ENVIRONMENTAL FACTORS AFFECTING HUMAN AND RAT PLACENTAL LACTOGEN

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Submitted to the Department of Nutrition and Food Science on December 28, 1974, in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

The placental lactogen or mammotropin hormone is a protein hormone produced by the placenta and found in the maternal blood. Its secretion increases progressively during human pregnancy and is estimated to be an indicator for the function of the trophoblast. Its role is not fully determined yet, nor how it is regulated during pregnancy.

The purpose of this study was to measure rat chorionic mammotropin level (RCM) under various nutrition states: low protein diet before and/or during gestation, and administration of PTU (hypothyroid animal) before and/or during gestation.

A rapid double radio immuno assay for HPL was developed but since the cross reactivity of RCM with the HPL radio immuno assay was very low, it was necessary to use a radio-receptor assay.

Thesis Supervisor: Dr. John Stanbury
Title: Professor of Experimental Medicine

"tango:

four steps forwards

three steps back

with an occasional turn around"...

ACKNOWLEDGMENTS

I would like to thank Dr. J. Stanbury for welcoming me into his lab, for guiding and advising me all along these months and for having given me the opportunity to see and learn so many things.

Thank you to Dr. P. White for her attention and also many special thanks to Dr. D. Talley for all the time she spent helping me when I was having a hard time, and for the proof reading of this thesis. What would I have done, too, without the technical assistance of Rusty Murray and Jutta Salinas and without the patience of all the members of this lab who had a difficult period with my accent (but now I am waiting for you in France!).

Thank you so much to all the friends of mine who made me know and love this co ntry and who with humor, initiated me to so many things, from spring skiing to this crazy game that is squash, without forgetting apple picking, Chinese cooking and concert life in Boston. They will never guess the importance they had for me just by the simple fact I knew they were there. It would be unfair not to say to my friends from abroad how I appreciated their fidelity through the mail, helping me not to loose contact with my roots.

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To finish (but is it ever possible to stop to say thank you) I would dedicate this study to my parents who, by their encouragement and warm understanding, have made this stay possible.

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CHAPTER I

LITERATURE SURVEY

Mental and physical retardation (endemic cretinism) has been shown to be much related to iodine deficiency (1). However, many observations have suggested that other environmental factors may be involved. We were very interested in studying the response of placental hormone production of the prolactin-like hormone, the placental lactogen in malnutrition and also in hypothyroid status, because of the possibility that these deficiencies might contribute to the retarded state, and that this could be indicated by a diminished level of this placental hormone in the blood. Accordingly, experiments were designed wherein rats were bred in the hypothyroid and in the protein-deprived state. Efforts were made to establish the appropriate assay method on rat blood.

Since the early studies on the placenta as an endocrine gland (2) and on production of human placental lactogen (3), different techniques have been used to assay the level of placental hormones in the blood. One of the earliest methods for lactogenic activity was a bioassay on the pigeon crop sac: the epithelial cells lining the crop sac of immature pigeons of 6 - 8 weeks of age, respond to lactogenic hormone by a spectacular mitotic rate: intradermal injections just above one side of the crop sac zone are made; two to five days after the first injection, the pigeons are beheaded and the area and thickness of the proliterative area are measured. One side of the crop sac is injected with standard, on the other one it is the unknown material, diluted until the response is equal to that of the standard. The details of this technique are slightly different from one laboratory to

another (4)(5).

Injections of extracts of rat placentae have been shown to maintain the progesteron-secretin function of the corpus luteum of pseudo pregnant rats (6). Thus, placental lactogen seems to have a luteotropic effect.

The stage of pregnancy is of great importance, the highest potency of lactogenic activity being present in day 12 placentae (7). The fact that abortion does not occur in hypophysectomized rats in mid or late pregnancy led to the conclusion of a production of a prolactin-like hormone with luteotropic activity by the placenta. To detect the luteotropic and mammotropic properties, use was made of the deciduoma reaction (8): female Long-Evans rats are brought to estrus and induced to form luteal bodies, by injecting on the 26th day of age 5 IU of pregnant mare serum gonadotropin (PMSG); they have been hypophysectomized on the 13th day of age and injected locally over the fourth mammary gland from the 13th to the 36th days. A thread is placed in the lumen of the uterus on day 33 and autopsy performed on day 37. Animals are examined for the presence of deciduomata (increase of the weight of the traumatized uterus) = positive response for the luteotropic activity, which is confirmed histologically. For studies of the mammogenic properties, changes after local injection into the male rat mammary parenchyma were observed (8).

Corroboration of these results has been shown with some modifications of the previous experiments: no hypophysectomy was performed on the injected animal and there was no use of pregnant mare serum gonadotropin but that of serum of pregnant rats (9).

Besides these bioassays, the technique of organ co-culture of placental tissue and mid pregnancy mammary tissue, and of transplantation

of mice placental fragments into mammary fat pads, have also been used to demonstrate the mammotropic activity of placentae. Results of different aged placentae were judged histologically (10).

Most of the studies on lactogenic hormone production have been performed on humans, for evident clinical purposes, on rodents, for the brevity of their reproductive cycle, and on ruminants, because of the economical importance of factors influencing milk production (11).

Until recently, there were no specific assays allowing a clear differentiation between all the prolactin-like, or growth hormone-like hormones.

First of all, isolation and characterization of the hormone has, for the moment, only been achieved for HPL (3), and for the monkey placental lactogen (13).

Most of the measurements for HPL are now achieved by radioimmuno assays which give great accuracy and sensitivity. This technique will be discussed in Part III.

The results obtained by measuring serum of normal pregnant women during pregnancy show the first detectable amount of the hormone around the fifth week of gestation. The production increases to a plateau at 35 weeks, where it reaches a concentration of 6.0 ± 1 µg/mg (14). The concentration decreases drastically within a few hours after delivery. The secretion of rat placental hormone does not follow this pattern and will be discussed later.

