

1959

Rapid disappearance of Progesterone from a subcutaneous site in mice

Sidney Mace Cohen
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RAPID DISAPPEARANCE OF PROGESTERONE FROM
A SUBCUTANEOUS SITE IN MICE

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Sidney M. Cohen

1959

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RAPID DISAPPEARANCE OF PROGESTERONE
FROM A SUBCUTANEOUS SITE IN MICE

SIDNEY M. COHEN
B.S. FRANKLIN AND MARSHALL COLLEGE
1956

A THESIS PRESENTED TO THE FACULTY
OF THE YALE UNIVERSITY SCHOOL OF MEDICINE IN CANDIDACY
FOR THE DEGREE OF DOCTOR OF MEDICINE

THE DEPARTMENT OF ANATOMY
YALE UNIVERSITY SCHOOL OF MEDICINE
1959



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TO MY WIFE

ACKNOWLEDGEMENT

I wish to thank Dr. Thomas Forbes for his encouragement, stimulation and guidance during the years which I spent in his laboratory. To Ann Burrow, who patiently taught me many of the necessary techniques, my sincere thanks.

This project was supported by grants from the Josiah H. Macy, Jr. Foundation.

TABLE OF CONTENTS

	Page
Introduction	1
Materials and Procedures	3
Experimental Results	6
Discussion	8
Summary	18
Bibliography	19
Graphs and tables	21

INTRODUCTION

Since the place of progesterone as a therapeutic agent has been firmly established, it is not difficult to understand the interest which has been shown in the fate of exogenous hormone. The problems of absorption, transportation, action, metabolism and excretion have been approached from many directions.

Absorption of a hormone, being the first step in the chain of events which hopefully will lead to a desired effect, is of primary importance. Many of the studies which have been carried out to clarify the fate of exogenous hormone have circumvented the absorption phase by utilizing the intravenous route. However, since the intramuscular and subcutaneous depots have been widely used in laboratory experiments and medical practice, knowledge of the rate of absorption from these depots is of obvious interest. An example of the current thought about this problem is contained in a paper by M. E. Davis and E. J. Plotz on the distribution of radioactivity in human maternal and fetal tissues following the administration of C¹⁴-4-progesterone: "There is no fundamental difference in the excretory pattern of pregnenediol in the urine after intramuscular or intravenous injections of progesterone, indicating that absorption from an oily I. M. depot must be a fast and efficient process."¹

The foregoing statement concerning absorption, although reasonable, lacks direct proof and the present study was designed to determine the rate of disappearance of progesterone from an oily depot in mice. It was reasoned that the rate of disappearance of hormone

could be determined if the material was administered in such a way that the oily solution could be recovered at varying intervals and its activity could be determined by bio-assay. At the same time, the plasma level of "free" progesterin was determined in an attempt to elucidate further the mechanism of disappearance.

Earlier, Forbes² had utilized a similar technique using intraperitoneal injection to investigate the rate of disappearance of activity from oil in the peritoneal cavity.

MATERIALS AND PROCEDURES

The progesterone was administered as a sesame oil solution. The latter was prepared by dissolving weighed amounts of progesterone in ether, and adding to this solution measured amounts of sesame oil. The ether was then allowed to evaporate, with the last small amount being removed by distillation in a vacuum. The resulting preparation was a progesterone solution with a concentration of 1000 micrograms/c.c. sesame oil.

One hundred and sixty-eight mice of the highly inbred CHI strain were used in this study. An inbred strain was employed in the effort to minimize biological variation. Twenty-eight mice ("test animals") received subcutaneous injections of the progesterone solution, while the other 140 mice were used for the bioassay ("assay animals"). Both groups were composed of sexually mature females not younger than 75 days nor older than 90 days. They were selected at random from the colony, and all appeared to be healthy.

The test animals were ovariectomized through a single dorsal midline skin incision and bilateral lumbar muscle incisions. The ovaries were removed with an electrocautery. Before the test material was administered, any animals which appeared not to have recovered completely from the operation were discarded. Although the muscle layer was not sutured at the time of operation, autopsy of these animals after the administration of the test material revealed that the muscle incisions had healed and that there was no communication between the subcutaneous depot and the peritoneal cavity.

After a rest period of 8 days following ovariectomy, each test animal was lightly anesthetized with ether and was injected with 1500 µg of progesterone (1.5 c.c. of the solution) in the subcutaneous tissue of the back. The injection was made through a fine (#27) hypodermic needle to prevent leakage. Any mouse which showed signs of having lost any of the test material through the injection site was discarded.

