The Detection of Auto-Immune Thyroiditis in Dogs

by

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Bioengineering

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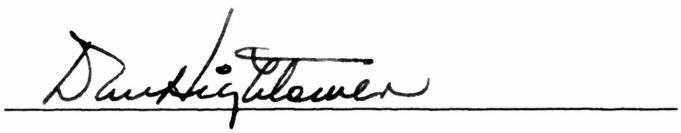
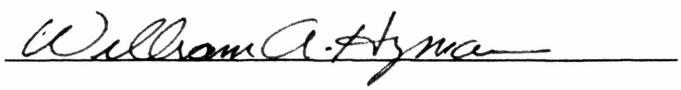
Approved by:



Dr. Dan Hightower



Dr. William Hyman

April 1980

Abstract

A micro Ouchterlony gel diffusion test was adapted for detecting auto-immune thyroiditis. The Ouchterlony test was standardized by testing crude thyroid extract against antiserum from an immunized rabbit. The rabbit was sensitized IV by three seperate injections of the crude thyroid extract and finally fully immunized with an injection IM by a 1:1 solution of crude thyroid extract and incomplete Freund's adjuvant.

A clinical sample of dog serums of low T4 and normal T3 levels which were suspect of thyroiditis and were close to being Hypothyroid were tested by the Ouchterlony test. No lines were produced when the antiserum from 16 dogs and crude extract were in the gel diffusion wells.

The negative results indicate that the dogs of clinical interest did not have thyroiditis and also that there exists a second mechanism for Hypothyroid development other than thyroiditis.

The validity of the results were not checked by actually testing a thyroiditis dog but by assuming values for the chances that the test worked; the probability that the test gave negative results when all dogs tested were actually thyroiditis turned out to be low(P=.5,.01% chance of wrong results and P=.2,4.4% chance of wrong results).

Some tests and further research is suggested and should be directed toward quantifying the thyroiditis test by inducing thyroiditis in a dog and using that as a model of the disease. Also a more sensitive test such as a radioassay

method (11) should be employed.

Acknowledgments

I would deeply like to thank all who helped me complete this project. Special thanks goes to Dr. Dan Hightower for "advising" me with his elucidating insights in thyroid diseases. Thank-Yous are also extended to my "other" advisors; Dr. James Smathers who struck it rich in the California gold rush(or is it blue and gold for UCLA) and to Dr. William Hyman, who allowed me maximum freedom in my research endeavor and never questioned why I was not doing an engineering project.

A special acknowledgment is extended to Dr. McConnell who provided much needed assistance and information.Along that same line, the help that Dr. Amos, Dr. Samoll, Mr. Riggs and the "crew" at the lab gave was extremly helpful.

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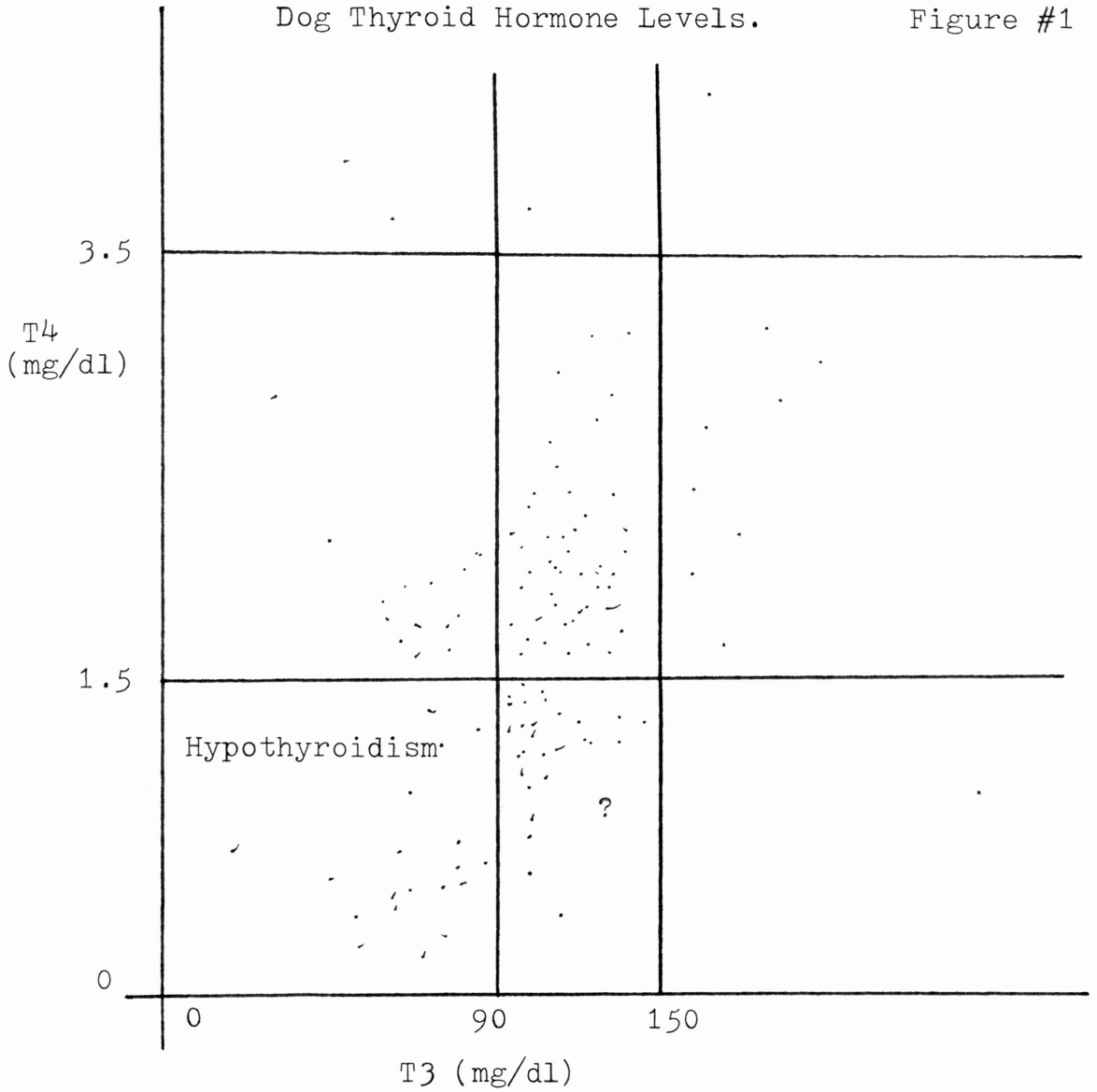
Introduction