The question of the role of placental lactogen has not been completely answered. It is possible to observe what happens in the absence of prolactin (studies in hypophysectomized or ergocornine treated animals)

where it seems that placental lactogen is powerful enough to replace the pituitary hormone, at least from a certain time of the pregnancy. Ergocornine is a peptide-containing ergot alkaloid which reduces serum prolactin level in rats (15). Partuition in this case is accompanied by lactogenesis but is not followed by galacto-poiesis (16). It is, of course, impossible to study the effect of a total lack of placental lactogen.

Human placental lactogen has been shown to be structurally very close to human growth hormone: 85% of their amino-acid sequences are identical (17). But in spite of similar biological activity, the amino-acid sequence is very different in human prolactin and in human placental lactogen (18).

Furthermore, the role of this placental lactogenic hormone versus prolactin does not appear to be the same among all the mammals: for instance, prolactin levels increase regularly during pregnancy in women but stay relatively low until partuition in monkeys (19). This raises the hypothesis of a different influence of these hormones in breast development.

Apart from a role of placental lactogen in mammary development, not yet entirely clarified, one of the main hypotheses is that this hormone could be a vestigial one, the physiological role of which may be only fully expressed in special cases. In case of hypoglycemia, for instance, there is a fast decrease in HPL (20). The fact that pancreatic cells in vitro show an insulin rise if HPL has been added to the medium, but not in the presence of glucose would be an argument in favor of a negative feedback control of glucose on HPL. Because the authors obtained the same results with pituitary growth hormone, the question arises as to whether this suggestion can be applicable to the rats where no somatotropic influence has been detected in

the placental lactogen, contrary to the one of human placental lactogen (21).

Some speculations about the meaning of this phenomenon would assume a maternal carbohydrate sparing, lipid mobilizing action of HPL (22). The fact that in total starvation HPL production seems to increase (23) lends support to this theory.

In spite of the increasing literature about HPL, many basic problems have not been solved probably because of the difficulties with most of the existing assays in man, and because of the lack of methods for rat placental lactogen measurements. It is well established that measurement of HPL may be a good indication of complicated pregnancy, thus, there is a good correlation between decreased HPL levels and spontaneous abortion (24).

CHAPTER II

INTRODUCTION

The use of radioimmuno assay was a major step in the study of protein hormones in general and placental lactogen in particular, because of its sensitivity and specificity. The principle of this type of assay is the competition between a radioactively labelled antigen (the tracer) and a cold antigen molecule, for a fixed concentration of specific binding sites which are the antibodies specific for that hormone.

Rat chorionic mammotropin has not been isolated yet and all the investigations so far have been done with bioassays, or more recently, with radioreceptor assays.

This hormone (RCM) was first shown to have a peak of plasma concentration at day 12 of rat pregnancy (total length = 21 days), with the deciduoma test (endometrial trauma with progestin secretin) (9), (8). No evidence has been found in favor of a growth promoting activity identical to that of HPL.

The pigeon crop sac method do s not seem to be sensitive enough for this hormone in plasma (8), but another technique derived from studies on ovine pituitary hormone (8), using immature male rats injected with placental extracts which eventually would stimulate the alveoli, proved to be very sensitive (1).

With a sensitive HPL assay, it might be possible to test RCM, assuming a certain amount of cross reaction, and then to measure the effects of low protein diet and hypothyroid state on the hormone production.

Laga's study (25) on the placenta has shown that an undernutrition state would diminish placental weight on the basis of a reduced trophoblast,

which is the active tissue in steroid and protein hormone syntheses, and connective tissue mass and volume of the interstitial space. It could be assumed that this fact could have some effects on the hormone production, but so far with a bioassay method the results seem to be different. W. G. Kinsey (26) suggests that the endocrine function of the placenta is not impaired by the absence of dietary protein. Indeed, in this study, placental mammotropic and luteotropic activities were similar in experimental group (protein free diet) and control group.

Hypothyroidism in rats reduces the radioimmuno assayable plasma growth hormone but not the plasma prolactin concentration (27).

On the other hand, in humans, the thyrotropin releasing hormone (TRH) stimulates the production of both TSH and PRL but not HPL or HCT (human chorionic thyrotropin (28)(29).

In rats, TRH does not stimulate prolactin release (30), but no study has been done on the effect of placental lactogen.

Since difficulties were encountered with the radioimmuno assay for RCM, a radioreceptor assay was tried. This procedure involves the specificity between a hormone and its target tissue and the competition between the radioactive and the cold hormone for a fixed quantity of specific binding sites: the membrane receptors (instead of the specific antibodies in the case of radioimmuno assays).

The microcomplement fixation method (Levine method cited in [31]) has been only used for HPL measurement and is not satisfactory for measuring small amounts of the hormone.

To summarize, the first objective of this study was to establish a precise measurement of RCM. This was first attempted by using a possible

cross reaction with HPL, but there was no significant cross reactivity. Accordingly, a rapid double radioimmuno assay was developed for this hormone, a radioreceptor method was then tested.

CHAPTER III

MATERIALS AND METHODS

In order to avoid unnecessary loss of time, both the studies on the setting up of the assays and the preparation of the animals were developed in parallel.

Preparation of the Animals

All animals were Sprague-Dawley* albinos rats. The first group weighed 150 g to 200 g and were put on a diet for ten days before being bred. The diet consisted either of 5% or 10% protein. In order to make the animals hypothyroid, prophylthiouracil (PTU) was placed in the drinking water (o.o5% PUT + 5% sucrose, to prevent the bitterness of PTU)/

Control animals were on normal rat chow and tap water. They were all fed "ad libitum". Both PTU water and 10% or 5% protein diets were well accepted by the animals. The composition of the different diets are given in Table I. The experiment was designed such that beside the control group one part of the animals was protein (2 groups: 5% and 10% protein in the diet) deprived and the other one made hypothyroid with PUT. Among each group of protein deprived animals there were the three different possibilities:

- the rats were on diet before being bred and not during gestation
- the rats were on diet before being bred and also during gestation
- the rats were on diet only during gestation.

The same scheme was used for the rats on PTU which had PTU added in the drinking water either before and during gestation, or only before or only

*Charles River Breeding Labs, Wilmington, Massachusetts

TABLE I

RAT DIET COMPOSITION (32)

	10% Protein	5% Protein
Casein	10.0	5.0
Dextrose	75.4	80.4
Corn Oil	10.0	10.0
Salt Mixture*	4.0	4.0
H_2O Soluble Vitamins**	0.5	0.5
Fat Soluble Vitamins**	0.1	0.1

* (34)

** Ramalingaswami, V., Vickery, G. L., Stanbury, J. B., Hegsted, D. M., personal communication.

after gestation.