At varying intervals of from 2 3/4 minutes to 8½ hours the mice were exanguinated by dividing the carotid sheath under ether anesthesia, the moment of the division being accepted as the end point of the experiment. The blood from each mouse was allowed to drain directly into a centrifuge tube containing 0.05 ml. of a 20% sodium citrate solution as an anticoagulant and was centrifuged for five minutes. The plasma was removed and added drop by drop with constant stirring to approximately 10 c.c. of acetone at 6° C. The precipitated plasma protein was stored together with the acetone at 6° C for varying periods of time. Immediately after exsanguination, the subcutaneous depot was tapped, and as much oil as possible was collected. This material will be referred to as "recovered oil." Recovery of the oil required from 2 to 4 minutes. Prior to assay, the recovered oil was centrifuged for two hours to remove any hair, water, or tissue fluid which it might contain.

The plasma was prepared for bio-assay for "free" (i.e. ether-acetone soluble) progestin according to the method adopted by Hooker and Forbes.³ The mixture of precipitated plasma proteins and acetone was centrifuged for 5 minutes, and the supernatant was pipetted off. The precipitate was then washed 3 times with approximately 10 c.c. of 1:1 mixture of ether and acetone, the mixture being centrifuged

and the supernatant removed after each washing. The washings were combined with the original supernatant, and to this was added 0.7 c.c. of sesame oil. The ether and acetone were then removed by distillation in a vacuum. Prior to bio-assay the progestin solution was centrifuged for 2 hours to remove any water or particulate matter. The resulting solution was assayed as "free" progestin.

Both the "free" plasma progestin and the recovered oil were separately bio-assayed according to the method of Hooker and Forbes.³ In this assay CHI mice are ovariectomized. An interval of 16 days is then allowed for complete involution of the stromal nuclei of the endometrium. At the end of this period the uterine horns are exposed and uniform segments are isolated by means of two ligatures, care being taken to preserve the blood supply to the segments. A measured amount of test material is injected into the isolated segment of each horn. Two days later the mice are killed, the uterine horns are fixed, and serial sections are mounted and stained.

The presence of a critical amount of progestin in the test material results in enlargement of the stromal nuclei of the endometrium. The nuclei assume a smooth, slightly elongated, oval outline. The chromatin particles are fine and evenly distributed, and the nucleolus is conspicuous. Presence of a single nucleus which meets all of the above criteria constitutes a positive test. Using this method it is possible to measure 0.0002 $\mu\text{g.}$ of progestin in 0.00079 c.c. of test material or 0.253 $\mu\text{g./c.c.}$

EXPERIMENTAL RESULTS

Progesterone disappeared rapidly from the sesame oil solution in the subcutaneous depot. The initial concentration of hormone in every case was 1000 P.E./c.c. Concentration of 101.2, 28.8, and 16.95 P.E./c.c. recovered oil were found in 2 3/4 minutes, 6 minutes and 10 minutes respectively. After 10 minutes the rate of disappearance was somewhat slower, and a concentration of 0.25 P.E./c.c. recovered oil was reached by 3 hours. Less than detectable amounts of progestin were present in all except 2 subsequent samples. Concentrations of 0.51 P.E./c.c. recovered oil and 1.01 P.E./c.c. recovered oil were found at 5 1/2 and 8 1/2 hours respectively (see table 1).

It was noted that in the mice killed within approximately 1 hour of injection the depot was small and compact, but that as the interval became longer the solution spread into a large area of the subcutaneous tissue. Therefore the surface area of the depot increased as the interval between injection and exanguination increased.

Plasma concentration of "free" progestin were quite low. An initial rise of 1.36 P.E./c.c. plasma was recorded at 2 3/4 minutes, followed by concentrations of less than 0.47 P.E./c.c. plasma, 0.48 P.E./c.c. plasma, and 0.47 P.E./c.c. plasma in 4 and 5 minutes. A concentration of 1.44 P.E./c.c. plasma was found at 6 minutes, the highest concentration obtained. All specimens collected between

12 minutes and three hours contained less than detectable amounts of progestin.

From 3½ hours to 8½ hours there were marked fluctuations in the concentrations found by assay. At 3½ hours, 4½ hours and 6½ hours concentrations of 0.61 P.E./c.c. plasma, 0.44 P. E./c.c. plasma and 1.44 P.E./c.c. plasma were found. However, all other samples during this time interval contained less than detectable amounts.

