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LOCALIZATION OF TREHALOSE SYNTHESIS AND DEGRADATION IN ASCARIS

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ABSTRACT

Tissues of Ascaris lumbricoides (variety suum), the large round worm of pigs, contain trehalose, a disaccharide composed of two molecules of glucose. Minces of various Ascaris tissues were incubated with C^{14} glucose to determine the site of trehalose synthesis. Chromatography and radioautography were used to identify C^{14} trehalose synthesized during the incubation period. It was found that the reproductive tissues of both male and female Ascaris possess a mechanism for trehalose synthesis. Intestinal tissue and muscle showed no capability for trehalose synthesis under conditions of the incubation. By incubation of Ascaris tissue homogenates with commercial trehalose it was found that only intestinal tissue was capable of hydrolyzing trehalose into two molecules of glucose. The significance of synthesis and degradation of trehalose is discussed with regard to the findings of previous investigations.

Localization of Trehalose Synthesis and Degradation in *Ascaris*

I Introduction

∝ Trehalose (1-(∝ D glucopyranosyl)∝ D glucopyranoside) is a disaccharide composed of two molecules of glucose. (See fig. 1) It is chemically distinguishable from maltose by its inability to reduce Fehling's solution and its ability to resist hydrolysis in strong hot alkali. This compound was first isolated from ergot in 1832 by Wiggers, and has since been found to have wide distribution in fungi, yeasts, algae and bacteria. In 1956 trehalose was described as the primary sugar in hemolymph of the locust, Shistocerca gregaria, and a number of other insects (Howden and Kilby 1956). Fairbairn and Passey (1957) reported that Ascaris eggs and tissues also contained this disaccharide. Investigation of 71 species indicated that trehalose was present in a wide variety of other Invertebrates (Fairbairn 1958). It was found in protozoans, sponges, coelentreates, platyhelminths, entoprocts, echinoderms, annelids, arthropods and molluscs. The amount of trehalose in fourteen species of parasitic helminths ranged from 0-2.3% of tissue solids. Nematodes investigated, with the exception of Ascaridia galli and Heterakis gallinae, contained considerably more trehalose than glucose. Cestodes and trematodes, on the other hand, showed very low and approximately equal concentrations of the two sugars. The one Acanthocephalan assayed, Moniliformis dubis, contained large amounts of both glucose and trehalose.

Distribution of trehalose in adult Ascaris tissues was

determined by Fairbairn and Passey (1957). Greatest quantities of this sugar were found in uterus and muscle from female worms; 1.5 and 1.8% of tissue wet weight respectively. Testis and seminal vesicle also contained amounts exceeding 1% of their wet weight. Trehalose comprised .5-.8% of the wet weight of hemolymph, male muscle and ovary tissue, but only trace amounts were found in intestinal tissue and integument. Glucose concentration, however, was highest in tissues with the lowest concentrations of trehalose. Intestinal tissue was particularly characterized by its low trehalose and high glucose concentration.

Major investigations of trehalose synthesis and degradation have been carried out with microorganisms and insects. Cabib and Leloir (1958) described an enzyme from brewer's yeast which catalyzed the reaction Uridine diphosphoglucose + glucose -6- phosphate-------> Uridine diphosphate + trehalose phosphate. They found that their enzyme preparations also contained a phosphotase specific for trehalose phosphate. Using Rhistocerca gregaria, Candy and Kilby (1960) discovered that trehalose synthesis was localized in the fat body and that the pathway of biosynthesis (see fig. 2) was similar to the one elucidated in yeast cells. Passey and Fairbairn (1957) demonstrated that trehalose was synthesized in embryonating Ascaris eggs from the 10th to 25th day of incubation. They concluded from their balance studies of lipid and carbohydrate carbon during this period, that degraded

fatty acids served as carbon source for trehalose synthesis.

The enzyme trehalase which is responsible for hydrolysis of trehalose has been described in microorganisms and insects. Kalf and Reider (1957) were able to obtain a fraction with fairly specific trehalase activity from homogenates of Galleria mellonella larvae. Further purification and characterization of this enzyme has been carried out by Friedman. A 1,000 times purified preparation from Phormia regina was specific for trehalose hydrolysis, had a pH optimum of 5.6, and a temperature optimum of 45°C. The Michaelis-Menten constant was calculated to be 6.7 x 10-4M (Friedman 1960).

Although considerable work has been done on the pathways of carbohydrate metabolism in adult Ascaris lumbricoides, trehalose synthesis and degradation have not been investigated. The purpose of the present study was to determine which tissues of Ascaris possess the capacity for synthesis of trehalose from glucose. Fairbairn (1960) suggested that conversion of glucose to trehalose in intestinal cells might serve to maintain a favorable concentration gradient for absorption of glucose from the intestinal lumen. The potential of gut tissue for trehalose synthesis was, therefore, of special interest. A secondary goal was to determine which tissues might show trehalase activity.

In an attempt to answer the first question Ascaris tissue preparations were incubated with C^{14}glucose under a variety of conditions. The presence of C^{14}trehalose in

incubation media or extracts of incubated tissue was used as a criterion for trehalose synthesis. Incubation of tissue preparations with commercial trehalose and subsequent assay for increased glucose concentration served to determine "trehalase" activity.

II Materials and Methods

A. Procurement and Handling of Ascaris

Ascaris were collected from fresh hog intestines at a local packing company. They were transported to the laboratory in Krebs-Ringer's salt solution buffered at pH7.2 with Tris(hydroxymethyl) amino methane-maleate (10mM.). The temperature was maintained at 37°C by use of a fiberglass insulated vessel. After arrival in the laboratory worms were allowed to equilibrate in fresh buffered Krebs-Ringer's salt solution for one hour at 37°C and were used within the hour following equilibration. Total time between collection and use of the live material did not exceed three hours.

B. Preparation of Material for Incubation

Whole worms were used following a brief rinse in Krebs-Ringer's salt solution. Usually worms were dissected and the various tissues rinsed in Kreb's Ringer's saline to remove perienteric fluid. The tissues were then blotted on a piece of hard filter paper and placed in dry, iced petri dishes until sufficient material was collected. Wet weight of tissue was recorded. In some experiments whole tissues were used without further treatment, for others, minces and homogenates were made. The tissue was either cut into 2-5 mm. pieces with a scissors or homogenized in a Tenbroeck homogenizer (Corning) which was kept in an ice bath during homogenization. Muscle tissue was occasionally minced in a Serval

omnimix prior to homogenization. Suspending media for homogenizing varied among experiments and each is indicated with the experimental results.

Perienteric fluid was collected by chilling the worms in an ice bath for 2-5 minutes, suspending them over an iced beaker and making a small slash in the posterior third of the body. This permitted body fluid, uncontaminated by tissue, to drip into the cold beaker.

For determination of "trehalase" activity, tissue was prepared according to the method of Friedman (1960). Twenty percent homogenates were prepared in 0.05 M Tris HCl buffer pH 7, strained through three layers of cheese cloth and centrifuged (1,300 x g) at 5°C for 15 minutes. Particulate fatty material was removed from the supernatant by pouring it through a layer of glass wool.

In one experiment female reproductive system homogenate and 50 percent perienteric fluid were centrifuged for 60 minutes at 25,000 x g in the high speed head of the International refrigerated centrifuge. Aliquots of supernatants from this centrifugation were dialyzed for 12 hours against 16 liters of 10°C deionized water. Dialysates were recentrifuged for 60 minutes at 25,000 x g. The pellets from the two centrifugations were resuspended in 5 ml. of cold distilled water and assayed with the two supernatant fractions.

C. Incubation with C^{14} Glucose

Uniformly labeled glucose C^{14} was obtained from California

Biochemical Corporation and New England Nuclear Corporation. The two samples were diluted to contain 2 μc/ml. (.0665 μM/ml.) and 1 μc/ml. (1 μM/ ml.) respectively. Incubations were carried out in Warburg flasks of five or ten milliliter capacity. The flasks were maintained at 37°C and constantly agitated during the incubation period. For anaerobic incubations, flasks were gassed for ten minutes with 95% N_2 -5% CO_2 after which C^{14}glucose was introduced from the side arm. Aerobic incubations were run in flasks under air. One milliliter of Krebs-Ringer's bicarbonate solution, pH 7.4 or pH 6.4, was used as a suspending medium for 80-430 milligrams wet weight of tissue mince. Perienteric fluid was incubated with C^{14} glucose without further dilution or, in one case, after 50 percent dilution with deionized water.

Incubation was generally for 60 minutes after the addition of glucose. Reactions were terminated by addition of 1½-2 volumes of 95% alcohol. Mince preparations were then centrifuged or filtered and the tissue extracted with 50% alcohol for at least 24 hours. Both the supernatant fluid and the tissue extract were retained for chromatography. In some cases the supernatant fluid from the reaction mixture was decanted before the addition of alcohol, frozen immediately and later lyophilized. Alcohol-treated homogenate preparations were centrifuged briefly and pellets washed with 50 or 70% alcohol. The two supernatant fractions constituted the sample for chromatography.

Control incubations were run simultaneously using tissue or homogenated previously boiled for three to five minutes. Zero time controls were also used. In these, the aliquot of 95% alcohol was added to the reaction mixture before the tissue.

D. Incubation with Trehalose

Assays for trehalase activity were performed with homogenates of fresh and frozen Ascaris gut, muscle and reproductive tissue prepared as previously stated. Reaction mixtures are shown in table 1.

Incubations were carried out aerobically at 37°C. Reactions were stopped after 60 minutes by the addition of 1 ml. of BaOH with agitation, followed by 1 ml. of neutralizing $ZnSO_4$. The tubes were centrifuged until clear and "Glucostat" used to determine the amount of glucose in several dilutions of aliquots of the supernatant. "Glucostat" (Worthington) contains glucose oxidase, peroxidase and a chromogen, o-dianisidine. The quantitative determination of glucose is based on the following reactions.

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{gluconic acid}$$

$$H_2O_2 + \text{reduced chromogen} \xrightarrow{\text{peroxidase}} \underset{\text{(colored)}}{\text{oxidized chromogen}}$$

Four ml. of glucose oxidase-chromogen mixture were used for each 0.5 ml. sample. These were incubated for 30 minutes at 35°C and the reaction was stopped with 1 drop of 6N HCl followed by agitation. The color was assayed in a Klett-Summerson photo-electric colorimeter. Standards contained

6.25, 12.5, 25.0, 50.0, and 100.0 micrograms of glucose. No difference was noted between glucose standards incubated and treated with $Ba(OH)_2$-$ZnSO_4$ and untreated standards.

