A DEMONSTRATION OF FLUORESCENT ANTIBODY STAINING TO LOCALIZE ENDOGENOUS JUVENILE HORMONE-LIKE MOLECULES IN SELECTED TISSUES OF THE AMERICAN COCKROACH, PERIPLANETA AMERICANA

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thesis entitled

A DEMONSTRATION OF FLUORESCENT ANTIBODY STAINING TO LOCALIZE ENDOGENOUS JUVENILE HORMONE-LIKE MOLECULES IN SELECTED TISSUES OF THE AMERICAN COCKROACH, PERIPLANETA AMERICANA

presented by

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ABSTRACT

A DEMONSTRATION OF FLUORESCENT ANTIBODY STAINING TO LOCALIZE ENDOGENOUS JUVENILE HORMONE-LIKE MOLECULES IN SELECTED TISSUES OF THE AMERICAN COCKROACH, PERIPLANETA AMERICANA

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Dicyclohexyl carbodiimide reacting with epoxy farnesenic acid and keyhole limpet hemocyanin yielded a conjugate with at least 6.4, and probably over 20, molecules of hapten per carrier protein molecule. Rabbit-formed antibodies to both hemocyanin and to the epoxy farnesenic acid hapten were identified. The epoxy farnesenic acid antibodies were quantified periodically to plot the decline from a peak 22.1 μg/ml after the last booster, to 4.6 μg/ml 5½ months later. The plotted decline was consistent with the general immunological predictions and further, yielded original data on how this specific small hapten responds in rabbits. Anti epoxy farnesenic acid serum was applied to indirect

fluorescent antibody staining of adult cockroach corpra allata tissues and hemolymph smears to produce specific fluorescence. Most likely, this specific fluorescence is localized at the sites of endogenous juvenile hormone and/or its metabolic precursors that have very similar molecular configurations.

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A DISSERTATION

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1974

Dedicated to Luanne and Scott: a wife whose encouragement and flexibility of life style were constant reinforcement throughout this research and a son whose budding enthusiasm for science is a source of delight and hope for the future.

ACKNOWLEDGMENTS

I would like to thank my Chairman, Dr. Roger Hoopingarner, for his willing counsel and advice throughout this research and for providing laboratory facilities for a substantial part of the work. For guidance and advice in immunochemical methodology, I would like to extend appreciation to Dr. Richard Patrick who also provided key assistance with assay problems, and to Dr. David Bing for overseeing the initial design of the immunological work. I would also like to recognize the contributions of Dr. George Nellis for providing juvenile hormone analogs and isotopes as well as advice on chromatography and epoxidation procedures, and Committee Members, Dr. Matthew Zabik and Dr. Stanley Wellso, who provided advice and guidance throughout the course of this work.

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available laboratory space, research equipment, specialized reagents and animal housing facilities to support this research.

A special debt of gratitude has been earned by my wife, Luanne, for her moral support as well as valuable contributions in drawing the diagrams, typing the manuscript and proofreading.

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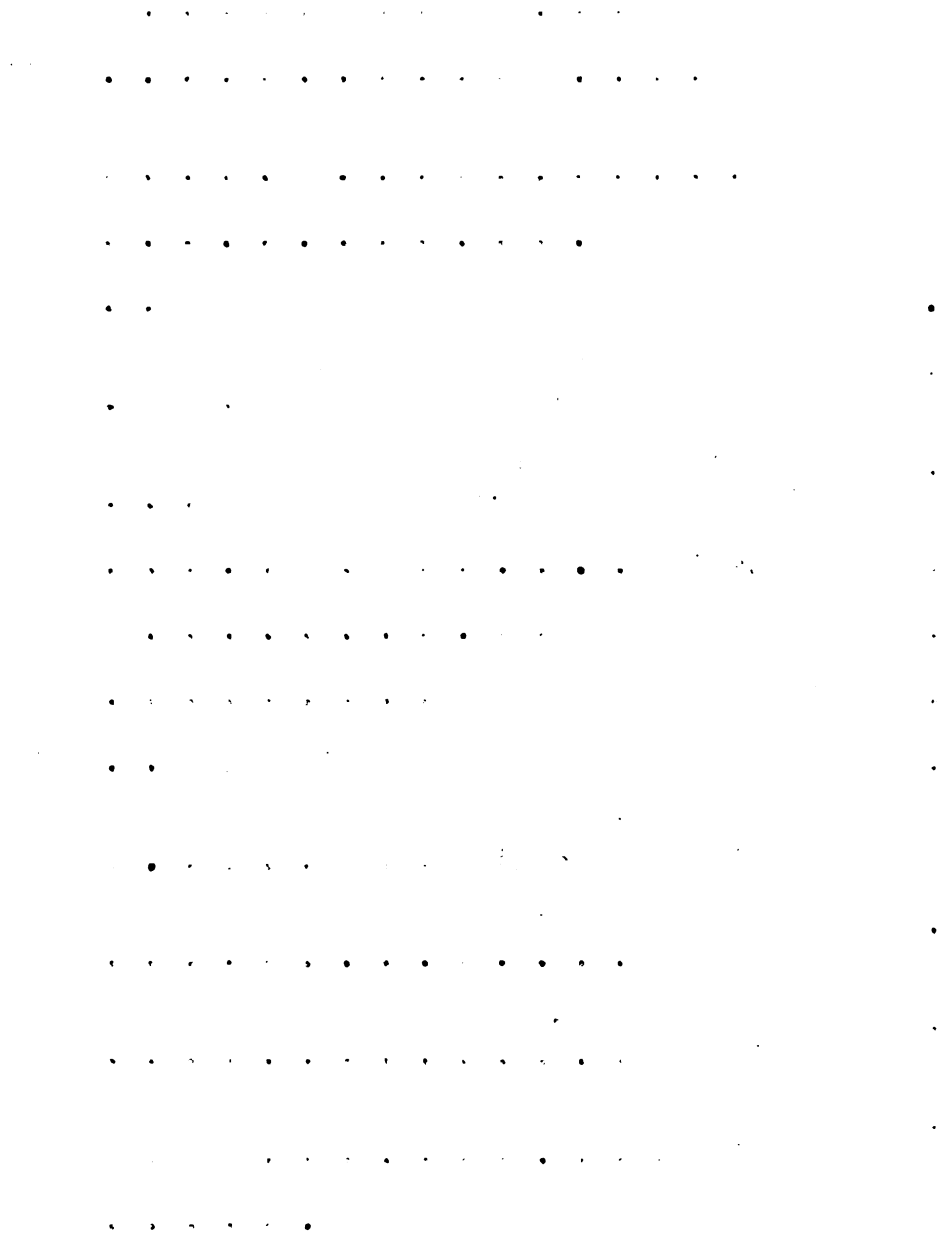

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INTRODUCTION

The ability of an antibody to selectively bind with a specific molecule or portion of that molecule (determinant) has given rise to its application as a tool of metabolic investigation. With a conjugated fluorochrome, the antibodies in such a solution can be used to produce a distinct fluorescence when excited by incident UV light. The location of antigenic molecules can be determined by controlled application of this tagged antibody which binds with the specific molecule to give a fluorescing site.

Fluorescent antibody staining is one of several techniques allowing research to transcend the gap between the cellular level of the histologist and the molecular level of the biochemist. Thus, under optimum conditions the tissue location of specified molecules can be viewed microscopically. This technique has the advantage of using endogenous molecules (tissue antigens) rather than introduced radioisotopes of the antigen, whereas an alternate localization technique such as autoradiography requires the assumption that an exogenous tracer

molecule will respond in a metabolically identical fashion to its endogenous homologue. Once set up, routine fluorescent antibody determinations can produce results within hours versus several days to weeks for bioassay development or autoradiographic exposure.

Early workers first developed fluorescent antibody staining technique for the identification of human pathogens (Coons and Creech, 1942). As clinical demand increased the commercial availability, its diversification as a research technique expanded. In the early 1960's fluorescent antibody localization was applied to many vertebrate metabolites. To cite a few examples: human anterior pituitary hormones (Cruickshank and Currie, 1958), pancreatic elastase (Moon and McIvor, 1959), chorionic gonadotropin (Midgley and Pierce, 1961), and vertebrate cytochromes (Reichlin, et al., 1966)--all of which were localized via fluorescent antibody microscopy. Emmart was one of a few to apply this to invertebrates as he investigated muscle enzyme localization in the cockroach (Emmart, et al., 1961).

Through this expanded research new or improved fixing, embedding, sectioning, and staining techniques were developed. As selected examples of the numerous

improvements, techniques were developed for freeze drying (Freed, 1955), paraffin embedding (Sainte-Marie, 1961), and albumin embedding (Rahmen, 1972) of tissues retaining antigenicity. More permanent plastic mounting media with no autofluorescence was developed (McCurdy and Burstone, 1965). Column chromatographic separation of blood proteins facilitated purification of antibody (Levy and Sober, 1960), while analysis of preparation techniques for fluorescent antibody reagents emphasized safeguards to minimize error (Kaufman and Cherry, 1960, and Lewis, et al., 1964) New applications were developed to use antisera for quantitative fluorescent antibody scanning (Mansberg and Kusnetz, 1966) and for electron microscopy immunoferritin staining (Pepe, 1961, and Tougard, et al., 1973).

The most useful methods of this new technology were consolidated in several books in the early 1970's. The most extensive is the four volume series on immunochemical methodology edited by Williams and Chase (1967-1971). Weir (1967) and Campbell, et al., (1970) produced less extensive texts on immunological techniques.

With immunochemical advances in conjugation techniques it has been proven possible to make specific antibodies to biologically active molecules

much smaller than macromolecular proteins and polysaccharides commonly involved in pathogen or enzyme identification. Lacey and Davies (1958) succeeded in making antibodies specific for insulin. In 1964 Churchill and Tapley reported antibodies specific for thyroxin. Midgley, Niswender and Ram (1969) used human steroid antibodies for routine radioimmunoassay. In 1972 Borst and O'Connor reported making antibodies to insectan ecdysterone for application to radioimmunoassay quantification. With the recent successes in forming antibodies to these haptens, it became feasible to attempt antibody production to the even smaller juvenile hormone analogue, 10-11 epoxy farnesenic acid (epoxy FA).

It was the goal of this research to first form antibodies to epoxy FA and then use the antisera to demonstrate fluorescent antibody staining of endogenous juvenile hormone or precursors in insectan tissue. These would be the first steps in developing a tool that potentially could have a diverse range of applications in juvenile hormone (JH) study.

Initially, the fluorescent antibody staining could confirm correct and/or expand upon the available information based on radioisotope localization of JH in tissues (Gilbert, 1967; Riddiford and Ajami, 1973;

Emmerich, 1973; etc.). Classical ligation experiments with confirmation and quantification of the JH in various developmental stages could be repeated (Wigglesworth, 1970).

Fluorescent antibody techniques promise to provide more rapid and potentially more accurate methods of quantification of JH. Picogram quantities of JH extracted from insect tissues could be measured within hours via isotope inhibition radioimmunoassay as compared to several days for bioassay results (Borst and O'Connor, 1972).

Application of similarly formed antibodies to tag molecules of the JH mimicking, potential third generation pesticides, could lead to more convenient assay methods when studying the metabolic effect of these chemicals on various animals (Williams, 1967). It could contribute to several prime areas of current academic interest in JH metabolic roles such as embryonic cell reprogramming and regulation of larval structural changes (Willis, 1974). The identity and role of JH as a gonadotropic hormone and its interrelationship between the sexes (Connin and Hoopingarner, 1971) are promising areas for application of fluorescent antibody localization techniques.