DISCUSSION

Although the rate of disappearance of progesterone from an oily solution had been previously studied by only one investigator,² the rapid clearance of progesterone from the blood after intravenous administration of the hormone is a well established phenomenon. Butt and Crooke⁴ administered 100 mg. of progesterone intravenously in 40% ethyl urea and 40% ethyl urethane to four women after hysterectomy. Polarigraphic determinations of blood levels of progesterone before and after injection were made. The pre-injection blood levels ranged from 0 - 1.5 $\mu\text{g./ml}$ plasma and peak levels of 2.5 - 11 $\mu\text{g./ml}$ were attained in four minutes after injection. The concentration of "free" progesterone had returned to the pre-injection level in 16 minutes.* "Bound" progesterone levels were also measured; these varied from less than measurable amounts initially to 2 $\mu\text{g./ml}$ after 16 minutes.

Zander⁵ reported the intravenous administration of progesterone in doses of 200 mgm. to postmenopausal and ovariectomized women. Blood progesterone levels were determined by chemical means. In three to five minutes the plasma hormone level reached 2.540 $\mu\text{g./c.c.}$ Gradually, over a period of 2 hours, the concentration fell to 0.197 $\mu\text{g./c.c.}$ and to less than detectable levels in 24 hours.

Intramuscular injection of progesterone in sesame oil into

* "Bound" progesterone is that fraction released by hydrolysis of plasma proteins in an acid solution.

castrate rabbits was studied by Zarrow et al.⁶ When doses of 10 mgm. of progesterone were administered, maximum concentrations of 14 µg./ml plasma were attained in 30 minutes. There was a decrease to 0.3 µg./ml in 1 hour, and no hormone was detected after 8 hours. If, however, doses of 40 mgm. were used, a peak level of 4 µg./ml was reached by the second hour, and twelve hours elapsed before the concentration fell below detectable levels.

Several other investigators, including Klein and Ober⁷ and Kauffman,⁸ reported that they were unable to maintain increased blood levels of progesterone despite the administration of large doses of exogenous hormone.

The rate of disappearance of injected hormone activity seen in the present experiments becomes more significant when it is realized that a similar dosage, based on weight, for a 70 kilogram man would be 4.2 grams. Actually, the average daily therapeutic dose is 5-25 mg. intramuscularly, depending on the condition being treated. The dose administered to the mice might well be calculated to overwhelm any mechanism available in the animal for disposing of or utilizing the hormone, but whether the limit of these mechanisms was reached is not known for sure.

Because of the limited amount of experimental data which bears directly on the rapid disappearance of progesterone from an oily solution as demonstrated in this study, I will make use of indirect evidence to attempt to explain this phenomenon. In discussing the possible mechanisms suggested by the previous work, I shall have to make certain assumptions concerning the applicability of reported observations to the present study. That these are only assumptions will be made clear.

Statistical analysis of the results revealed facts which were not immediately evident from gross examination of the raw data. Had a constant proportion of the injected progesterone disappeared per unit time, the resulting curve, when these figures were plotted on semi-logarithmic paper, would have been a straight line. It was found, however, in plotting these data that if the time interval were sufficiently long, the graph was in fact curved. The rate of decrease of P.E./c.c. was rapid at first and for about 10 minutes was reasonably constant. After that interval, the rate of decrease changed more slowly until the values of P.E./c.c. became small and approximately constant.⁹ It is possible that at different concentrations the absorption is occurring by two different mechanisms.

Three factors which may play a role in the change of rate of disappearance of progesterone from the depot suggest themselves. Assuming that the rate of disappearance is directly dependent upon the concentration of hormone present, it could be predicted that as the concentration decreased the rate of decrease would fall. The fact that for the first ten minutes the rate of decrease remained constant suggests that within a wide range of concentrations there is not a direct relationship between concentration and rate of disappearance but that such a relationship is manifest when the concentration reaches a critical level.

The possibility must also be considered, that the mechanism operative in removing the hormone from the solution has, in fact, been overwhelmed or saturated, and that continuing rate of removal from the depot is dependent upon removal of the hormone from the system. Use of a larger initial concentration might clarify this point.

Finally, it was noted that although the injected oil remained

in a relatively small area when the interval between injection and exanguination was short, the surface area of the depot increased markedly as this interval increased. One might expect that since the surface area available for absorption varied directly with the length of time the solution was in the body, the absorption would be more rapid as the interval increased.

It is impossible to say that these are the only three factors involved in the changing rate of disappearance, or in fact that they actually are operative at all. However, in accepting them one would have to assume that since the change is a decrease in rate, the factors of concentration and/or saturation would be more important than increased absorption area.

Several mechanisms have been suggested which would be available in an animal to handle large doses of exogenous hormone. The rapid disappearance from the blood after intravenous administration suggests the possibility that hormone moves quickly from the depot to the blood and is rapidly transported to distant sites where it is metabolized. The fact that large amounts of progesterone were never found in the blood would suggest that if the disappearing hormone was carried in the blood, the mechanism for its removal was both fast and efficient.

