1973

Gas chromatographic analysis of succinate in the face fly, Musca autumnalis De Geer.

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GAS CHROMATOGRAPHIC ANALYSIS OF SUCCINATE IN THE FACE FLY, *MUSCA AUTUMNALIS* DE GEER

A Thesis

By

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WARREN B. MEEKS

Submitted to the Graduate School of the
University of Massachusetts in
partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May, 1973

Major Subject: Entomology

"The first step to understanding is asking a question."

Anonymous

ABSTRACT

An extraction and analysis technique of succinate using the face fly, Musca autumnalis De Geer, is presented. The fly tissue is homogenized in perchloric acid and cleaned up by ether elution from silica gel. The recovered succinate is esterified with boron-trifluoride in methanol. Quantitation is made on a dual flame ionization gas chromatograph with glutarate used as the internal standard. The succinate concentrations found in the major stages are 48-60 µg/gm during the larval period, 92-277 µg/gm during the pupal period, 53-149 µg/gm for the adult female and 50-163 µg/gm for the adult male.

The possible correlations between succinate concentrations and published values for the activity of succinate dehydrogenase and the respiratory rate reported during the pupal period of the face fly are discussed.

ACKNOWLEDGEMENTS

I wish to acknowledge the helpful patience of my advisor, Dr. Lawrence J. Edwards. His encouragement was the major factor in completing the work behind this thesis.

Of my wife, Mila, I wish to acknowledge the understanding and confidence that she never failed to show me.

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I. INTRODUCTION

Succinate is the substrate for the enzyme succinic dehydrogenase which exists in the mitochondria of insects. It is known that the activity of this enzyme changes during development (Rousell, 1967) and recent work has indicated that inhibition of this enzyme may explain the mode of action of CO_2 in producing knockdown in insects (Edwards, 1971). It is a reasonable assumption that the succinate concentration might vary as the insect develops since succinic dehydrogenase is one of several enzymes in intermediary metabolism which catalyze the release of energy for endergonic processes. If a "pool" or reserve of succinate is available for the enzyme, this may rise or fall during activity and during development. Also, inhibition of the enzyme for whatever reason may lead to an accumulation of succinate (Webb, 1966) which can be detected. Thus, the effects of inhibitors <u>in vivo</u> upon succinic dehydrogenase might be realized with an effective method of succinate analysis.

II. OBJECTIVES

A. Develop a succinic acid analysis technique for use with insects.

B. Measure succinic acid levels during development.

C. Interpret the significance of these levels during development.

As will be explained in the thesis only succinate was subjected to extensive analysis, as the other acids did not lend themselves to the analytical method used.

III. LITERATURE REVIEW

A. Rearing and Breeding Habits

The face fly, Musca autumnalis De Geer, was used for all work in this thesis. The duration of the life cycle from egg to adult is 11½-12 days at temperatures of 25°-30°C and 50%-70% relative humidity (Wang, 1964). Adults fed blood, sugar, and milk will mate and oviposite 4-5 days after emergence. The three active larval stages total 3-4 days and the pupal stage approximately 7½ days. The adults lay eggs in fresh manure where the developing larvae live and feed. The third instar crawls from the manure pat to form the puparium in the soil.

B. Metamorphic Development

Holometabolous development is the most dramatic form of development occurring in insects. Wang's study (1964) illustrates the obscure use of terms that are used to describe developmental events. In cyclorrhaphous Diptera, the pupa should not be called one instar as is frequently done. A search of the literature for information clarifying the events of the pupal period reveals a more concise picture. Following are some of the terms with appropriate definitions. Molting is the process through which an

insect goes from one instar to the next (Jenkin, 1965; Jenkin and Hinton, 1966). The molting process is divided into two distinct events. Apolysis is the actual separation of the epidermis from the previous instar's cuticle. Following apolysis is the partial digestion of the old cuticle and deposition of the new one. After apolysis, ecdysis occurs (Jenkin, 1965; Jenkin and Hinton, 1966). The pharate stage is the phase of the insect existing between apolysis and ecdysis within the old cuticle (Hinton, 1946). The duration of the pharate phase is variable depending on juvenile age, stage of development, and species. Fraenkel and Bhaskaran (1973) suggest reserving the term "pharate" to describe the adult stage after the pupal-adult apolysis and "cryptocephalic pupa" instead of "pharate pupa" for the stage following the larval-pupal apolysis in cyclorrhaphous diptera. "Pupariation" should be used distictly from "pupation" (Fraenkel and Bhaskaran, 1973). Pupariation is the formation of a hardened case from the cuticle of the third instar larva. After pupariation the face fly has a larval-pupal apolysis and a pupal-adult apolysis followed by ecdysis of the adult. Table 1 presents a summary of developmental events observed for the face fly. The terminology is that of Jenkin (1965), Jenkin and Hinton (1966), and Fraenkel and Bhaskaran (1973). The symbols at the left are used for consistent reference to age of flies throughout the thesis.

TABLE 1

SEQUENCE OF STAGES AND DEVELOPMENTAL EVENTS.