E. Subsequent Treatment of Samples

1. Drying

Supernatant fluid and alcohol extract samples were usually placed in 30 ml. beakers and evaporated to dryness in an air current under a hood at a temperature of 40°C. To check this method a number of samples were frozen on dry ice and evaporated in vacuo over concentrated sulfuric acid. The two drying methods produced the same results. Beakers containing dry samples were covered and stored in the freezer.

2. Desalting

Chromatography of non-desalted samples was impossible. An attempt was made to desalt electrolytically using ion-exchange membranes but this method was not satisfactory. Combinations of various ion-exchange resins were tested. Double columns of Dowex 50(H form)-Amberlite IR 45 (OH form), Dowex 50W (H form)- Dowex 21K (OH form) and single columns of Amberlite MB 1 and MB K were evaluated for their capacities in salt removal and glucose retention. MB K, a bulk resin, presumed to be a mixture of strongly acidic cation exchanger and weakly basic anion exchanger, proved most satisfactory and was used to desalt all samples cited.

A combination of the slurry and column method increased the desalting efficiency. One milliliter of water and about .05 $in.^3$ of mixed resin were added to dried samples. Slurries were agitated frequently over a 15 minute period and then poured into 3/8 inch columns of Pyrex tubing containing one inch of the same resin. Six five-milliliter aliquots of deionized water followed each slurry and the effluent was collected and evaporated in 30 ml. beakers.

3. Chromatography

After some experimentation with standards of glucose, trehalose and glucose-6-phosphate, it was determined that descending chromatography utilizing n-propanol, ethyl acetate, water (7-1-2 by volume) (Baar and Bull, 1953) produced the best separation. Further separation of the three standards could be gained by using a second dimension of ethyl acetate, pyridine, methylethyl ketone, water (50-36-36-30 by volume). For one dimensional chromatography, graduated five and twenty-five microliter pipettes were used to apply samples previously redissolved in 25-100 microliters of deionized water. Whatman #1 filter paper sheets were cut to 8 x 17 inch size and marked.(see fig.3) Multiple aliquots of about 0.5 microliter were applied to the same spot; each aliquot was dried with a hairdryer before applying the next aliquot. Results did not depend on the spot drying method. Chromatograms on which the applications were allowed to dry without

treatment appeared similar in all respects to those on which the hairdryer was used. If the redissolved sample was of thick consistency or the chromatogram was to be used for elution, the spotting was done according to the method of Dimler et al. Five or ten aliquots of a sample, about 2 microliters each, were applied in a row of spots about 3 mm. apart along the origin line. This type of application was advantageous when it was desirable to separate a large amount of sample on a limited amount of paper. A mixture of standard glucose, trehalose and glucose-6 or glucose-1-phosphate was applied at one or two sites on the origin line.

When all sample and standard spots were dry, the folded ends of sheets (see fig.3) were placed in troughs suspended in chromatography cabinets. A pyrex dish containing 150-200 ml. of solvent was put in to saturate the chamber, the troughs filled with the remaining solvent and the cabinet closed. After 24-36 hours chromatograms were removed and dried at room temperature.

Whatman # 1 filter paper of the same size was utilized for two dimensional chromatograms. Ten to fifty microliters of samples, previously dissolved in 25 to 100 microliters of 2×10^{-2}M trehalose solution, were applied at a point on the origin line one inch from the left margin of the paper. Descending chromatography was carried out as described above. After sheets were dry they were stapled into cylinders so that the previous origin line was parallel to the axis of the

cylinder. These were placed, left side down, into about 1/2 inch of solvent. Usually within three hours the solvent level had reached a height of seven inches and the cylinders were removed and dried.

Various reagents for carbohydrate color development were tested. Periodic acid in acetone followed by benzidine in glacial acetic acid and acetone (Gordon, et al.,1956) worked well in detecting standard samples of trehalose but was not satisfactory when used on chromatograms of experimental samples. The most useful detection method was that of Trevelyn, Procter and Harrison (1950). Chromatograms were dipped into a solution of silver nitrate in acetone (0.1 milliliter of saturated aqueous $AgNO_3$ to 20 milliliters of acetone plus enough water to dissolve the precipitate). After drying at room temperature, chromatograms were sprayed with NaOH in ethanol (2 milliliters of saturated aqueous NaOH per 100 milliliters of 95% ethanol). Glucose, trehalose, and most of the sugar phosphates appear as yellow to brown spots as do many other non-carbohydrate reducing substances. The greatest problem, the rapid darkening of the sprayed chromatograms, was solved by tracing the spots immediately after their visualization. Generally chromatograms were not treated with silver nitrate until they were analyzed for radioactivity because silver impregnation decreased the amount of detectable radiation emitted from the filter paper. If treated chromatograms were not to be used for

further analysis of radioactivity, their color was preserved by dipping them in 6 N NH_4OH (Trevelyn *et al.*) or Ansco liquafix and rinsing them for several hours in running water. This prevented further fading but tended to completely remove the color from faint spots.

Detection of sugar phosphates was carried out by the Bandurski and Axelrod (1951) modification of the Haynes-Isherwood technique. Haynes-Isherwood reagent (1 gram of ammonium molybdate, 8 ml. water, 3 ml. concentrated HCl, 3 ml. of 70% perchloric acid mixed and diluted to 100 ml. with acetone) was sprayed onto chromatograms. These were dried at 85°C and then exposed to ultraviolet light (G.E. germicidal) at a distance of 10 cm. until blue phosphate spots were clearly visible.

4. Elution

The method proposed by Dimler *et al.*(1952) was used to elute materials from chromatograms. Samples designated for elution were spotted in several series of two microliter aliquots. The usual method is shown in fig. 4. One dimensional chromatography was carried out as previously indicated. When the paper was dry it was cut into three parts (see fig. 4) and Trevelyan's method used to develop the color in parts I and III. Part II was then cut horizontally into four sections depending on the levels of the compounds on parts I and III. (see fig. 5) If eluates were

to be counted on the flow counter (see assay of radioactivity) ten microliters of the sample were spotted in two microliter aliquots onto a separate piece of paper 1 x 3 in. (fig.4) This was not chromatographed but was eluted with the chromatogram strips to give an indication of total activity.

Straight ends of strips to be eluted were clamped between edges of pairs of two inch glass squares and these fastened together with chromatography clips. (fig. 6) The double squares were placed in the elution tray, leaning against the edge so the paper strips hung directly over collection vessels below. Boiling water was poured into the bottom dish of the aquarium fitted as an elution chamber (fig. 6) and a glass plate, in which a small hole had been drilled, used to seal this chamber. When the chamber was fully saturated, the plug was removed from the hole in the lid and distilled water poured through a funnel into the elution tray. Water traveled between the plates, down the paper carrying all water soluble materials to the point of the strip and finally dripped into the containers below. In four hours about twelve drops of eluate were collected. Tests with glucose indicated that 95-100% was eluted in four hours. Eluates to be hydrolyzed were collected in teflon watch glasses and those to be counted with the flow counter and subsequently rechromatographed were collected in 30 ml. beakers. All eluates were dried as previously mentioned.

5. Hydrolysis

Hydrolysis of eluates followed by chromatography of hydrolysates was used as a check on their composition. A modification of the method of Porter and Hoban (1954) was found to be most satisfactory. Evaporated eluates on teflon watch glasses were dissolved in 25 microliters of 1 N HCl. This was taken up in capillary tubes which were then sealed on both ends. The tubes were boiled for two hours and the hydrolysates spotted directly from the pulled and broken ends of the capillaries onto Whatman #1 filter paper. Chromatography and color development were carried out as previously mentioned.

It was determined during trial hydrolysis of standard samples of trehalose that 1 N HCl at 100°C for two hours would hydrolyze 90-100% of samples equivalent in weight to those likely to be encountered in eluates. It was also found that 25 microliters of acid added to an eluate dried on a teflon watch glass would permit removal of most of the sample to a capillary tube. Second aliquots of acid added to the watch glasses contained no carbohydrate detectable by chromatographic methods. Controls for hydrolysis were samples eluted from the same strips but redissolved in water rather than acid.

F. Assay of Radioactivity

1. Strip Counting

The distribution of radioactive compounds on one dimen-

sional chromatograms was analyzed by use of a strip counter (Actigraph II, Nuclear-Chicago) combined with an analytical count rate meter and a rectilinear recording milliammeter. A scanning speed of three inches per hour, sensitivity of 1 second, slit width of 1/8 inch and maximum scale of 300 counts per minute was the combination of settings generally found most satisfactory. Because of the many variables involved in the absorption of radiation by the paper, data obtained were not quantitative. The advantage of strip counting was the relative speed in determining which sets of samples should be selected for more thorough investigation by radioautography and elution. The presence of a peak of radioactivity corresponding exactly to the trehalose spot on the chromatogram was considered to be a tentatively positive result for trehalose synthesis. The disadvantage of this assay method was the total dependence of identification upon trehalose standards and equality of the dimensions of the chromatogram strip and the scanning record.

2. Counting of Eluates

Eluates from specific areas of one dimensional chromatograms, obtained and dried as mentioned under elution, were redissolved in 5 milliliters of 70% alcohol. Aluminum planchets were washed in 95% alcohol and dried before receiving 0.5 ml. of each sample. Plated samples were then dried under a heat lamp and counted with a model D47 Nuclear-Chicago gas flow counter. The remainder of each dissolved

eluate was rechromatographed to verify its identity.

3. Radioautography

The technique of "fingerprinting" as described by Roberts et al (1955) was used for trehalose identification. This method involves the exact correspondence of radioactivity localized by radioautography with a known spot on the radioautographed chromatogram. A known spot is produced by the addition of a large quantity of the compound to be identified to the sample before chromatography. In this case trehalose was to be identified so 189-756 micrograms of trehalose were added to each sample. Two-dimensional chromatograms of these samples were stapled onto sheets of Ansco non-screen X-ray film and kept in sealed envelopes for six to ten weeks. At the end of the exposure time the X-ray films were developed and the chromatograms treated with silver nitrate and NaOH. It was noted that chromatograms which had been stapled to X-ray film for long periods often reacted in a peculiar manner when silver nitrate was applied. Large dark areas appeared which obliterated carbohydrate spots. On some occasions color development was carried out and tracings made of the chromatograms before they were radioautographed even though this necessitated longer exposure times.