Actually this proposition suggests two separate problems, transportation and metabolism. Transportation of progesterone in the blood has been studied by Hooker and Forbes.¹⁰ These investigators have shown that the hormone appears in the blood in two forms designated "free" and "bound". The "free" fraction represents progesterone which is extractable in an active form with an ether:acetone solution. The technique, which has been described at length earlier

in this paper, is a modification of a method used earlier for the extraction of other steroids from the blood. The "bound" fraction consists of progesterone which is biologically inactive unless freed from some, as yet not definitely characterized linkage, by hydrolysis in an acid solution.¹¹ Hooker and Forbes showed that approximately 90% of the blood progesterone was in the "free" fraction while only 10% was "bound". It is possible that binding is not important as a mechanism of transportation, but rather that its chief function is to remove active hormone from the blood.

Thus most of the hormone seems to be transported in a "free" state. It is of interest that concentrations of "free" progesterone have never been found that were greater than might be explained by the solubility of progesterone in water. Therefore, it is quite likely that "free" progesterone is carried in simple solution in the plasma.

Although the concentration of "free" hormone in the blood is small at any given moment, it is possible that large amounts of hormone could be transported some distance in a very short time if clearance were rapid enough. Possible sites of clearance include the liver, kidney, and fat depot.

Certainly all of the metabolic processes in the liver involving progesterone are not known. However, there are three processes occurring there which play a part in removing the hormone from the body. The first is "binding" which was already mentioned, and although this is believed to occur in the liver, the mechanism has not as yet been described.

A second metabolic process is the formation of pregnanediol, once considered the most important metabolite of progesterone. However, excreted pregnanediol has been shown to account for only a

small portion of exogenous hormone. Sommerville and others¹² for example, administered progesterone to young men, post-menopausal women, and women who had undergone hysterectomy. They found that the pregnanediol recovered in the urine represented only 10-16% of the administered progesterone. However, of some importance to our discussion is the fact that this metabolite continued to be excreted at high levels over a period of 2 to 3 days.¹³

Finally, there is evidence that progesterone or its metabolites are excreted in the bile and leave the body in the feces.¹

The importance of the liver in the removal of progesterone from the blood has been debated. Butt et al¹⁴ have reported that ligation of the blood supply to the liver caused a decrease of only 0.8 per cent in the rate of disappearance of progesterone. Haskins,¹⁵ however, reported a highly significant decrease in the rate of loss of progesterone from the blood of hepatectomized rabbits. This is made all the more confusing by the fact that Zarrow et al⁶ found that although there was a decreased rate of disappearance of progesterone from hepatectomized and nephrectomized animals, rabbits suffering traumatic shock showed similar changes.

The kidneys, too, have been implicated in the metabolism of progesterone. Forbes et al,¹⁶ working with monkeys and pseudo-pregnant rabbits, showed that there was a renal artery-renal vein difference in the level of "free" progestin of 16-94%, but that there was no significant or constant change in the level of "bound" hormone. Zarrow⁶ calculated from these data that in the rabbit this mechanism alone could account for the disappearance of 600µg. of progesterone/minute. However, it is believed that this progesterone is not irretrievably lost to the body, since such loss

would require greater production of progesterone in the body than other available evidence suggests.

The final area in which progesterone could be cleared from the blood is the fat depot. However, since this plays an important part in the final analysis of the present problem and does not involve metabolism in the same sense as the processes in the liver and kidneys, we will reserve the fat depot for later discussion.

Probably the most promising approach to this problem lies in the conjecture that the hormone diffuses into the fat either locally or at a distance and is subsequently slowly released.

Zarrow, et al⁶ determined plasma volumes of gonadectomized rabbits then injected the animals with a quantity of progesterone calculated to produce a concentration of 10 $\mu\text{g}/\text{ml}$. This level was taken as the initial concentration, and the rate of disappearance was calculated from this amount.

When nephrectomized, traumatized and hepatectomized rabbits were used, it was found that the rate of disappearance in the nephrectomized and traumatized animals was equal, but was slower than in the controls. It was also found that hepatectomy decreased the rate of disappearance even more than nephrectomy and shock.

Protein "binding" did not appear to play a significant part in the initial rapid disappearance of the hormone, as this fraction constituted only a small part of the total circulating hormone.

Fat and muscle tissue were assayed for progestin. Fifteen minutes after injection a concentration of 100 $\mu\text{g}/100\text{ gm}$. of fat was found. This increased to 230 $\mu\text{g}/100\text{ gm}$. of fat by 30 minutes, and then fell to zero within 1 hour.