Day	Stage	Molting event	Landmark event
L_1	egg		oviposition
L_2	first instar	hatching	
L_3	second instar	apolysis-ecdysis	
L_4	third instar	apolysis-ecdysis	
L_5			
L_6			crawl into the sand
P_1		pupariation	
P_2	cryptocephalic pupa	apolysis	
P_3			
P_4			insertion of the prothoracic horns
P_5	pharate adult	apolysis	
P_6			
P_7			
P_8		ecdysis	
A_1	adult		emergence from puparium

C. Developmental Physiology

The literature contains many reports of biochemical research in the area of insect development. The emphasis in this area is on respiratory fluctuations and enzyme patterns. Oxygen consumption during the pupal stage of insects follows a U-shaped pattern (Wigglesworth, 1965). Face fly respiration also follows the U-shaped pattern during the pupal stage (Guerra and Cochran, 1970). Succinic dehydrogenase activity during the face fly pupal period follows this same graphic pattern (Rousell, 1967). A U-shaped enzyme activity pattern with the low occurring near the end of the pupa stage and at the beginning of the pharate adult stage occurs in the blowfly, Calliphora erythrocephala (Agrell, 1949), the Mediterranean flour moth (Diamantis, 1962) and the mealworm and house fly (Ludwig and Barsa, 1956, 1959; Sacktor, 1951). A U-shaped pattern occurs in the activity of the glycolytic enzymes during the pupal period in the blowfly, Lucilia cuprina (Crompton and Birt, 1969). The route taken by injections of labelled glucose during the pupal stage of the blowfly, indicates that the drop in concentration of the glycolytic substrates is due to their incorporation into the cuticle of the developing pharate adult (Crompton and Polakis, 1969). This would seem to set a precedent for a low level concentration to occur in the Krebs cycle

components during pupation of the face fly. There is not, however, any literature that examines this point.

D. Organic Acid Analysis Techniques

Chromatographic procedures have been widely used for separating and quantifying compounds of a similar biochemical nature. Below is a summary of some of the basic procedures.

1. Thin-layer chromatography. Thin-layer cellulose plates are spotted with a prepared sample containing the organic acids. Using the flow of mixtures of solvents and aqueous solutions in two dimensions, the respective acids separate into defined areas on the plate. With an appropriate indicator, usually an acid-base reaction, the acids appear as individual spots which can be compared with standards for identification. This is primarily a qualitative test as acid concentrations must be in the area of 10 millimoles (Myers and Huang, 1969).

2. Ion-exchange chromatography. Organic acid analysis by ion-exchange chromatography is based on an acid gradient elution system. The columns are composed of inert resins containing either cationic or anionic groups that attract functional groups of compounds having the opposite ionic form. The elution order is a function of the pK's of the individual acids. Indicators are generally acid-base

titrations. Unless large quantities of the acids are present, sample loss is a constant problem with the large volumes of elutants that are used. The acid content of the samples must be in the area of 5 to 200 micromoles (Von Korff, 1969).

3. Partition column chromatography. Partition column chromatography is not unlike ion-exchange. It is also based on a gradient elution system but in this case the gradient is formed from the changing composition of chloroform and t-amyl alcohol in the elutant used on acidified, hydrated silica gel. Photometric recordings of an indicator changed to the hydrogen form by the acids are compared with a standard to give a quantitative measure of the organic acids present. Sample content of the acids requires concentrations of 0.05 to 3 micromoles (Kesner and Muntwyler, 1969).

Both ion-exchange and partition column chromatography can take extended periods of time (less than 5 hours) to analyze one sample not including the tissue preparation time preceding the analysis.

4. Gas chromatography. Gas chromatography is based on the separation of compounds in the gaseous state. An inert carrier gas moves the volatile components through a column packed with a solid support coated with a liquid stationary phase that has the characteristics necessary for separating the sample's components. The thermal or ionic change that occurs at the detector as a component elutes

from the column is electrically and graphically recorded. The sensitivity of ionization detectors permits analysis of samples containing acids in the nanomole levels (Alcock, 1969). Analytical use of gas chromatography on biological material presents two immediate advantages over any of the other procedures. First, large numbers of samples may be analyzed in a relatively short period of time. Second, analysis of low levels of organic acids that occur are easily accomplished by the sensitive nature of gas chromatographic detectors.

E. Application of Gas Chromatography to the Organic Acids of the Tricarboxylic Acid Cycle

1. Extraction of acids. When working with biological material, a homogenizing media that extracts all of the available organic acids is needed. Burchfield and Storrs (1962) found extraction of non-volatile acids from plant material possible using aqueous alkali or hot 80% ethanol with vigorous homogenization. Rumsey et al. (1966) extracting metabolic organic acids from forage and silage samples used only distilled water during homogenization. On ruminal fluid samples they used a 1:1 mixture of acetone and ethanol. On animal tissues Kuksis and Prioreschi (1967) used 0.6 N perchloric acid.

2. Clean-up of extracts. Crude acid extracts which contain material other than the organic acids need to be further purified. Kuksis and Prioreschi (1967) made a comparative study of five procedures. The use of perchlorate in tissue homogenization followed by sample elution from an anion exchange resin was the most satisfactory procedure followed by ether elution from silicic acid.