Because of the small size of the hapten (one of

the smallest biologically active molecules to form antibodies to date), it would be a promising candidate for use in immunochemical studies on intrasite binding influences and limiting factors in cross-reactivity. If future work can demonstrate the limits of its cross-reactivity and increase its purity, antibodies to JH and juvenile hormone analogues could significantly expand the technology available for research in this area.

METHODS AND MATERIALS

I. Epoxidation of Farnesenic Acid (FA).

A quantity of 360 mg (1.5 mM) of farnesenic acid (Chemical Procurement Laboratories) was added to 10 ml dichloromethane (ca. 0° C) and maintained in an ice bath. To this was added 280 mg (1.6 mM) m-chloroperbenzoic acid, after stirring $1\frac{1}{2}$ hr at 0° C. The reactants were filtered through a Buckner funnel with a Wattman #1 paper disc and the soluble portion evaporated under reduced pressure to a residue. The residue was treated with minimal hexane and again filtered to remove any remaining m-chlorobenzoic acid. The concentrated residue was then passed through a 40 cm column (Fischer-Porter), 10.5 cm long and 1.5 cm in diameter, using silica gel (30-70 mesh) eluted with a 1:1 hexane: ethyl ether solvent. The flow rate was ca. 60 drops/min with 30 ml fractions collected and stored in the freezer until GLC identification and purity of the 10-11 epoxy farnesenic acid (epoxy FA) fractions could be determined. In all, ten fractions were collected (Bowers, 1965).

II. Conjugation of 10-11 Epoxy Farnesenic Acid (Epoxy FA) to Hemocyanin.

A preliminary trial and error series established that a 50% ethanol in distilled H_2O would dissolve all three components of the conjugation reaction without interfering with the reactants.

One hundred fifty mg (0.6 mM) of the previously synthesized epoxy FA was dissolved in 1.0 ml 100% ethanol with vigorous mixing. One ml of distilled H_2O was added and mixed. The resultant solution appeared clear with a uniform slight yellowish overcast that indicated complete solubility. An amount of 400 mg (0.5 mM) of a lyophilized keyhole limpet hemocyanin (Cal Biochem "A" grade) was dissolved through a stepwise addition of 88 ml of a .04 N NaOH solution in distilled H_2O. Eighty-five ml of 100% ethanol was gradually added with continuous mixing. The resultant solution was nearly clear with no sign of a precipitate forming after 12 hr at 4^{o} C. The epoxy FA solution was slowly added to the dissolved hemocyanin with continuous stirring. A nearly clear solution resulted with no evidence of precipitation. One thousand mg of dicyclohexylcarbodiimide (8.3 mM) was dissolved in 30 ml of a 70% ethanol in distilled H_2O. This was slowly added to the hemocyanin epoxy FA

solution with gentle stirring for a period of 30 min at room temperature. The reaction continued for 24 hr at room temperature.

Partition separation to remove the bulk of the less polar reaction products was carried out by the addition of 4 additions of 200 ml of ethyl ether and this reduced the aqueous phase to 88 ml. The aqueous phase was then dialyzed for 36 hr against 3 liters of borate buffered saline (BBS) at 4° C. A precipitate of hemocyanin formed in the dialysis bag during this time. A 9.7 mg/ml protein concentration determination of the precipitate-bearing portion of the dialysis bag content (33 ml) indicated that most of the hemocyanin (320 mg of 400 mg) was precipitated at this point. (Precipitation was probably due to reassociation of the molecule as the pH returned to the 8.4 of the BBS.) Both the precipitate and the liquid fraction from the dialysis bag were sealed and frozen for storage at -20° C.

III. Hydrolysis of 10-11 Epoxy Farnesenic Acid (Epoxy FA) from Hemocyanin.

One ml of the conjugated hemocyanin epoxy FA precipitated hemocyanin fraction, having a protein concentration of 9.7 mg/ml $\frac{9.7 \text{ mg}}{7.5 \times 10^6 \text{ mg/mM}} = 1.2$ nM

was added to 1.0 ml of a 20% conc HCl in distilled H_2O (2.2 N). This was maintained for 30 min at 50° C in a water bath with a foil cover over the test tube to minimize evaporation. The cooled hydrolyzed FA products were separated from the aqueous fraction by ethyl ether extraction using three 1 ml repetitions.

IV. Immunization.

The initial injection material was prepared using a 1:1 volume ratio of antigen to adjuvant. The antigen, 24 mg of the conjugated hemocyanin-epoxy FA in 3.0 ml buffered saline, and 3.0 ml of the warmed (38° C) mixed Freund's complete adjuvant were combined by using a sterile emulsifying needle and a 10 ml syringe. Alternate 0.5 ml aloquots were introduced and emulsified until all 6.0 ml had been added. Emulsification continued 15 to 20 min to yield a homogenous product of sour cream consistency that would not separate and would not disperse when placed on a drop of water. The resultant 6.0 ml volume, having 4 mg/ml concentration of conjugate, was refrigerated at 4° C in a covered vial until use 4 to 14 days later.

After preliminary ear tatooing for identification, clipping of toenails and overall health inspection, generous normal blood samples were collected via ear

bleeding (ca. 50 ml x 2, yielding ca. 50 ml whole sera) from each of four 8 month old New Zealand white female rabbits. The rabbits had an average weight of 2.5 kg. The pre-immunization normal blood was coagulated and centrifuged, and the resultant whole serum was stored in sealed tubes at -20° C for future use as control serum.

Prior to injection, the fur was clipped from the hind foot bottoms over a 1 cm^2 area and the foot pad was swabbed with 80% ethanol. The first rabbit (JH-1) was injected with a sterile #20 gauge needle and a disposable 2.5 ml syringe. An injection of 0.25 ml of the 1:1 Freund's adjuvant conjugated hemocyanin epoxy FA emulsion was introduced into each foot pad, taking care to avoid bones or large blood vessels. The raising of a bleb on the surface indicated that the material was localizing. At the same time, 0.25 ml of the emulsion was injected subcutaneously into each side of the neck. Close observation of the animal's health was taken for the next 48 hr. Some swelling of the foot pads and perceptible lumps of swelling in the neck region were evident, but the overall health of the rabbit appeared to be satisfactory.

This procedure was repeated with JH-2 and JH-3 eight days later and subsequently with JH-4 after

another three day interval. The stepwise initial injection sequence was used to allow for observation of any deleterious effects of immunization in time to prevent any possible loss of test animals. The rabbits were inspected daily for signs of ill health but with the exception of short term minor nasal discharge or conjunctivitis on several occasions, they remained in good health.

Booster adjuvant of a 10% aluminum hydroxide in H_2O was made by dissolving 5.0 g aluminum ammonium sulfate in distilled H_2O and adding 0.1 N sodium hydroxide until the pH reached 7.2. The resultant precipitate was centrifuged and washed twice in phosphate buffered saline (PBS) with a pH of 7.2. A quantity of 0.41 ml of the conjugated hemocyanin epoxy FA was added to 0.82 ml of the aluminum hydroxide and centrifuged to bring the total volume to 0.60 ml and thereby increase the viscosity to a gel consistency. This was used the same day for booster injections of 0.15 ml (1.0 mg antigen) which were placed subcutaneously in the neck of each rabbit. The booster procedure was repeated each 6 to 8 weeks (see Table 1. for the schedule of immunization).

Table 1. Schedule of Immunization.

Rabbit	JH-1	JH-2	JH-3	JH-4
Initial injection	2/03/73	2/11/73	2/11/73	2/13/73
Booster #1	4/10/73	4/12/73	4/12/73	4/12/73
Booster #2	5/21/73	5/21/73	5/21/73	5/21/73
Booster #3	6/11/73	6/11/73	6/11/73	6/11/73
Booster #4	7/24/73	7/24/73	7/24/73	7/24/73

V. Blood Collection.

Blood collection via ear bleeding was selected as the least hazardous method to guard against rabbit loss over the anticipated long term collection period of a year or more (Campbell, 1970). The ear was dry shaved in a 1 cm strip back from the lower margin to expose the marginal veins. The shaved area was coated with a light layer of vaseline and swabbed with 80% ethanol. A 1 to 2 mm incision was made longitudinal to the vein with a sterile lancet and 40 to 50 ml of blood was collected. For each subsequent collection a new incision was made about 5 mm distally. After approximately ten collections the series was repeated using the other ear.

Methods of pressure at the ear base, a warm water bottle, and xylene on the ear tip were all used to enhance vein dialation and blood flow if required.

When xylene was used, care was taken to remove all traces with an 80% ethanol wash followed by soap, water and vaseline to prevent chapping.

Blood samples were collected about once a week from each rabbit. The blood was collected in clean 50 ml test tubes and allowed to coagulate at room temperature with a parafilm covering for 2 hr. The clot was then dislodged from the tube sides and allowed to shrink in the refrigerator at 4° C for 24 hr. The cool serum was carefully pipetted into centrifuge tubes and any remaining red blood cells were removed by centrifugation for 30 min at 1000 x g. The whole serum was pipetted into screw top test tubes and stored at -20° C in parafilm double sealed caps. Initial samples were separated by rabbit source, but due to the added work, later samples were pooled, storing all four rabbits' sera for a week in the same container.

Initially, samples were taken ten days after the original immunization and seven days after the first booster. After the second booster samples were collected nearly every week from each rabbit. The regularity of the early blood sampling varied somewhat with the time available, but following the second booster, a weekly and occasionally bi-weekly collection was carried out.

VI. Antibody Purification.

Serum albumin removal by ammonium sulfate precipitation of the immunoglobulin (Ig) protein fraction was routinely carried out using 10 ml fresh serum (or freshly thawed). With continuous stirring 10 ml of a cold saturated ammonium sulfate solution was slowly added. Stirring continued on the cooled solution for 15 min at low speed to eliminate frothing. The resultant precipitate was removed by centrifugation at ca. 1200 x g for 20 min. After removal of the supernant the precipitate (composed mostly of Ig--the antibody fraction of blood proteins) was dissolved in 5.0 ml cold distilled H_2O. An equal volume of saturated ammonium sulfate was added and the process of mixing, precipitation and separation was repeated twice. The final dissolution was in PBS. The resultant volume (ca. 5 ml) was passed through a 21 x 1 cm column (Sephadex G-25) to separate any remaining ammonium sulfate salts and to concentrate the predominantly Ig serum proteins remaining. Fractions of 3.0 ml were collected using a 1 ml/min flow rate (Williams and Chase, 1967). Protein containing fractions and resultant protein concentrations were determined by UV spectrophotometry.

This procedure was repeated for each antiserum sample used in titer determinations and later fluorescent staining. Identical and often immediate repetition of the procedure was carried out for normal serum used in controls. For the earlier inconclusive titer testing attempts using solid phase radioimmunoassay (Peron, 1970), a DEAE cellulose ion exchange technique (Williams and Chase, 1968) was used to isolate the gamma globulin fraction (IgG).

VII. Protein Concentration Determinations.

Protein concentration determinations were made with a double beam spectrophotometer (Beckman Spec 20) reading absorption at 278 nM, using a carefully measured bovine serum albumin in PBS at concentrations of 10.0 mg/ml, 5.0 mg/ml, 2.5 mg/ml, 1.0 mg/ml, and 0.5 mg/ml as reference standards. Normal serum and antiserum protein containing fractions from the Sephadex gel filtration were each determined. The protein containing fractions for each were combined and the resultant "starting concentration" was diluted with PBS buffer to a carefully equated 1.0 mg/ml total protein. Readings were taken at several voltage settings to additionally support the measurement of normal sera and antisera concentrations.