Although it was found that incubating progesterone with serum at 37° C resulted in loss of 95% of the activity in four hours, this mechanism would not be sufficient to explain the early, rapid disappearance of hormone.

It was therefore reasoned that the initial rapid disappearance of progestin activity resulted from the diffusion of the relatively small molecule into the tissues and especially the fat.

Davis and Plotz,¹ using C¹⁴-4- progesterone, studied the distribution of radioactivity in human maternal and fetal tissues following intramuscular administration. The highest concentration of radioactivity was found in the maternal fat tissue. Assuming that body fat constituted 18% of the body weight and that the radioactivity was evenly distributed, 17.7%, 33.7% and 19.6% of the administered dose were present 12, 24, and 48 hours after administration. It was therefore apparent that progesterone and/or its metabolites diffuse promptly from the circulating blood into the fat of the body after intramuscular administration. Although it was not determined that the radioactivity in the fat was derived from the intact molecule it appears very likely that this is indeed the fact. Support for this idea comes from the study of Kauffman and Zander¹⁷ in which they found relatively high concentrations of progesterone in fatty tissue of pregnant women.

The above discussion might be considered adequate to explain the rapid disappearance of hormone from the subcutaneous depot if it were known for certain that the hormone reached the blood stream. However, in this experiment we are faced with the fact that blood levels of "free" progestin were consistently quite low, reaching a maximum of 1.44 P.E./c.c. of plasma in 6 minutes and dropping below measurable levels by 12 minutes. As stated earlier, this might

be explained by rapid clearance. However if we are going to accept diffusion of the small progesterone molecule into the tissues from the blood, we can with some assurance postulate also a direct diffusion from the rather large depot into the surrounding tissue. Diffusion from the depot, an area of high concentration of hormone, to the surrounding tissue, an area of negligible concentration, through a large interface, could indeed be expected to be quite rapid. It is possible that diffusion into the surrounding tissue is so much more rapid than the diffusion into the vascular system that the concentration of progesterone available to the blood stream is actually quite small and low blood concentrations of hormone result.

That there is subsequent slow release of either progesterone or its metabolites is suggested the study of Davis et al¹⁸ which showed that pregnanediol continued to be excreted in the urine in appreciable amounts after production of hormone had ceased. The same possibility had been suggested by Sommerville and Marrian after they had studied the pregnanediol excretion pattern in ovariectomized women given progesterone.¹³

There were two obvious sources of possible variation inherent in the experiment as it was designed. In the first place, the length of time between exanguination and the completion of recovering the oil could not be kept constant. There was certainly no reason to believe that death of the mouse by exanguination necessarily signaled the end of absorption of hormone, whatever the mechanism involved. On the contrary absorption may well have continued until the solution was removed from the depot. An attempt was made to keep the variation in time as small as possible; the recovery was completed in every case in from 2 to 4 minutes.

A second possible source of variation was not recognized until late in the experiment. It was then noted that while the injected material in those animals killed within 10 minutes formed a small, compact bolus, the material allowed to remain in the animals for longer periods had spread into the subcutaneous tissue of the whole back. Obviously the surface area available for absorption in these latter mice was much greater. As pointed out previously, however, although for this reason the disappearance might be expected to be faster it was in fact slower. An acceptable explanation for this fact is not readily apparent.

Finally it would seem worthwhile to point out that caution must be used in applying the conclusions from this experiment to any attempt at evaluating subcutaneous therapeutic or experimental administration of progesterone. In spite of the fact that progesterone disappears rapidly from the depot there is no evidence that it is not subsequently slowly released from other tissue in an active form. Indeed, the effects of administering the hormone both subcutaneously and intramuscularly would suggest that this is probably true.

SUMMARY

The present study was designed to measure the disappearance rate of progesterone from an oily solution injected subcutaneously in mice and to determine the resulting plasma levels of progestin.

Progesterone (1000 $\mu\text{g./c.c.}$) was dissolved in sesame oil. Twenty-eight adult CHI mice were ovariectomized. Eight days later, 1.5 c.c. progesterone solution was injected under the dorsal skin. At intervals of 2 $\frac{3}{4}$ minutes to 8 $\frac{1}{2}$ hours the mice were exsanguinated, the plasma was separated, and the oil was aspirated from the subcutaneous depot. Plasma and oil were separately assayed for progestin (Hooker-Forbes method). Results are expressed as "Progesterone Equivalents"/c.c., a P.E. equalling the activity of 1 μg progesterone in the Hooker-Forbes bio-assay.