3. Esterification. In the acid form the tricarboxylic acids will decompose before the necessary temperatures are reached for volatilization. Gas chromatographic analysis of the acids requires conversion to their more volatile ester form. Consideration of the temperatures used is still important since the esters dimethyl-succinate and dimethyl-malonate, show increasing thermal decomposition as temperatures rise above 140°C (Ackman et al., 1960).

Esterification with diazomethane is the most expedient procedure but the literature contains contradictory findings over its use. The yield of succinate, malate and citrate methyl esters from diazomethylation is reported to be quantitative at 25°C and -70°C (McKeown and Read, 1965). Low yields occur in etheral solutions but the presence of methanol in the reagent mixture enhances the yields. McKeown and Read (1965) report that fumarate esterification with diazomethane at 25°C does not form the dimethyl ester but an addition product, 4,5-dicarbomethoxy -pyrazoline, which has

a retention time longer than citrate. When esterification is conducted at -70°C and with limited exposure to excess diazomethane, the loss of the methyl ester of fumarate is halted. This last result is in contrast to the comparable yield of both dimethyl-fumarate and dimethyl-succinate using diazomethane found by Estes and Bachmann (1966) with no special treatment. Their reactions are run in etheral solutions devoid of methanol. They state that the low yield of the fumarate methyl ester that occurs is not due to side reactions or addition products across the ethylene bond. The observed loss of fumarate is probably the result of its thermal decomposition when the column temperature rises above 90°C as indicated by the increased area of the methanol peak (Estes and Bachmann, 1966).

Despite the ease in the use of diazomethane, other procedures have been found to display more consistent results. Alcock (1965) and Kuksis and Prioreschi (1967) using boron trifluoride in methanol obtained consistent methylations of most of the acids of the tricarboxylic acid cycle, but Alcock (1969) recommended selective procedures for the different acids owing to their variable nature of esterification.

4. Chromatographic conditions. The liquid phase most widely used for acids of the tricarboxylic acid cycle is

diethylene glycol succinate (DEGS) (Estes and Bachmann, 1966; Alcock, 1965; Rumsey et al., 1964). Comparable resolution of these acids can be obtained by mixing 6% DEGS with 4% Carbowax 20M, a slightly more apolar coating (Kuksis and Prioreschi, 1967). If one or even two acids with similar boiling points are tested, isothermal oven temperatures are used. When more are tested in one sample, the oven temperatures must be programmed to start near 100°C and finish near 200°C.

5. Quantitation. Two means of sample quantitation are commonly used. Comparing the chromatograms of test samples with chromatograms of acids mixed in standard solutions (Rumsey and Noller, 1966), and direct comparison of the sample components to an internal standard added near the beginning of the sample preparation (Salminen and Koivistoinen, 1967; Kuksis and Vishwakarma, 1963). Methyl esters of the tricarboxylic acids show a linear relationship for concentrations of 0.01 to 10 μg (Kuksis and Vishwakarma, 1963). However, quantitations between chromatograms do not assure a way of measuring the amount of acids lost during preparation before GC analysis.

Salminen and Koivistoinen (1967) show that a relative detector response value, calculated from peak area and weight ratio comparisons to the internal standard, can be used, provided the standard curve of a particular ester is linear.

They report a linear regression with fumarate, succinate, malate, and citrate, but not with oxalacetate and trans-aconitate. The lowest responses come from malate and oxalacetate. This variability in response by the detector seems to be the result of the varying oxygen content in each ester.

IV. MATERIALS AND METHODS

A. Analytical Procedure for Succinate

1. Introduction. The numerous procedures reported in the literature were examined for their suitability for analyzing succinate in flies. The reader who is familiar with such may wish to go on to section IV. B. (p. 24), "Succinate Levels in the Face Fly," which deals directly with the quantitative tests made on the face fly.

2. Face fly rearing. The stock and experimental populations are maintained in a culture room fitted with three 8-foot banks of fluorescent lights set on a 16:8 light-dark cycle. The temperature is kept between 23°-26°C by a thermostatically controlled space heater and an air conditioner. An automatic humidifier keeps the relative humidity between 40%-60%.

Fresh cow manure is offered to the flies one to three times a week. The adult flies are permitted access to the manure in plastic half-gallon ice cream containers for no more than 4 hours.

Three days after egg laying, the manure pat is transferred to one end of a porcelain pan and dry sand is placed at the other end. At age L_6, the larvae burrow into the sand and pupariate (p. 4). One day later the puparia are sifted from the sand and transferred to clear plastic

containers until the adults emerge. The young flies are transferred to cages 2x3x3 feet in size and are constantly supplied with water wicks, dry powdered milk and sugar. Besides serving as an egg laying medium, the manure supplies supplemental nutrients for the adults.