When the outline of an exposed area on the X-ray film could be matched exactly to the outline of the known trehalose

spot on the chromatogram it was regarded as a positive result for trehalose synthesis in the sample.

G. Protein Determination

Protein content of homogenate preparations was determined by the method of Lowry et al (1951). Five milliliters of 5% trichloroacetic acid were used to precipitate the protein in each .5 ml. sample. The resulting precipitates were redissolved in one milliliter of 1 N NaOH and three dilutions made. Five milliliters of alkaline copper solution (50 ml. 2% $NaCO_3$ plus 1 ml. .5% $CuSO_4.5H_2O$ in 1% sodium tartrate) were added to 1 ml. of each dilution. After 10 minutes, 0.5 ml. of Folin phenol reagent (1.0 N phenol solution) was pipetted into each tube and immediately mixed with the sample. Thirty minutes after the addition of the Folin reagent, resulting colors were read using a Klett-Summerson colorimeter fitted with a 660 mu filter. Eight concentrations of plasma albumin, between 4 micrograms/milliliter and 500 micrograms/milliliter served as standards.

H. Dry Weight Determination

After alcohol extraction, tissue was transfered to tared foil pans and dried at 100°C for at least 12 hours before pans were reweighed.

III Experimental

A. Incubations of *Ascaris* and *Ascaris* tissue preparations with C^{14}glucose

1. Result of anaerobic incubations of whole *Ascaris* with C^{14}glucose

Two immature female and two male worms were incubated anaerobically with 90 µg of C^{14}glucose (.208 µc.) in 5.5 ml. of Krebs Ringer's bicarbonate buffer pH 7.4. Both incubation media and alcohol extracts of the incubated worms were chromatographed and assayed for radioactivity. Evidence for C^{14}trehalose was lacking in these samples.

2. Result of anaerobic incubations of *Ascaris* tissue minces with C^{14}glucose

Ascaris muscle, ovary-oviduct, male reproductive system and gut minces were incubated with C^{14}glucose under anaerobic conditions. Results of these experiments are recorded in table II. Experiment I was carried out at pH 6.4 and zero time controls used. Strip countings of one-dimensional chromatograms indicated that radioactive trehalose was present in both the incubation media and alcohol extracts from ovary-oviduct preparations. (fig. 7) Muscle, gut and control samples were negative. Radioautography of two dimensional chromatograms confirmed the identity of C^{14}trehalose in the ovary-oviduct incubation media. (fig 8) An area of weak radioactivity on radioautograms of incubated ovary-oviduct alcohol extracts could be matched with trehalose in one

sample but not in the other. This was also true of radioautograms of chromatographed muscle mince incubation media. Sample 190 was weakly positive for radioactive trehalose while sample 191 was negative. All gut samples were negative, however an unidentified area of activity (Rg or $\frac{\text{motility of x}}{\text{motility of glucose}}$ = 1.68 in the descending solvent) was noted on alcohol extract chromatograms.

Experiment II was carried out at pH 7.4 and minces previously boiled for three minutes were used in control incubations. Strip counting and radioautography of chromatograms from heat treated preparations and gut samples gave no indication of radioactivity from C^{14} trehalose. Incubation media from both ovary-oviduct and male reproductive tissue mince preparations showed approximately equal activity in C^{14}glucose and C^{14}trehalose. (fig.9) The presence of C^{14} trehalose was not noted on strip countings of chromatograms of tissue extracts from these incubations. Tracings obtained by counting one-dimensional chromatograms of incubation media and alcohol extracts from muscle preparations showed peaks of radioactivity corresponding to trehalose. After radioautography of two-dimensional chromatograms it was observed that, although trace amounts of C^{14} trehalose were present in the incubation media, the activity of the alcohol extract was due to an unidentified compound.

The third experiment was also carried out at pH 7.4 but larger tissue samples were used.(see table II) When aliquots containing 1% of dried incubation media samples were

chromatographed, strip countings showed activity in the trehalose areas of male and female reproductive system samples. (fig 10a) Tracings of chromatograms of 5% aliquots, however, gave indication of trehalose activity in the muscle and glucose control samples as well. No activity was detected in the trehalose area of gut sample chromatograms. Aliquots of eluates from the trehalose areas of female and male reproductive tissue incubation medium chromatograms had respective activities of 819 and 337 counts/minute above the count of similar eluate from the glucose control chromatogram. The activity of the trehalose area eluate from the chromatogram of muscle incubation medium was 123 counts/minute above the control count. The eluates from trehalose areas of chromatograms of gut and glucose control samples were approximately equal in activity.

Hydrolysis of eluates from trehalose areas provided further evidence for the identity of the radioactive material. Strip countings of chromatographed hydrolyzed eluates from male and female reproductive system samples showed a decrease in activity of the trehalose area to background level and a corresponding increase in glucose area activity. No other significant areas of radioactivity were noted after the hydrolysis. (fig 10 b-c-d-e.) A silver nitrate reactive substance (R_g = .44) appeared on chromatograms of hydrolyzed samples. The activity of this area was not above that of controls (fig. 10 e) A small increase in glucose activity after hydrolysis of trehalose

area eluate from the gut sample was equivalent to that of the control. Similar eluates from muscle incubation medium chromatograms showed greated increases in radioactive glucose after hydrolysis, but activity in the non-hydrolyzed eluate was not located in the trehalose area.

Radioautographs of two dimensional chromatograms of male and female reproductive system incubation media demonstrated excellent correspondence of radioactivity and trehalose spots. (fig.11) Low level, diffuse activity was indicated in the general trehalose area on radioautographs of gut, muscle, and glucose control samples, but this activity did not correspond to the well defined trehalose spots on the chromatogram.

3. Result of Aerobic Incubations of *Ascaris* Tissue Minces with C^{14} Glucose

A preliminary experiment was performed in which muscle, female reproductive system and male reproductive system tissue minces were incubated with C^{14} glucose. The suspending medium for each sample was one milliliter of Krebs-Ringer-bicarbonate buffer pH 6.6 to which 0.5 ml. of 5×10^{-2}M C^{14} glucose (1 µc/ml.) was added. Strip countings of chromatographed muscle incubation media showed no C^{14} trehalose. Male and female reproductive tissue media samples were also negative for C^{14}trehalose. They did, however, show activity in an unidentified substance of mobility slightly less than trehalose.(Rg trehalose .695, Rg unknown .555)

Later aerobic incubations of muscle, female reproductive tissue and gut sacs were carried out at pH 7.6. Each sample was suspended in two ml. of Krebs-Ringer-bicarbonate and 1 ml. of 1 x 10^{-3}M C^{14}glucose (1 µc/ml.) added. Results were similar to those of anaerobic incubations. Activity was present in the trehalose area on chromatograms of female reproductive tissue incubation media but not on those of muscle or gut media samples. The radioactivity in female reproductive system media was further identified as trehalose by the fingerprint technique. Radioautographs of gut and muscle samples were negative for C^{14} trehalose.

4. Result of Incubation of Homogenate Preparations of *Ascaris* Tissue with C^{14}Glucose

Only one aerobic incubation of homogenate preparations was completed. Results were not obtained from several early experiments of this type due to lack of a satisfactory desalting method. *Ascaris* female reproductive system homogenate (20%, in deionized water) and various products of centrifugation and dialysis of this homogenate were tested for the ability to synthesize trehalose from glucose. One milliliter aliquots of whole homogenate, supernatant after 60 minute centrifugation, resuspended pellet after centrifugation, dialysate and centrifuged dialysate were incubated aerobically with 0.5 ml. of C^{14} glucose (1 x 10^{-3}M., (.417 µc/ml.)). Other additions to each sample were 0.1 ml. 1 x10^{-2}M $MgSO_4$, 0.05 ml. 2 x 10^{-2}M adenosine triphosphate (ATP) 0.05 ml. 9 x 10^{-3}M

uridine diphosphoglucose (UDPG) and 0.2 ml. 2.5 x 10^{-2}M Tris (hydroxymethyl) amino methane maleate buffer pH 6.6. Tissue preparations boiled for three minutes were used in control incubations. After samples were processed and chromatographed, strip countings and radioautograms were made. Although several radioactive compounds were formed, C^{14} trehalose could not be identified in the incubation samples.

Two milliliter aliquots of muscle, female and male reproductive system homogenates (20%, in Krebs-Ringer's-bicarbonate pH 6.6) were incubated anaerobically with 0.1 ml. 2 x 10^{-2} M ATP, 0.1 ml. 1.8 x 10^{-2} M UDPG, 0.5 ml. 5 x 10^{-2} M (1 µc/ml.) C^{14}glucose, and 0.2 ml. of buffer. Boiled homogenate controls were used as in the previous experiment. Strip countings of chromatographed media from both experimental and control incubations showed peaks in the trehalose area but radioautograms of samples chromatographed two dimensionally gave no indication of the presence of C^{14}trehalose.

A second anaerobic experiment utilized only 15% female reproductive tissue homogenates. Two types of preparations were made; one in Krebs-Ringer-bicarbonate pH 6.6, the other in deionized water. Since it had been noted in previous experiments that heat treated homogenates often gave rise to samples with scattered activity, aliquots of each homogenate were boiled for two minutes or ten minutes. A portion of each homogenate was also frozen to be incubated later. The results of incubations using boiled homogenates

were compared to those of non-boiled frozen and trichloroacetic acid precipitated preparations. (table III). The only chromatograms showing activity near the trehalose area were those of the untreated and frozen homogenates. Further investigation indicated, however, that this peak was not caused by C^{14}trehalose. Several similar peaks of radioactivity corresponding to unidentified compounds appeared on chromatograms of incubation media from heat treated, frozen and non-treated homogenate samples. One of these also appeared on trichloroacetic acid precipitated control and glucose control chromatograms. There were no major differences in activity distribution on chromatograms of incubation media from untreated and frozen homogenates.