The females were put with the males in the breeding cages at night. Vaginal smears were checked the following morning, day 1 of pregnancy being the day of the positive vaginal smear.

When necessary, the animals were bled by cardiac puncture under light ether anesthesia. The blood was collected in heparinized tubes, centrifuged at 1000 xg during 10 mn and the plasma stored at -20°C.

Iodination of HPL

Iodination of HPL has been prepared by a modification of the method of Greenwood and Hunter (33) using ^{125}I* instead of ^{131}I because of the longer half life of the former.

The reaction was carried out in the rubber capped vial in which the ^{125}Iodide was received. To neutralize the pH ($Na^{125}I$ was diluted in NaOH) phosphate buffer 0.5 M pH 7.6 was injected into the vial in order to give a total volume of 50λ; then, immediately, the human placental lactogen** (5μg in 10λ) and 25λ of chloramine T (20 mg dissolved in 10 ml of phosphate saline buffer: PBS) were added just before use. Chloramine T was the oxidizing agent. After rapid and vigorous shaking, 50λ of sodium metabisulfite (25 mg dissolved in 10 ml buffer) was immediately added to stop the reaction.

The separation of the labelled hormone from damaged labelled material and unreacted ^{125}I was done on a G50 Sephadex (length 10 cm - diameter 1 cm) column, equilibriated with 30 ml PBS 0.01 M pH 7.6 and presaturated with albumin to prevent adsorption (1 ml of PBS - 2% BSA); 10

* Purchased from New England Nuclear, Boston, Massachusetts
** Purchased from Nutritional Biochemical Company, Cleveland, Ohio

drops (≃0.5 ml) of the elution solution were collected in 100λ of BSA 2%. (The total volume of elution was ≃ 15 ml.) 10 of each of these tubes was counted in the Packard Gamma scintillation spectrometer (Model 578 - efficiency 30%).

The pattern of elution is shown in Figure 1. This method was also followed for the iodination of the rat prolactin* (10μg/20λ).

In this case, we observed a higher peak for the free iodide (≃65% of the total count).

Radioimmuno Assays

Several techniques have been described for the radioimmuno assay of HPL and the separation of free from bound hormones. In most of the cases, the double antibody method (33) was not used because of the time needed for the incubation, time which will be shown to be possible to shorten.

Standard Curve

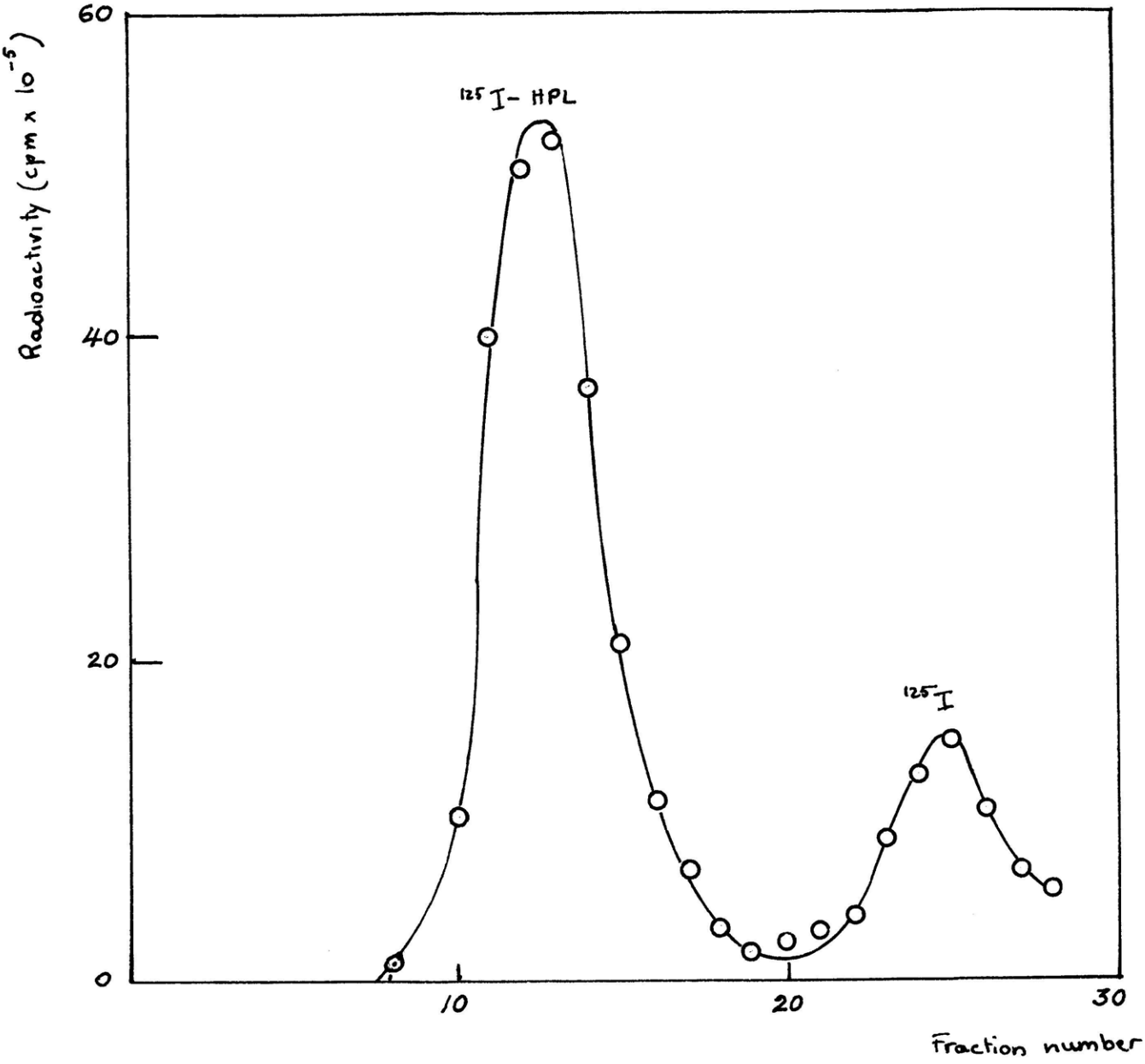
Reagents

First Antibody** (1st Ab): rabbit anti-human antibodies to HPL, added to the assay at a dilution of 1500, dilution which was determined by antibody titration: the antibody at different concentrations is added to a constant amount of HPL, then after the first incubation time, a constant amount of 2nd Antibody is added, before a second incubation time. The concentration of 1st Ab is plotted against the percentage of binding and the concentration of 1st Antibody which is chosen is the one giving a binding of approximately 50% of iodinated hormone.