In clinical observations the correlation between diseases and symptoms observed is most important. Based on clinical data obtained by Dr. Dan Hightower(TAMU Veterinary College), there was some indication of a correlation between suspected thyroid disease in canines, hypothyroidism, and thyroiditis.

Thyroiditis is an auto-immune disease most probably caused by leakage of thyroglobulin(18) which initiates a reaction in the body to deteriorate the thyroid gland. This results in a lower body metabolism, much like that of hypothyroidism as the thyroid gland is the site of metabolism control(9). The principle active secretions are 3,5,3' - triiodothyronine(T3) and thyroxine(T4). T3 is the most active of the two for an increase in body metabolism. T3 and T4 are secreted from the follicles of the thyroid gland where thyroglobulin(an iodinated glycoprotein) is found. It is the breakdown of the follicles and the resulting release of thyroglobulin which is the catalist for thyroiditis(18). When thyroglobulin is released and thyroiditis develops, degradation of the gland occurs caused by the reaction between antibodies in the blood stream and the thyroglobulin protein. Since the T3 and T4 levels most probably would decline in this condition, it is hypothesized that thyroiditis is a precurser to hypothyroidism. In hypothyroidism, normal functions of the thyroid gland are inoperable which result in a lower iodine uptake and hence a low T3 and T4 output in the body as the iodine is an essential chemical which initiates development of T3 and T4

Dog Thyroid Hormone Levels. Figure #1



?= undefined area

90-150(mg/dl) is the normal range of T3.

1.5-3.5 is the normal range of T4.

Data represents typical clinical results.

in the thyroglobulin protein.

The clinical sample of interest as shown in diagram #1 was a group of dogs that have low T4 and normal T3. In the clinical data, the sample of low T4 and normal T3 dogs are close to the dividing line of the hypothyroid criteria and the hypothyroid dogs lie close to the low T4 and normal T3 undefined box.

It has been shown also that in the humans there is a decreased level of T4 and near normal level T3 in hypothyroid Hashimoto thyroiditis affected people(7). It is thought that this may well be the case with dogs. If this is the case, then the dog sample of low T4 and normal T3 will have a thyroiditis condition. It has already been established that thyroiditis does occur in dogs but proven only in a laboratory controlled population. It is therefore important to see if this large group of clinically undiagnosed dogs are thyroiditis for a better understanding of thyroiditis, hypothyroidism, thyroid hormone levels and generally a modeling and understanding of thyroid diseases in dogs.

To test the clinical group, first a test for thyroiditis had to be developed. This test was developed by using an established method for antigen antibody detection which is also used extensively in clinical laboratories already, consequently, in could be adapted for thyroiditis detection. The test of choice was the Ouchterlony gel diffusion test which gives precipitin lines specific to each antigen antibody system present.

A Review of the Literature

Past research on dog thyroiditis has focused primarily on establishing the existence of thyroiditis through its frequency in laboratory controlled populations of dogs. The first known study was by Tucker 1962(17), who studied thyroiditis occurrence in 167 beagles and found a 16.2% thyroiditis population in his purebred colony. Mussler and Gaham(Feb.'68)(12) also studied the occurrence of thyroiditis and found a similar percentage with the majority of the affected population descending from one female who had thyroiditis.