VIII. Equilibrium Dialysis Radioimmunoassay (EDRA) Titer Testing.

Dialysis tubing, seamless, 6.4 mm diameter by 8.8 cm in length was soaked 24 hr in a vacuum chamber to condition and remove surface bubbles. One end was double-tied with #20 white cotton thread. The tubing was checked for leakage by filling with buffer and applying gentle pressure after clipping the bottom ends of the bags uniformly at 6.4 mm. The bags were stored in buffer until used (usually within 24 hr). Before filling with the test solutions the bags were uniformly stripped to squeeze out remaining buffer.

For the regular titer testing replications of three were used, adding in each case 1.00 ml (±.01 ml) of normal serum (1.0 mg/ml), anti JH serum (1.0 mg/ml), phosphate buffered saline, and in later assays, inhibited anti JH serum (1.0 mg/ml), for a total of twelve bags. The dialysis bags of PBS were to provide a measure of the completeness of isotope diffusion through the membrane of a non-binding solution, thus accounting for differences of diffusion-related, not protein-antibody-related, isotope counts. The inhibited anti JH serum was prepared by reacting 0.225 ml of a 10 µg/ml (2.37 µg/ml) methyl juvenate which was not isotopically tagged to 4.0 ml of the

1.0 mg/ml anti JH serum and allowing it to incubate for 1 hr at room temperature in a scintillation vial. Plain buffer in the amount of 0.225 ml was added to 4.0 ml samples of normal serum, and the anti JH serum to keep total protein concentration exactly equal throughout (i.e., at this point, 0.95 mg/ml for each). The twelve bags were placed in a 33 mm diameter straight side vial and 9.0 ml buffer added. To this was added 9.0 ml of a 1.28 μg/ml ^{14}C labelled methyl juvenate isotope with a specific activity of 140 μCi/mM (and a measured 1150 cpm/ml). The contents were carefully mixed and covered with parafilm. Dialysis was carried out in the refrigerator for 48 hr.

The dialysis bags were removed one at a time in a hood with double-covered surfaces and proper isotope waste disposal containers to prevent contamination. Each bag was opened by cutting off the top where a 1 cm air space was allowed to prevent solution loss during opening. The bag content was carefully mixed with the pipette, then 0.900 ml (±.002 ml) was withdrawn with a volumetric pipette and placed in a scintillation vial. Only 0.900 ml was used to provide a constant volume in the event that slight changes had occurred in dialysis or that folds at the bottom of the bag prevented withdrawing the entire

sample without getting air bubbles in the pipette. The pipette was allowed to drain 30 seconds and any accumulation was added to the vial.

The same pipette was used for each bag, but was rinsed thoroughly: first, with distilled H_2O, then 50% ethanol, then five times with distilled H_2O, and a buffer rinse followed by a 60 second drain and blotting of any accumulation. Testing of this procedure for any isotope carryover showed none detectable by the scintillation system in use. However, there is some evidence that slight dilution of the first of each series occurred from small amounts of remaining buffer. This was uniform and small in each case. A sample "error" of 8 µl (a length of 1 cm on the pipette) was run with each EDRA. This was roughly twice the real error in pipetting, and when above background, was in the 3 to 5 cpm range.

Three samples of the solution remaining in the dialysis container ("antigen" solution) was also pipetted into scintillation vials to provide data on remaining unbound ^{14}C methyl juvenate. Using this in conjunction with the PBS counts and the serum count, nearly 90% of the total isotope introduced could be accounted for with a reasonable presumption, that the membrane surfaces could contain a large portion of the remainder.

IX. Liquid Scintillation Counting Techniques.

New optimally low background glass liquid scintillation vials (Nuclear Chicago, Amersham/Searle #003326) were each given a triple distilled H_2O rinse followed by triple acetone rinse-drying and were then oven dried for at least 30 min at 60° C. The foil lining of each cap was wiped with an acetone damp Kimwipe to remove any loose dust. Into each vial was pipetted 0.900 ml of the buffer dissolved serum antigen antibody solutions from the equilibrium dialysis. To this was added 10.0 ml of a water solubilizing fluor. The fluor was made by mixing 4.0 g PPO and 50.0 mg POPOP in 667 ml toluene (Mallinckrodt Scintilar Grade) to which was added 333 ml Triton-X 100 (Packard). The solution was stirred 14 hr in a covered flask then stored in a brown glass 2 liter jug. After fluor addition the sample vials were capped and swirled to thoroughly mix the contents. Identifying code for date and sample was recorded on the cap. A second mixing and vigorous wiping of the vial sides with clean Kimwipes to remove fingerprints accompanied a second check of the caps for tightness before placement of the samples in the liquid scintillation counter.

The vials were set up in the counting series as follows:

1. A commercially prepared (Nuclear Chicago) toluene reference standard background vial (labelled Ref).

2. Two currently prepared background vials containing the same batch of fluor as used in the series (Bk-1, Bk-2). A third current background vial was placed at the end of the series to give indication of slight background changes which could occur over the 7½ to 8 hr total counting.

3. Next, the three buffer vials were placed in sequence (Buf-1, Buf-2 and Buf-3).

4. These were followed by the three antigens remaining in the tube vials (Tube-1, Tube-2, Tube-3).

5. The test sera vials were alternated: normal serum, antibody serum, and inhibited antibody serum (NS-1, Ab-1, InAb-1; NS-2, Ab-2, InAb-2; NS-3, Ab-3, InAb-3), thus providing a closer comparison of small variations in background and a similarity to any pipetting variations such as slight dilution of the first of each series by buffer carryover.

6. The pipetting "error" sample was added.

7. Two 1.00 ml standard samples of the 1.28 µg/ml methyl juvenile ^{14}C from the original antigen solution were finally included (Std-1, Std-2) and between these was placed the third background vial (Bk-3).

The series was counted for 20 min counts with a repetition 20 min count within 24 hr of the first. The vials were maintained at 4.4° C throughout the initial and recount sequences. Initial instrument settings of D-50, LC-20, and UC-59 were employed by using balance point determination of the optimal net cpm-background ratio for the serum quenched test vials. This method was utilized in order to obtain maximum counts of the low levels of isotopes used under the higher but quite constant protein quenching of the serum samples.

The quenching would predictably decline in the non-protein containing buffer, tube antigen, standard isotope and background vials. Therefore, separate counting efficiencies were determined for each quenching situation (71% for protein containing samples and 77% for the non-protein samples).

X. Insect Tissue Dissection and Sectioning.

Adult male or female American cockroaches (Periplaneta americana) at least two weeks past last larval moult were selected from cultures maintained at room temperature with relative humidity around 70%. The roaches had been reared for many generations (over ten) on Purina Puppy Chow and water. After a

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superficial check to confirm the typical and healthy appearance of the specimen the head was removed with a razor blade. Number 00 insect pins were pushed through each mandible from the posterior to provide a handhold on the head. Using a shearing cut to minimize tissue trauma, the dorsal 1.5 mm of the cranium was removed. The head was then mounted with the dorsal opening upwards on the concave side of a 1 cm deep depression in the wax of a small dissecting pan (2.4 x 7.5 x 10 cm).

The depression was filled with saline solution and the pan oriented under a dissecting scope (Bausch and Lomb) with a 2x supplementary objective to give a working magnification range of 14 to 60x. Using a pair of microforceps, the cranium and dorsal cervical region was pulled apart and pinned with minutin pins. This exposed the brain with corpra cardiaca projecting posteriorly along the esophagus and the corpra allata slightly posterior and lateral to this, lying close to the surface of the esophagus. The corpra allata were yellowish rounded glands about 200 μ in diameter in contrast to the bluish-white roughly ellipsoidal corpra cardiaca.

A corpus allatum was freed by seizing the nervous allatus from the corpra cardiaca using microforceps. A sharpened minutin pin was used to cut around the slightly lifted corpra allatum until it was free. The

dissected corpus allatum was transferred to a drop of embedding medium (Tissue Tek, Ames). The drop was enclosed on a tape cone mounted on a cork for easy handling. After using the dissecting scope to orient the corpus allatum, it was flash frozen using a spray freezing agent (National Tissue Freeze, Allied Chem.) in a styrofoam container. This was transferred to a freezer (-20° C) for storage until sectioning. After numerous preliminary dissections, the overall time of cranium opening to freezing was reduced to about 10 min, with any one corpus allatum being frozen within 1 to 2 min after removal from the cockroach brain. At no time was the tissue allowed to dry. The actual freezing time of the sprayed refrigerant on the small mountant drop was 5 to 10 seconds.

The frozen corpra allata were maintained below 0° C during the transfer from storage freezer to cryostat and when mounting on sectioning discs. Six, four, and ultimately three micron sections were cut, using a microtome-cryostat (International Equipment Co., CTF) with tissue and blade maintained at -12 to -18° C. The sections were mounted on #1 cover glasses (Corning) and usually four good sections were centered in the four quadrats of a square cover glass. Affixation was accomplished by allowing the embedding media

and section to melt as it touched the room temperature cover glass. The tissues were allowed to dry with no applied fixative solutions, and the mounted sections were stored at room temperature in a tape sealed plastic slide box.

XI. Indirect Fluorescent Antibody Staining.

Sections 3 μ thick of corpra allata were affixed with one section in each quadrat of a square coverslip and encircled with a paint line ("Deco-write"oil base, Craftint Co., Cleveland) to prevent interchange of solutions during selective staining. Two drops of cold PBS were added to each section for 5 min to remove the embedding and affixing materials. The pre-rinse buffer was then removed using a bent blunt ended #24 gauge needle and syringe to draw off the liquid while avoiding the tissue section. Serum incubation proceeded immediately to prevent drying of the tissues.

The test section was flooded with two drops of an anti JH serum Ig fraction (10 mg/ml total protein). The normal serum and stain control sections received two drops of normal serum Ig fraction (10 mg/ml total protein) while the autofluorescence control and the stain only control sections received two drops of cold PBS. Incubation continued at room temperature for

15 min in a humid chamber made by placing a PBS wetted filter paper in a petri dish and inverting the dish over the sections. Each solution was drawn off and the coverslip was placed in a gently flowing cold (10° C) PBS bath to wash the tissues for 15 min. Upon withdrawal from the bath excess PBS was drawn off and staining proceeded without allowing the tissues to dry.

To the test section and the normal serum and stain control sections, two drops each were added of a 1:10 (PBS diluted) fluorescein isothiocyanate conjugated anti-rabbit globulin that had been extracted from a sheep (Nutritional Biochemicals Corp., Cleveland, Control #4343 with 1:10,000 merthiolate). Two drops of PBS were added to each of the other two non-stain control sections and the coverslip was again placed in the humidity chamber to stain for 15 min at room temperature. The stain solutions were removed by suction and an additional two drops of a PBS primary rinse was added and withdrawn to minimize any stain contamination of the main rinse bath. Washing of all sections in the gently flowing cool PBS bath continued for 15 min and was followed by a 10 second rinse in distilled H_2O to remove excess buffer salts. The excess liquid was removed by suction and a small drop of buffered glycerol (10% PBS) was added to the

sections. The coverslip was inverted on a slide, taking care to prevent bubble trapping. A strip of cellophane tape was placed on the vertical sides to keep the coverslip in place. The slide was then labelled and observed.

Preliminary tests using a range of washing times from 5 to 90 min and a range of stain concentration from 1:1 to 1:20 were tried before selecting the experimental parameters described above. Several trials using "Fluoromount" (Gurr Ltd., London) as a more permanent mountant showed some promise, but this method was not incorporated into the routine since the resultant data was to be recorded photographically shortly after staining.

