopped growing and shrivelled up and disappeared. You will recall that with no corpora lutea present the endometrial proliferation discovered by Ancel and Bouin also failed to take place. It was only natural, therefore, to assume that the embryos died because the uterus had not been properly prepared for their reception, and that presumably one of

the functions of the proliferated endometrium was to nourish the embryos prior to implantation.

I have described these experiments of Fraenkel, Loeb, Ancel and Bouin, and Dr. Corner in some detail because each adds an important link in the chain indicating that the corpus luteum plays no small rôle in the processes of reproduction. To complete the chain of evidence that it was an endocrine gland it would only be necessary to prepare an extract of the corpus luteum which would induce in an animal without corpora lutea all the changes which the corpus luteum itself was known to produce. Specifically, an active corpus luteum extract would be expected to produce endometrial proliferation in the castrated rabbit, maintain pregnancy in a castrated pregnant rabbit and sensitize the uterus of a castrated guinea pig so that deciduomata would be produced following mechanical trauma, as in the experiments of Leo Loeb.

With these objects in view in 1927 Dr. Corner and I began extracting pigs' corpora lutea hoping to assay the extracts by their capacity to produce endometrial proliferation in the immature rabbit. (We chose the immature rabbit because young animals never have corpora lutea in their ovaries, and hence no difficulty would be experienced with the rabbit's own corpus luteum.) The method of preparation was deliberately planned after a procedure used by Hermann in 1915, since apparently he obtained active extracts in some cases.

At this point I shall have to digress once more to explain that a large number of people, in the interim following the work of Fraenkel, Loeb and Ancel and Bouin, had prepared extracts of placenta, ovaries and follicle fluid and many had obtained preparations which would bring the uterus of an immature or castrated animal into full sexual maturity after only a few days' injection. This work is fully as fascinating and important as that dealing with the corpus luteum, but I shall have to leave it by saying that the work of Edgar Allen and Doisy led to the isolation in pure form (1930) of a hormone from the urine of pregnant women which had all the properties of the earlier extracts, in so far as their ability to cause growth of the uterus and vagina were concerned. However, study of these oestrogenic extracts, called that because they induced characteristically those changes found during oestrus or heat, by Asdell and Marshall (1927) and Corner and Allen (1929) in the rabbit, and Loeb and Kountz (1928) and Ebhardt (1928) in the guinea pig, showed that they do not produce the changes in the uterus which are characteristic of the corpus luteum phase of the cycle. It was apparent, therefore, that the extracts of Hermann (1915), which according to his own illustrations must have contained an active corpus luteum hormone in some cases, had something in them quite different from the substance isolated by Doisy. We were led, therefore, to attempt to repeat Hermann's work, but this proved to be quite impossible because he was not aware that in most cases his extracts contained the oestrogenic hormone rather than a special corpus luteum hormone.

By using an ordinary alcoholic extract we were able after only about two or three months' work to prepare a crude extract which occasionally produced endometrial proliferation in young rabbits, but the results were so irregular, even though the method of preparation was kept constant, that in desperation we assumed that the fault lay with the test animal and not the extract. We then changed to an adult, recently castrated rabbit and when such an animal was injected with adequate doses of the extract perfect proliferation was obtained in every case, and if the animals were mated 18 hours before castration, and then injected daily for 5 days, normal embryos, as well as good proliferation, were obtained. In short, the evidence that the corpus luteum was an endocrine gland whose chief function was the preparation of the uterus for the implantation of the embryos was complete. At the same time Tatelbaum and Goldstein studied the effects of these extracts on the guinea pig's uterus following the experimental method of Leo Loeb. They found that it also sensitized the endometrium so that deciduomata could be produced following artificial trauma. Dr. Corner and I were also able to carry rabbits whose ovaries had been removed 18 hours after mating through to full term by the daily injection of the extract. The active principle was able therefore to substitute for the corpus luteum in every respect as far as its function was known at that time.