3. Reagents and apparatus.

a. Succinate, fumarate and glutarate (Sigma) as pure acids are used as powders of known weight and as aqueous or etheral solutions of standard concentrations.

b. Organic solvents: ether, methanol, ethanol, iso-propanol, n-propanol, acetonitrile, and chloroform (Fisher, Baker, Matheson, Coleman & Bell, and Mallinckrodt).

c. Perchloric acid (Mallinckrodt) in 2 N concentrations and sodium or potassium hydroxide (Baker) in 10% concentrations. Saturated aqueous ammonium sulfate (Fisher).

d. Silicic acid powder, 100 mesh, (Sigma) prepared for etheral elution with acid (HCl) wash. Elution column is 15 mm (i.d.) by 400 mm overall length with sintered glass disc.

e. Diazomethane generated from Diazald (Aldrich, 1968) and boron trifluoride in methanol (10%, Matheson-Coleman & Bell; 14%, Applied Science Laboratories, Inc.).

f. Fly tissues are homogenized on Ten Broech ground glass or smooth glass-teflon homogenizers. A rheostat

controlling a low torque fan motor is used to drive the pestles.

g. Rotary evaporator with temperature controlled water bath (Buchi). Automatic refrigerated centrifuge RC2-B (Sorvall).

h. Research gas chromatograph 5750B equipped with a dual flame ionization detector (Hewlett-Packard). Stainless steel columns 1/8-inch o.d. by 6 or 8 feet in length.

i. Sample injections into the gas chromatograph are made using 10 ul gas tight syringes (Hamilton). The injection technique involves flushing the syringe with several rinses of ether leaving approximately 1 µl remaining on the last draw followed by a draw of 3 to 6 µl of sample.

j. Chromatographic conditions. All of the analyses are conducted using the dual flame ionization detector on the gas chromatograph. The columns are packed with diethylene glycol succinate (DEGS) in percentages of 3, 6, or 15% by weight alone or in combination with 3% Silicone Gum Rubber (OV-17), coated on Diatoport-S 80-100 mesh or Chromosorb WAWDMCS (High Performance) 80-100 mesh and conditioned at 200°C for 2-4 hours (Hewlett-Packard). The carrier gas is helium set for an inlet flow rate of 30-50 cc/min. under 45-47 p.s.i. The injection port temperature is held at 180°-195°C and the flame detector at 280°C with a hydrogen flow rate of 25 cc/min. at 10 p.s.i. and an air

flow rate of 500 cc/min. at 33 p.s.i.

Range and attenuation settings, and oven temperatures (programmed or isothermal) vary with the needs of the specific tests.

The chart speed is run at .25 inch per minute.

4. Standard curve. Standard concentrations of succinate and glutarate in aqueous solutions are used for the detector-response determinations in establishing the linear calibration curve. The various mixtures of the two acids are evaporated to dryness and the acid residue methylated with boron trifluoride in methanol adapted from the procedure of Kuksis and Prioreschi (1967).

Quantitations are based on area ratios from triangulations of eluting peaks using glutaric acid as the internal standard (Barbato, et al., 1966; Salminen and Koivistoinen, 1967).

5. Extraction and clean-up. The determination of a suitable organic acid extraction procedure from insect tissues centers around three types of homogenizing media: acid ($HClO_4$), base (NaOH or KOH), and organic solvent (acetone). Groups of individuals from the metamorphic or adult stages are the tissue sources. Following are the particular steps followed in each of the major types of extraction. In the hyphenated phrases, the first term indicates the extracting solution, the second indicates the

clean-up procedure.

a. Perchloric acid-silicic acid, ether elution.

1) Fly tissue homogenized in 0.6 M or 2 M $HClO_4$ (20-30 ml).

2) Homogenate centrifuged at 5000-10,000xG for 10 minutes (pH 1.1). Discard particulate matter.

3) Supernatant neutralized to pH 5.5 with 2 N KOH.

4) Supernatant centrifuged at 5000-10,000xG for 10 minutes. $KClO_4$ precipitate discarded.

5) Supernatant evaporated to 1 ml <u>in vacuo</u> at 40°-60°C. Water discarded.

6) Residue acidified with 9 N H_2SO_4 (1-2 ml).

7) Acid residue combined with 6 g of silicic acid and eluted with 100-250 ml of ether.

8) Ether evaporated to dryness, residue esterified, and esters chromatographed.

b. Sodium hydroxide - no clean-up.

1) Fly tissue homogenized in hot 0.05 N NaOH (15-50 ml).

2) Homogenate centrifuged at 3500xG for 20 minutes, pH 10.5. Particulate matter discarded.

3) Supernatant acidified to pH 1 with 9 N HCl.

4) Supernatant evaporated to dryness <u>in vacuo</u> at 60°C. Water discarded.

5) Dried residue esterified and esters chromatographed.

c. Sodium hydroxide - ether liquid-liquid extraction.

1) Fly tissue homogenized in hot 0.05 N NaOH (15 ml).

2) Homogenate centrifuges at 3500xG for 30 minutes, pH 10.5. Discard particulate matter.

3) Supernatant extracted with 2x25 ml ether. Solvent phase discarded.

4) Supernatant acidified with 9 N HCl, pH 1.1.

5) Acid solution extracted with 2x50 ml ether. Aqueous phase discarded.

6) Ether evaporated to dryness, residue esterified and esters chromatographed.

d. Acetone - no clean-up.

1) Fly tissue homogenized in acetone (100 ml).

2) Homogenate centrifuged at 5000xG for 10 minutes. Particulate matter discarded.