* Kindly supplied by Dr. A. F. Parlow, (Harbor General Hospital, Torrance, California) for NiH

** Kindly supplied by Dr. S. K. Varma

Fig 1. Iodination of HPL



Second Antibody* (2nd Ab): goat anti-rabbit IGg serum was used at full concentration.

All dilutions were made in phosphate buffered saline and bovine serum albumin (PBS - 1% BSA).

Experimental Procedure

To 100λ of freshly prepared standard (different ilutions of the cold hormone) were added 200λ of 1st Ab (dil: 1/500), then 100λ of labelled HPL (approximately 100,000 cpm). After each addition, the tubes were carefully vortexed.

Non specific binding tubes were prepared by adding to 300λ of buffer, 100λ of ^{125}I-labelled HPL.

Maximum binding tubes were prepared by adding 200λ of 1st Ab (1/500) and 100λ of ^{125}I-labelled HPL to 100λ of buffer. The tubes were then incubated for varying periods of time, as indicated in results, and then the 2nd Antibody (100λ) was added and well mixed.

After a 2nd incubation time, the tubes were first counted to determine the total count, then centrifugated a x 1000 g for 10 minutes. The supernatant fraction was adjusted and the precipitate was counted.

The ratio $\frac{\text{Count for the precipitate}}{\text{Total count}}$ give the percentage of binding which is plotted against the concentration of cold hormone after subtraction, from each tube, of the non specific binding which has nothing to do with the reaction itself and then is not taken into account. Every concentration is run in duplicate or triplicate.

In no case was the hormone frozen more than once.

** Purchased from Biotek, Inc., St. Louis, Missouri

Radioreceptors

The methods, followed step by step, were those of Shiu, Kelly and Friesen (35). Two mid pregnant rabbits were injected daily, for 4 days with intra-muscular injections of 10 mg HPL and 5 mg hydrocortisone. Different amounts were tested; in the case of the control animals, the mammary glands were developed with no evidence of lactation. When only placental lactogen was injected, there was a marked development of the gland and a moderate degree of lactation but with placental lactogen (10 mg) + hydrocortisone (5 mg) there was a marked breast development and a very important milk production (36).

Briefly, cell membrane fraction from the mammary gland has been rinsed, cut, homogenized with a Virtis homogenizer at medium speed, at 4°C for 5 minutes, filtered through cheesecloth and centrifuged in different conditions until the last centrifugation (100,000 g, 90 min., 4°C) where this time only the pellet had been kept. Then it was resuspended in 0.025 M tris-Cl, pH 7.6, 10 mM $CoCl_2$ in aliquot such that the protein concentration, determined by the Lowry protein assay (using BSA as a standard) (37) was 475µg/100λ. All the samples had been frozen at -30°C.

CHAPTER IV

RESULTS

I. Radioimmunoassays of HPL

Different variables were tested to maximize the radioimmunoassay of HPL. One of them was to investigate the effect of an amount of serum equivalent to that in an unknown sample, in the binding of HPL to antibody, versus standard curve made just in buffer.

In this case, the serum was normal male rat serum which would contain no lactogenic hormone to affect the competition. The average rat prolactin level given in the literature is around 2.3 ng/ml and would not cross react. In the results shown in Fig. 2, it is possible to see that the maximum binding is higher when no serum is present. The difference tends to be more marked when the nonspecific binding is subtracted. Indeed, this nonspecific binding is slightly higher in the case of the serum. The curve is also less sensitive in serum. This assay has been reproducible. In these cases, the incubation conditions in Fig. 1 were for the first incubation: one hour at 37°C plus overnight at 4°C, followed by a second incubation of one hour at 37°C.

In one experiment, the dilution of the first and the second Ab was with normal rat serum (which was then in great excess) instead of with buffer. In both cases the standards were made in buffer; all the assay conditions were otherwise identical. It was interesting to note that the standard curve with the buffer was similar to Fig. 2, but there was no binding at all for the standard curve where assay tubes contain huge quantities of normal male rat serum: cf. Fig. 3. This is consistent with

