Endocrinology. — ERNST LAQUEUR, P. C. HART, S. E. DE JONGH and I. A. WIJSENBEEK: "*On the preparation of the hormone of the estrous cycle, and its chemical and pharmacological properties.*" (Communicated by Prof. R. MAGNUS.)

(Communicated at the meeting of December 19, 1925).

A few months ago we announced in a brief communication [1]) that we were able to confirm the important findings of ALLEN, DOISY, RALLS and JOHNSTON [2]) on a hormone of the estrous cycle, and that, moreover, we had succeeded in preparing a protein-free, water-soluble form of a substance identical with or at least very much like the American product in action. This enabled us to investigate its chemical and pharmacological properties, and, moreover, its clinical and therapeutic action.

ALLEN and DOISY c.s. in their paper repeatedly mention the fact that their ovarian extract was *insoluble in water*. and could only be injected into mice when dissolved in oil. The same investigators, in collaboration also with PRATT, afterwards published many important data regarding the presence of the hormone in human organs, but did not mention any further technical progress.

ZONDEK and ASCHHEIM [3]) declare to have prepared a water-soluble product. LOEWE [4]) too announced the preparation of a substance able to produce the estrous phenomena, and which could also be demonstrated to be present in the blood of female rabbits. Quite recently STEINACH, HEINLEIN and WIESNER [5]) published a paper in which they say to have prepared extracts from ovaries and placentae which can produce the development of the secondary sexual characteristics, can reactivate the organism of female rats showing signs of long-standing senility, and finally can produce the estrous cycle in castrated mice.

ZONDEK c.s. nor LOEWE nor STEINACH c.s. do not mention anything about the method of preparation of their extracts.

In a recent paper by DICKENS, DODDS and WRIGHT [6]) the authors say that their extract is only soluble in alcohol, ether, acetone and olive oil. To complete our review of the present state of the problem, we may add that ALLEN and DOISY mention a dry residue of 2 milligrams per rat unit

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1) Deutsche Med. Wochenschr. N°. 41, 1925.

2) Am. Jl. Anat. **34**, 133, 1924; Jl. biol. Chem. **61**, 711, 1924; Am. Jl. Physiol. **69**, 577, 1924; Proc. Soc. Exp. Biol. a. Med. **21**, 500, 1924; ibid. **22**, 303, 1925.

3) Klin. Wochenschr. N°. 29, 1388, 1925.

4) „ „ „ „, 1407, 1925; Zentralbl. f. Gynäkol. N°. 31, 1735, 1925.

5) PFLÜGER's Arch. **210**, 4/5, 588, 1925.

6) Bioch. Jl. **19**, N°. 5, 853, 1925.

with their usual mode of preparation, which may be lowered to 0.13 mg. by a certain process of purification, that DICKENS and DODDS describe the substance as a brown oil, of which in the purest preparation 25 mg. correspond to one rat-unit, whereas STEINACH's mouse-unit weighs 9—13.5 mg. or more. This may be considered pretty well to be the present condition of things.

Therefore we think it progress that we are now able to prepare a substance which brings about very extensive cyclic changes, in a much simpler manner than the rather complicated method of the American and English investigators : moreover, our product has a dry residue of considerably less than 0.1 mg., even less than 0.01 mg. per mouse unit ; it is quite free from proteins and is water-soluble. Up to this moment there is no publication containing any proof that the active principle, dissolved in water, is in true solution.

Definition and standardization.

To prevent the repeated use of the term "relatively pure hormone of the estrous cycle" we propose the name "Menformon" for the substance in question, but with the restriction that we understand by it only a substance *which contains at least 10 mouse-units per 1 mg.*

As a mouse-unit (thus included in the definition of "menformon") we define the smallest quantity which is able to produce undeniable cyclic changes of the vaginal epithelium (at least surpassing, at their maximum, the stage described by ALLEN c.s. as "pro-estrus") within 72 hours after the last injection in at least 2 of every three castrated mice, on simultaneous injection.