The stage was set for finding a test to see if it exists clinically and how to distinguish it from other goiters and tumors. Fritz et. al.(6) studied thyroid ^{131}I metabolism on a kennel group of dogs to determine if it could be used as a test for Lymphocytic thyroiditis. They concluded that it could be used as a diagnostic tool. Their project was based on a beagle colony once again from a laboratory controlled environment.

Even though researchers were still using dog colonies, they seemed to be at least aware that there are similarities to human Hashimoto's struma. This was established in Bierwalter's et. al.(2) research. Their research was once again based on a beagle colony and also established that thyroiditis does occur(much like Mussler and Graham,1968(12)).

Meanwhile in human Hashimoto's struma research, the methods of clinical detection were fairly well established by the '60's. This was established by Doniach et. al.(5) in 1960.

Research on further tests were also performed. Research on "Precipitin Tests in Thyroid Disease", by Gouldie et. al., (8), showed that reaction of crude thyroid gland extract tested in a one dimensional precipitin test using agar gel in a test tube against serum of Hasimoto's disease, resulted in precipitin lines. The lines indicate the presence of antibodies. When checked with an Ouchterlony plate, two distinct lines appeared indicating two antibody sets.

Later in 1970, Reginald Hall(10) wrote on the various up to date test methods available. These tests included the precipitin tests, Coons technic and tanned red blood cell agglutination.

Another Hashimoto's clinical studyin 1972 by Gharib et. al. (7) found connections between hypothyroidism and Hashimoto's disease by looking at the thyroid hormone levels. It was found that Hashimoto patient's with hypothyroid disease had serum thyroxine decrease early and evidence for actual increaseof T_3 while the gland was failing but "no abnormality in the serum concentrations of the thyroid hormones specific for Hashimoto's thyroiditis was found"(7).

Other studies to better understand thyroid disease was performed earlier by Witebsky et. al. (16). The study had beendone on inducing thyroiditis and the cross reaction of thyroid antibodies with thyroid extracts from other species of animals. The results showed a positive reaction with thyroid autoantibodies of rabbits(produced from dog thyroid extract) and dog's crude thyroid extract.

No research had been done to date on the detection of auto-immune thyroiditis in dogs in the clinical realm, nor on the clinical group of interest here(low T4 and normal T3 serum levels).

Methods

To begin the research, thyroglobulin was needed because this particular glycoprotein is the antigen in the auto-immune response of thyroiditis. Thyroglobulin is a large iodoprotein(660,000 Daltons)(14) found within the thyroid follicle suspended in a colloidal solution. To liberate thyroglobulin, any technique that disturbs the follicles in the thyroid gland and thusly frees the colloidal suspension within, will provide thyroglobulin emacipation.

Using this concept, and after obtaining thirty biopsied dog thyroid glands , the method of liberation of the thyro-globulin was chosen. The procedure used was adopted from Derrian 1948(4) and is a crude thyroid extraction by use of a phosphate buffered saline solution. The process began with trimming the fats away from the gland and then mincing the gland up into small pieces. This process exposed the colloidal suspension where the thyroglobulin was stored in the gland. To make the solution, 46 ml of 7.6 ph .01M PO_4 buffered saline solution was added to 15.2 grams of minced gland for a solution of 3 ml of PBS to 1 gram of tissue. This was left over night in the refrigerator. The next day(21 hours and 45 minutes) the mixture was centifuged at 3,000 r.p.m. for 30 minutes. The supernatant(33.9 ml) was decanted into a beaker and frozen for storage. The supernatant(crude thyroid extract) appeared as a pink clear liquid which is what Gouldie et. al.(8) suggested that their crude extract looked like.(Note: at this point it is assumed thyroglobulin is present; no qualitative

tests were performed.)

A word about the choice of PBS and the crude extraction method; it was chosen because it was simple to do and did not require alot of time. The first attempt to obtain pure thyroglobulin took too long. Basically, the procedure started with a crude extract only this time in distilled water. A Sephadex column(G-200) was used to try and isolate the large iodoprotein.The flow rate was near .5ml a day a procedure all to slow for a project of this magnitude to utilize, so it was aborted. This process theoretically should have worked but therewere too many variables to look at and not enough time to try and correct the procedure.

The next prcedure was to immunize the rabbit to build antibodies to thyroglobulin. The technique employed was recommended by Dr. McConnell(TAMU Vet. College). To immunize, care was taken not to induce shock in the animal, so it was important to sensitize the animal first before massive immunization could take place.