3) Supernatant evaporated to dryness <u>in vacuo</u>.

4) Residue esterified and esters chromatographed.

e. Acetone and H_2O - hexane liquid-liquid extraction.

1) Fly tissue homogenized in a mixture of 40 ml acetone and 40 ml H_2O.

2) Homogenate centrifuged at 8000xG for 10 minutes.

3) Particulate matter dried <u>in vacuo</u>; esterified and chromatographed.

4) Acetone evaporated from supernatant <u>in vacuo</u> at 30°C, pH 6.7.

5) pH lowered to 2.9 with 9 N HCl and the supernatant extracted with 3x175 ml hexane.

6) Hexane evaporated *in vacuo*, residue esterified and esters chromatographed.

7) Aqueous phase evaporated *in vacuo*, residue esterified and esters chromatographed.

f. Hot perchloric acid - silicic acid, ether elution check. Fly tissues are processed by the procedure described under 5.a. In addition, the perchloric acid homogenate is heated to 100°C for 1 hour. One sample is not purified with the ether elution of silicic acid. Another sample receives an additional 100 ml ether elution of the silicic acid. The prepared samples are esterified and chromatographed.

g. Perchloric acid - anion exchange compared to silicic acid. Fifty, third instar larvae, age P_1, are processed to the centrifugation step preceding evaporation *in vacuo* in the perchloric acid procedure described in 5.a. One half of the supernatant (36 ml) is continued through the remainder of the perchloric acid procedure. The second half of the supernatant is passed through 25 ml of a strong anion exchange resin (Dowex 1x8-200) that had been generated to the formate form with 1 N formic acid and rinsed with distilled water. After application of the supernatant, the resin is washed with 25 ml of distilled water followed by elution with 100 ml of 6 N formate solution. The elutant is dried and both sample preparations esterified and chromatographed.

None of the extraction and clean-up procedures eliminate an unidentified compound which elutes near or overlapping the point expected for fumarate on a GC chromatogram. The results for this are not reported. If fumarate does occur, it is usually in much lower concentrations than succinate.

6. Esterification procedures.

a. Description of procedures.

1) Acid-methanol methylation: samples containing the prepared organic acids are dissolved in 5 ml of 1.9% hydrochloric acid in methanol or 5% sulfuric acid in methanol set in screw cap test tubes. The reaction mixtures are placed in varying temperature conditions for varying time periods. At the end of the reaction period the mixtures are evaporated *in vacuo* at 22°C to 1 ml. Five ml of distilled water is added to the aqueous phase and extracted with 5x5 ml volumes of ether. The combined extracts are evaporated to dryness and the esters collected in a defined volume of solvent (acetonitrile or ether).

2) Diazomethane methylation: prepared organic acid samples are dissolved in 3-5 ml of methanol or ether and enough ethereal diazomethane added until the yellow color persists (2 to 5 ml). The temperature of the reaction mixture and the length of time the acids are exposed to diazomethane varies with each test. After the reaction

conditions, the volume is reduced by evaporation in vacuo or by a stream of nitrogen and brought back to a defined volume with solvent (acetonitrile or ether).

3) Boron-trifluoride-methanol methylation: prepared organic acid samples are dissolved in 1 ml of 10% or 14% boron-trifluoride in methanol. The mixtures are allowed to stand overnight at 24°C or incubated at 100°C for 10 minutes. Four ml of saturated ammonium sulfate are added to the mixture after the reaction conditions. The esters are extracted with 1 ml of ether and reduced to a small volume by a stream of nitrogen.

b. Esterification experiments.

1) Effect of time and temperature on acid-methanol and diazomethane. The acid-methanol solutions (HCl or H_2SO_4 in methanol following the procedure IV.A.6.a.1), p. 21) are run for either long duration (4 hours) and low temperatures (55°C) or short duration (10 minutes) and high temperature (100°C). Succinate methylation with diazomethane following the procedure IV.A.6.a.2) is conducted for a duration of 3 hours and room temperature (22°C) in both the presence or absence of methanol in the etheral solution.

2) Effect of time on diazomethane-methanol. Esterification with diazomethane following the procedure a.2) are run at room temperature (22°C) and exposure times of 3 minutes or 90 minutes.

3) Effect of evaporation temperatures on esters. Methylations of succinate following procedure a.2) at 22°C and 30 minutes are subjected to evaporation <u>in vacuo</u> at temperatures of 24°C or 65°C.

4) Effect of time and temperature on diazomethylation. The test samples contain 3 mg of succinate and fumarate. Reaction conditions with diazomethane using the procedure a.2) are run for short durations of 0 to 8 minutes and temperatures of either 25°C or -60°C. The reactions are halted by evaporation with a stream of nitrogen. Exposure time is taken to be 0 minutes when evaporation with a stream of nitrogen is initiated the moment after diazomethane has been added. A reference mixture of the succinate and fumarate methyl esters is included using the boron-trifluoride-methanol procedure IV.A.1.a.3).

7. <u>Organic acid analysis of manure - procedure</u>. All of the larval stages up to age L_6 contain observable quantities of manure in the gut. As a source of error in the absolute levels of succinate that might be present during this stage, an analysis of 4 grams of manure is made using the extraction and clean-up procedure of 5.e. and esterification procedure 6.a.1) using sulfuric acid at 22°C standing overnight.