XII. Observation and Photography.

The fluorescent antibody stained corpra allata were observed and photographed using a research microscope (Leitz Ortholux Trinocular) with a 200 watt super pressure xeon mercury lamp (Osram HBO) as the light source for the UV unit. The objectives used were 10x/0.25, 25x/0.50, Fl Ocl 54x/0.95, and Fl Ocl 95x/A 1.10-1.32 adjustable (Leitz). A periplan GF 10x ocular set was used for viewing and a WF 10x ocular (Bausch and Lomb) was used for photography. The binocular prisms swung out to direct all light through the

camera tube during photography.

Through trial and rejection all available exciter and barrier filter combinations (exciter filters BG-38-4 mm, BG-12-3 mm, UG-1-2 mm, and barrier filters K430, K460, K470, K490, K510 and K530) were tested for optimal sensitivity, using both lightfield (Leitz 0.90 AS) and darkfield (Leitz D 1.20 A) condensers. Darkfield, using BG-12-3 mm (320-490 nM) exciter filter with a K530 (530-700+ nM) barrier filter, allowed the use of the short wavelength blue visible light to enhance the fluorescein isothiocyanate fluorescence (with more light entering near its maximum absorption range of 490 nM). This was the filter combination most used for observation and photography. Darkfield, using UG-1-2 mm (300-400 nM) with the K530 barrier filter gave moderately better contrast between stained and control sections with only UV incident light. However, the low levels of total light at the ocular required much longer film exposure times and was consequently a less used combination.

Photographs were taken using a ED-10 Polaroid film back with a manual shutter. Both color 108 (ASA75) and black and white 107 (ASA3000) 8.1 x 10.6 cm prints were taken. The early trials were photographed with color film that required extended

exposure times ranging from 6 to 12 min. Switching to the black and white film allowed for reduction of exposure time to well under one min. Usual exposure time using the BG-12, K530 filter combination (dark-field) was from 3.5 to 5.0 seconds. When the UG-1, K530 filters were used the times were typically about ten seconds with the range being from 5 to 50 seconds. The shutter was hand operated and timed with a hand held stopwatch (1/10 second gradations). Observed range of timing error was ±2% (0.1 seconds).

Test and control sections as previously described had been mounted on the same coverslip to allow close comparison without adjustment of incident light and with minimal change of focus. Initial scanning was conducted at 250x to view the entire tissue under each of the test and control procedures. Morphologically typical and matched areas of the sections were compared briefly at higher magnifications of 540x and/or 950x using oil immersion. Viewing time was kept nearly equal to minimize the effect of slow fluorescence fading observed with the bright incident light. Again, critical focus exposure time just before each photograph was kept equal. Focusing was directly through the camera tube with the camera removed. The exposed prints were labelled with date, time, exposure time, tissue type, filter system and magnification.

These were coated with the polaroid print coater and stored in a notebook.

RESULTS

I. Results of the Farnesenic Acid Epoxidation.

A gas liquid chromatograph (Packard Model 840) with a flame ionization detector was used to analyze the FA epoxidation products. A 2 m length by 3 mm diameter column packed with a 3% XE-60 coated Chrom Sorb-Q (60-80 mesh) was conditioned for 18 hr at 250° C, then equilibrated at 180° C. Nitrogen carrier gas was adjusted to a flow rate of 100 ml/min and the hydrogen for the flame maintained at a constant 40 ml/min. After stabilizing the recorder and injecting ether, hexane and FA standards, 0.5 µl injections from the epoxidization reaction fractions were tested.

Of the ten 30 ml fractions collected from the silica gel separation fractions, #2 was shown to contain nearly all of the epoxy FA. Two small peaks in the 2:30 and 3:40 min range (total 87 mm^2) were due to unreacted FA with a double major peak (3850 mm^2) in the 8:20 to 9:35 min range, indicating roughly 2.3% unreacted FA. Two narrow peaks in the 1:10 and 1:35 min range were identified as m-chlorobenzoic acid and m-chloroperbenzoic acid not completely removed during

previous purification (see Fig. 1).

Control solution containing ethyl ether, dichloromethane, hexane, and m-chloroperbenzoic acid was run showing all control components coming off by 1:15 min. Using the same settings, an FA standard was run which yielded a double peak in the 3:10 and 3:30 min region. The two very sharp peaks at 1:25 and 1:30 min were possibly due to incomplete cleaning of the needle and opening from the previous control injection of m-chloroperbenzoic acid (see Fig. 2). Fraction #2 was concentrated under reduced pressure to remove remaining solvents and stored at -20° C until use in the conjugation reaction with hemocyanin.

II. Results of 10-11 Epoxy Farnesenic Acid Hydrolysis from Hemocyanin.

The ether extract containing hydrolyzed FA products was analyzed by GLC under approximately the same conditions as described in the epoxidation results (see page 31). After stabilizing the recorder and injecting controls of hexane and ether, a series of 1 µl injections of 1 ml starting portion of the ether extract was serially concentrated by vacuum evaporation of the ether at room temperature to halve the solvent volume at each step (see Fig. 3).

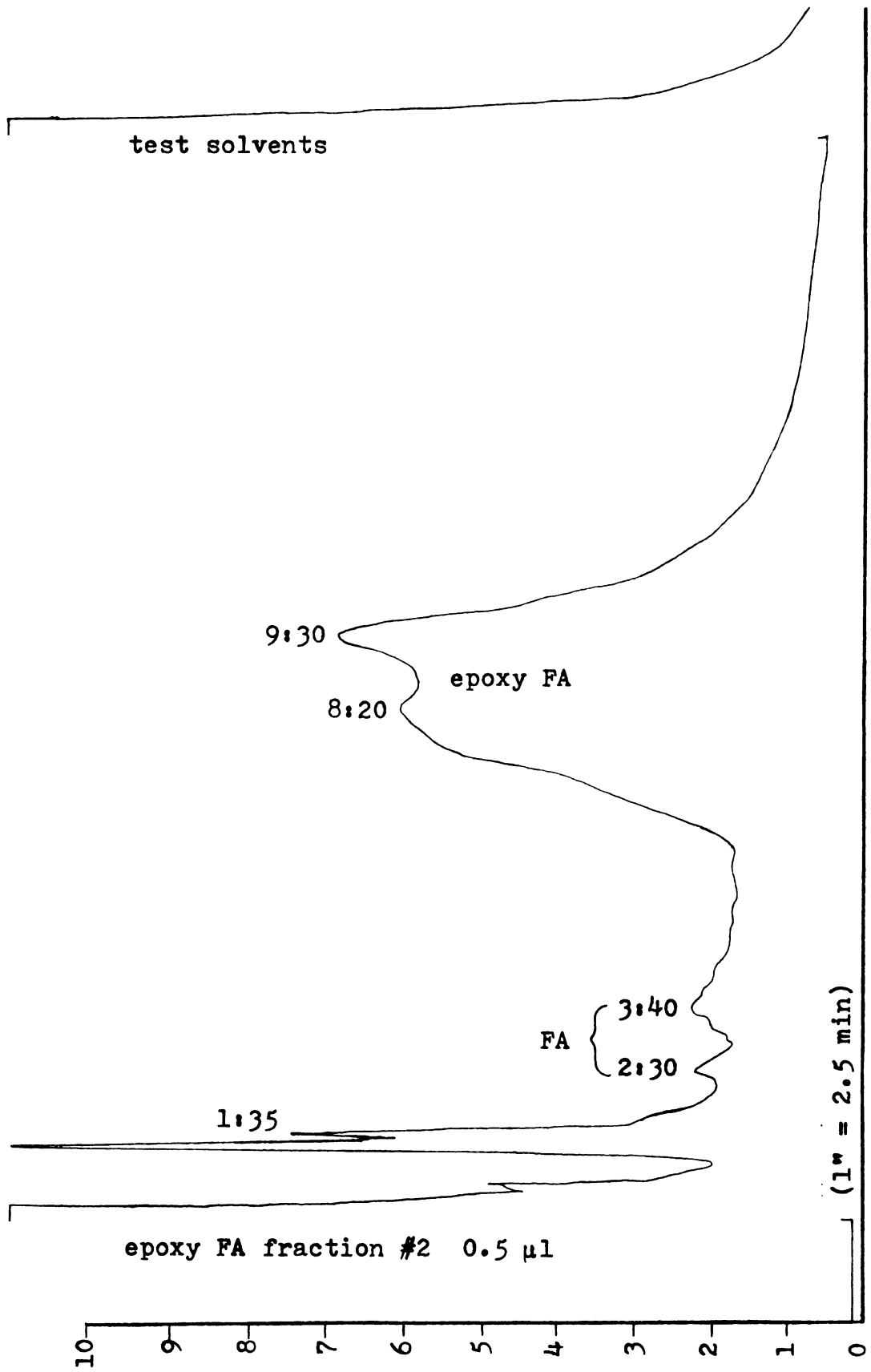

Figure 1. Gas Liquid Chromatograph Data Sheet (10/4/72-1)

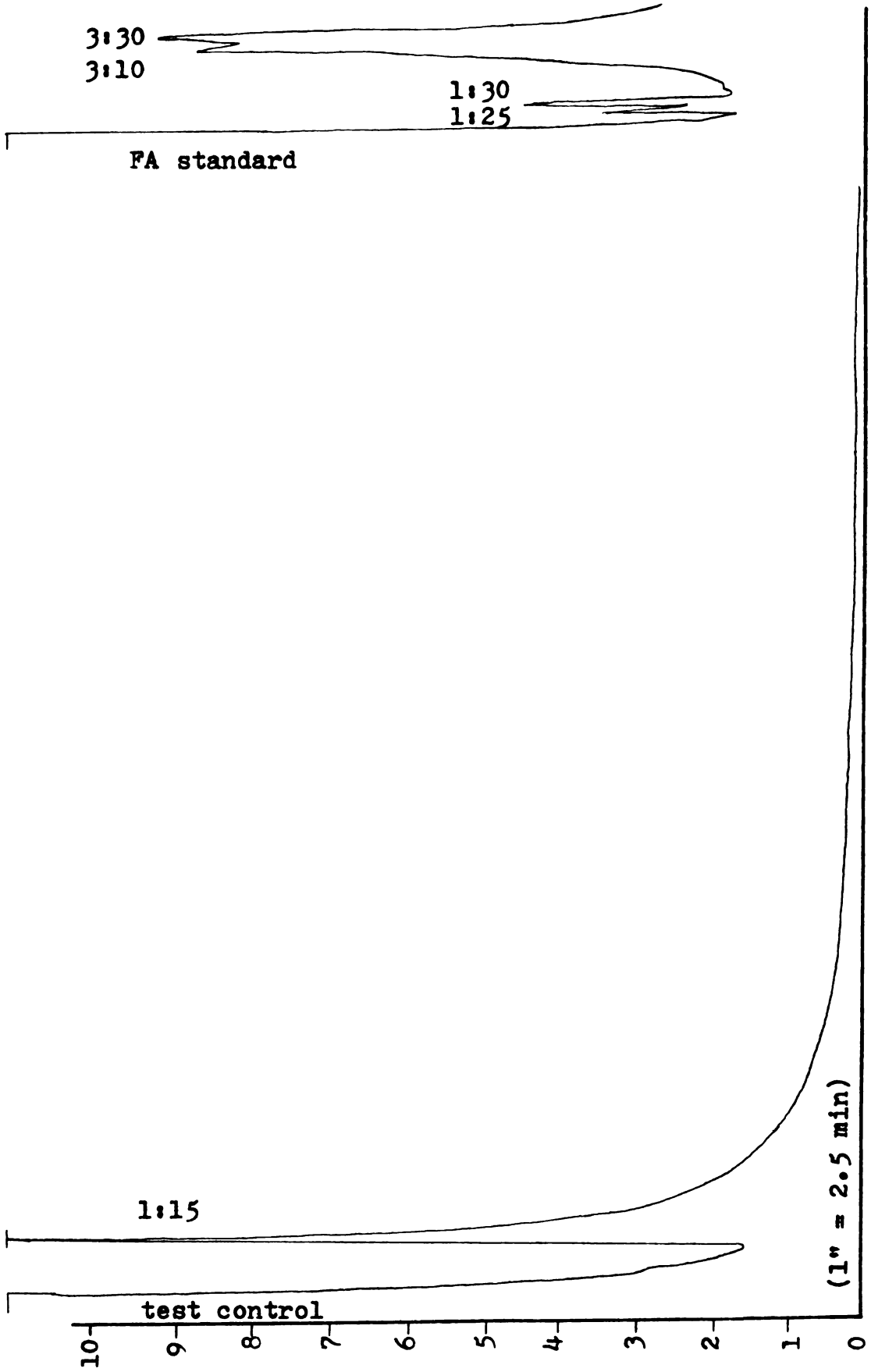

Figure 2. Gas Liquid Chromatograph Data Sheet (10/4/72-2).