The animal used was a big white rabbit(15-18" long, weight unknown). The injection method called for three beginning shots intra ven ously in the ear. This was a delicate procedure and the rabbit had to be still to accomplish it. First the rabbit was caught. In this procedure care had to be used sincethe rabbit had long sharp claws and powerful front legs. Grabbing the ear/neck region and simultaneously grasping the hind legs and sweeping them behind the animal (to immobilize it), the rabbit was quickly brought out of the

cage. A rabbit box was used to hold the animal in place. The box(about 2' long and 8" wide) had a neck restraining area made of a "u" shaped retainer on the bottom where the rabbit's neck is placed and a bar is then placed over the neck to lock the rabbit in position. The hind legs were brought out behind the animal and a bar was put over its rump area to further restrain the rabbit.

The injection was then made IV in the ear. The hard cartilage was felt for first and(after the area was shaved) a vein was located. The needle(TB size) was inserted into the vein and the plunger slowly depressed. Those veins were so small that when the injection was correctly inserted, the shot contents displaced the blood and the vein turned to a white color which indicated a successful injection.

The schedule of injections started with .2 cc of the crude thyroid extract followed by .5 cc(on the third day) and 1 cc(four days later) all IV. Then to boost up the antibody production, 7 days after the last IV shot, 2 cc of crude thyroid extract mixed with 2 cc incomplete Freund's adjuvant was injected IM. The adjuvant is a water in oil emulsion that enhances antibody production(3). The location of injections were in the rump, the left and right upper leg and finally in the right back muscle. The procedure was different from IV in that now the needle was considerably larger and the muscle was tougher, so the jabbing technique was used to quickly pierce the muscle (in one quick dart throwing motion) and inject. The rabbit actually yelped on this injection set.

Nine days after the last injection, cardiac puncture was performed to gain some serum for antibody titre studies. The holding box could not be used for this procedure since now the animal had to be laid on its back(supine position). Instead of in the box, the rabbit was held securly by hand by grasping its hind legs and front legs and stretching and pinning the rabbit down. For cardiac punture, the location of the heart was crucial to prevent mutalation of the animal. First, the Xiphoid process was found, then the last sternal rib to the left of the midline was located. Holding the needle at a 30^{o} angle, entry into the heart is accomplished. Dr. Samoll (TAMU) helped with the procedure to collect 7 cc of blood.

The 7 cc of blood had to be prepared so the antibody content could be analized. The serum of the blood was where the antibodies could be found, so serum was obtained from the sample. The method here was to first wait one hour for agglutination to occur and then to slice up the clots and leave it sit over night at $4^{o}C$. The liquid was then decanted off and centrifuged at 35Xg for 15 minutes. 4.4 ml of serum was gotten from this procedure.

Testing

To test the presence of the antibodies, both in the immunized rabbit(simulating an auto immune condition) and the clinical sample of dog serums, the Ouchterlony gel diffusion was chosen. This test was highly specific for different antigen antibody systems and was useful in determining kinds of antibodies present. Positive results in this test were characterized by the formation of a white precipitin line. The micro procedure chosen started with a microscope slide on which a warm agar(.9-1%) solution was poured(from a pipet). After it turned into a gel, cylindrical holes were bored out to form recepticles for the antigen and the antibody. The antigen and antibody then diffused outward until they formed a precipitin line. The observations were made for up to three days with the use of a magnifying glass and a optical light. This test had been used in past Hashimoto's studies(10) and was also in use in clinical laboratories, consequently, in could be adapted for thyroiditis detection.

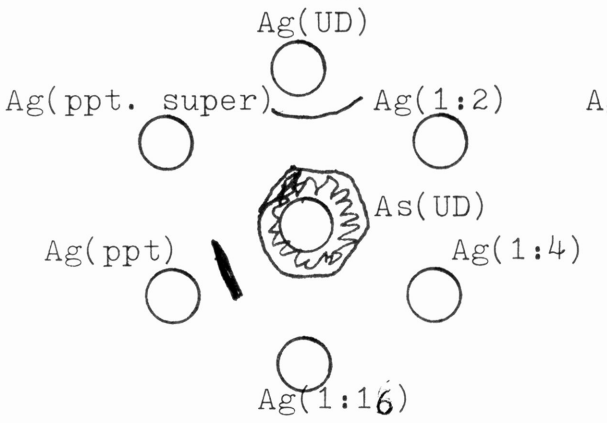
To standardize the Ouchterlony test to see what kind of results to look for, the rabbit serum was tested against the dog crude thyroid extract. The results showed a continuous line around the center well(the rabbit serum) and two lines by the extract solution(in undiluted form) and by the precipitated concentrate derivative of the extract. The precipitated form was made by adding Polyethylene Glycol to the crude extract solution to precipitate out some of the proteins. The precipitate was put back into solution to make a very