5. Result of Incubation of Ascaris Perienteric Fluid with C^{14}Glucose

Aerobic incubations of 50% perienteric fluid and products of high speed centrifugation and dialysis of this fluid were carried out at pH 6.6. The reaction mixture was identical to that of the aerobic incubation of female reproductive system homogenate (page 23). As far as could be ascertained by strip counting and radioautography, no C^{14} trehalose was formed during any of these incubations. When undiluted perienteric fluid was incubated with C^{14}glucose anaerobically, similar results were obtained.

B. Incubations of Ascaris Tissue Preparations with Trehalose

Assays for trehalase activity were performed on four

dilutions of muscle homogenate (20%, 10%, 2%, .2%) and on two dilutions of frozen muscle homogenate (20%, 4%). All were negative. Similar assays of fresh and frozen reproductive tissue were also negative. With gut homogenates, however, there was evidence of trehalose hydrolysis during sixty minute incubations with trehalose. (table IV) Trehalase activity was calculated to be .086 uM of trehalose hydrolyzed/mg. protein hr. for 20% fresh gut homogenate and 0.105 uM for the diluted preparation. Frozen gut homogenates exhibited over six times the activity of the non-frozen preparation. Activity of 20% frozen gut homogenates was 0.54 uM of trehalose hydrolyzed/ mg. protein/ hr. and that of the diluted (4%) homogenate was 0.67 uM.

IV Discussion

Date obtained through anaerobic incubation of Ascaris tissue minces with C^{14}glucose indicate that both male and female reproductive tissues possess the enzymes necessary for synthesis of trehalose from glucose. There was little evidence for trehalose synthesis in muscle tissue and no evidence for its formation in intestinal cells or hemolymph. Limited experimentation precludes conclusion that in vivo synthesis occurs only in reproductive tissue. However, it does not seem likely that intestinal tissue serves in this capacity. Data are compatible with Fairbairn and Passey's analysis of trehalose and glucose distribution in Ascaris tissues. They found that trehalose was most abundant in reproductive tissue and in lowest concentration in intestinal tissue, while glucose had an inverse distribution. The high glucose and low trehalose content of intestinal tissue and the inability of this tissue to synthesize trehalose under conditions which were satisfactory for synthesis in reproductive tissue, suggest that rapid conversion of glucose to trehalose in gut cells is not a mechanism for glucose absorption from the gut lumen. By incubation of gut sacks filled with C^{14}glucose solution and analysis of media and tissue extracts for C^{14}trehalose more conclusive evidence might be obtained. Fisher (personal communication) has recently shown that glucose is the compound appearing on the anti-luminal side of gut tissue during glucose absorption.

The activity of female reproductive tissue is not particularly surprising. Fairbairn and Passey reported that trehalose constituted 7.9 ± 1.2% of decoated unembryonated egg solids. Considering data obtained, it seems plausible that trehalose is synthesized in ovarian tissue and incorporated into the eggs as they are formed. Significance of the synthesis in male reproductive tissue, however, will remain an enigma until Ascaris sperm have been analyzed more completely.

The absence of trehalose synthesis in homogenate preparations of reproductive tissue probably indicates loss of a labile factor during homogenization. Incubations of homogenate with an ATP generating system and further experimentation provide a starting point for purification of the enzyme system (s).

Despite reported high trehalose content of female Ascaris muscle, only trace amounts of the disaccharide were formed during incubation of muscle minces in C^{14}glucose. Either trehalose is transported by the hemolymph from the site of synthesis to the muscle or it is synthesized in muscle under conditions not duplicated by the incubation. Clegg and Evans (1961) found that flight muscle of Phormia regina had little capacity for trehalose synthesis and that blood played a major role in distribution of trehalose formed in the fat body. Further experimentation might demonstrate that a parallel situation pertains in Ascaris.

Differential effects of aerobic and anaerobic conditions on trehalose synthesis are not indicated by data

obtained. Radioautograms from two experiments at pH 7.4-7.6, differing only in presence or absence of air from reaction vessels, were qualitatively similar in all aspects. Results from another set of experiments, these carried out at pH 6.4-6.6, were not comparable. These aeorbic samples contained a large percentage of activity in an unidentified compound which was not present in anaerobic samples. In vivo synthesis of trehalose most certainly occurs under conditions of low oxygen tension so that it might be considered that anaerobic incubations more closely approximate the normal situation.

The distribution of "trehalase" activity presents an interesting problem. In insects trehalose serves as an energy source and muscle is the primary locus of its degradation. Ascaris muscle, however, did not exhibit this activity. Data indicated trehalase activity in Ascaris intestinal tissue. It is possible that the enzyme causing this hydrolysis is a digestive enzyme of wide capabilities for carbohydrate degradation. Further testing and purification of this activity would indicate whether a specific trehalase was present in the intestinal tissue. The activation by freezing and increased activity in dilute preparations merits further study.

Passey and Fairbairn (1957) demonstrated that trehalose disappeared in embryonating eggs between the first and tenth day of development. Trehalose metabolism is possibly more

important during embryonic development than in adult Ascaris. Administration of C^{14}trehalose to adult worms and assay of tissues for labeled compounds after a period of incubation might provide additional information on trehalose metabolism.

V Summary

1. Incubations of muscle, intestine, male and female reproductive system tissue minces and hemolymph of adult Ascaris lumbricoides with C^{14}glucose and subsequent assay for C^{14}trehalose demonstrated that only reproductive tissue was capable of synthesizing trehalose under the experimental conditions.
2. No C^{14}trehalose was synthesized when tissue homogenates were incubated with C^{14}glucose.
3. Muscle and reproductive tissue homogenates did not hydrolyze trehalose. Fresh and frozen gut homogenates, however, hydrolyzed 0.105 and 0.67 uM of trehalose/ mg of protein/ hour respectively.

VI References

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TABLE I

Reaction Mixtures used for Assay of Trehalase Activity

	homog. or homog. dil.5X	trehal. $2x10^{-2}$M	citrate buffer .1M 5.6 or 4.7	water
control for glucose in trehalose and trehalase in glucose oxidase	____	.5 ml.	.6 ml.	.4 ml.
control for glucose and trehalose in homogenate	.4 ml.	____	.6 ml.	.5 ml.
zero time control*	.4 ml.	.5 ml.	.6 ml.	____
experimental	.4 ml.	.5 ml.	.6 ml.	____

* One ml. of NaOH was added to the tubes before addition of the homogenate. Immediately after homogenate addition samples were neutralized with one ml. of $ZnSO_4$.

Table II

Anaerobic Incubation of Tissue Minces with C^{14} Glucose

A

Incubation: 37° C., 60 minutes, constant shaking
suspending medium: 1 ml. Krob's-Ringer's bicarbonate, pH6.4
additions: .5 ml. C^{14}glucose(1 µmole/ml.,.417 µc/ml.)
gas phase: 95% N_2-5% CO_2

#	tissue	mg. wet wt.	C^{14}activity in trehalose area strip count media	strip count alc.ext.	radioautogram media	radioautogram alc.ext.
190	muscle	435.	trace	-	trace	-
191	muscle	455.	-	-	-	-
199	muscle (0 time)	620.	-	-	-	-
193	ovary-oviduct	230.	+	+	+	-
194	ovary-oviduct	340.	+	+	+	trace
201	ovary-oviduct (0time)	180.	-	-	-	-
196	gut	150.	-	-	-	-
197	gut	80.	-	-	-	-
200	gut (0 time)	120.	-	-	-	-

Table II

B.

Incubation: 37°C, 60 minutes, constant shaking
suspending medium: 1 ml. Krebs-Ringer's bicarbonate, pH 7.4
additions: .5 ml. C^{14} glucose (1 umole/ml.-.417 uc/ml.)
gas phase: 95% N_2-5% CO_2

#	tissue	mg. dry wt.	C^{14} activity in trehalose area strip count		radioautogram	
			media	alc.ext.	media	alc.ext.
231	muscle	81.	trace	trace	trace	-
232	muscle (heat treated)	76	-	-	-	-
238	ovary-oviduct	63.4	+	-	+	-
237	ovary-oviduct (heat treated)	112.8	-	-	-	-
236	gut	24.	-	-	-	-
235	gut (heat treated)	40.	-	-	-	-
234	testis-seminal vesicle	10.3	+	-	+	-
233	testis-seminal vesicle (heat treated)	27.7	-	-	-	-

Table II

C.

Incubation: 37°C., 60 minutes, constant shaking
suspending medium: 2 ml. Krebs-Ringer's bicarbonate, pH 7.4
additions: 1 ml. C^{14}glucose (1 umole/ml.- luc/ml.)
gas phase: 95% N_2-5% CO_2

#	tissue	mg. dry wt.	C^{14}activity in trehalose area strip count media	radioautog. media	eluate ct. media
342	muscle	314.3	-	-	267. ct/min.
340	ovary-oviduct	211.	+	+	963.
343	gut	102.1	-	-	139.
341	testis-seminal vesicle	95.6	+	+	481.
	glucose control		-	-	144.