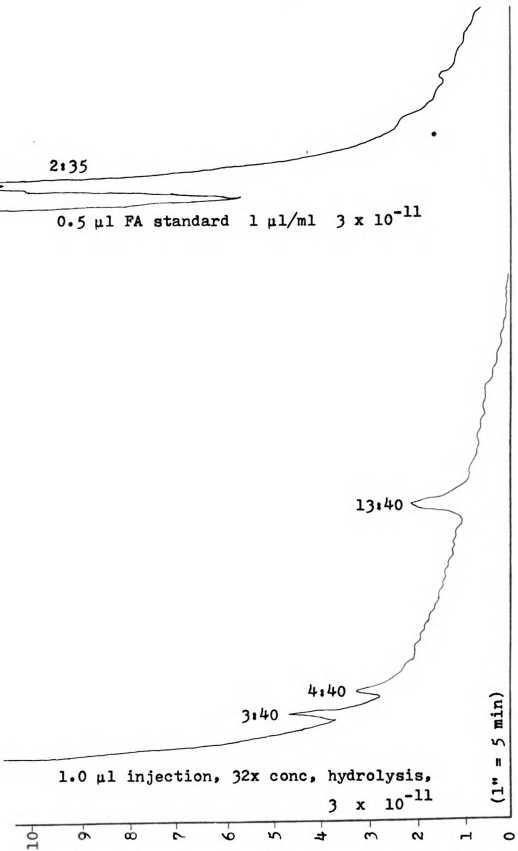

Figure 3. Gas Liquid Chromatograph Data Sheet of JH Hydrolyzed from Conjugated JH Hemocyanin (2/6/73).

The earliest appearance of a peak in the FA region occurred at the 6x concentration of the sample with a detector voltage setting of 1×10^{-10} AFS. At 32x concentration and 3×10^{-11} AFS attenuation three distinct peaks were evident. The first peak at 3:40 min had a 13 mm height and a 2 mm base. The second at 4:40 min had a 7.5 mm height and a 3 mm base. The third peak at 13:30 min with a 13 mm height and an 8 mm base was out of the typical FA range, but could quite possibly represent hydrolysis degradation products from the epoxy FA, such as closed ring molecules. This possibility is supported by an earlier test hydrolysis of a sample of FA in which a large peak was also obtained in the 13:30 min area.

The quantities were calculated from peak area of the known FA standard concentration (0.88 µg/ml) with a 0.5 µl injection. The combined peak area of 266 mm^2 was divided into the 0.44 µg injection and yielded .0017 $\mu g/mm^2$. The hydrolysis extract peaks were then quantified and divided by the concentration factor (64x) to yield the total micrograms of each in the original 3.0 ml sample from a 1.0 ml starting sample. The results are shown in Table 2.

Table 2. Hydrolyzed Farnesenic Acid Products.

Peak (min)	=	mm^2	=	µg/ml	or	µg/ extract sample
3:40		13.00		0.345	or	1.04
4:40		11.25		0.299	or	0.89
13:40		52.00		1.380	or	4.14

Thus, from a starting 1.0 ml of conjugated hemocyanin epoxy FA (1.2 nM), at least 1.94 µg (peaks 3:40 and 4:40 min) and probably 6.04 µg (total peaks) of the epoxy FA products were recovered. The figure, 1.94 µg divided by .252 µg/nM yields 7.68 nM of FA. This, divided by the 1.2 nM of the hemocyanin would show at the minimum an average of about 6.4 molecules of epoxy FA to be conjugated to a molecule of hemocyanin. More likely, the 6.04 µg (23.9 nM) yielding 20 molecules of FA per molecule of hemocyanin is a more accurate estimate. In either case, this demonstrated a minimally (to moderately, ca. 20) adequate uptake of the epoxy FA to predict probable success in stimulation of hapten antibody formation.

III. Antibody Titer and Testing Results from Equilibrium Dialysis Radioimmunoassay (EDRA).

Initial titer testing using a solid phase radioimmunoassay was attempted. This technique involved coating a polyethylene tube with an antibody monolayer to detect selective uptake of an isotopically labelled antigen (Peron and Caldwell, 1970, pp. 87-147). After eight weeks and six experimental runs incorporating a diversity of purification and design variation steps, this method was rejected since most trials yielded inconclusive results. The major problem areas seemed to be low titer levels, antibody purification, and low isotope specific activity for this type of assay.

Standard alpha and beta precipitation tests and ring tests were run to demonstrate the presence of hemocyanin antibody as would be expected. Early attempts to detect anti-juvenile hormone via these tests did not yield positive results. Changing to equilibrium dialysis radioimmunoassay (EDRA) as a method to quantify anti JH allowed the use of much larger quantities of both sera and isotope. This resulted in the detection and quantification of positive anti JH titers.

To equate the titer testing data over a 5½ month period when EDRA's were being run, molar antigen

uptake equivalents of the anti JH (Ab) were calculated and the results are shown in Table 3. The net CPM from the average normal serum control (NS) was subtracted from the average net anti JH CPM samples, thus yielding the net selective binding (NSB) CPM. This was divided by 0.90 ml (sample volume counted) to give NSB CPM/ml. The NSB CPM were divided by 71% efficiency of the counting system to yield the NSB DPM/ml. This, in turn, was divided by the specific activity of the methyl juvenate ^{14}C (MJ ^{14}C) at 140 μCi/mM (= 1170 DPM/μg) to give the weight of antigen (MJ ^{14}C) taken up by the 1 mg/ml Ig solution containing the antibodies. The nanogram quantities of MJ ^{14}C were divided by 266 ng/nM of methyl juvenate to give the molar uptake of antigen.

In a reaction with generous excess of antigen the antibodies (primarily IgG class) will bind with two molecules of antigen per molecule of antibody so the halving of the nanomolar antigen would give the nanomolar equivalent of antibody involved in antigen binding. The molar antibody concentration multiplied by 166000 ng/nM of IgG yielded the nanograms of anti JH. This was plotted as the μg of anti JH per 1.00 ml solution of 1 mg/ml serum Ig against two week time intervals. The graph in Figure 4. shows the titer

Table 3. Titer Results of Equilibrium Dialysis Radioimmunoassay.

Date		Samples	Ave. Net CPM	Ave. NSB	Molar Binding Equivalent of Ab
7/19/73	NS	(1.0 mg/ml	93.5	50.2	20.9 µg/ml
	Ab	(1.0 mg/ml)	143.7		
7/19/73*	NS	(IgG 0.75 mg/ml)	111.0	60.0**	25.0 µg/ml
	Ab	(IgG 0.75 mg/ml)	156.0		
8/08/73	NS	(1.0 mg/ml)	262.0	53.0	22.1 µg/ml
	Ab	(1.0 mg/ml)	315.0		
8/28/73	NS	(1.0 mg/ml)	450.0	29.0	12.1 µg/ml
	Ab	(1.0 mg/ml)	479.0		
	InAb	(1.0 mg/ml)	426.0		
10/20/73	NS	(1.0 mg/ml)	275.0	18.0	7.5 µg/ml
	Ab	(1.0 mg/ml)	293.0		
	InAb	(1.0 mg/ml)	285.0		
12/06/73	NS	(1.0 mg/ml)	239.0	11.0	4.6 µg/ml
	Ab	(1.0 mg/ml)	251.0		
	InAb(1/10)	(1.0 mg/ml)	245.0		
	InAb(1/20)	(1.0 mg/ml)	243.0		

* Secondary purification test. ** 45.0 NSB = 60.0 at 1.0 mg/ml.

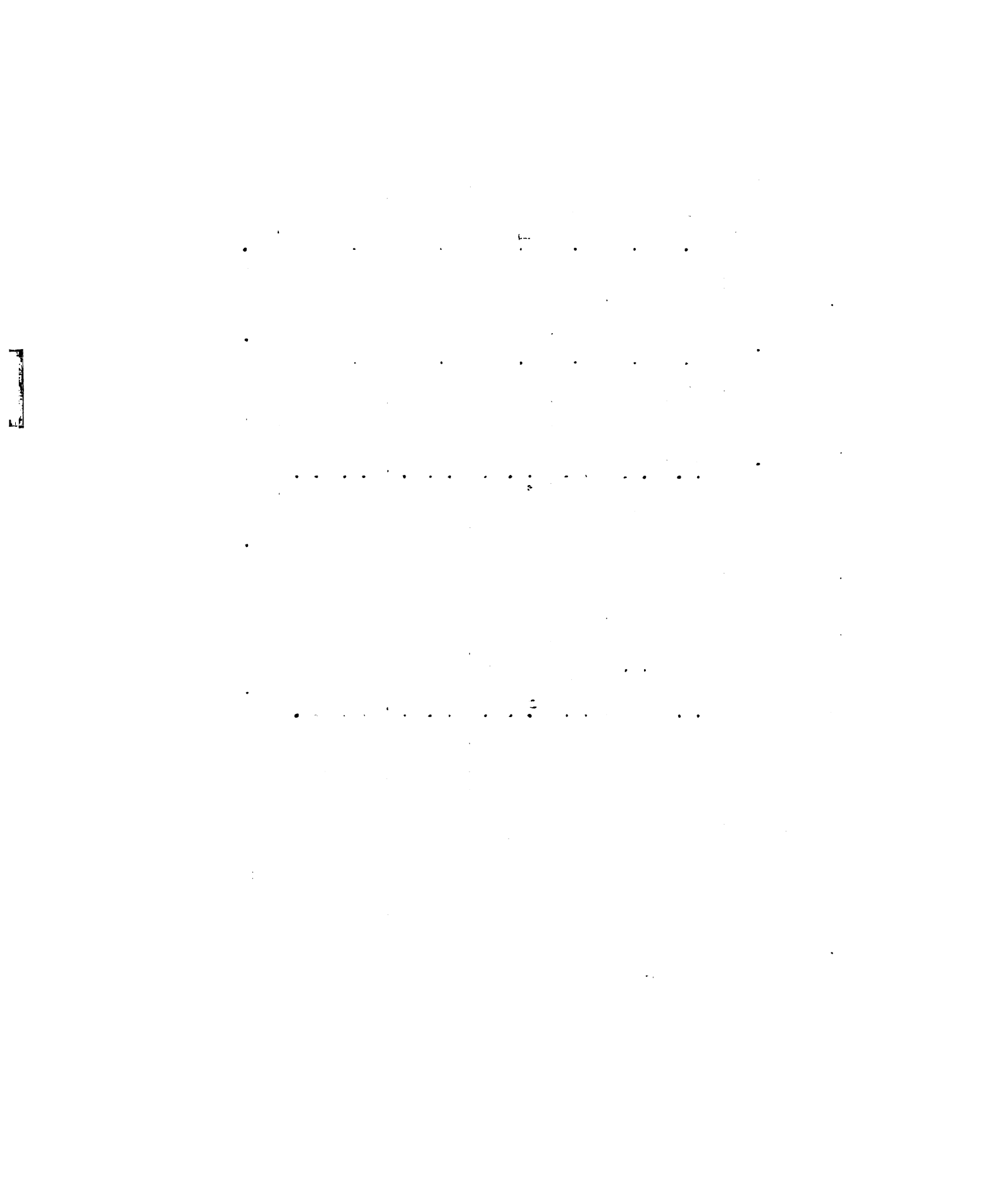

Figure 4. Molar Antigen Uptake Equivalents.

levels and the expected decline after booster injections were stopped. The titer data illustrates specific antibody levels from 4.6 to 22.1 μg of anti JH per mg of Ig protein, thus making up from 0.46% to 2.09% of the total protein.