With this convincing evidence that the extracts really contained a hormone or active principle, we set about the task of trying to isolate the compound in pure form, assaying it by its ability to induce progestational proliferation. We then developed a standard biological unit, named the hormone progestin, *i.e.*, a substance necessary for gestation, and began to fractionate the crude extract into different fractions. You will recall that we used alcoholic extracts. This means that we were dealing with the lipid fraction. The purification consisted essentially in separating all the known lipids without the use of alkali, because saponification caused complete inactivation. In this respect the hormone was quite different from the oestrogenic hormone isolated by Doisy because that could be treated vigorously with moderately strong alkali without any deleterious effect. The alcohol is boiled off and the residue extracted with ethyl ether, and an excess of acetone is added to the ether. This precipitates the phospholipids and causes marked puri-

fication. When the acetone ether is boiled down to dryness a thick syrupy oil is obtained which contains about 1 rabbit unit per half gram of solids, the solids being cholesterol, neutral fat and fatty acids. This oil is next dissolved in a fairly large volume of 70 per cent. methyl alcohol and chilled at $-5°$ C. for several hours, practically all the cholesterol and neutral fat being removed. The 70 per cent. alcohol is then distilled off and the aqueous remainder extracted with ethyl ether or petroleum ether, the hormone being obtained in a relatively pure condition, 1 rabbit unit equalling from 20 to 40 mg.

At this point it should be stated that these extracts really contained two hormones rather than one. We were assaying only the progestin, but at the same time we were extracting oestrin as well as progestin, since occasional tests by the Allen and Doisy method for oestrin showed that considerable amounts of this substance were invariably present. This meant that no physiological results obtained with these extracts could be said to be due to progestin alone, since both hormones were present. We, therefore, desisted from purification and developed a method for separating the oestrin from the progestin. The key to this separation was to be found in some data obtained on the distribution of progestin between 70 per cent. EtOH and petroleum ether (Allen and Meyer, 1933). We found that about 75 per cent. of the progestin remained in the alcohol and 25 per cent. was lost along with the majority of solids to the petroleum ether. Now, Doisy already had shown that the oestrogenic compound was retained in the alcohol even better. In fact, when the relative solubilities were computed, using the formula ordinarily used for the distribution of a substance between two immiscible solvents, we found that progestin was four times as soluble in 70 per cent. alcohol as in petroleum ether, and when Doisy's figures were subjected to similar mathematical analysis oestrin was found to be about twenty-five times as soluble in 70 per cent. alcohol as petroleum ether. This difference in solubility made a separation theoretically possible, but rather tedious because of the sparing solubility of progestin in petroleum ether, as compared to alcohol. However, a few additional facts such as the solubility of oestrin in dilute alcohol and its insolubility in petroleum ether led us to try separation between 35 per cent. alcohol and petroleum ether. When these maneuvers were carried out taking care to work over the petroleum ether twice, an excellent separation of oestrin and progestin was obtained. In fact, 95 per cent. of the oestrin was removed with no loss of progestin whatsoever.

Having thus removed the oestrin we returned to purification of the progestin fraction. This was accomplished by further distributions between dilute alcohol and petroleum ether and by freezing from absolute ether-petroleum ether at $-70°$ C. When this is done a copious yield of crystals is obtained, but they were no more potent than the mother liquors. Consequently we distilled the non-crystallizable oils in high vacuum in a specially designed apparatus which I'm sure Hill and Carruthers would recognize as a modification of their subliming apparatus. This has several advantages. The upper half, including the condenser, can be lifted out and the oil introduced into the well in ether solution. The ether can be evaporated off and the last traces pumped off, using a large rubber stopper to plug the upper end. After all solvent has been removed and all danger of splashing has passed, the upper half is replaced and the chamber evacuated. When the vacuum is about 0.0005 mm and temperature about 100°–120° a thick yellow wax sublimes. When this is dissolved in ethyl-ether-petroleum ether a considerable amount of crystals is obtained, and these are much more potent than the first lot. However, they are usually worked up together for convenience.

The crude crystals were quite potent, 2.5 mg usually being a rabbit unit. These were then subjected to fractional crystallization from ethyl ether-petroleum ether mixtures. Two different types of crystals were obtained. The less soluble ones were obviously impure and melted with decomposition at about 160°. From the mother liquors were obtained long prismatic needles which melted at 116° without decomposition and which were about three times as potent as the higher melting ones. This was quite encouraging, so we worked up another large batch of tissue, only to find that no needles were found, but that short thick prisms melting at 125° this time were very potent, and curiously enough these seemed to have the same potency as the needles, *i.e.*, about 1 mg to the rabbit unit. At this point we entered into a partnership with Dr. Wintersteiner, of Columbia University, whose expertness in micro analysis we hoped would help solve these apparent difficulties. When he fractionally crystallized a lot of 400 mgs of crude crystals he obtained four different compounds, which for the sake of convenience we called A, B, C and D. A melted at 190°. These were found to be quite inactive. B melted at 128° when pure, the prisms mentioned above, and were very potent. C melted at 120.5–121° when pure, the long needles I originally obtained and they were also very potent. D melted at 65–74° and was inactive.