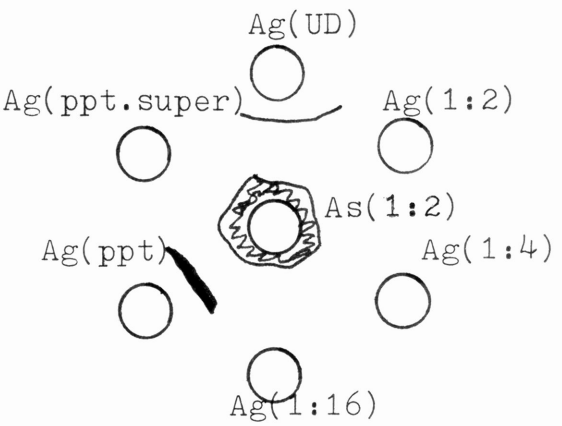
Rabbit Ouchterlony test. Figure #2

Ag=Antigen
As=Antiserum

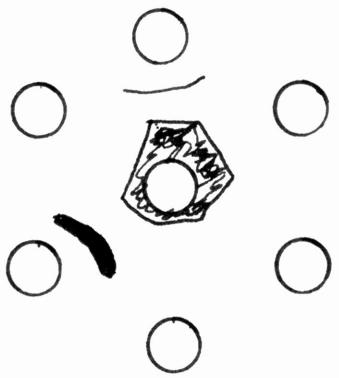
( )= dilation amount
ppt= precipitated derivative.

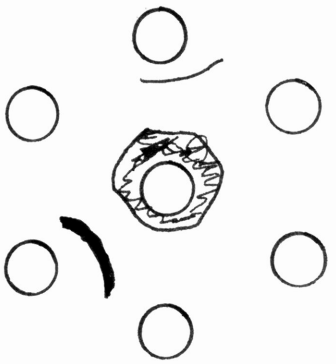
In a PBS agar.





Wells are the same. The agar is now Borate buffered.





concentrated form of the extract. The supernatant was used also. The two seperate lines indicate a possible thyroglobulin antibody precipitin line since it appeared by the concentrated precipitated version and the other most concentrated, the undiluted extract(of which the line was very faint). This being the case, the seperate lines were the positive result to look for when testing the dogs.

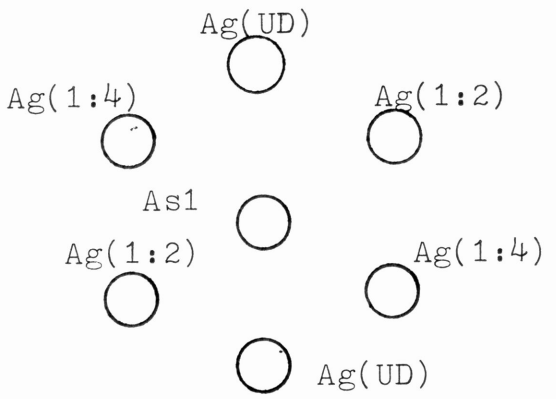
The first test performed was with three dogs, two of the clinical group of interest and one normal dog. No lines appeared(see illustration 3) between the dog serums and the crude extract, but a strange result was encountered; lines appeared between the two dogs of the low T4 and normal T3 group. Later these two dogs were tested against each other again and no lines appeared between the two. Certainly these results are mysterious and should be looked at more carefully in the future. One possible and logical reason a line formed was that the two dogs were in different stages of development of thyroiditis and so one had more thyroglobulin in the blood stream(the initiator of the auto-immune disease) and the other had already developed the antibodies. If this were true, then lines should have appeared between the lines of extract as well. No explanation can be thought of for why the lines disappeared in the second running of the test.

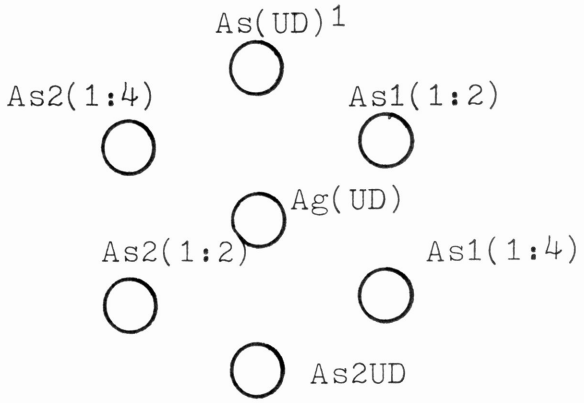
In the second running of the first two dogs, two new ones were added and these two had no lines produced.