The injection should be performed with intervals of 4 hours, giving $^1/_3$ of the total dose at a time ; the animals must be castrated at least 25 days before, and they must have been submitted thereafter to a daily control to show that spontaneous cyclic changes are completely absent. In our laboratory the histologic preparations are controlled by two persons independently of each other and without knowledge of what has happened with the animals.

In a few words we shall explain what we mean by "complete absence of cyclic changes". The anatomical investigations by STOCKARD and PAPANICOLAOU [1]) furnished a means by which further progress in this field was made possible.

It is well known that the vaginal epithelium in the period of rest of the mucosa consists of 2—4 layers of cells, through which large numbers of leucocytes find their way to the lumen of the vagina. During the estrus the epithelial cells multiply until there are to be seen 14—18 layers of them. The upper layers are cast off as cells and plates without nuclei ; the passage of leucocytes is prevented. *This absence of leucocytes* is the most

[1]) Am. Jl. Anat. **22**, 225, 1917.

important criterion ; yet a mere decrease of leucocytes and the massal appearance of epithelial cells which ordinarily are only to be found here and there, constitute an unmistakable change as compared with the negative finding in castrated animals not subjected to the treatment. We consider the action of a substance as *positive* (+) when in the microscopic preparation nearly all leucocytes have disappeared and the epithelial cells predominate, of which last category there must be about as many cells with a nucleus as without one. Frequent occurrence of epithelia *without* nuclei warrants that the pro-estrus has been *passed,* as required in our definition of the mouse-unit. Maximally positive (++) we call the stage in which epithelial cells with nuclei have completely disappeared, and in which moreover most of those without nuclei are cornified.

Up to this moment our experiments have shown that a "maximally positive" (+ +) reaction (on distributing one mouse-unit over one day) is more often obtained with the original follicular fluid than with the substances prepared from it. These often only give the (+ +) reaction on repeating the injection the second or third day, even with less than one mouse-unit. This is not merely a question of dosage, for giving 2 or 3 mouse-units in one day needs not produce the same effect. Probably quicker absorption plays a part combined with quicker excretion of the purer preparations, so that only by repeated injections a sufficient concentration during a certain time may be obtained. Thus to obtain a certain effect two factors are concerned : concentration and time.

Important though this problem be, it does not in the least influence the fact that on injecting thrice in the course of one day an unmistakable effect may be obtained, which instead of the continually negative findings in untreated animals doubtlessly shows a change in the sense of the typical estrous cycle, possibly only in a more rapid succession of its stages.

Our diagnoses made in this manner in smears have been confirmed by histologic control of some mice killed for the purpose : when the smear had been labelled (+) their vaginal epithelium showed 10—14 layers.

We require that the animals be castrated at least 25 days before, and that for every experiment at least 3 animals be used, because only the suppression of several cycli seems to make complete castration largely probable ; further, because chance can only be excluded when more than one animal, and even at least three, are being used. On using only two animals, different results can compensate each other.

Preparation.

Our first preparations were made from follicular fluid, which, if unchanged, contains in our experience between 600 and 1200 mouse-units per kilogram. ALLEN and DOISY with their method found 2000 rat-units maximally, DICKENS and DODDS c.s. with their extraction-method only 200 rat-units, an amount corresponding to that obtained from ovaria

without follicles. The English investigators therefore decline the particular part ascribed by the Americans to the follicular fluid: we ourselves are inclining toward the American point of view, though we have been able to prepare menformon from whole ovaries and placentae too. Often the follicular fluid, when not quite fresh and sterile, is toxic, so that the animals sometimes die within 1—3 days after the injection. The follicular fluid we sucked from the follicles with a syringe, as far as possible under aseptic precautions.