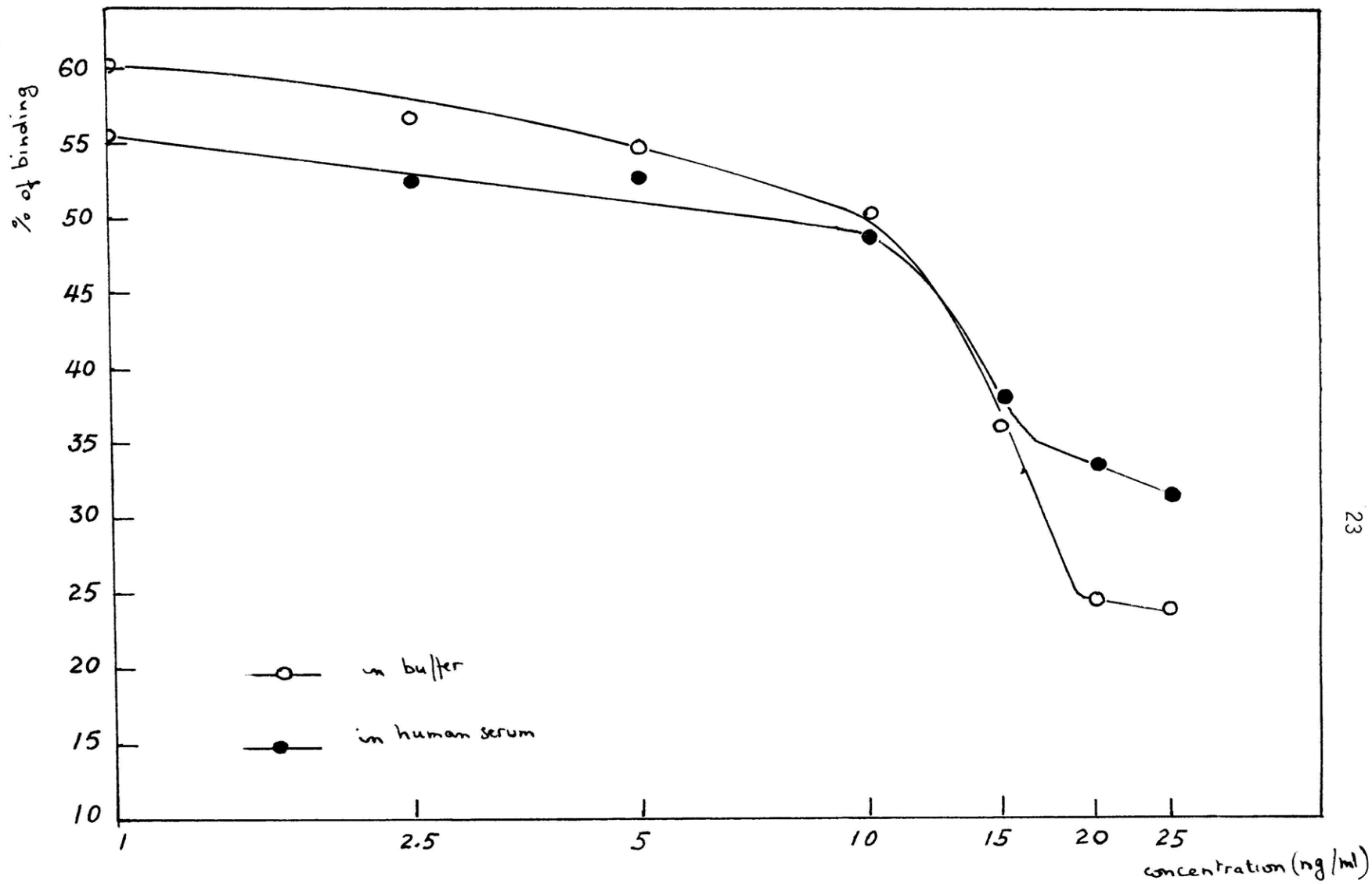


Fig 2 dilution of the hormone (HPL)

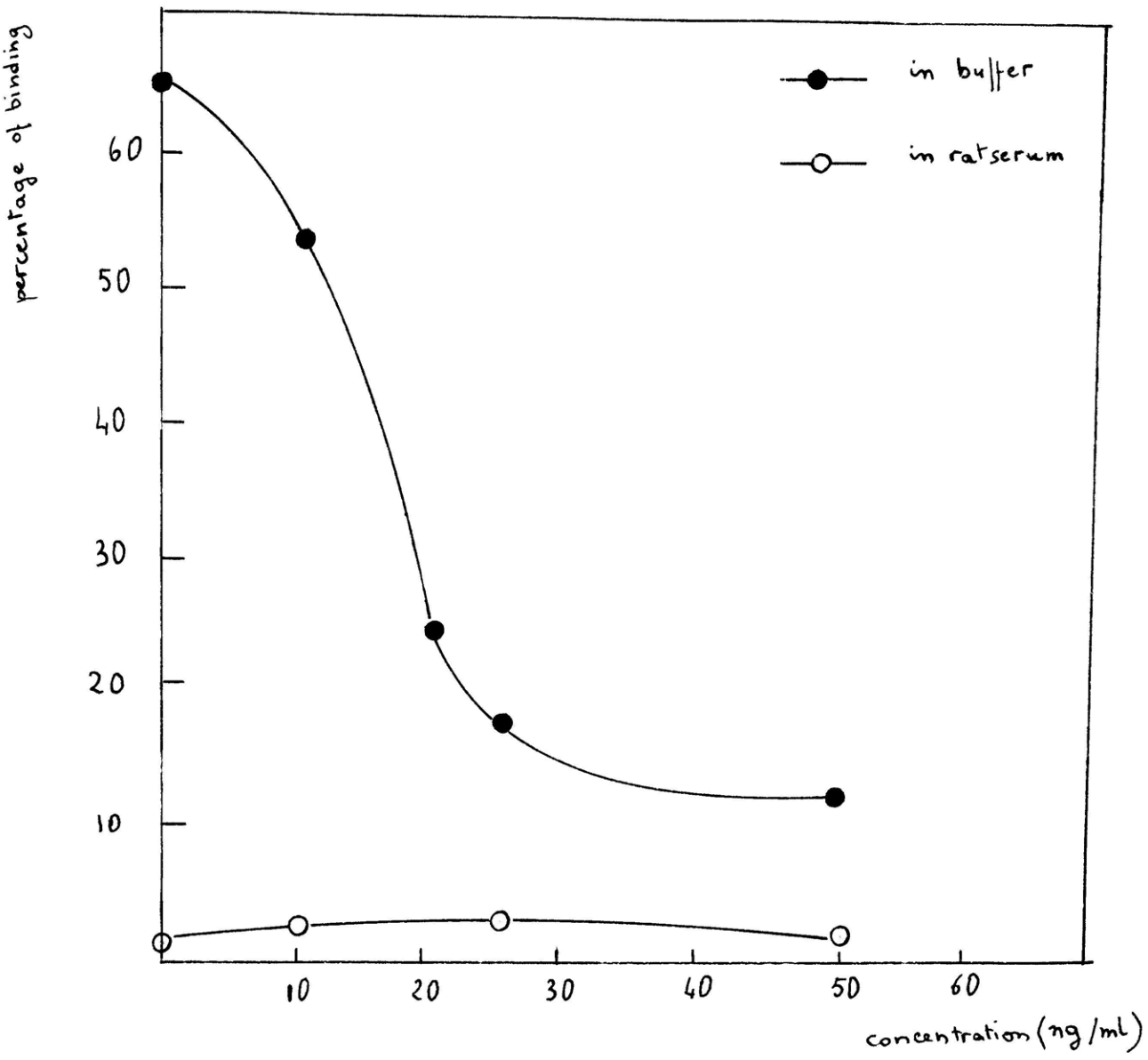


Fig 3 Dilution of the Antibody

our results in Fig. 2.