B. Succinate Levels in the Face Fly

1. Introduction. Presented here is a listing of the procedures from IV.A. which give the most consistent results for succinate. These procedures are used to obtain the data of the succinate levels in Musca autumnalis.

2. Rearing and collecting samples. Larvae, pupae and adults are collected as described in IV.A.2. to get samples of insects the same age for analyses. Each test culture originates from a single batch of eggs collected from stock cultures. Fresh weights of all the samples are taken before treatment. The larvae are pretreated before weighing by placing them in a beaker filled with moist tissue paper where they crawl actively, consequently, washing off adhering manure liquids and solids. Puparia are gently rubbed with dry tissue paper prior to weighing to scrape off adhering substrate particles. For adults no pretreatment prior to weighing is done.

3. Extraction and clean-up. Intact organisms in groups of 25, 50, or 75 are processed in Experiment I using hot perchloric acid as described in IV.A.5.f. and using perchloric acid as in IV.A.5.a. for Experiments II-V.

4. Esterification. Boron-trifluoride-methanol IV.A.6.a.3).

5. Gas chromatographic analysis. Conditions as in

IV.A.3.j. with variations according to test.

6. Internal standard. Immediately prior to homogenization, glutarate in standard concentrations with a final amount of 250 or 500 µg is added to the fly-reagent media. This amount is based on succinate levels found in earlier experiments and allows the chromatograph to run without change in attenuation.

7. Calculations.

a. Fly weight during the pupal period. A biochemical component takes on value when compared to its original source. The succinate level in the face fly compared to the body weight will present a clearer picture by knowing the changes that occur and difference between the fresh and dry weight over the developmental period.

Round, plastic, half gallon, ice cream containers with lids are used as chambers for the tests. Drierite crystals spread on the bottom are used to maintain a dry atmosphere in one container and a half-inch layer of water with Kimwipe tissues adhering to the inside walls to serve as wicks maintain a wet atmosphere in the other. In the lids a 2-inch hole is made and loosely plugged with Kimwipes to allow passage of fresh air, to keep out flies and dust, and to prevent a sudden surge of moisture into or out of the containers. The prepared chambers are kept in the rearing room under the temperature and lighting conditions

established there. No attempt was made to measure the relative humidity in the containers.

1) Determination of the change in weight during the pupal period consists merely of weighing groups of individuals contained in plastic disposable petri dishes. No group of individuals is out of its chamber for more than three minutes while being weighed.

2) Determination of the water content during the pupal period consists of taking the fresh weight of groups of individuals followed by 60 minutes of drying at 100°C and cooling to 25°C in a desiccator for 30 minutes. Then, the weight of each test group is taken.

b. Conversion of chromatogram peak area to µg/gm fresh weight. Below is a sample determination of succinate concentration from the values of Experiment I, Samples 1 and 2, age P_1 from Table 7.

Signal ratio.

$$\text{Sample 1} = \frac{\text{succinate (peak height x width at half height)}}{\text{glutarate (peak height x width at half height)}}$$

$$= \frac{50 \times 6}{336 \times 5} \ (64^{\text{ths}} \text{ of an inch}) = .179$$

Sample 2 = .183

Succinate content.

Sample 1 = signal ratio x weight I.S. (µg)

= .179 x 500 = 89.5 µg

Sample 2 = 91.5 µg

Succinate per unit fresh weight (µg/gm).

$$\text{Sample 1} = \frac{\text{succinate content}}{\text{fresh weight of pupae}}$$

$$= \frac{89.5}{1.598} = 56 \text{ µg/gm}$$

$$\text{Sample 2} = \frac{91.5}{1.612} = 58 \text{ µg/gm}$$

Average for age P_1.

57 µg/gm

V. RESULTS

IV. A. Analytical Procedure for Succinate

4. Standard Curve. The detector response data for succinate with glutarate as the internal standard is presented in Table 2. There is less than a 1:1 response as indicated by the signal ratio. However, a linear ratio seems to be retained (Figure 1) between the signal ratio and the weight ratio over the range of values found for the fly assays.

5. Extraction and clean-up. The major problem encountered in the extraction procedures is the presence of high levels of fatty acids. In the procedures which yield high levels of fatty acid esters, there is often no indication of the presence of the desired organic acids.

a. Perchloric acid-silicic acid, ether elution. This procedure leaves succinate and glutarate free of any interfering compounds. In addition the long chain fatty acids which elute after glutarate are cleaned from the resulting sample (Figure 2).

b. Sodium hydroxide - clean-up. The saponifying action of NaOH releases large quantities of myristate, a palmitate derivative, and stearate. Organic acids do not appear in the preparation (Figure 3).