After observing repeatedly the activity of the unchanged follicular fluid, we tried the purification process of ALLEN and DOISY a few times. This too yielded positive results, though we did not reach the low dry residue as recorded by ALLEN and DOISY in a few cases. Yet we did not spend too much time upon it, because the method is extremely complicated.

We have the impression that, for the first and decisive phase especially, the authors have been strongly influenced by the principles of insulin preparation, and, secondly, by the idea that the active principle would be insoluble in water. This we thought without proof, for though most previous investigators, e.g. FRAENKEL and HERMANN [1]) maintained the exclusive lipoid-solubility of the ovarial hormones, others, e.g. L. ADLER already in 1912, mentioned the activity of aqueous extracts. Possibly the insolubility in water was only caused by the fact that in the various methods the active principle is extracted together with water-insoluble substances, which prevent the active hormone from dissolving in water.

Thus we aimed at obtaining a water-soluble product.

We thought it one of the most important problems how to free it from proteins. For this purpose, we tried several well-known methods.

After boiling with dilute acid the remaining dry residue was very high, but this experiment confirmed the *thermostability* of the hormone as shown by ALLEN and DOISY. Precipitating with trichloro-acetic acid gave much less dry residue, the solution was perfectly clear, but showed to be very toxic, even after neutralizing. In control experiments with solutions of the sodium salt of trichloro-acetic acid however (of a concentration corresponding to that used in precipitating the proteins) the animals died in much the same manner (perhaps a chlorine intoxication?).

Removing the proteins by the FOLIN-WU-method (as usual in determining the blood-sugar content) proved unsatisfactory.

Better were the results on using colloidal ferric hydroxide.

To one part of follicular liquid 4 parts of physiological saline and about 1½ parts of a 3 % solution of colloidal ferric hydroxide were added, the mixture was then centrifugalized. This yields a turbid yellow fluid which apart from some colloidal $Fe(OH)_3$ contains the active principle. On evaporation at low temperatures (35° C.) the yellow turbidity precipitates as minute particles, and there results an almost water-clear liquid, faintly

[1]) (German) Patentschrift N⁰. 309482. Klasse 12.0. Gruppe 26, Ausgegeben 23. 11. 1918.

opalescent and with a dry residue of about 3.3 % : 0.5, 0.75 and 1 ccm. of this liquid, injected into castrated mice, constantly gives positive results (0.5 cc. of this liquid corresponded to 0.3 cc. of the original follicular liquid).

The application of this method to larger quantities of follicular fluid, followed by removal of $Fe(OH)_3$ by means of evaporation and H_2S showed its usefulness. Such a solution may for instance show a dry residue of 3.35 %, an ash content of 1.06 % and a nitrogen content of 0.47 %.

From such a larger batch we injected, among others, 9 mice, every animal three times ; three mice got 0.5 cc., three others 0.75 cc. and the remaining three 1.0 cc. All except one showed a positive reaction. Repeating the experiment with 0.5 cc. gave strongly positive reactions. Possibly the limit value was even less. But — when prepared by this method one mouse-unit contained about 50 mgr. of dry residue, which compares very unfavourably with the results of the American method and even with those of the English mode of preparation. The only advantage of our method consists in its simplicity.

In view of this unsatisfactory dry residue we further simplified our technic, combining the precipitation of the proteins with the extraction, and then trying to make the hormone pass over into water again. The leading principle must be : to extract the menformon as completely as possible with the least impurities (lipoids included) possible.

To give an example : 10 cc. of follicular fluid and 10 cc. of chloroform are shaken together. The proteins coagulate in part. Then the liquids are separated, the chloroform is evaporated and the residue is taken up in about 5 cc. of destilled water. A perfectly clear watery solution results, having an unweighable content of solid matter. Its activity is rarely more often less than half that of the original follicular liquid, that is to say, if, c.g. 0.6 cc. of follicular liquid contained one mouse-unit, 0.6 cc. of the aqueous solution again contained one mouse-unit.