The concentration of progestin in the recovered oil decreased very rapidly, for example to 101 P.E., 50 P.E., and 3 P.E. in 2 $\frac{3}{4}$, 4, and 20 minutes, respectively. By 3 $\frac{1}{2}$ hours there was less than 0.25 P.E./c.c. recovered oil.

Some plasma samples contained "free" progestin, but no consistent correlation was observed between time interval and plasma level. A rise to 1.44 P.E./c.c. plasma was found at 6 minutes, but after 10 minutes "free" plasma progestin was not detected consistently. Concentrations of 0.6 P.E., 0.4 P.E., and 1.4 P.E. were found at 3 $\frac{1}{2}$, 4 $\frac{1}{2}$ and 6 $\frac{1}{2}$ hours respectively.

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TABLE I

<u>TIME</u>	<u>P.E./c.c. Recovered Oil</u>	<u>P.E./c.c. Plasma</u>
2 3/4 Min.	101.2	1.4
4 Min.	50.6	
4 Min.	50.6	<0.5
5 Min.	38.0	0.5
5 Min.	38.0	0.5
6 Min.	28.8	1.4
8 Min.	17.7	1.3
10 Min.	17.0	0.4
12 Min.	5.0	<0.9
14 Min.	4.8	<0.5
17 Min.	<2.5	<0.6
20 Min.	3.0	<0.6
25 Min.	3.5	<0.6
30 Min.	1.5	<0.6
35 Min.	1.5	<0.6
49 Min.	1.0	<0.5
64 Min.	1.0	<0.5
86 Min.	0.8	3.3
125 Min.	0.2	<0.4
151 Min.	0.2	<0.5
180 Min.	0.2	<0.5
210 Min.	<0.2	0.6
240 Min.	<0.2	<0.5
270 Min.	<0.2	0.4