Table III

Anaerobic Incubation of Anemia Female Reproductive System Homogenates with C^{14} Glucose

#	prep.	treatment prior to incub.	reaction mixtures homog.	glucose	susp. med.
320	5 g. tissue homog. in 30 ml. KRB pH 6.6	5° C -10 min.	1 ml.	.5 ml.	1 ml. KRB pH 6.6
321	"	100°C-2 min.	1	.5	1
322	"	100°C-10 min.	1	.5	1
323	"	1 ml. TCA added	1	.5	---
324	"	-5°C-36 days	1	.5	1
325	5 g. tissue homog. in 30 ml. delon. water	5° C-10 min.	1	.5	1 ml. HOH
326	"	100°C-2 min.	1	.5	1
327	"	100°C-10 min	1	.5	1
328	"	1 ml. TCA added	1	.5	---
329	"	-5°-36 days	1	.5	1

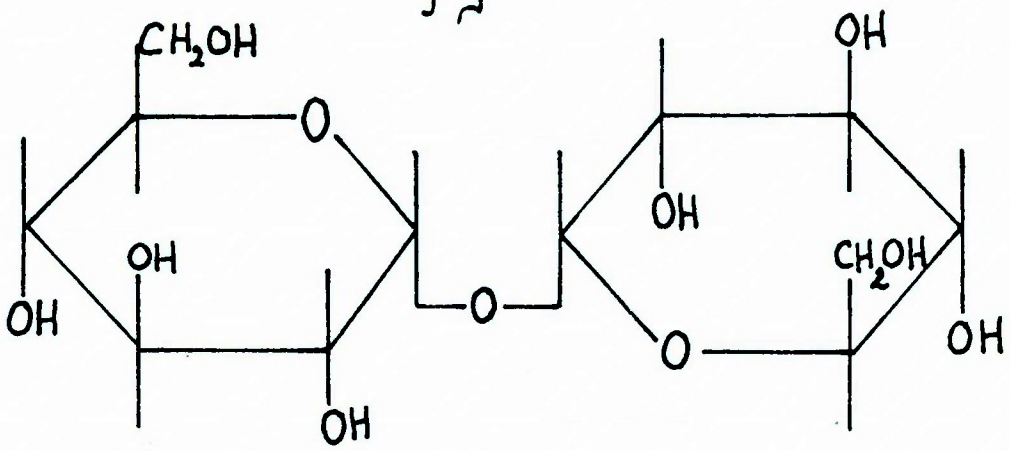
KRB-Krebs-Ringer's bicarbonate
TCA-trichloroacetic acid

Table IV

Incubation of Fresh and Frozen Gut Homogenates with Trehalose

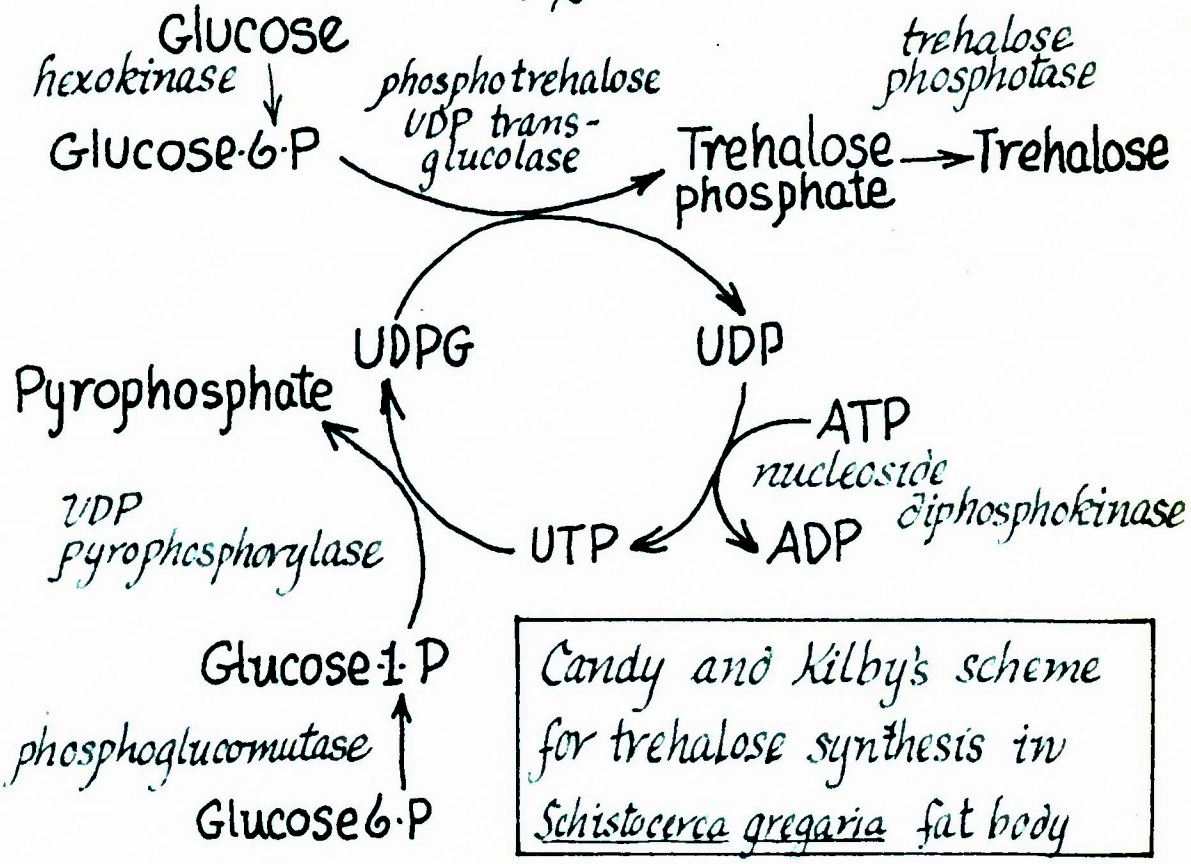
FRESH GUT	homog. ml.	homog. %	2×10^{-2} M treh. ml.	cit. buff. ml.	KCN ml.	µg. gluc. formed/ incub.	uM. treh. hydrol. /samp. /hr.	uM. hydrol. /mg. prot /hr.
reagent control	-		.5	.6	.4	--	--	---
	-		.5	.6	.4	--	--	---
tissue control	.4	20%	--	.6	.5	--		
	.4	20%	--	.6	.5	--		
	.4	4%	--	.6	.5	--		
	.4	4%	--	.6	.5	--		
0 time control	.4	20%	.5	.6	--	--		
	.4	20%	.5	.6	--	--		
exper.	.4	20%	.5	.6	--	294.	.396	.086
	.4	20%	.5	.6	--	280.		
	.4	4%	.5	.6	--	70.	.097	.105
	.4	4%	.5	.6	--	70.		
FROZEN GUT								
reagent control	-		.5	.6	.4	--		
	-		.5	.6	.4	--		
tissue control	.4	20%	--	.6	.5	--		
	.4	20%	--	.6	.5	--		
	.4	4%	--	.6.	.5	--		
	.4	4%	--	.6	.5	--		
0 time control	.4	20%	.5	.6	--	--		
	.4	20%	.5	.6	--	--		
exper.	.4	20%	.5	.6	--	1,442.	2.0	.54
	.4	20%	.5	.6	--	1,484.		
	.4	4%	.5	.6	--	336.	.474	.67

fig. 1



α α trehalose

fig. 2



Candy and Kilby's scheme for trehalose synthesis in *Schistocerca gregaria* fat body

Fig 3

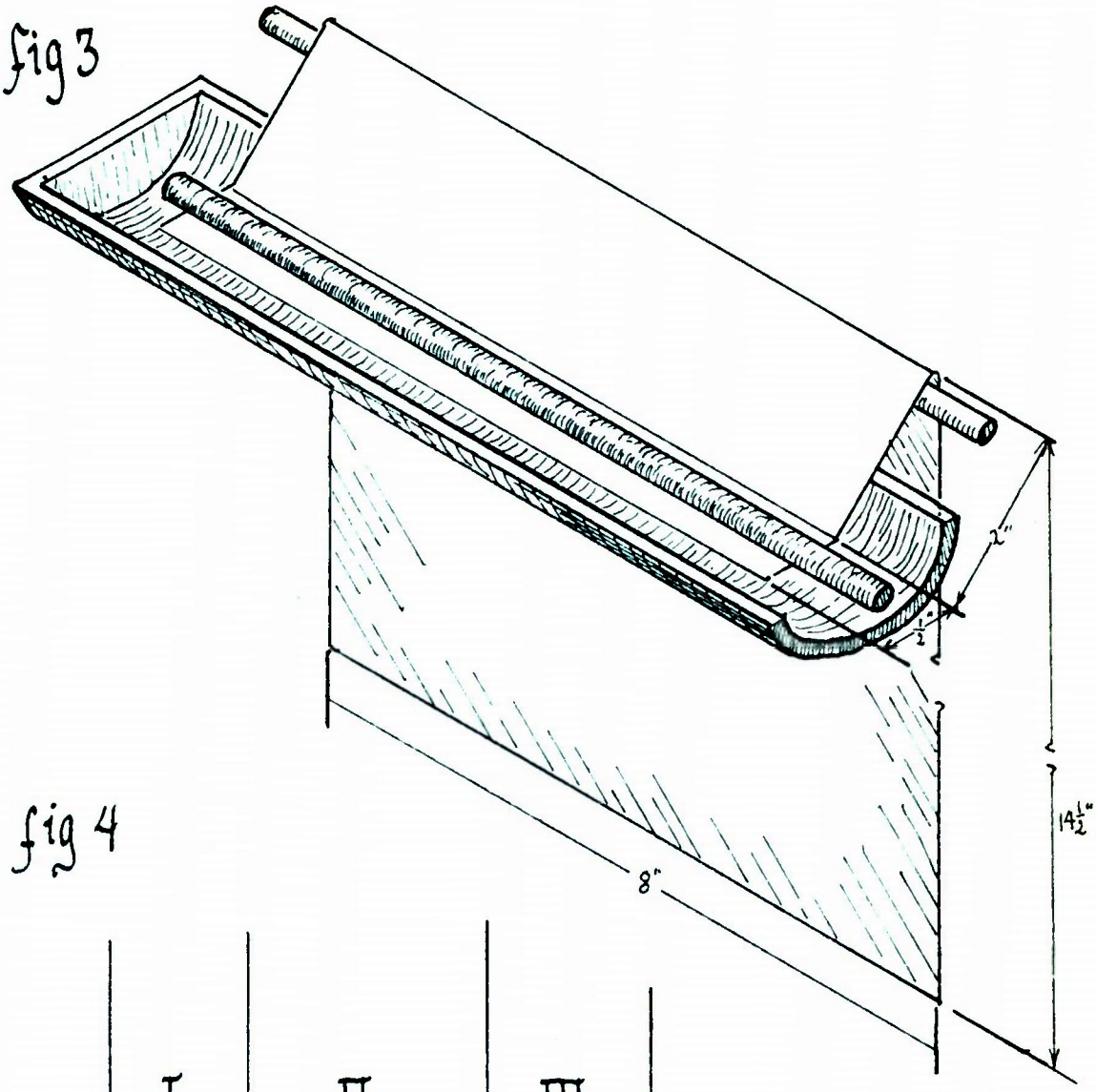
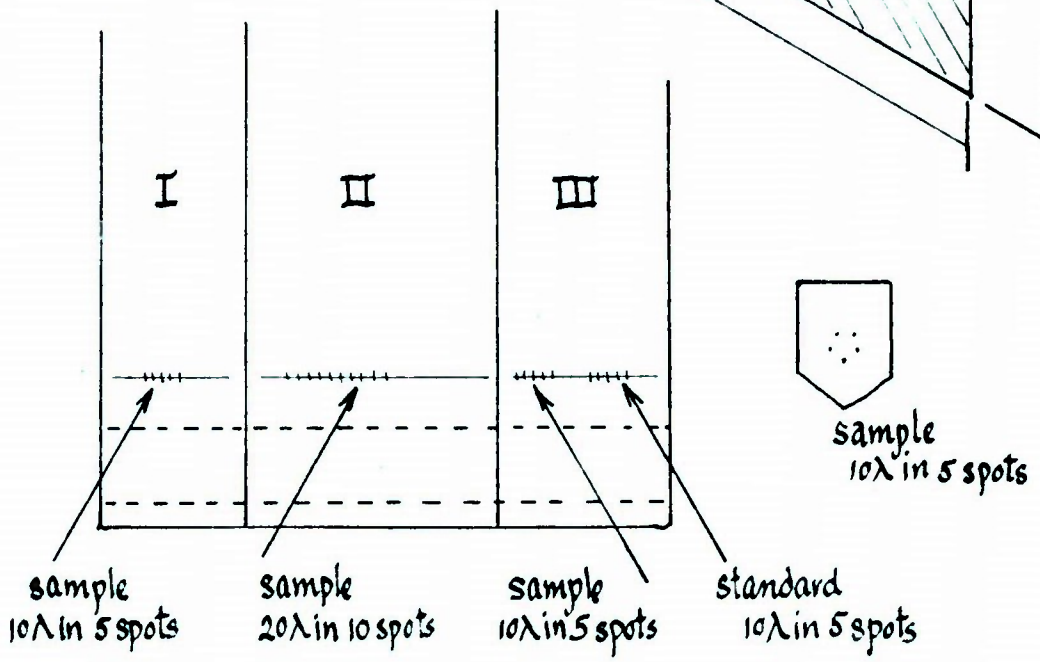