The third testing group of twelve new dogs(this time run with the rabbit serum in one well to make sure the test was

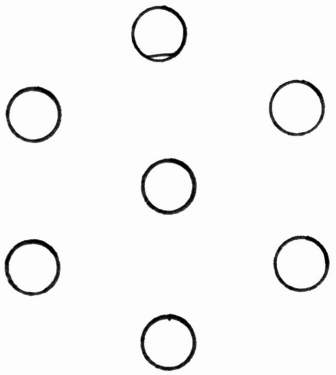
Test set number one. Figure #3

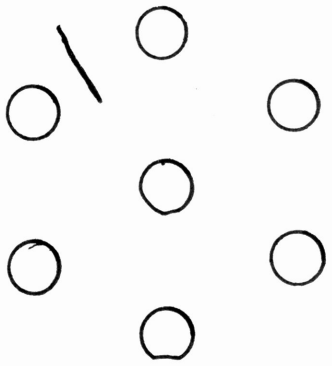
0.9% agar



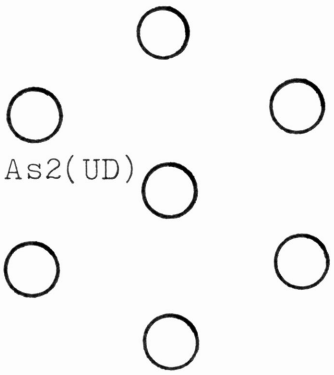


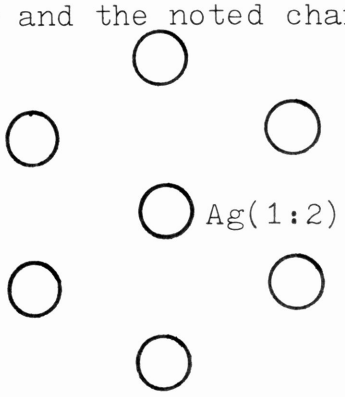
Same Wells but now in 1% agar.





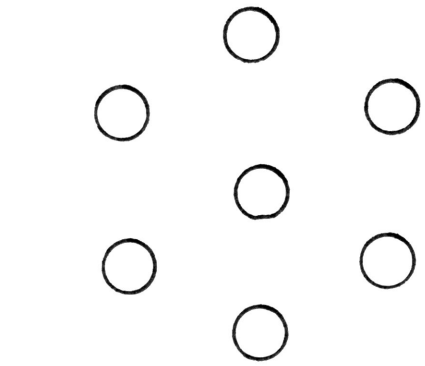
Same Wells again but now in .9% agar and the noted changes.

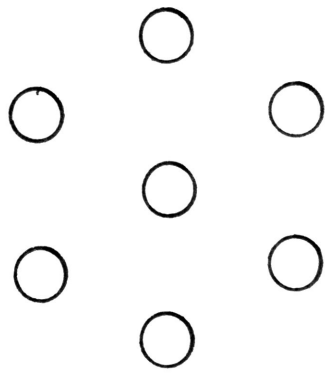


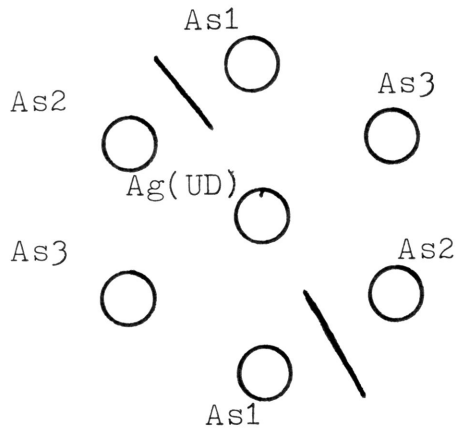


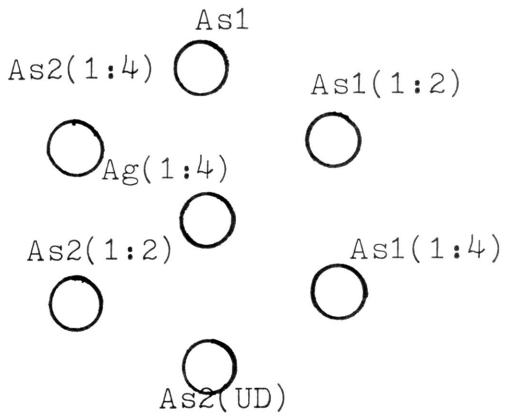
(Test set #1)(Figure #3 continued).

Same wells as last two,but now in 1.0%agar.





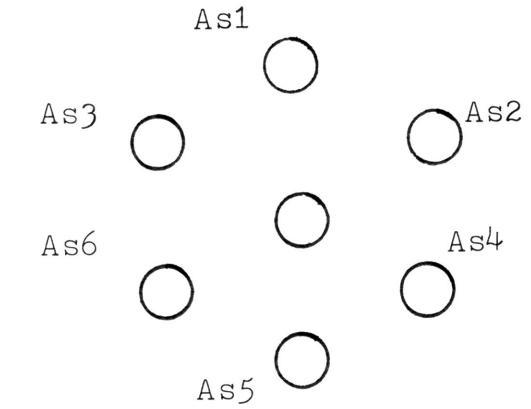




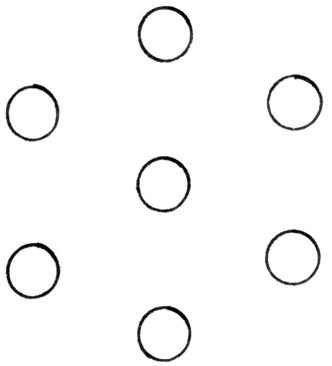
Test set number two.