In all the radioimmunoassays, the concentration of the antibody was determined by titration to give a maximum binding occurring at around 50%. The greatest sensitivity of the assay (i.e., greatest displacement of hot hormone on antibody sites by cold hormone) occurs approximately at 50% binding of hot hormone at this level of antibody.

The only accurate condition to read the unknown sample is in the linear portion of the standard curve.

At the beginning, the assay was done in plastic tubes and with PBS buffer containing no protein. In this case, the minimum binding was high. The nonspecific binding decreased drastically when glas tubes and albumin-containing buffer (PBS - 1% BSA) were used. For instance, the minimum percentage of binding was 14.2% in glass tubes but with no bovine serum albumin and 6.5% when BSA was present. The use of plastic tubes in the presence of BSA gave a nonspecific binding at 11.4%.

Once the conditions of the assay had been well defined, serum samples with known amounts of HPL were assayed to assure the accuracy and the reproducibility of the method. In preparing the standard curve, all the conditions were identical to those of Figure 2. However, in these assays, all the dilutions of the hormone were done in normal human male serum.* Two samples from the plasma of normal pregnant women (37 weeks and 38 weeks of pregnancy) were tested. The average HPL plasma found was 4.6 µg/ml. The value of term has been reported to be around 5 µg/ml (14).

Then the assay was tested to see whether it could be used for the assay of rat placental lactogen. For the standard curve, HPL was diluted

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* Thanks to all the gentlemen of the lab.

in normal male rat serum. Samples of serum of day 12 pregnant rats were tested, undiluted and diluted at 1/2, and also samples of normal male rat (diluted 1/10). In these cases, the results of the binding (∿60%) were not significantly different. This means that there was no competition for binding, i.e., no detectable amount with this method. A further attempt was to assay 400 of unknown 12 day pregnant rat serum samples, to increase the concentration of the hormone in the tubes. The antibody concentration was adjusted so that it would give the same final concentration of antibody, 10λ of labelled HPL were used (70,000 counts per 0.1/ mn) and 100λ of 2nd Ab. The maximum binding was still very high and revealed a lack of competition, thus no measurable amounts of rat placental lactogen by this technique.

II. HPL Radioimmunoassay: Time Study

Besides the fact of its price, one of the major limitations of assaying HPL by a double antibody technique is the time needed for the incubations: 4°C during 96 hours for the first one and 4°C during 24 hours for the second (34). Even in the case of the use of charcoal dextran separation, or organic solvent separation, for the last step of the separation of free from bound hormone, the time of the first incubation is still a limiting factor in the use of RIA: 37°C during 18 hours (38) or 4°C during 16 hours (24). The purpose of this part of the study has been to check the effects of different incubation times, especially in the bindings of HPL to antibody.

A standard curve was made for every different incubation time. The reagents of the reaction and their quantity were the same as the one

described in materials and methods, p. 20. All the dilutions were made in normal human male serum, and for the maximum and minimum bindings they were also made in buffer (PBS-BSA), to compare the effect of the serum with the buffer.

Minimum binding: 300λ of buffer (PBS+BSA) + 100λ of labelled HPL then 100λ of second Ab after the first incubation of 300λ or serum + 100λ of labelled HPL, then 100λ of second Ab after the first incubation.

Maximum binding: 100λ of buffer (PBS+BSA) + 200λ of first Ab + 100λ of labelled HPL, then 100λ of second Ab after the first incubation or 100λ or normal male rat serum + 200λ of first Ab + 100λ of labelled HPL then 100λ of second Ab after the first incubation. The different incubation times were the following:

1st incubation at 37°C for one hour + eight hours at 4°C, then a second incubation time at 37°C for one hour.

1st incubation at 37°C for one hour plus eight hours at 4°C, then a second incubation at 37°C for one + fourteen hours at 4°C.

1st incubation at 37°C one hour + 16 hours at 4°C then a second incubation at 37°C for one hour.

1st incubation at 37°C, one hour + 16 hours at 4°C, then a second incubation at 37°C for one hour + 16 hours at 4°C.

This procedure allows a comparison between a long and a short incubation time for the first incubation, the second incubation period remaining constant, either short or long, and a comparison between a long and a short second incubation time, the first one being constant, either long or short. The results are shown in Figure 4. Surprisingly enough,