TABLE 2

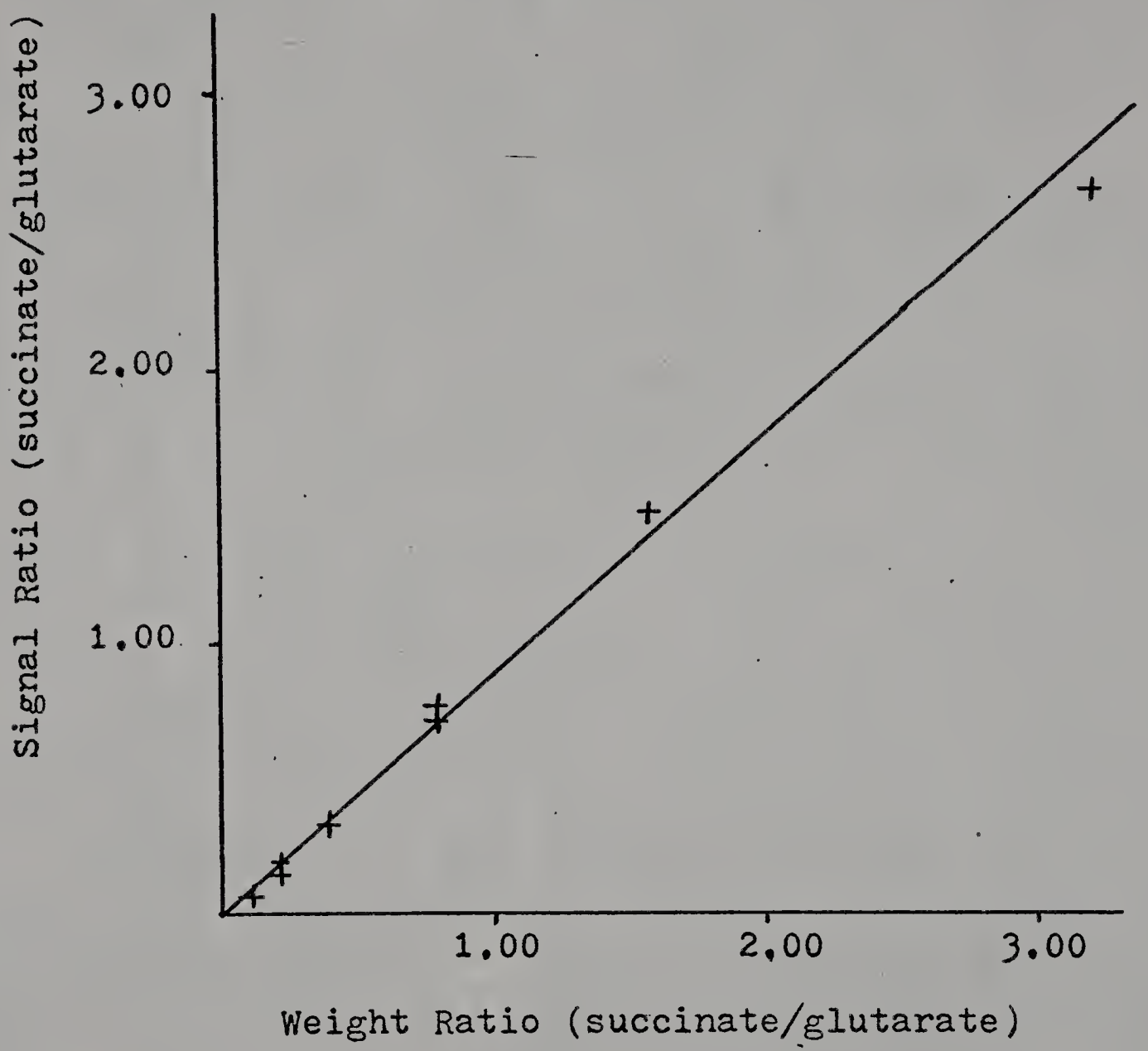
DETECTOR RESPONSE DATA FOR SUCCINATE WITH GLUTARATE AS THE INTERNAL STANDARD.

Sample	Succinate (μg)	Weight Ratio Succ/Glut	Signal Ratio* Succ/Glut
Exp. I			
1	10	.10	.08
2	20	.20	.17
3	40	.40	.36
4	80	.80	.78
Exp. II			
1	20	.20	.325
1a	20	.20	.335
av			.330
2	80	.80	.801
2a	80	.80	.696
av			.748
3	160	1.60	1.666
3a	160	1.60	1.322
av			1.494
4	320	3.20	2.603
4a	320	3.20	2.755
av			2.679

*Each sample contains 100 μg of glutarate from a stock solution of 50 μg/ml; final sample volumes range from 0.5-1 ml; esterification by BF_3-MeOH overnight; chromatography on 6% DEGS and 3% OV-17 on Diatoports-S; column oven temperature, programmed 80° to 180°C at 4°C/min; injection port temperature, 180°C; flame detector temperature, 278°C; range 10^5 to 10^2; attenuation 32 to 4.