In a test of further purification DEAE cellulose ion exchange was used to isolate the IgG fraction from the Ig sera (Williams and Chase, 1967, Vol. I., pp 322-326). A 0.75 mg/ml IgG fraction yielded a molar antigen binding equivalent to 18.8 μg/ml (or 25.0 μg/mg). Thus, purification concentrated the antibody from 2.09% total protein to 2.51% in a 1.0 mg/ml solution (see Table 3, 7/19/73*).

IV. Fluorescent Antibody Staining Results.

Initial photography using color film was generally less satisfactory than black and white, due to the fluorescence fading during film exposure along with somewhat reduced contrast and higher cost of materials. After initial trials of staining with serum dilutions from 10.0 to 0.01 mg/ml Ig, observations and photographs were taken of slides stained with 1.0 to 10.0 mg/ml Ig. Photographic data is further delineated in Table 4 and Figures 5 to 19 show the actual photographic results.

In the first series of photos (Fig. 5 and 6) Ab and NS demonstrated good contrast and specific cytoplasmic fluorescence in the corpra allata tissue area of whole brain sections 6 μ thick. The Ab and NS photos cannot be directly compared in this set because the longer exposure time of Ab (Fig. 5) increased the fluorescence intensity somewhat. However, the presence of specific fluorescence is evident.

The next series (Fig. 7, 8 and 9) was of whole mount corpra allata stained and photographed at 250x with 3.5 sec exposure time. A non-stained control was added to the series. Both the normal serum control (Fig. 8) and the non-stained (Fig. 9) show markedly reduced cytoplasmic fluorescence when contrasted to the antibody stained (Fig. 7). Note that the brightly fluorescing rectangular structure on the upper surface of Fig. 7 is a piece of tracheol intensely autofluorescing.

With improving cryostat technique, sections containing only corpra allata were cut at 3 μ thickness. Sections of Ab, NS and 00 (Fig. 10, 11 and 12) were stained with more dilute sera (1.0 mg/ml) and more dilute FITC antiglobulin 1:20 in an attempt to diminish the nonspecific fluorescence. Exposure time was increased to 5 sec and photos were taken at 540x.

Again, in closely comparable sections, somewhat greater cytoplasmic fluorescence is evident in Fig. 10 with similar and diminished fluorescence in the controls (Fig. 11 and 12).

Using another slide stained under the same conditions, photos (Fig. 13 and 14) were taken at 950x with only UV incident light. Exposure time was increased to 10 sec for each. While the overall intensity was diminished by the use of only UV light, the contrast between Ab and NS control (Fig. 13 and 14) was enhanced.

Hemocytes had been implicated as a likely area for early concentration of exogenous isotopically labelled juvenile hormone (Emmerick, 1973) so hemocyte smears were prepared and stained with the 10 mg/ml sera and 1:10 FITC-antiglobulin photographs were taken at 540x with blue and UV incident light. Antibody stained hemocytes (Fig. 15 to 19) showed distinct cytoplasmic fluorescence at 5 sec exposure. Normal serum stained control showed no fluorescence at 5 sec (Fig. 16), only a most faint blur at 20 sec (Fig. 17), and a more pronounced blur at 40 sec (Fig. 18). The non-stained control (Fig. 19) hemocytes showed blurred images at 50 sec exposure time.

The reason for this much greater contrast in hemocyte antibody staining as compared to the results

of the corpra allata whole and sectioned staining await further investigation. Yet, in both tissues a difference in the binding of the fluorescence isothiocyanate molecule via an anti rabbit globulin antibody, to an attached rabbit antibody, to an insect material was selectively demonstrable.

Table 4. Photographic Data for Fluorescent Antibody Staining Results.

Fig. #	Photo Code	Tissue	Sera	Stain	Incident Light	Exp. Time (sec)	Mag.
5	11/09-Ab	Roach brain 6 μ	10 mg/ml Ab	1:10 FITC	UV & blue	4.0	540x
6	11/09-NS	Roach brain 6 μ	10 mg/ml NS	1:10 FITC	UV & blue	3.0	540x
7	12/22-Ab	Roach corpra allata wm	10 mg/ml Ab	1:10 FITC	UV & blue	3.5	250x
8	12/22-NS	Roach corpra allata wm	10 mg/ml NS	1:10 FITC	UV & blue	3.5	250x
9	12/22-00	Roach corpra allata wm	None	None	UV & blue	3.5	250x
10	1/16-Ab	Roach corpra allata 3μ	1 mg/ml Ab	1:20 FITC	UV & blue	5.0	540x
11	1/16-NS	Roach corpra allata 3μ	1 mg/ml NS	1:20 FITC	UV & blue	5.0	540x
12	1/16-00	Roach corpra allata 3μ	None	None	UV & blue	5.0	540x
13	UV-1/15-Ab	Roach corpra allata 3μ	1 mg/ml Ab	1:20 FITC	UV only	10.0	950x
14	UV-1/15-NS	Roach corpra allata 3μ	1 mg/ml NS	1:20 FITC	UV only	10.0	950x
15	H-12/22-Ab(5)	Hemocyte smear	10 mg/ml Ab	1:10 FITC	UV & blue	5.0	540x
16	H-12/22-NS(5)	Hemocyte smear	10 mg/ml NS	1:10 FITC	UV & blue	5.0	540x
17	H-12/22-NS(20)	Hemocyte smear	10 mg/ml NS	1:10 FITC	UV & blue	20.0	540x
18	H-12/22-NS(40)	Hemocyte smear	10 mg/ml NS	1:10 FITC	UV & blue	40.0	540x
19	H-12/22-00(50)	Hemocyte smear	None	None	UV & blue	50.0	540x

Figure 5. Antibody Roach Brain, 6 μ
UV and blue light, 4 sec, 540x mag.

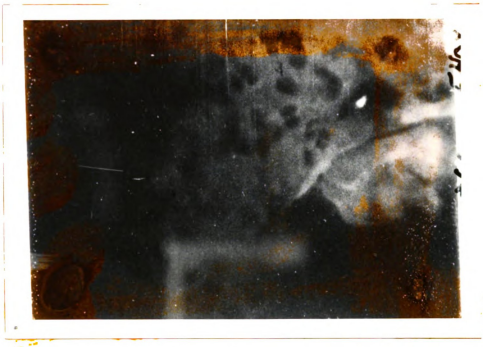

Figure 6. Normal Serum Roach Brain, 6 μ
UV and blue light, 3 sec, 540x mag.

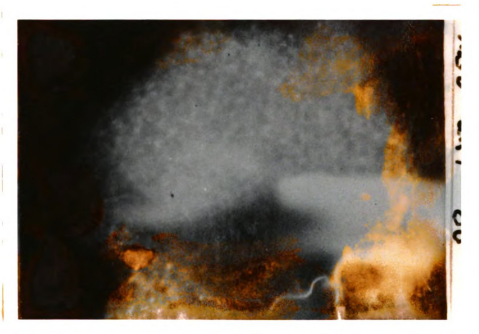

Figure 7. Antibody Roach Corpra Allata, wm.
UV and blue light, 3.5 sec, 250x mag.

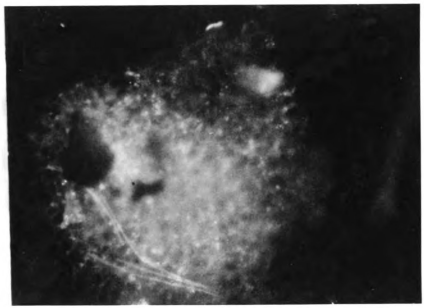

Figure 8. Normal Serum Roach Corpra Allata, wm. UV and blue light, 3.5 sec, 250x mag.

Figure 9. Stain Only Roach Corpra Allata, wm. Uv and blue light, 3.5 sec, 250x mag.

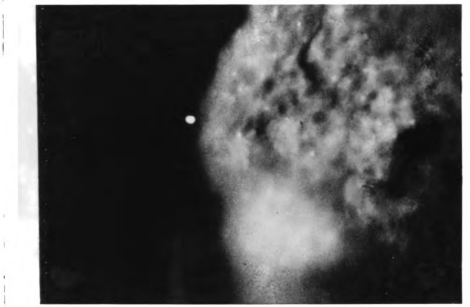

Figure 10. Antibody Roach Corpra Allata, 3 μ
UV and blue light, 5 sec, 540x mag.

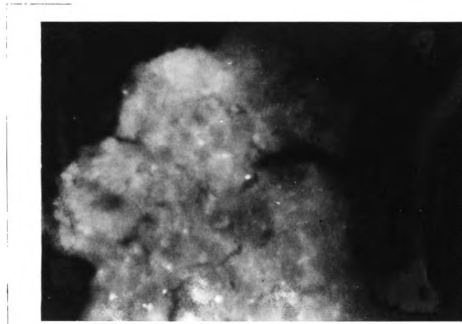

Figure 11. Normal Serum Roach Corpra Allata, 3 μ UV and blue light, 5 sec, 540x mag.

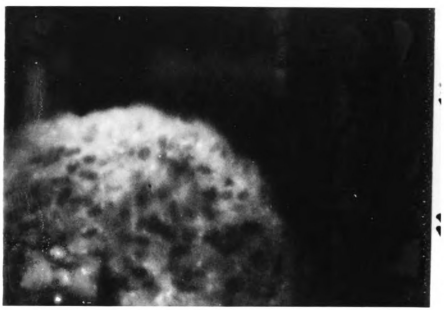

Figure 12. No Stain Roach Corpra Allata, 3 μ
UV and blue light, 5 sec, 540x mag.

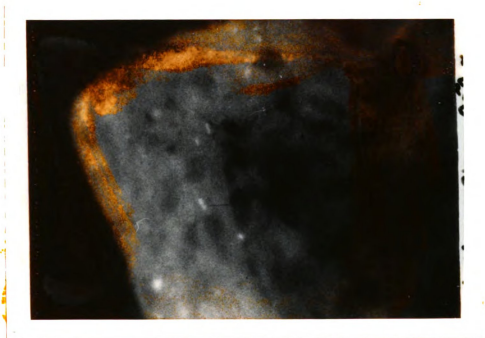

Figure 13. Antibody Roach Corpra Allata, 3 μ
UV light only, 10 sec, 950x mag.

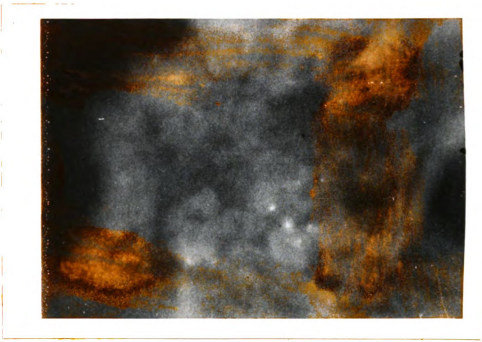

Figure 14. Normal Serum Roach Corpra Allata, 3 μ UV light only, 10 sec, 950x mag.