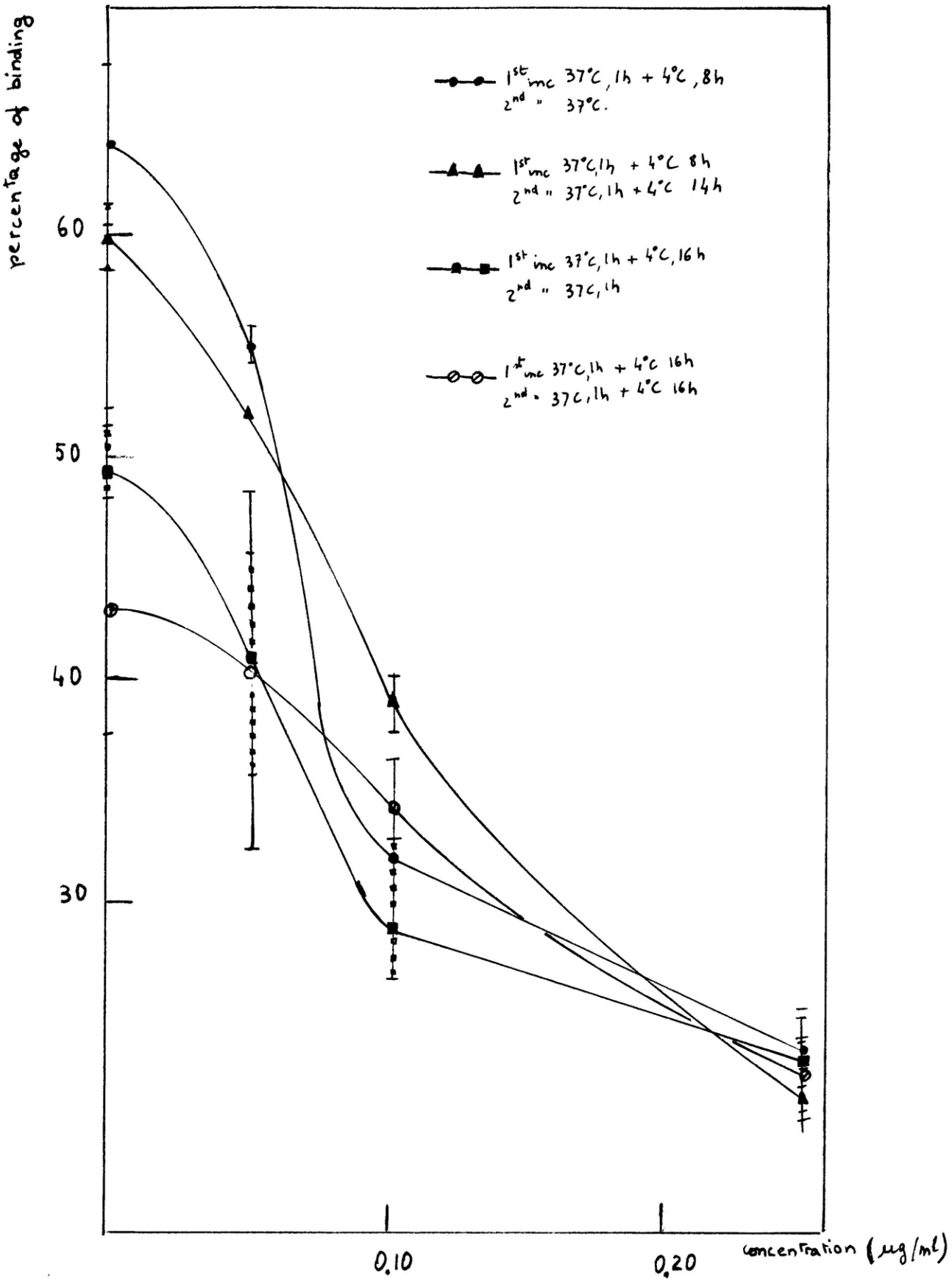

Fig. 4 Radioimmuno assay : time study

under these conditions, there was no improvement of the binding with the increase of incubation time and it seemed that it was even quite the contrary;* increasing with the time, the maximum binding in the case of the use of serum for the dilutions decreases from 60.0% for the shortest incubations to 44.3% to the longest one. This was true all along the sensitive part of the curve (from a concentration of 0 to 0.25 µg/ml). When dilutions of cold HPL were made in the buffer, there is no such a striking difference. In fact, this did not seem to have any negative effect on the maximum binding.

III. Radioreceptor Assays

Once the 100λ aliquots have been thawed, the particles have been resuspended, just before use, with a glass rod. To the 100λ of membrane receptor were added 100λ of unlabelled rat prolactin hormone (from 0 to 500 ng/500λ in the tube), and 100λ iodinated rat prolactin hormone + 100λ of normal rat serum (this was different from the assay of Shiu, Kelly and Friesen, but it seems absolutely necessary to have a standard curve made under the same conditions as those used for assaying the unknown). For the first experiment, it was not the right membrane which was used for the Millipore filtration (right size of the pores but not a special membrane for a low binding). 20% was the maximum binding (which is the number given in the literature) but also, the minimum binding was not lower. On the other hand, the time of passage through the membrane was far and away exceeding

*The experimental errors increase with the time but it is possible that the differences are not statistically significant. However, we never obtained better results with a longer incubation time and that is the result we want to point out.

the one of the same publication.

After the use of the right membrane, it has been observed that there was a difference between the results given with the membrane method and the centrifu ation and aspiration of the supernatant. The maximum binding was even lower in the second procedure.

To check whether or not there was a nonspecific binding to the membrane, some tubes with a minimum percent of binding have been tested (everything in the tubes except the mammary preparation). The nonspecific binding was very low then.

In order to see if the higher protein concentration in the tubes would have affected the receptors (aggregates), the same experiments have been done with a 1/2 dilution of the mammary receptor solution, which was the protein concentration requested in the paper. There was no improvement.

The fact that most of the preparation has been stored in 100λ aliquots could also have changed the experimental conditions, so part of the preparation which has been frozen at a protein concentration of 90µg/ml, and in a larger amount (30 ml) was tested with no better results.

The problem could also have been an iodinated rat prolactin solution damaged by refreezing, so some fresh hormone was reiodinated and used at once. That did not change the results.

To see if the provided hormone was a component of the problem, the same experiments have been done with some fresh iodinated HPL which has been proved reactive by radioimmunoassay. It was not possible to obtain a standard curve, either.

CHAPTER V

DISCUSSION

I. Animal Breeding

When female rats weighing from 15 oz. to 200 g. were bred, the percentage of successful breeding was very low after a week of low protein diet, compared to the normal group. The difference was not so important with heavier animals, but still existed.

The histological observation of the vaginal smear allowed one to see that the cycle was very much disturbed (anestrus). On the other hand, and especially for the animals on the lowest protein intake (5%), even in the case of a positive vaginal smear, the autopsy performed after every bleeding showed, in some cases, resorption sites of the fetus in the uterus, or no fetuses at all.

These facts show the importance of a lack of protein which can be detrimental to various hormone productions, acting either through the hypophysis or the ovary (39). It seems, however, that the endocrine function of the rat placenta is not impaired by the absence of dietary protein, as it has been shown in bioassays (26), that the difference of reaction among heavier and older animals can be explained by the fact that the second group has much more protein in reserve, so protein deprivation would not have an effect so quickly as in young animals.

Also, these older animals have their reproductive cycle much more well-established, and thereby are less sensitive to any external stress.