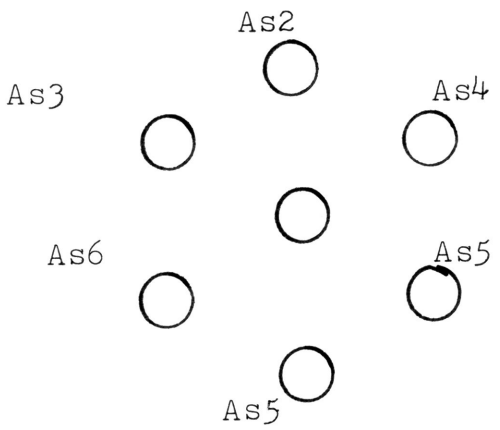
Figure #4

Center well= Ag(ppt)

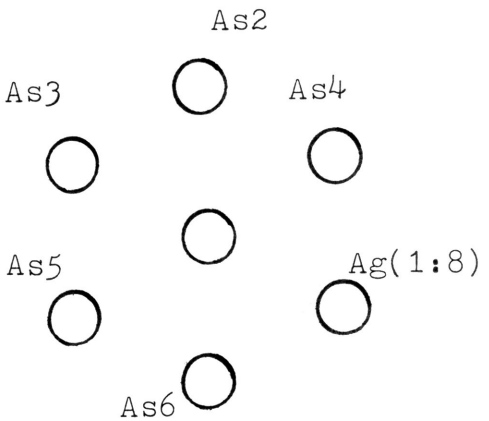


Same well set up.
Center well= Ag(ppt super)



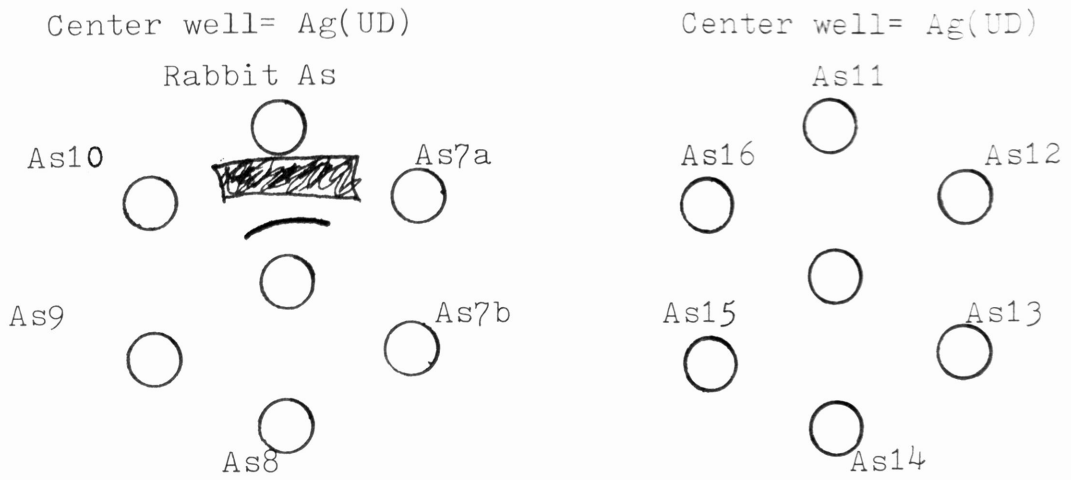


Center well Ag(1:16)