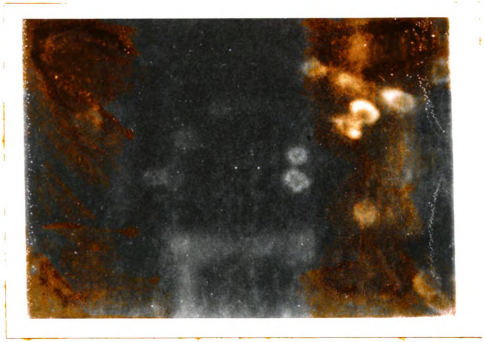

Figure 15. Antibody Hemocyte Smear
UV and blue light, 5 sec, 540x mag.

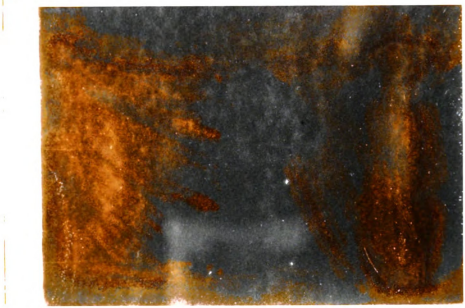

Figure 16. Normal Serum Hemocyte Smear
UV and blue light, 5 sec, 540x mag.

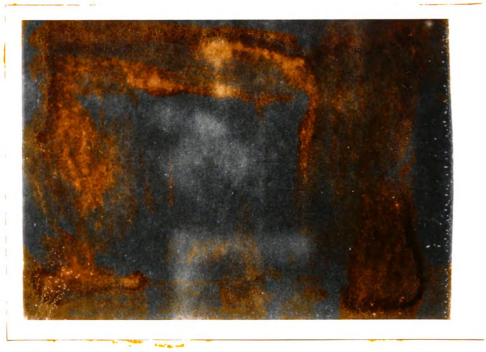

Figure 17. Normal Serum Hemocyte Smear
UV and blue light, 20 sec, 540x mag.

Figure 18. Normal Serum Hemocyte Smear
UV and blue light, 40 sec, 540x mag.

(Note the arrow indicating an artifact.)

Figure 19. No Stain Hemocyte Smear
UV and blue light, 50 sec, 540x mag.

DISCUSSION

Before the actual research to make an antibody against epoxy FA could start, it was necessary to answer two preliminary feasibility questions:

First, acting on the unlikely possibility that rabbit serum contains naturally occurring antibodies that would selectively bind a juvenile hormone-like molecule, normal serum was tested for methyl juvenate uptake using methods similar to those described in the Equilbrium Dialysis Radioimmunoassay section. The normal serum was shown to have only low levels of uptake which should not interfere with the immunization procedure.

Secondly, the small size of the proposed antigen, epoxy FA (266 daltons), would very likely require a large protein carrier molecule if an immune response was to be stimulated. The absence of amino groups on the epoxy FA hapten required the adoption of a less commonly used conjugation technique. The reaction makes use of a carbodiimide to link the epoxy FA carboxyl to one of the hemocyanin amino acid units which has an available amino group (*i.e.*, lycine-aspergine). Before investing the time and money into

synthesis and purification of the epoxy FA a preliminary conjugation and subsequent hydrolysis reaction using FA was completed using the procedure described for the epoxy FA. This also gave positive results that deemed further investigation feasible.

The choice of epoxy FA as the juvenile hormone analogue to use as a haptenic source of antibody was based on its relatively close molecular configuration to naturally occurring Cecropia juvenile hormone (C-18) trans trans cis methyl 10-11 epoxy-7-ethyl 3,11 dimethyl 2,6-tridecadienoate (Roller, 1967) and its closer similarity to the likely naturally occurring (C-16) methyl 10-11 epoxy 3,7,11 trimethyl 2,6-dodecadienoate. The C-16 has been demonstrated as a probable corpra allata synthesis product based on work with Orthopterans and Lepidopterans (Riddiford and Ajami, 1973 and Judy, 1973).

The available isotopic source for radioimmunological testing was MJ ^{14}C (10-11 epoxy methyl farnesoate) which has the same molecular configuration except for the methyl ester which is located in a homologous position to the carrier attachment site on the epoxy FA molecule. Therefore, it would be expected to contribute very little, if any, to the antibody-antigen binding reaction (Boyd, 1962). The

epoxy FA molecule has the necessary carboxyl group available for participation in the carbodiimide conjugation reaction. The carbodiimide conjugation reaction was modified from a procedure for protein to protein linking via carboxyl groups (Williams and Chase, 1967, pp. 155-158).

The epoxy FA carboxyl reacted with a carbodiimide which undergoes rearrangement, then couples with a second molecule of epoxy FA to form the acid anhydride. This, in turn, combines with an available amino group from the hemocyanin chain to form a peptide bond and releases the second epoxy FA molecule (see Figure 20).

The immunization procedure designed was a modification of that used by Borst to make ecdysone antisera (Borst, 1972). The procedure used was one which would give the maximum opportunity for antibody formation through secondary immune response (IR) enhancement of a booster series. To further enhance the IR, the hapten-carrier conjugate was injected with an adjuvant in which attenuated bacteria heighten IR sensitivity and the oil emulsification of the antigen prolong its metabolic availability. Subcutaneous injection and a non-antigenic booster medium were utilized to minimize the hazard of rabbit loss to

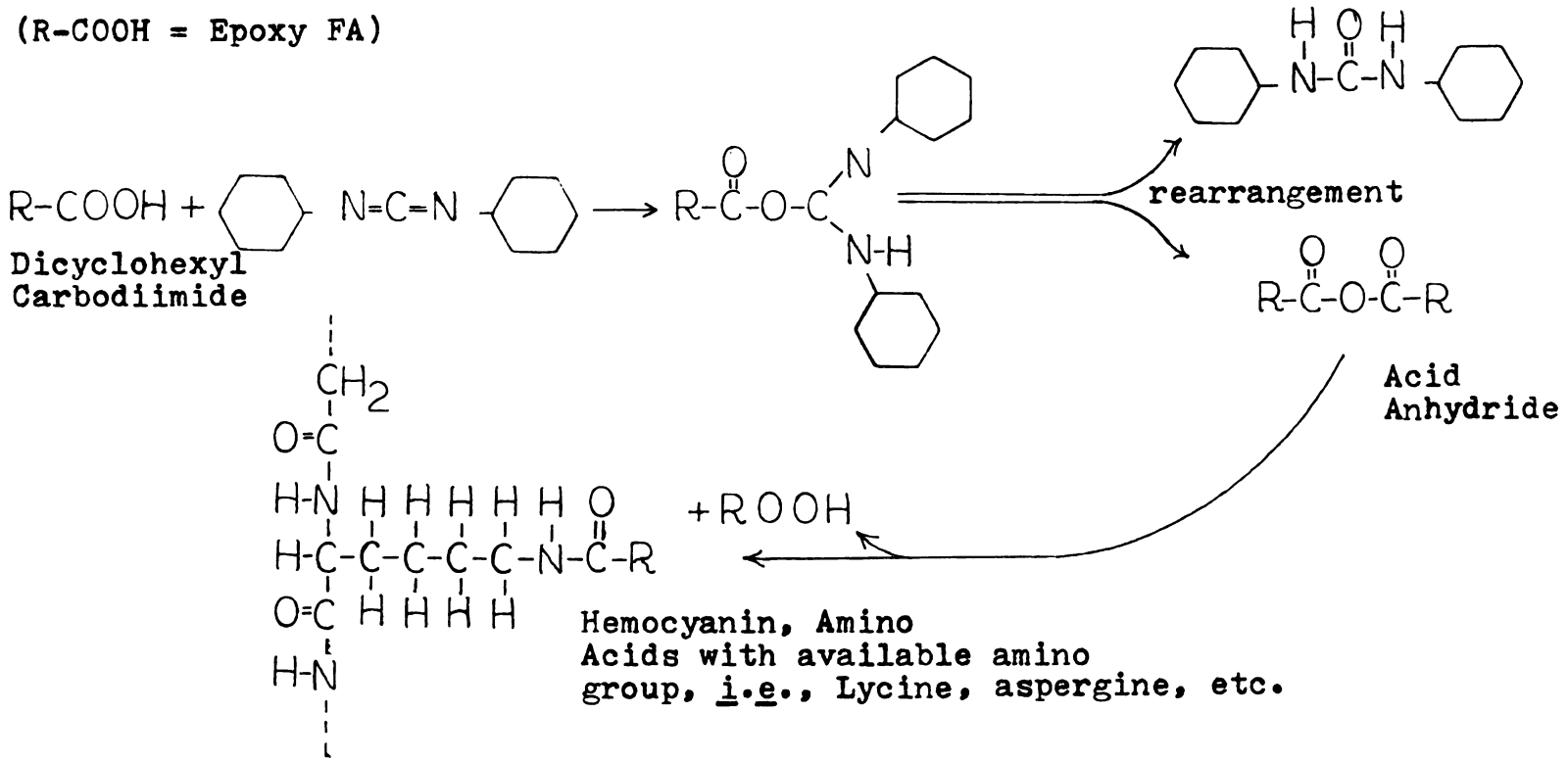

Figure 20. **Epoxy** FA Conjugation to Hemocyanin.

infection or anaphylactic shock. For this same reason, ear bleeding for serum harvest was chosen in place of the more rapid, but more hazardous, cardiac puncture.

Classical titer testing methods such as precipitation, hemagglutination or hemolysis were bypassed because the anti JH titer levels were expected to be very low, and both the radioisotopic antigen and scintillation counting equipment provided a more sensitive quantitative assay technique. For initial titer testing a solid phase radioimmunoassay procedure was designed which has the promise of high sensitivity and low volume use of a sparse radioisotopic antigen supply (Peron and Caldwell, 1970). Unfortunately, this did not produce significant results.

A greater time- and isotope-consuming equilibrium dialysis radioimmunoassay was designed, following the general procedures outlined in Weir (1967). This was possible because the small molecular size of the antigen (MJ ^{14}C) allowed it to pass freely through the dialysis membrane while the serum proteins could not. This free flow was confirmed in buffer control samples. Because the biologically active half life of the synthesized MJ ^{14}C was around six to twelve

months (personal communication) some isotopically labelled degradation products would be in the antigen solution.

While further isolation and testing of these products would be required to confirm their antigenic activity, it was assumed in this research that selective uptake would be almost entirely by those molecules retaining their bioactive configurations, because in any reaction there would be a massive excess of available antigen molecules for any one site. Thus, if a specificity preference is demonstrated, there would be a strong tendency to establish an equilibrium in which the most site-compatible molecules would be found in those sites. The other, if binding at all, would do so less selectively. Even if there was selective binding of some of the degradation molecules, for the purpose of this research, it could still represent an antibody binding site specific to only a part of the antigen molecule. Hence, it would add to the titer level a small cross-reaction factor, but should not significantly change it.

Corpra allata and hemolymph were selected from adult cockroaches (in most cases, males) at least two weeks past their last moult. Based on JH bioassays of corpra allata and hemolymph in Locusta and

Schistocerca (Johnson, 1973) as well as the less reliable corpra allata histology-activity estimates of Leucophaea (Scharrer, 1958), this would be sufficient time to expect a return to higher and relatively constant endogenous JH production.