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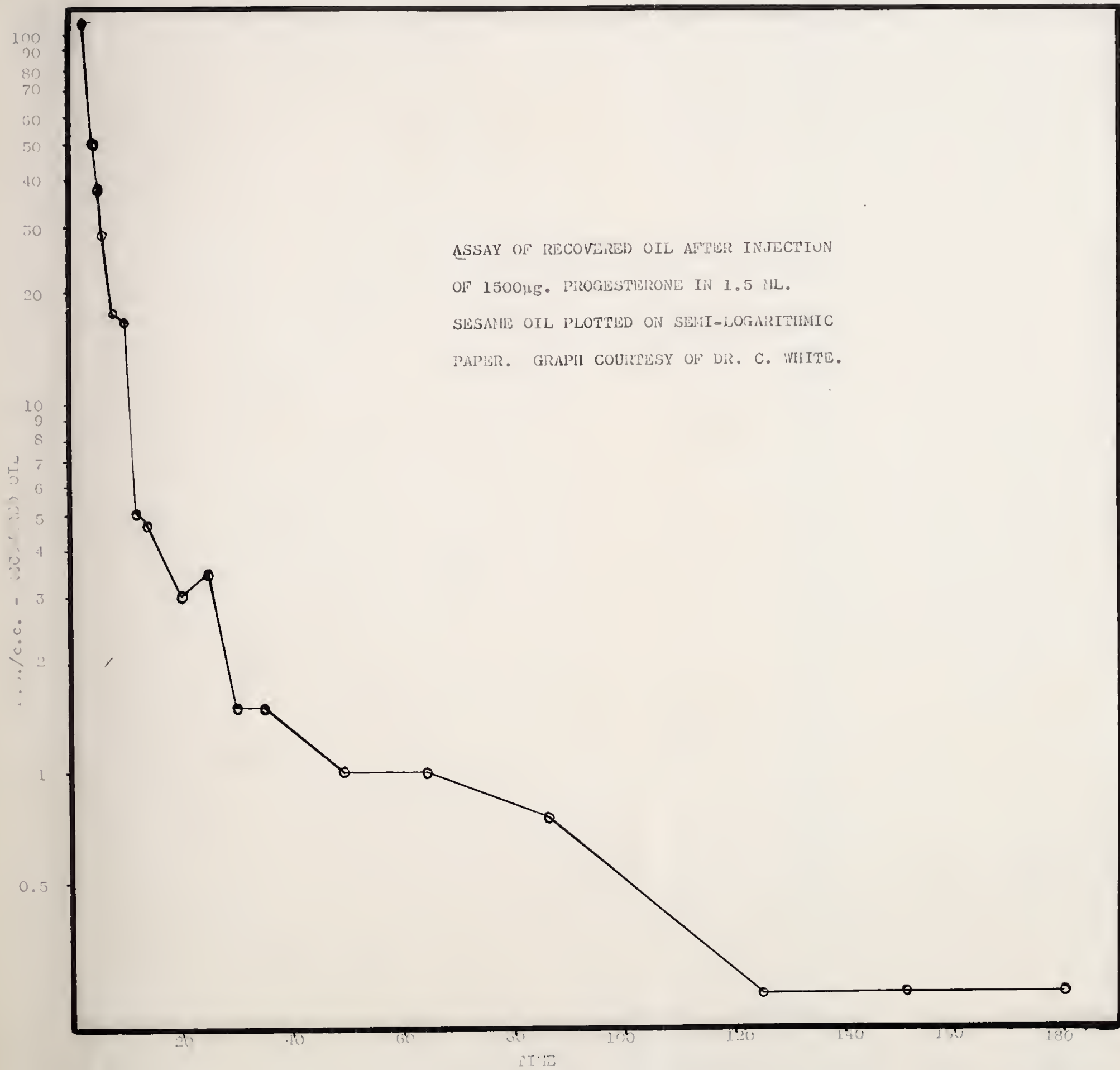
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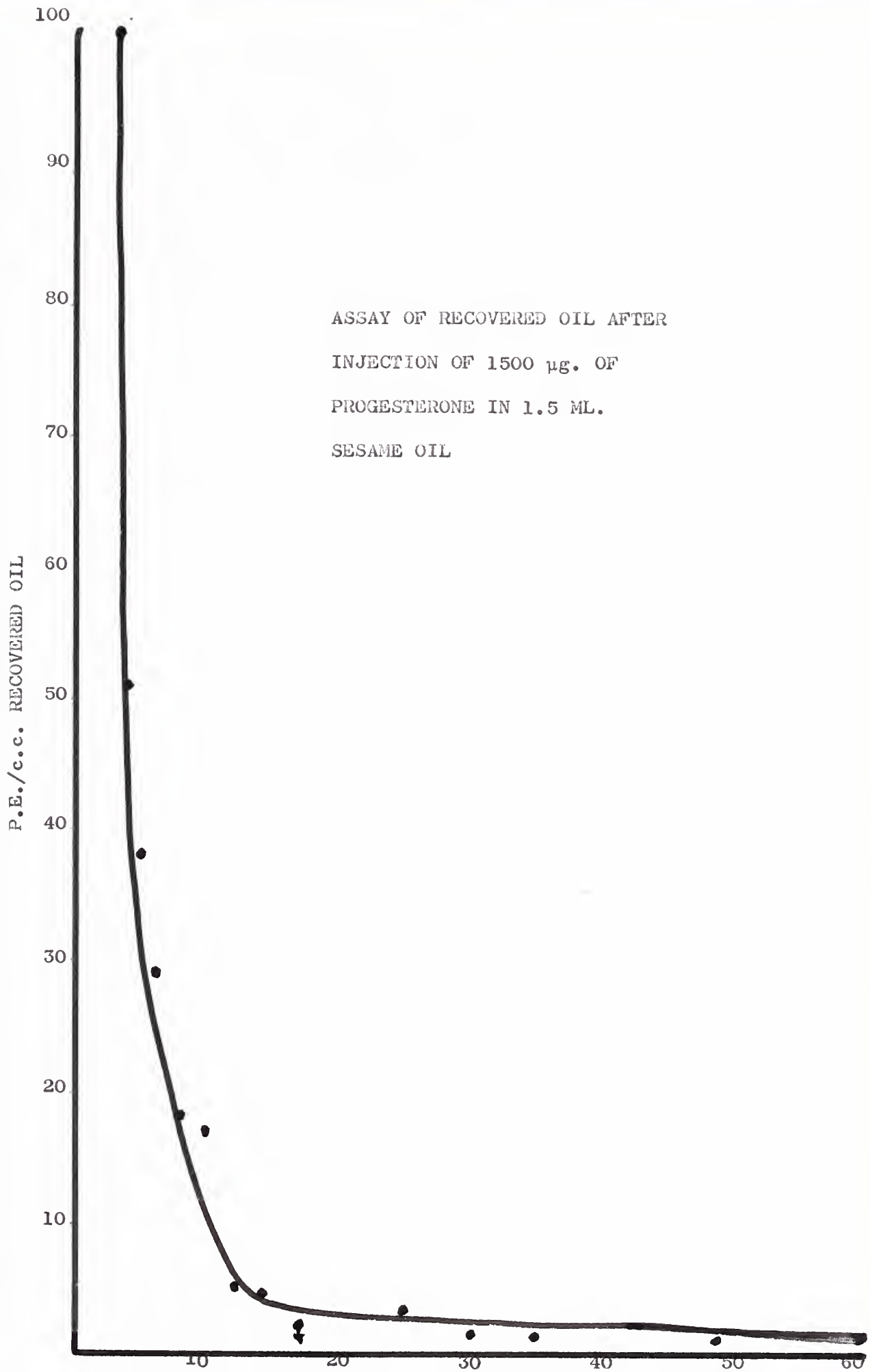
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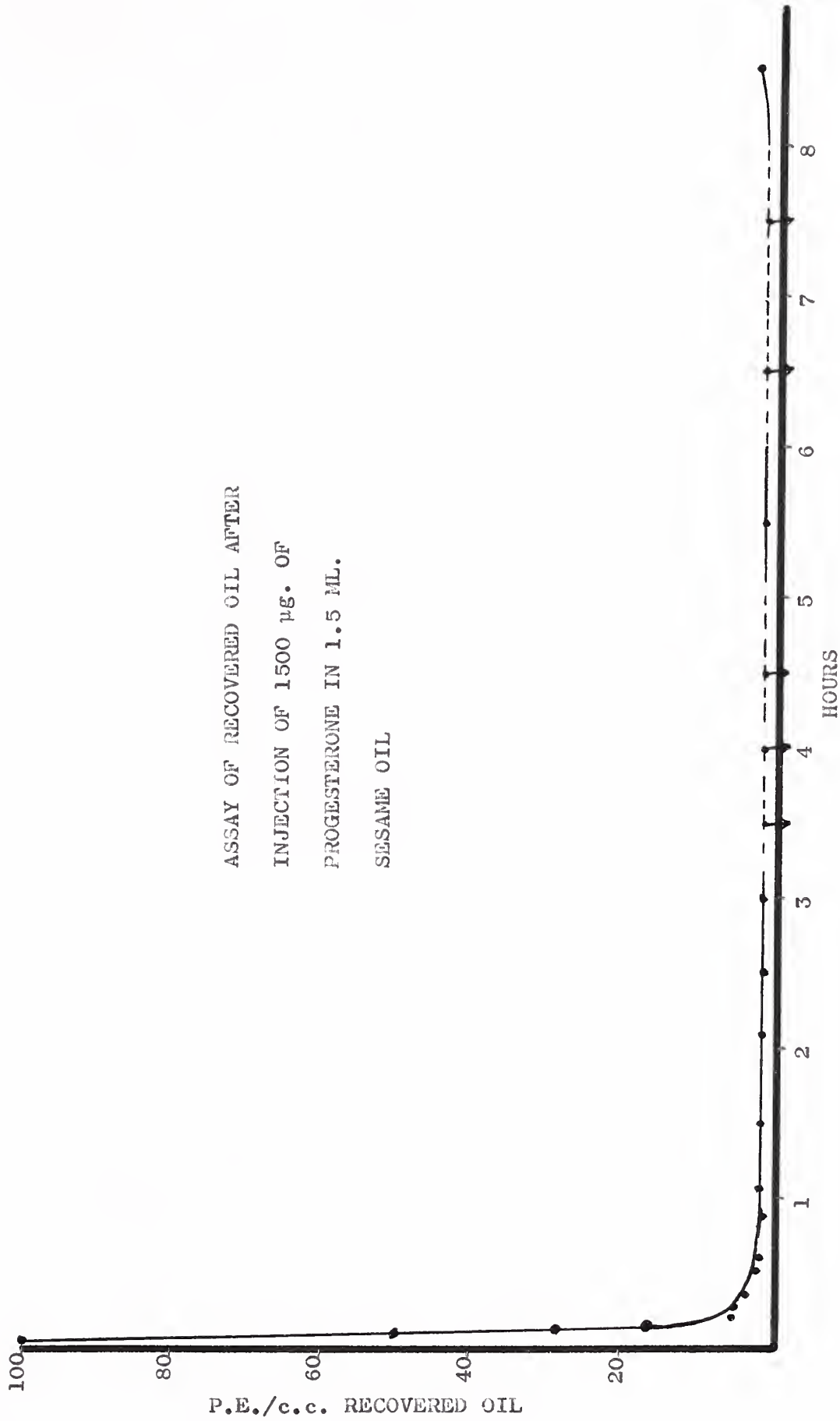
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<u>TIME</u>	<u>P.E./c.c. Recovered Oil</u>	<u>P.E./c.c. Plasma</u>
329 Min.	0.5	<0.8
390 Min.	<0.2	1.4
450 Min.	<0.2	<0.4
510 Min.	1.0	<0.5





ASSAY OF RECOVERED OIL AFTER
INJECTION OF 1500 μ G. OF
PROGESTERONE IN 1.5 ML.
SESAME OIL



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