2"
1"
14½"
8"

Fig 4


I
II
III
sample
10λ in 5 spots
sample
10λ in 5 spots
sample
20λ in 10 spots
sample
10λ in 5 spots
standard
10λ in 5 spots

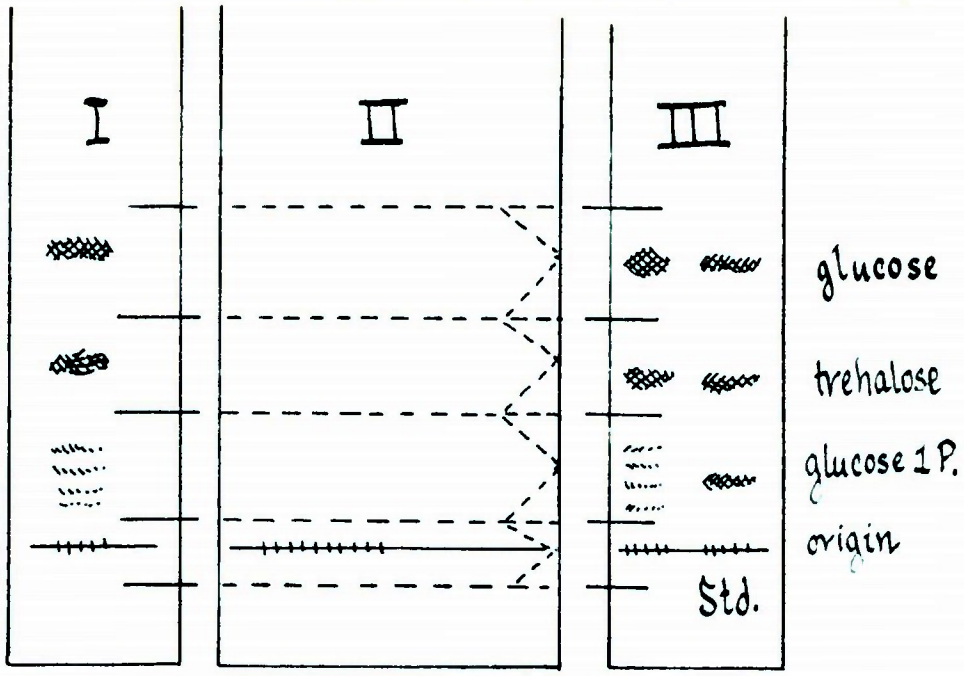

I
II
III
glucose
trehalose
glucose 1 P.
origin
Std.

fig 5

fig 6

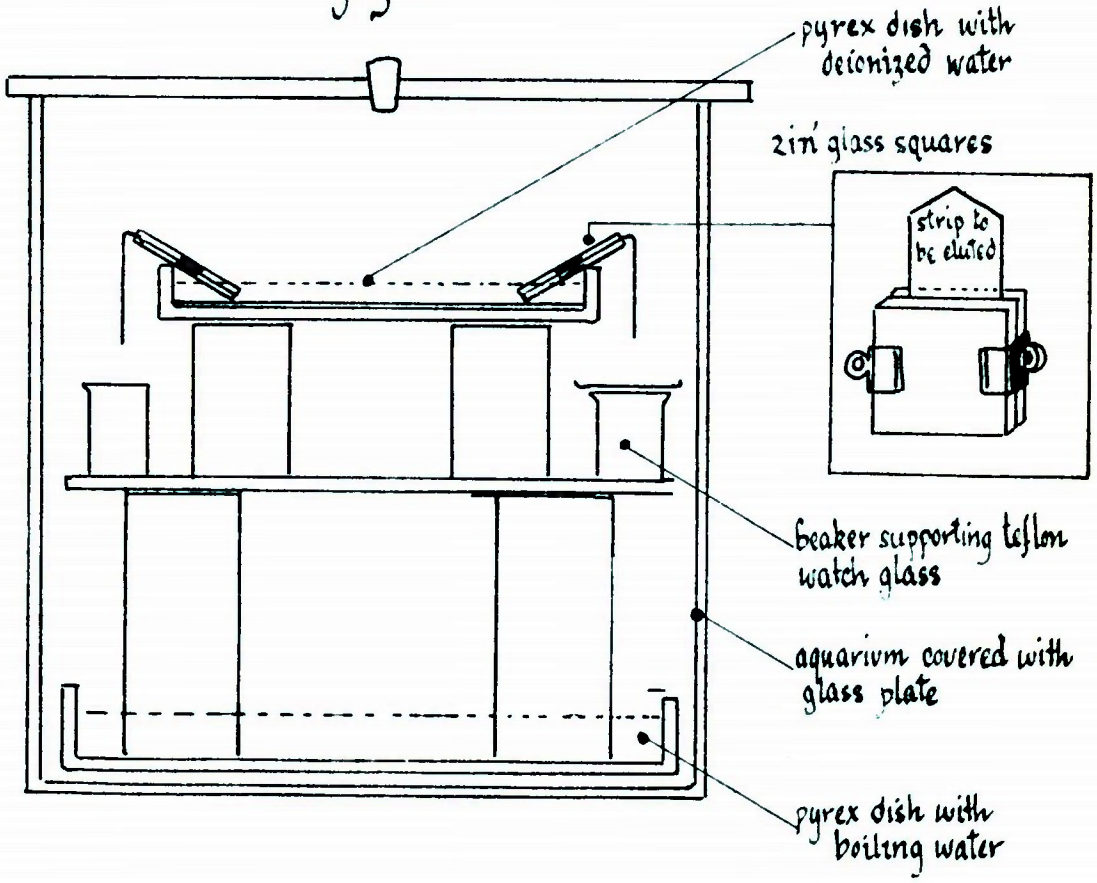
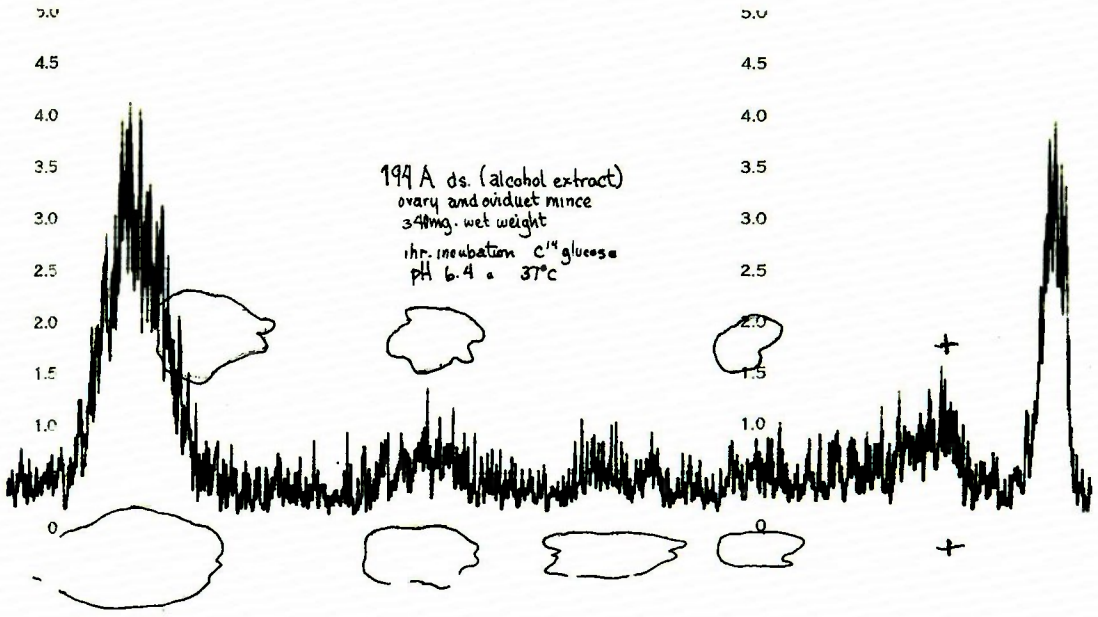

pyrex dish with deionized water
2in glass squares
strip to be eluted
beaker supporting teflon watch glass
aquarium covered with glass plate
pyrex dish with boiling water