Though we have already a rather large number of experiments at our disposal we are not yet prepared to say anything definite about the number of mouse-units which may be obtained in this way, nor do we predict anything about the possibility of obtaining by further purification a larger amount of units per cc. than are present in the original follicular liquid, for instance by removing substances with an opposite action (anti-menformon). To settle these questions the number of experiments must be still much greater than it is at present, and, moreover, the limit of accuracy of the method of standardization must be much better known.

Besides chloroform we used carbon sulphide, carbon tetrachloride, benzene, petroleum-ether, tetralin, ligroin, ether, acetone and ethyl acetate. In principle all these yielded identical results, but our experiments are still too few in number. Often the residue from the volatile solvent at first formed an emulsion with the water : in most cases however a simple

filtration made the fluid clear ; often the solution was water-clear from the first, and then its content of solids was minimal. Probably this depends, besides on the nature of the solvent, (which must dissolve menformon with as little water-soluble impurities as possible) on the freshness of the follicular liquid, and further on the completeness of the precipitation of the proteins, which then take the other colloidal constituents with them. Finally the completeness with which the volatile solvent is removed plays a part, eventually also the temperature at which this has been done. (We worked at atmospheric pression, in vacuo and in an air current at about 35°).

All these factors, and many more, are still to be investigated much more completely.

Besides shaking out the follicular fluid directly and eventually coagulating the proteins at the same time, we first dried the follicular liquid itself or solutions prepared from it by the $Fe(OH)_3$-method mentioned above, then we extracted the dry residue with chloroform, which in its turn was evaporated, after which the residue was dissolved in water. This solution too yielded identical results, with minimal amounts of solids. This leaves no doubt that *menformon is water-soluble.*

Our method may be briefly called the "water-method". When starting from larger quantities, e.g. 1 Litre, of follicular liquid, we found it useful to prevent the forming of emulsions when shaking out with the volatile solvent : this may be done by completing the coagulation of the proteins by adding salt or acids, by centrifugalizing etc.

Solubility, dialysis.

Whether the clear solution obtained by extracting the residue from the volatile solvents with water is a true solution or only a colloidal suspension, we tried to decide by means of dialysis. In previous experiments on purification of our preparations made by means of colloidal ferric hydroxide we found that the activity is lost by dialysis against running water through parchment or collodion membranes. On dialysis of preparations made by the "watermethod" and containing in 10—20 ccm. about 40—80 mouse-units we found in the exarysate (15 and 25 cc. respectively) a rather considerable amount of mouse-units, but less than (about one-fifth of) the calculated amount. But the dialysate too had become weaker than should be the case if the menformon had spread evenly over both exarysate and dialysate. The membrane must thus have retained part of the menformon. This hypothesis was confirmed by the result of efforts to extract it from the membranes again.

Chemical Properties.

The difficulty with which at the present moment larger amounts of the purest "menformon" are available makes it impossible to say much

about its composition. Results with impure preparations are only valuable if they are negative. If, for instance, the impure preparation does not contain phosphorus, the pure product will certainly have none, etc.

Dry residue.

Because, on evaporating unto dryness in the usual manner at 100—110°, amounts of, say 2 cc. of an aqueous solution, containing 20 or more mouse-units, often leave only unweighable quantities of solids, we thought of the possibility of sublimation, and for that reason we dried at temperatures not over 50°. But with these precautions too the residue of 10 cc. (= 160 mouse-units) was not more, und no sublimate was demonstrable with certainty on a watchglass kept above it.

On evaporating 5 ccm. (= 18 mouse-units) in an exsiccator at 37° and at 15°, there was left less than 0.1 mg., probably even less than 0.01 mg. of residue (roughly estimated on comparison with known amounts of other substances). The unweighable residue from other preparations, present, for instance, as rings on a watchglass, did not show any change on further drying at 98°.