Although the number of animals observed (150 rats) does not allow one to draw any statistical conclusions, it seems that anestrus becomes

permanent in the younger female rats put on a low protein diet for more than three weeks, even if they are put back in a normal diet.

Piasek and Meites (40) have shown that rats made anestrus by restricted food intake could be made to go into estrus by 24 hours light (through hypothalmic action). Steroid therapy can also reverse the effect of this deprivation.

It seems also that a hypothyroid state can also cause some perturbations to the reproductive system. This is well known in humans, but there is no evidence to show whether it is a specific effect or the result of a general effect of the thyroid on tissue metabolism. It is assumed that the gonadotropin levels decrease in hypothyroid rats, and also that hypothyroidism could effect directly the endometrium (27).

In both cases (hyperthyroidism and low protein diet), when the breeding was successful, there was no difference in the size of the litter when compared to the normal group.

II. Radioimmunoassay

The first point which appears clear is the importance of setting up the standard curve in exactly the same condition as the one in which the unknown sample is going to be.

It seems that there is an inhibitory effect of a substance present in the serum, on the percent of binding of the hormone to the antibody, but this effect would increase with the time of the incubation. This would involve a destruction of the protein hormone which would be unstable under the experimental conditions. It may also be possible to say that the sensitivity of the standard curve decreases in the case of dilution of the

total hormone in srrum instead of buffer, although this sensitivity of the method is much higher than that necessary to assay HPL in blood, and would allow us to detect some minute changes.

III. Radioimmunoassay: Time Study

In the case presented in Figure 4, the negative effect of a long incubation is very striking. This difference, which appears only in the case of dilution in serum and not in buffer, is consistent with the results shown in Figure 3 and leads to the hypothesis of the presence of an "inhibitor" in the serum which is revealed or reacts only after a certain amount of time. (At least 16 hours of incubation are necessary.) It would act either by destroying the protein hormone, denatured because of the special medium conditions created by the serum, or could possibly be another hormone which would compete for the binding.

It is known, for instance, that the clinical structure and the biologic and immunologic activity of the growth hormone is very close to the one of HPL (17), but there is no evidence to say why it would not react with the same kinetics.

IV. Rat Placental Lactogen and Radioimmunoassay

The fact that rat placental lactogen could not have been assayed in this case is due to a problem of cross-reactivity. A study of this problem was done b y J. Gusdon et al (41), using hemagglutimation inhibition. They determined the relative degree of antigen sensitivity to human placental lactogen in placental extracts of different animals.

To detect a minimum amount, 174 times more rat sera than human was necessary. It seems also that this protein is very easily degradable, the

testing of its activity being less after six months storage at -20°C.

Thus, a radioimmunoassay for the rat placental lactogen will only be possible when the hormone is isolated and purified.

V. Radioreceptors

Every step after the resorption of the membrane once it has been thawed has been checked: from the assay of different protein concentrations, different conditions of storage, and use of HPL already tested by radioimmunoassay. Also the material used has never been frozen more than once because we saw a very important decrease of the maximum binding in this case (from 50% to 16%). Then it seems that the difficulty took place during the membrane preparation itself. In this case, different factors were analyzed. In spite of the fact that this method was very time consuming, it seems that there was no destruction due to the temperature, for instance, because every step was performed in ice pocket. On the other hand, another hypothesis could be that there were some changes because the mammary suspension was made in two times. Indeed, the volume was too large to allow, for instance, centrifugation of the whole amount together, but as far as everything was kept in ice, that should not have made any difference. Also, the rotor used for the last centrifugation was not of the same dimension as the standard one, but the average radius was very close. The only other possibility would be a gross experimental mistake such as incorrect preparation of the buffer, but that also can be reasonably excluded.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The first goal of this study was to see the response of the placental lactogen production to different environmental conditions: malnutrition, more specifically, protein deprivation; and hypothyroidism. Because of a very fast reproductive cycle, the rat was chosen as a model for studying these different factors. Rats were put on special prepared diets and successfully bred. Some specific problems which developed are discussed. One of them was anestrus due to the diets. The animals were bled at different times during the pregnancy in order to see whether the hormonal production pattern was either retained, displaced, or suppressed as a consequence of the modification of the diet. Sera were collected and kept frozen at -30°C so as to be available for assay.

Assuming a cross reactivity between HPL and rat placental lactogen, an effort was undertaken to modify and shorten the HPL assay, with good results, but it was found that the cross reactivity between the two hormones of these species was not sufficient to allow the assay of the rat hormone by the method set up for the human placental lactogen.

The inhibitory effect of the serum on the radioimmunoassay reaction was explained and is discussed.

Radioreceptor assays were found to be very disappointing, but they probably can be improved and be more reliable, and then can be used to assay the collected samples.

CHAPTER VII

SUGGESTIONS FOR FUTURE RESEARCH

It would be interesting to study on a large scale the effects of environmental factors such as hypothyroid status and low protein diet, or both, on fertility in order to determine exactly where and when the influence of the factor appears: lack of ovulation, lack of maintenance of pregnancy, either due to a central control, or a local one or both. Some of these questions may be answered when the assays on our present collection of samples from many rats have been completed. Other questions and experiments will doubtlessly arise from these findings when we have them in hand. Further studies could also be done systematically to try to shorten the incubation time of the RIA in genera. The time needed is very much dependent on the hormone. This could be more specifically its relationship with the temperature at which the reaction is incubated. It should be admitted, too, that no RIA would be possible as long as the rat placental lactogen has not been isolated.

The promises of the radioreceptor assay in this particular study have not been sustained thus far. Further studies are in progress in our laboratory to define the optimal conditions and make it more reliable in order to assay the serum samples which have been stored. The direction of further experiments for the radioreceptor assay could probably be done by using another rabbit strain (the one used was New Zealand albinos). It would be useful also to be careful in eliminating the fibrous part of the mammary tissue, during the Virtis homo generation step. It could have been possible that this elimination was too exclusive and so eliminated a part of

the membrane to give less receptor available. One of the first tests to perform could be also to see whether the method, once a new inbane preparation will be available, works when no serum or a very small quantity is added, although the sensibility of inbane receptor should be much lower than the RIA.

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BIOGRAPHICAL NOTE

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