fig. 7

A

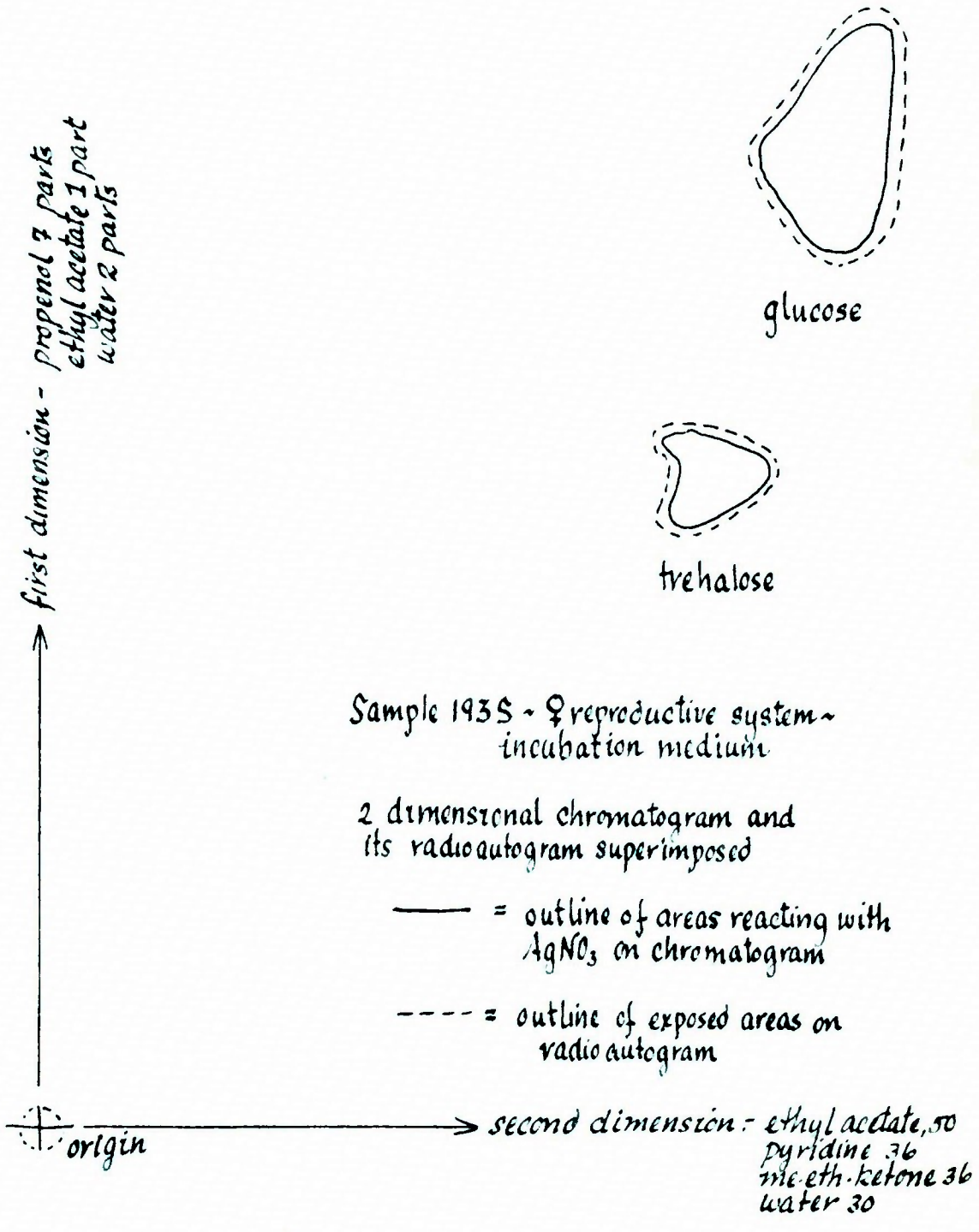
Strip counting of 1-dimensional chromatogram of incub. medium from incub. of ♀reprod. tissue with C^{14} glucose



B

Strip counting of 1-dimensional chromatogram of alcohol extract from ♀reproductive tissue incub. in C^{14} glucose

fig. 8 a.



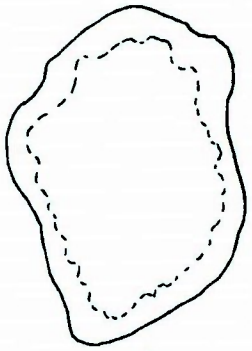
Sample 1935 - ♀ reproductive system - incubation medium

2 dimensional chromatogram and its radioautogram superimposed

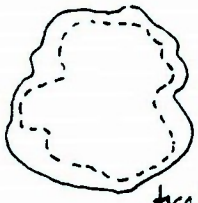
——— = outline of areas reacting with $AgNO_3$ on chromatogram

- - - - = outline of exposed areas on radioautogram

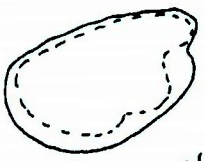
fig. 8b.



glucose



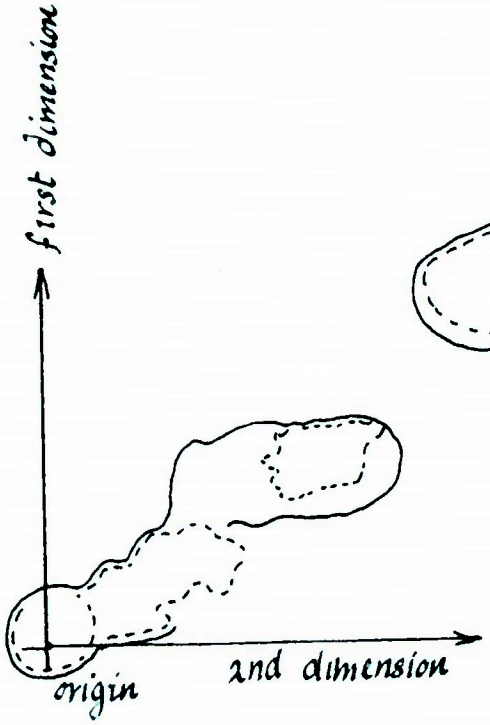
trehalose



Sample 194A

♀ reproductive system

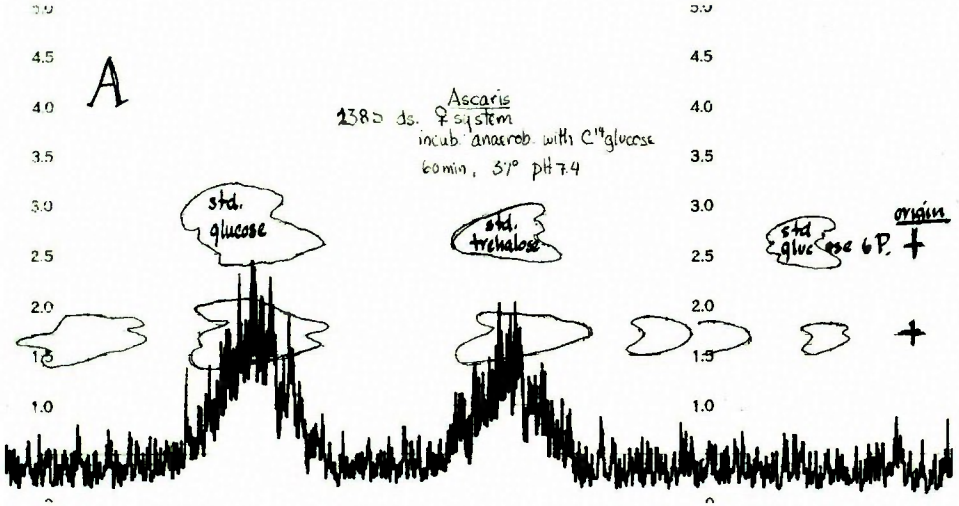
alcohol extract of tissue after incubation with C^{14} glucose

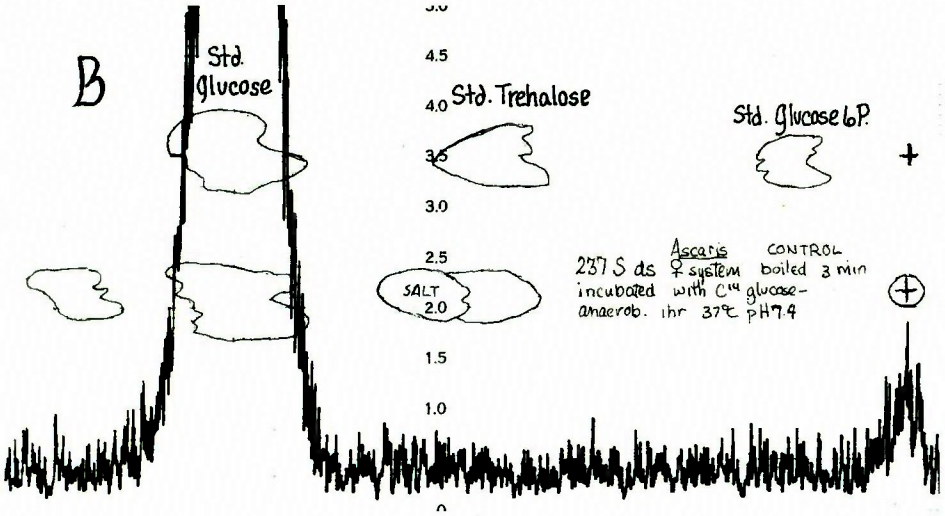


2 dimensional chromatogram and its radio autogram superimposed

—— = outline of $AgNO_3$ reactive areas on chromatogram

---- = outline of exposed areas on radio autogram





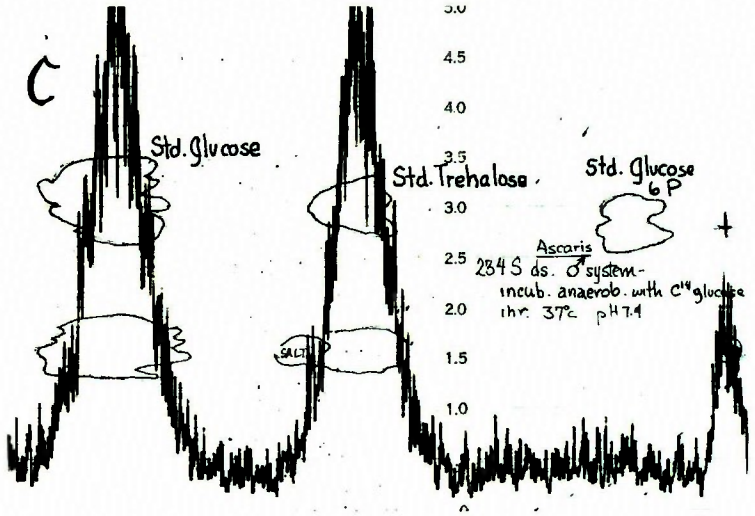


fig. 9 countings of chromatograms of incubation media from;