In those cases where hardly any residue was obtained (e.g. from 10 cc., containing at least 33 mouse-units) dissolving it again in 10 cc. of water yielded an active (though perhaps somewhat weakened) preparation. From a chemical point of view we thought this preparation somewhat suspect, but it should be borne in mind that also 0.01 mg. of adrenaline and even less are active in adult men.

Slightly impurer preparations (0.06 mg. per mouse-unit), obtained in an exsiccator at 37° (2.2 mg. from 10 cc.) showed a few crystals. Up to this moment we have been unable to identify these. In view of our experience that much purer preparations exist, we think it improbable that *these* crystals have anything to do with menformon itself.

Reactions on proteins, NH_2- or OH-groups; N-, P-, S- and cholesterol-content [1]).

Relatively impure preparations with 0.04 % of dry residue (7—10 M.U. per ccm.) did not show the least *protein*-reaction (Heller's ring-, sulfosalicylic-, ninhydrin-, biuret-reaction). 2 cc. of a similar preparation yielded no *nitrogen* on determination with the micro-Kjeldahl-method; diazobenzene sulfonic acid gave a negative reaction on phenol- or aromatic NH_2-groups.

An impure specimen with 3—4 M.U. per cc. and a dry residue of 0.076 % (i.e. about 0.22 mg. per M.U.) yielded 6.6 mg. from 30 cc. on drying at 50°. One-third of this quantity was analysed and showed a nitrogen-content of 2.4 %.

[1]) Experiments by Miss E. DINGEMANSE, Ch. D.

It was impossible to demonstrate the presence of *phosphorus* in 2.2 mg., whereas 3.5 mg. of casein with about 0.027 mg. of phosphorus yields a positive reaction.

The nitrogen-content could point to a lecithin-like constitution, but the absence of phosphorus excludes this. Probably the nitrogen too is due to the presence of an impurity.

About the *sulphur*-content we cannot yet say anything : the impure product does not contain demonstrable amounts, but in the same quantity of protein it is quite as impossible to show its presence.

Cholesterol : 2.2 mg. of the impure product, dissolved in chloroform, give a negative cholesterol reaction on addition of acetic anhydride and sulfuric acid, whereas 0.1 mg. of cholesterol gives an unmistakable red or violet colouration. SALKOWSKI's reaction yielded the same negative result. The cholesterol content of the impure substance, if at all present, is thus certainly below 5 %. Addition of 0.3 % of *trikresol,* or of a 0.9 % solution of *sodium chloride* do not alter the aspect of a solution of menformon, nor its potency.

Keeping qualities : After being kept for 3 weeks in an incubator at 37° the solution did not show any change of potency.

We are of opinion, that in menformon we found a new substance, of which we hope to be able to publish something more positive in the near future. This makes it necessary for us to have larger quantities at our disposal which is a matter of considerable trouble considering the difficulty in obtaining the original material.

Physiological action and pharmacological assay.

Repeated injections into *mice* of one or more mouse-units (up to this moment we gave about 4—8 M.U. every day) seem to have no action, apart from the specific effect.

In *rabbits* subcutaneous or intravenous injection of 8—80 M.U. (= 1 to 10 cc), at one time or within 10 minutes, is supported without any ill effects, even when repeated within a few days.

In *men* injection of 1 cc. (= 3—45 M.U.) even when repeated, does not produce any general effects, as experiments on ourselves, as well as those taken by clinical men among our friends on female persons have shown. For this purpose we used solutions to which 0.3 % trikresol and 0.9 % NaCl had been added, and of which the sterility had been proved.

Blood-pressure and Heart.