Rapid dissection and immediate freezing were employed to minimize any enzymatic degradation of the JH. Cryostat cut frozen sectioning and direct mounting made it convenient to avoid the organic reagents (i.e., ethanol, xylene, paraffin) which would most probably dissolve the tissue JH and remove the antigen source for staining.

An indirect fluorescent antibody staining (IFAS) technique was used in which an antibody (immunoglobulin) is first bound to an antigen in the tissue, and then an antiglobulin which has the fluorochrome attached is reacted to bind with the antibody molecule. This results secondarily in having several fluorescing tags attached to the site of the antigen. This indirect method has the advantages of: 1) allowing the use of standardized commercial fluorochrome tagged antiglobulin solutions at a considerable saving of time, and 2) enhancing the fluorescence of the antigen site by allowing the attachment of several fluorochrome-bearing antiglobulin molecules rather

than a single antibody molecule as with direct fluorescent antibody staining. But any fluorescent antibody staining method and especially the IFAS, requires precise controls to interpret the resultant fluorescence.

In the preparations of reagents, protein concentrations of antisera and normal sera were equated to keep the normal occurrence of some nonspecific protein binding the same. Any nonspecific protein bound in the initial incubation would bind antiglobulin molecules at the staining step and appear to be tissue antigens. It was also necessary to have complete washing of the tissues after both incubation and staining steps to remove as much of the nonbound proteins as is possible. Additional controls treated with only stain or only normal serum were incorporated to quantify the resultant nonspecific fluorescence. Placement of the controls on the same coverglass assured equal treatment at this step.

Another source of nonantigen related fluorescence is autofluorescence. Autofluorescence of various tissue components (some proteins, nucleic acids, etc.) was taken into consideration by the inclusion of a control that was treated with neither serum nor stain. For the hemocyte staining a second test area was

included to compare the repeatability of the staining from one section to another in the same series.

To maximize the contrast, both sera and stain solutions were tested through serial dilution to find the optimum level of maintained specific fluorescence with nonspecific fluorescence at a minimum. This resulted in a three way trade-off situation in which the minimal total light requirement took precedence in order to have low photograph exposure times, thereby limiting the fluorescent fading effect. Within these limiting factors the level of optimum contrast for photography was somewhat less than that for visual observation, but correlated through the range.

With autofluorescence varying in different tissues it was also necessary to match the histology of test and control tissues carefully when working with multi-tissue sections. This was much reduced after sections of only corpra allata or only hemolymph were utilized.

Finally, the focus of incident light often requires adjustment when changing slide or magnification levels, easily introducing intensity error. This was offset by placement of test and control

tissues on the same slide which facilitated comparisons at the same magnification without alteration of light adjustment between sections.

CONCLUSIONS

In conclusion, it was found that dicyclohexyl carbodiimide reacting with epoxy farnesenic acid and keyhole limpet hemocyanin did yield a conjugate with at least 6.4, and probably over 20, molecules of hapten attached to the carrier protein molecule. Through an adjuvant assisted immunization and periodic booster enhancement, the rabbits formed antibodies to both hemocyanin and to the epoxy FA hapten. This was quantified and periodically determined to plot the decline from a peak 22.1 μg/ml after the last booster, to 4.6 μg/ml 5½ months later. The plotted decline was consistent with the general immunological predictions and further, yields original data on how this specific small hapten responds in rabbits.

The application of anti epoxy FA serum to indirect fluorescent antibody staining of adult cockroach corpra allata tissues and hemolymph smears produces specific fluorescence. Most likely, this specific fluorescence is localized at the sites of endogenous juvenile hormone and/or its metabolic precursors with

very similar molecular configurations.

Explanation of the observed increase of hemocyte fluorescence intensity over that of the corpra allata tissues awaits further investigation. There are several possibilities worthy of consideration, the first being that much of the juvenile hormone material is in the form of a storage precursor molecule which does not cross-react with the antibody. The second possible area to investigate would be the relationship of the JH in association with carrier lipoproteins in the hemolymph, and membrane surface association with the hemocytes. Either of these would contribute to the physical availability of JH to the antibody or might provide a more stable surface for attachment and resultant higher survival of the antibody antigen complex through the several washings.

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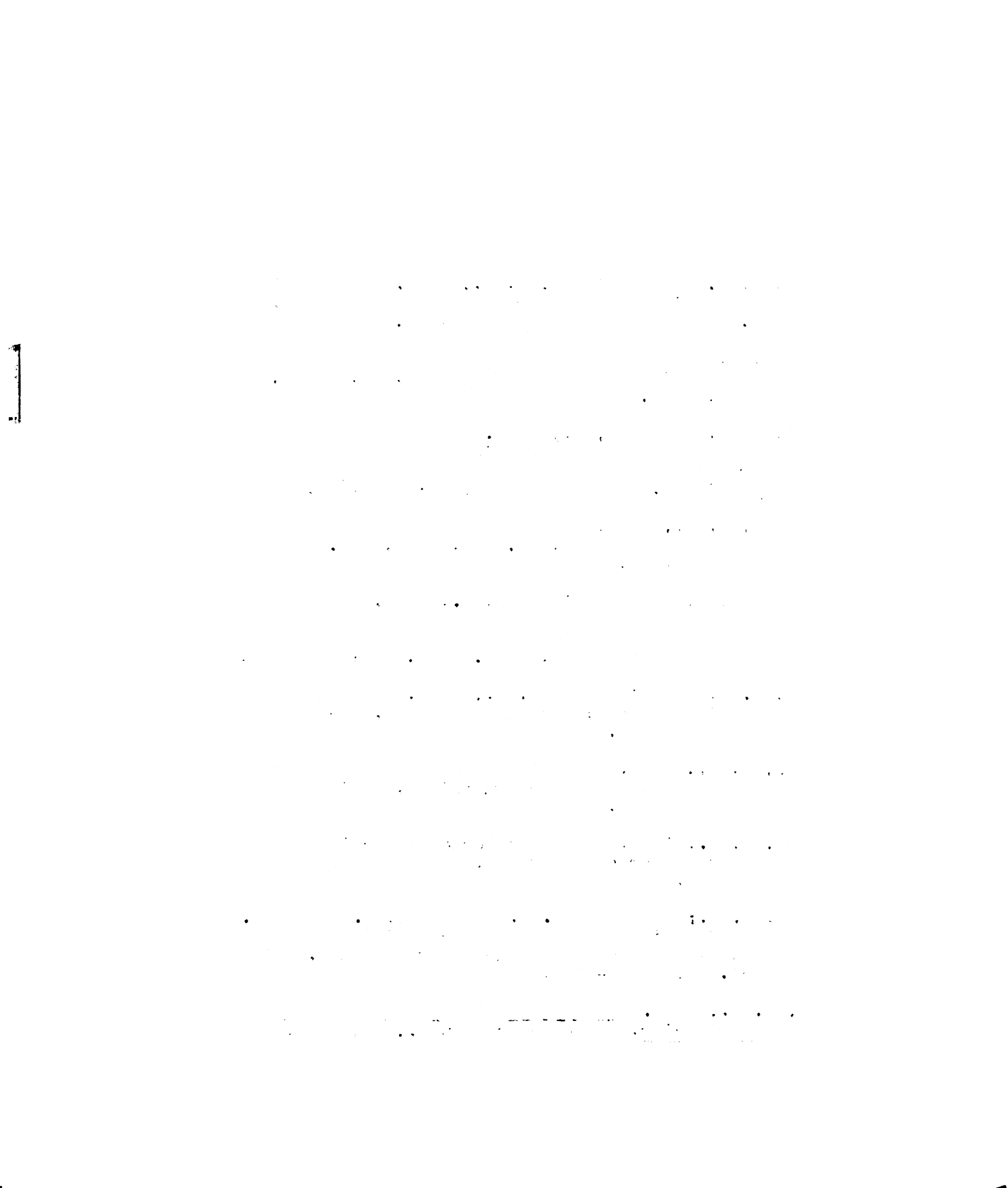

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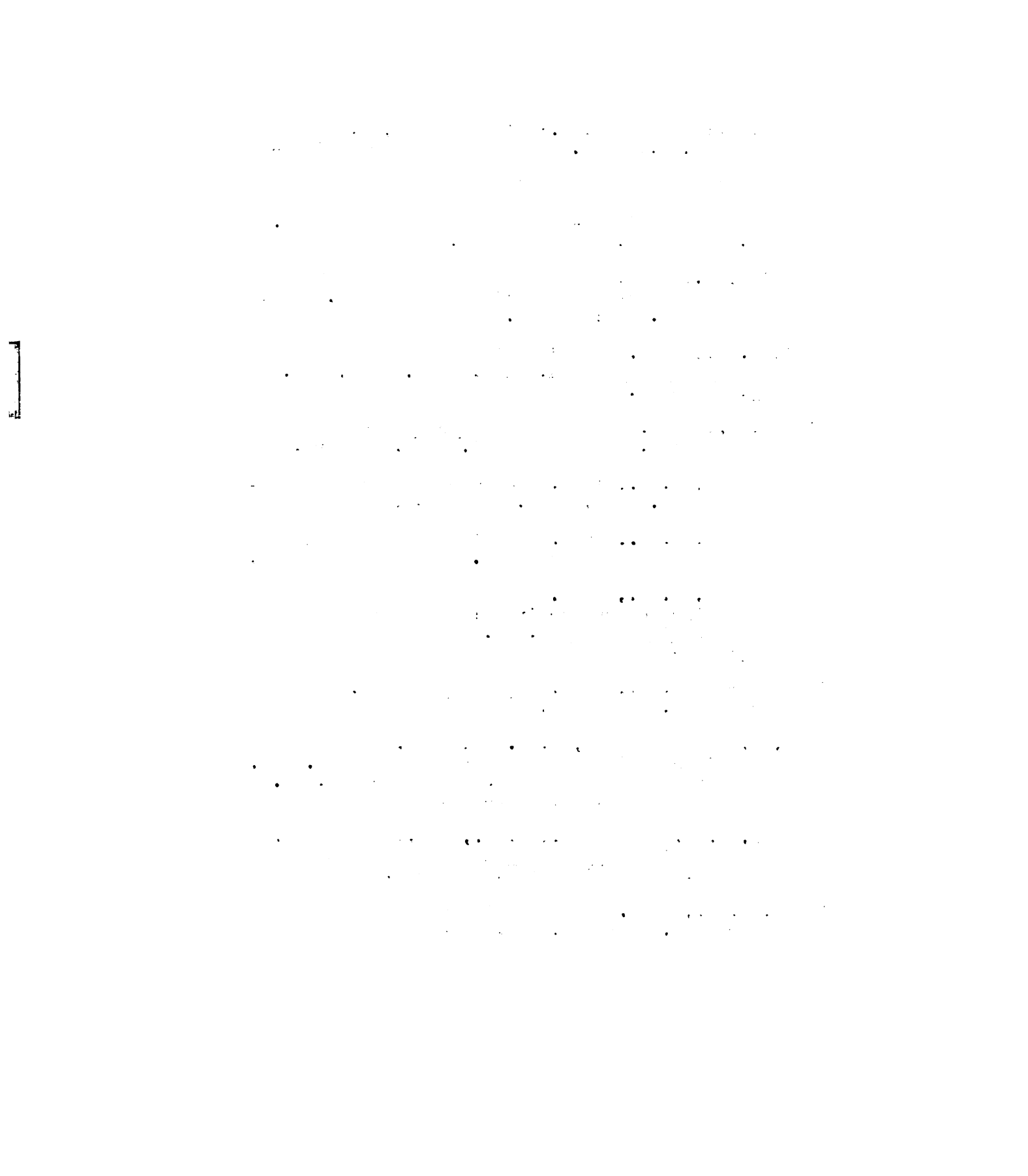

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