A. ♀ reproductive system incubation

B. heat treated ♀ reprod. system incubation

C. testis and seminal vesicle incubation

340
A $\frac{1}{20}$ of sample ~ 1 and 2 = areas eluted & counted
B eluate from area 1 ~ glucose
C eluate from area 1 ~ hydrolyzed
D eluate from area 2 ~ trehalose
E eluate from area 2 ~ hydrolyzed

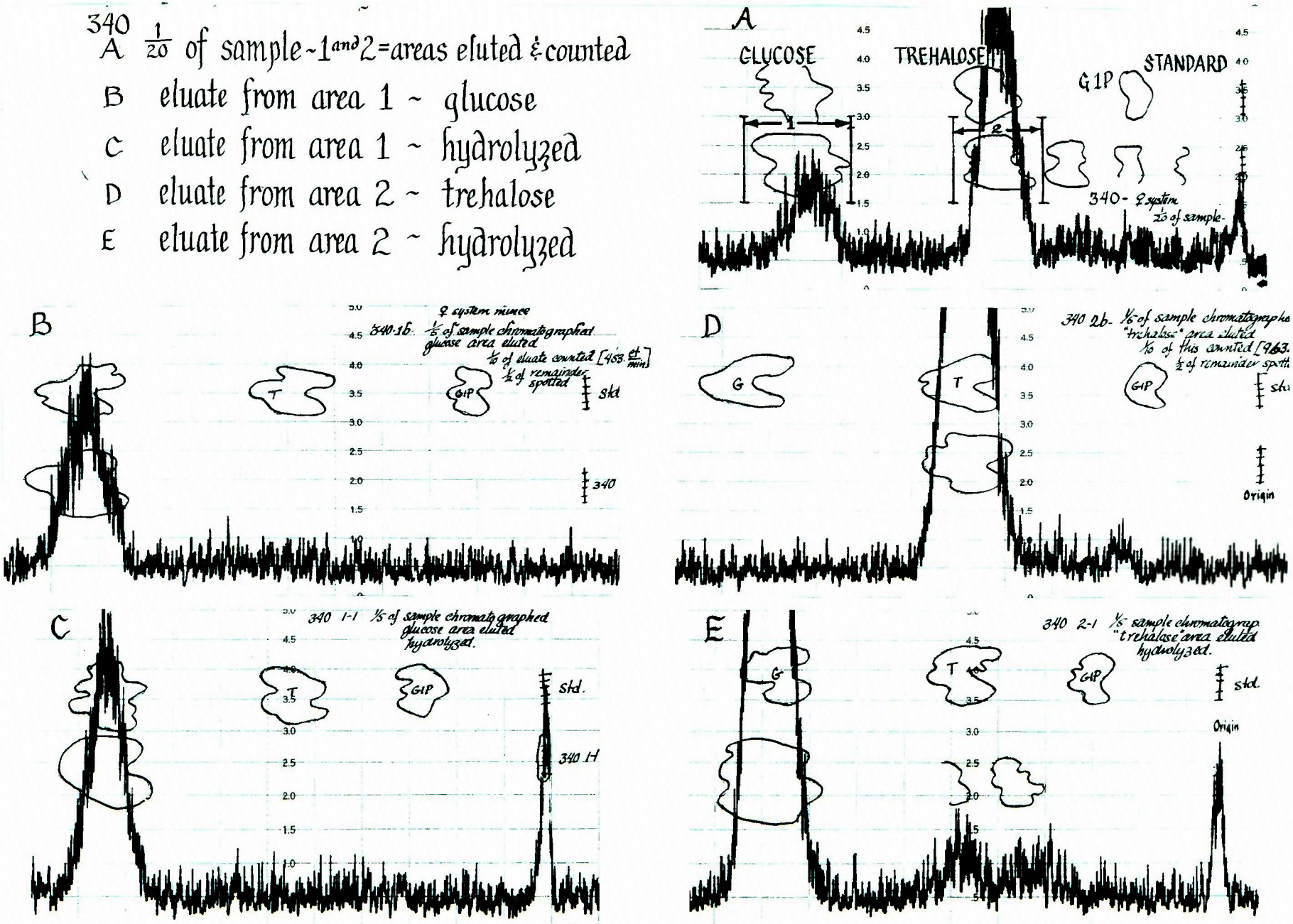
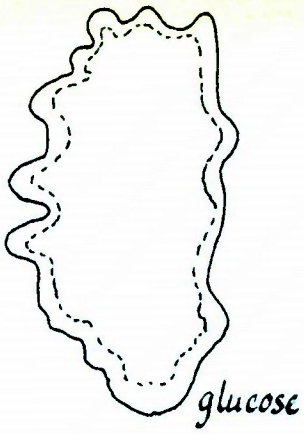


fig. 10 ~ Preproductive system ~ incubation medium

fig. 11a



Sample 341S- testis-seminal vesicle incubation medium

2 dimensional chromatogram superimposed on its radioautogram

——— = outline of $AgNO_3$ reactive spots on the chromatogram

- - - - - = outline of exposed areas on the radioautogram

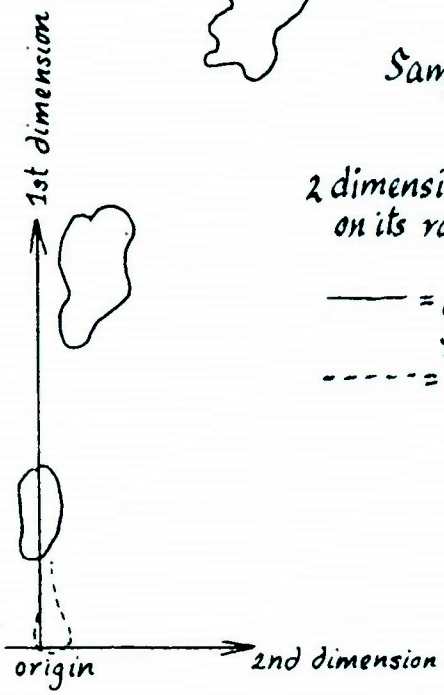
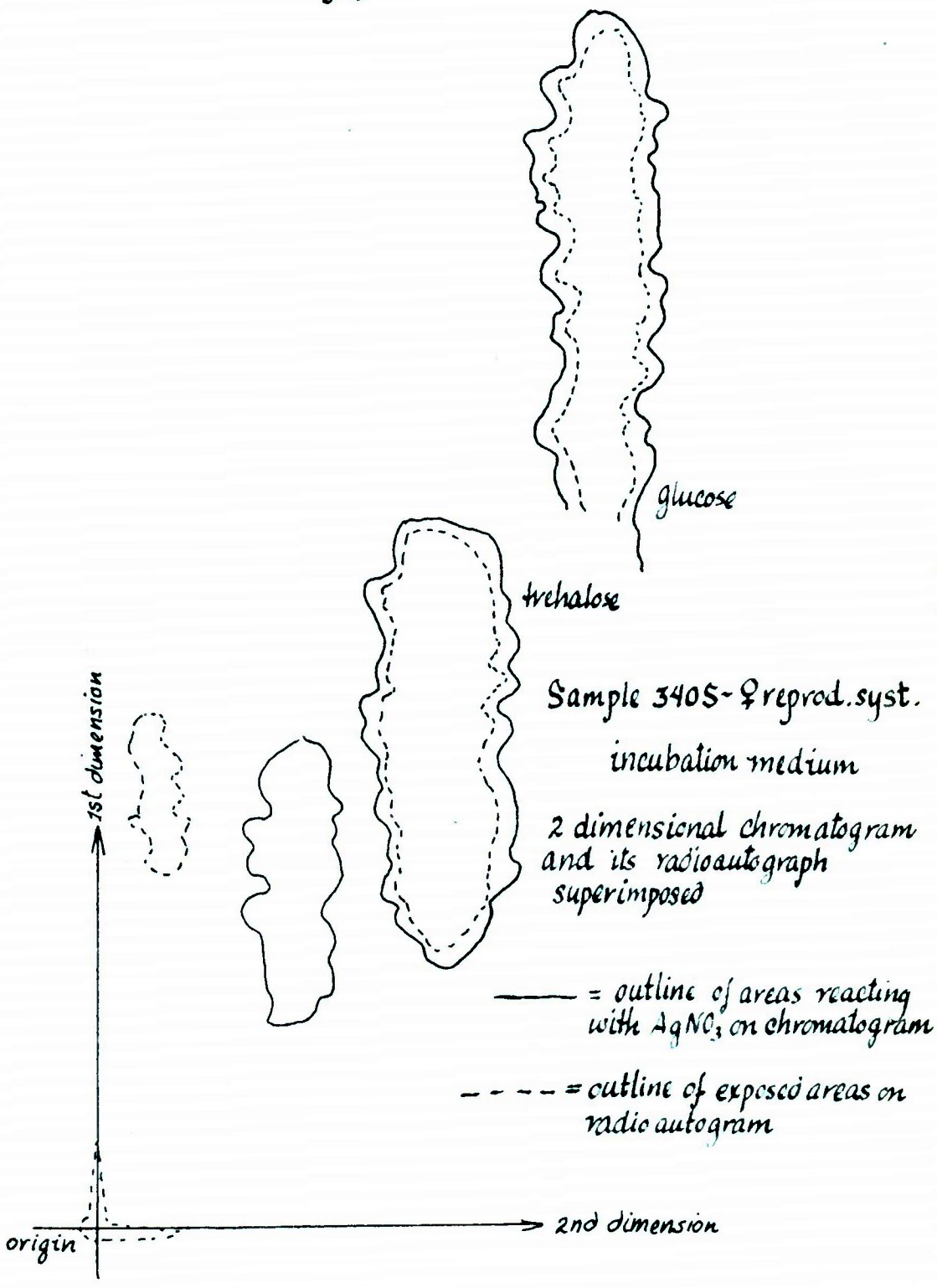


fig 11 b.



Sample 3405 - ♀ reprod. syst.

incubation medium

2 dimensional chromatogram and its radioautograph superimposed

——— = outline of areas reacting with $AgNO_3$ on chromatogram

– – – – = outline of exposed areas on radio autogram