Impure preparations (such as contained more solids than is tolerated by our definition of menformon), when injected intravenously into rabbits in urethane anesthesia, produced a steep lowering of their blood-pressure : on repeating the injection they died. The curves recently published by DICKENS and DODDS c.s. showing repeated lowering of blood-pressure

after intravenous injection are obtained with preparations of considerable impurity as compared with menformon. Only somewhat purer preparations, containing about 5 M.U. per mg. and therefore still too impure as to be comparable with our definition of menformon were *completely devoid of any action* upon the blood-pressure of rabbits or of cats decerebrated with novocain, even after injecting three times 1 cc., i.e. all together 6.6 mg. of solids and about 15 M.U.

Three and more M.U. did not show any definite effect on the isolated frog heart, beating at the Straub cannula.

Blood vessels.

On perfusing a LAEWEN—TRENDELENBURG—KOCHMANN preparation (frog or guinea-pig) we were unable to find definite changes of the width of the blood vessels.

Respiration.

An impure preparation showed some effect upon the respiration of rabbits in urethane anesthesia, along with a lowering of their blood-pressure. Pure preparations, however, do not produce the least alteration of the respiration.

Uterus.

Doses of about 17 M.U. often produce an unmistakable *contraction* in the *isolated uterus* of virgin guinea pigs. The amount of solids in this dose was far below 0.1 mg.; smaller doses did not act with certainty.

The *uterus in situ*, as studied by means of the abdominal window (through which several foeti could be clearly seen), did not show any change on intravenous injection of about 45 M.U. Repetition of the experiment 3 days afterwards remained negative too. The young were born the following day, i.e. about 2—3 days before the normal end of pregnancy. Of course this does not necessarily lead to the conclusion that menformon was the cause, for a similar early birth often occurs without any injections.

Blood sugar.

Injection of 15 M.U. was *without any influence* upon the blood sugar content of rabbits.

Growth.

In spring we performed some experiments with unchanged follicular liquid upon tadpoles. An addition of 10 % of the liquid to the growing medium killed the animals whereas a content of 2 % was without any

influence. On the contrary an amount of only 8 M.U. in total is capable of causing within 10 days an enlargement of more than 200 % of the generative organs in young female rats scarcely capable of holding their own in absence of their mother, as compared with the control animals.

Quite similar results were obtained with young guinea pigs. The control animals were injected with a liver extract prepared in quite the same way and in doses quite equal as regards the quantity of raw material (follicular liquid and liver) to which they corresponded. The animals got 7 injections of 0.2 cc. each within a week. The control animal got alltogether 0.06 mg. of solids, the others about 23 M.U. with unweighable residue. In this case too the generative organs of the menformon-animals had a weight which by more than 100 % surpassed that of the controls. This holds good for the animals killed immediately after the last injection, as well as for those which were killed a few days afterwards. This last fact, we think, pleads more in favour of a stimulation of the growth of the generative organs than for the induction of temporary estrous changes.

Summary.

A simple method is described (the "water-method") to prepare a substance (from follicular fluid especially) which is able to induce in castrated mice unmistakable changes of the vagina and uterus that are probably identical with those of the spontaneous estrous cycle. This substance is defined as one in which 1 mgr. of solids represent at least 10 of the so called mouse-units = M.U. These mouse-units are defined, and the conditions for standardization are given.

Provisionally chemical analysis shows the substance to be protein-free, probably also free from nitrogen, from cholesterol, from phosphorus and possibly also from sulphur. Besides in volatile solvents it is readily soluble in water. The name *menformon* is proposed.

Pharmacologically menformon in doses up to 45 M.U. showed perfectly non-toxic in men and animals; intravenous injections up to 80 M.U. did not affect blood pressure nor respiration in any way. Doses of 17 M.U. often caused contractions in the isolated virgin uterus. Up to this moment our observations on the uterus in situ (rabbits with abdominal window) did not show any influence. The effect upon the growth of the generative organs in young, normal female rats and guinea-pigs is evident.

The wide-spread belief that the substances which cause changes similar to the spontaneous estrous cycle are not in true solution in water and the observation that they act upon the blood-pressure both probably took their origin in a failure to free them from impurities, from lipoids especially.

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