These same compounds were then micro-analyzed and their molecular weight determined. The inactive compound was found to have the empirical formula $C_{21}H_{34}O_2$ and the two active compounds $C_{21}H_{30}O_2$ within limits of error of the methods. The two active

compounds gave identical disemicarbazenes and dioximes, and they had identical absorption curves with a maximum at 240 mμ. The compounds appeared, therefore, to be polymorphous forms of one and the same diketone, presumably containing at least one double bond. The inactive compound A was found to be a hydroxy ketone since it gave a mono-semicarbazone and a ρ nitro benzoate.

These results, therefore, were of considerable significance when we recall that pregnandiol $C_{21}H_{36}O_2$, a dialcohol, is found in the urine of pregnant women. What would be more logical than a series of compounds; progestin $-C_{21}H_{30}O_2$, a diketone with one double bond; pregnandione $-C_{21}H_{32}O_2$, a diketone, already known with no double bond; our oxyketone, $C_{21}H_{34}O_2$, and pregnandiol, $C_{21}H_{36}O_2$, all reduced products of the hormone. If so, our oxyketone should give pregnandione a known compound melting at 123°, but on oxidation it gave a diketone melting at 193°.

Once more I should like to digress and mention that at about the same time as our work on the pure substance appeared two German chemists, working independently, Dr. Butenandt and Dr. Slotta, reported the same chemical compounds. Their analyses and identification of the oxygens agreed perfectly with ours. Slotta's physiological studies of the potency of these two compounds were at variance with ours, rather seriously so. He found that full endometrial proliferation could not be obtained without the proper combination of the two different types of crystals, either form being inactive alone. Butenandt had no published data on the matter at that time. Recently, however, he has confirmed our results by showing that both crystals have the same potency. This adds very convincing evidence to the chemical evidence that the two crystalline forms are merely polymorphous modifications of one and the same substance.

I should like to show a few photographs of the two forms to show how different the same compound may appear. In general the needles are obtained when crystalizing from fairly dilute alcohol and prism when crystalizing from stronger alcohol. The melting points of these substances are also abnormal. The high melting variety on remelting will frequently melt at 120° and the low melting form will melt several degrees higher the second time. These findings do not occur regularly, however.

This about concludes the story as far as we are concerned, but I can not close without describing briefly the beautiful conclusion which Dr. Butenandt has brought to the subject by developing in the space of only a few months the structure of progestin and a method of synthesis. I mentioned above the close similarity between the empirical formula of progestin and pregnandiol. Butenandt had already worked out the detailed structure of pregnandiol. This substance was then converted by oxidation to a diketone, pregnandione, then brominated and HBr removed, the result—progestin. And curiously enough the active substance also occurred in two crystalline forms which were identical in every respect with that isolated from pigs' ovaries. An even more beautiful synthesis was carried out using stigmasterol, a wax obtained from soybeans, as a starting point. This was changed to 3-oxy bis-nor cholenic acid by the method of Fernholz and then converted to an unsaturated oxy-ketone, which we may call pregnenolon. The double bond was protected by bromination and the resulting dibrom compound oxidized to a diketone. Again when the bromine was split out progestin was obtained. These findings leave little doubt that the compound isolated from the natural source is really the hormone and further that the same active substance has been synthesized from inactive compounds of known structure. The formula must be correct, unless those for pregnandiol and stigmasterol are incorrect, something which is rather unlikely.

Surely no person a few years ago would have predicted that the hormone progestin would ever be made from such a non-human source as soy-beans.

One final question, What will the hormone be used for? Only time can tell. If it is found never to have any therapeutic value the result will have been worth the chase, for it has helped immeasurably to clarify some phases of the reproductive processes and at the same time contributed something to the chemistry of the human body.

In conclusion, I wish to take this opportunity to express my appreciation to Dr. Corner, who provided the stimulus for my early interest in the subject, and who, by his patience, has encouraged me to continue with it; and to Dr. Wintersteiner who, because of his remarkable technical skill and chemical ingenuity, helped to bring order out of chaos. I also wish to thank most gratefully the Eli Lilly and Company for their part in making the award possible and the committee which saw fit to make me the first recipient of the award.

OBITUARY

JOHN WEINZIRL

JOHN WEINZIRL, professor of bacteriology and director of the McDermott Foundation at the University of Washington, died, after a week's illness, on June 26, 1935. He was a native of Wisconsin, having been born on September 10, 1870. He was educated