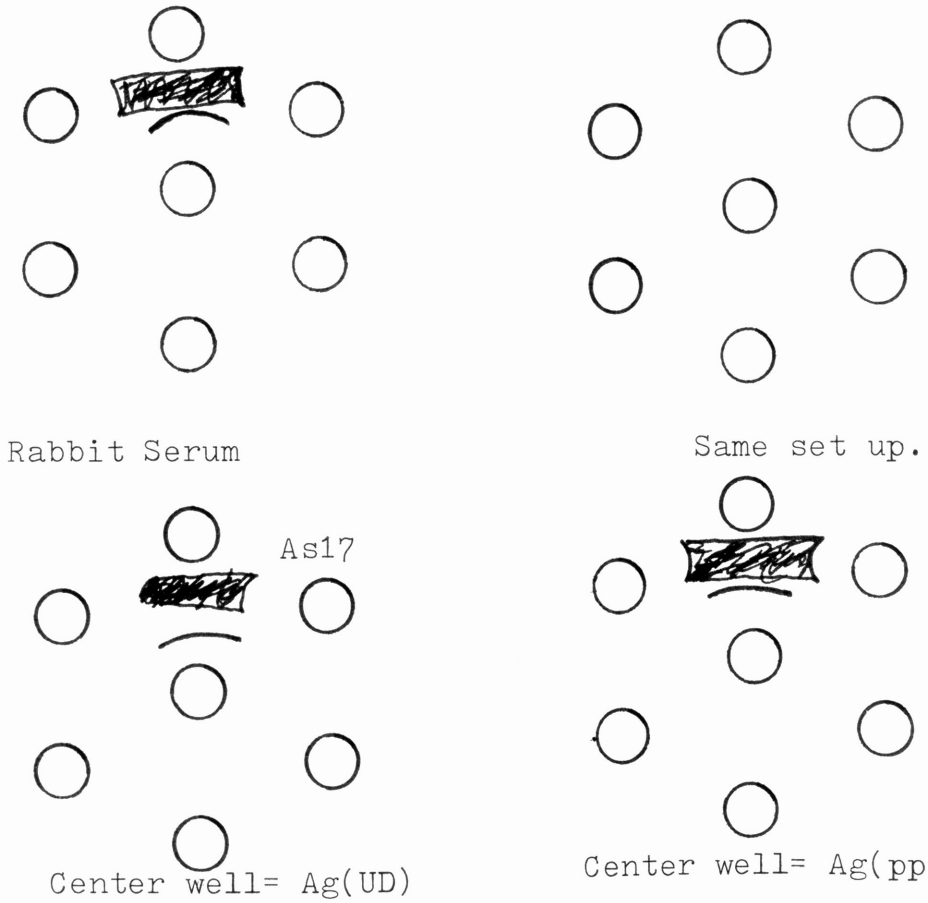


Center well= As1

Test set number three. Figure #5



Same well set up, but now center well is Ag(ppt).



Test dog's Thyroid hormone levels. Table #1

As	T3	T4	
#1	75	2.15	
#2	80	1.85	
#3	105	1.85	(Normal test dog)
#4	113	1.30	
#5	185	1.30	
#6	123	.800	
#7a	35	0.55	
#7b	39	0.40	
#8	100	1.15	
#9	49	0.65	
#10	163	2.0	
#11	138	2.50	
#12	111	2.45	
#13	92	2.00	
#14	129	1.60	
#15	88	1.45	
#16	75	0.75	
#17	100	3.90	

working) produced once again, no white precipitin lines.

Conclusion

The results indicate that the clinical group of dogs of low T_4 and normal T_3 are not thyroiditis. This conclusion further indicates that thyroiditis is not the mechanism in this case to develop hypothyroidism. These conclusions are based on the premise that the tests were valid. The validity of the tests to detect auto-immune thyroiditis has not been proven to work for dogs, but it can be concluded that indeed the test is valid since it worked for the rabbit. Also, one can not assign a number to how accurate the test was since a large sample of dogs was not tested and the actual disease of the dogs was not known for certain(it would help to test a known thyroiditis dog).

Mr. Riggs of the Statistics Institute of Texas A&M University suggested a probability analysis of the results obtained. The basis was to assume a probability of the test and then to calculate the probability that the test gave negative results when the dogs were actually thyroiditis. Letting

P=Probability that the test detects thyroiditis in dogs(an assumed value)

then with a population of 14 dogs(discounting the first two because of their strange results), the probability that negative results were obtained when all dogs tested were thyroiditis is

$$(1-P)^{14}.$$

Using a programmable calculator (Hewlett Packard 19C) the results were computed(shown in Table #2). The numbers indicate that if the test were 50% accurate, then there is a .01%

Probability Analysis. Table #2

Programmed on a Calculator.

PA= Probability of test.

PW= Probability that the results are wrong.

Results

PA
PW

Results

PA
PW

chance that the test gave wrong results and for 20% accuracy there is a 4.4% chance that wrong results were obtained. These results are encouraging but once again not conclusive.

The results and conclusions leave the veterinary world with two implications. The first is that the low T4 normal T3 group is still undefined and has some other disease. The second implication is that there must be another mechanism to hypothyroidism. For the latter implication, two possible mechanisms are suggested by Werner et. al.(18); that of the blockage of the pituitary which releases TSH(a hormone to stimulate production of T3 and T4) and a blockage of the Hypothalamus so it cannot release TRF(which regulates the pituitary release of TSH to some extent). Therefore, the disease in the low T4 normal T3 group could be a pituitary malfunction or a nervous system malfunction(Hypothalamus) or some other thyroid disease.

Many questions have been raised and now hopefully more research will be performed to study the results, conclusions, and implications of this study. Several modes can be suggested for future research. The first to be presented is a recommendation that the thyroiditis tests be verified by some means such as possible induction of thyroiditis in a dog to see if the test works. Another study should also be directed in using a different more accurate test. The test in mind is a solid state competitive Binding Radioassay technique used in 1970 by Mori, Fisher and Kriss(11) for studying LATS. They concluded it could easily be adapted for thyroglobulin research.

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