FIGURE 1

LINEAR CALIBRATION CURVE FOR SUCCINATE ANALYSIS WITH GLUTARATE AS THE INTERNAL STANDARD.



c. Sodium hydroxide - ether liquid-liquid extraction. Ether extraction of the acidic aqueous phase reduces the fatty acid carry over. Malate is the only organic acid collected in any quantity (Figure 4).

d. Acetone - no clean-up. High molecular weight fatty acids but not organic acids are obtained with this technique (Figure 5).

e. Acetone and H_2O - hexane liquid-liquid extraction. Neither fatty acids nor the organic acids are extracted with this procedure (Figure 6).

f. Hot perchloric acid - silicic acid, ether elution. Hot acid incubation does not increase the yield of succinate (Figure 7). Comparison with Figure 2 reveals the production of another peak which interferes with the resolution of succinate. Although the results are not presented, the first 200 ml volume of ether used to elute the silicic acid preparation draws off nearly all of the succinate and glutarate.

g. Perchloric acid - anion exchange compared to silicic acid. The silicic acid preparation contains three additional compounds not determined as components or impurities. The anion exchange preparation contains a lesser amount of succinate relative to glutarate by approximately 40% (Figure 8).

FIGURE 2

PERCHLORIC ACID-SILICIC ACID, ETHER ELUTION IV.A.5.a.

Chromatographic conditions: 6% DEGS 3% OV-17 on Diatoports-S; column oven temperature, programmed 80°-180°C, 6°C/min, 10 min post injection interval; injection port temperature, 190°C; flame detector temperature, 280°C; range, 10^5 to 10^2; attenuation, 32 to 8. Peak 1, fumarate; peak 2, succinate; peak 3, glutarate.

FIGURE 3

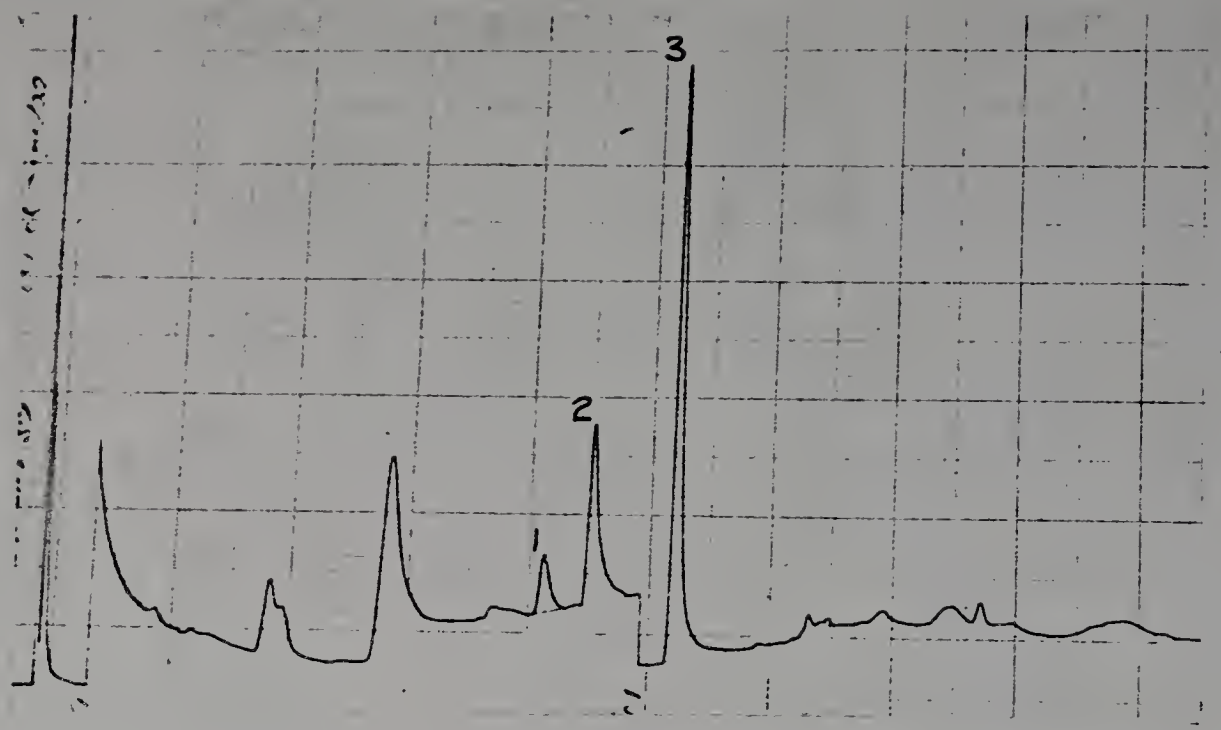
SODIUM HYDROXIDE-NO CLEAN-UP IV.A.5.b.

Chromatographic conditions: 15% DEGS on Diatoports-S; column oven temperature, programmed 82°-180°C, 4°C/min, 10 min post injection interval; injection port temperature, 196°C; flame detector temperature, 283°C; range, 10^5 to 10^2; attenuation, 32 to 8. Peak 1, malate; peak 2, myristate; peak 3, palmitate derivative; peak 4, stearate.

FIGURE 4

SODIUM HYDROXIDE-ETHER LIQUID-LIQUID EXTRACTION IV.A.5.c.

Chromatographic conditions: 15% DEGS on Diatoports-S; column oven temperature, programmed 91°-195°C, 4°C/min, 10 min post injection interval; injection port temperature, 236°C; flame detector temperature, 288°C; range, 10^5 to 10^1; attenuation, 16 to 8. Peak 1, malate.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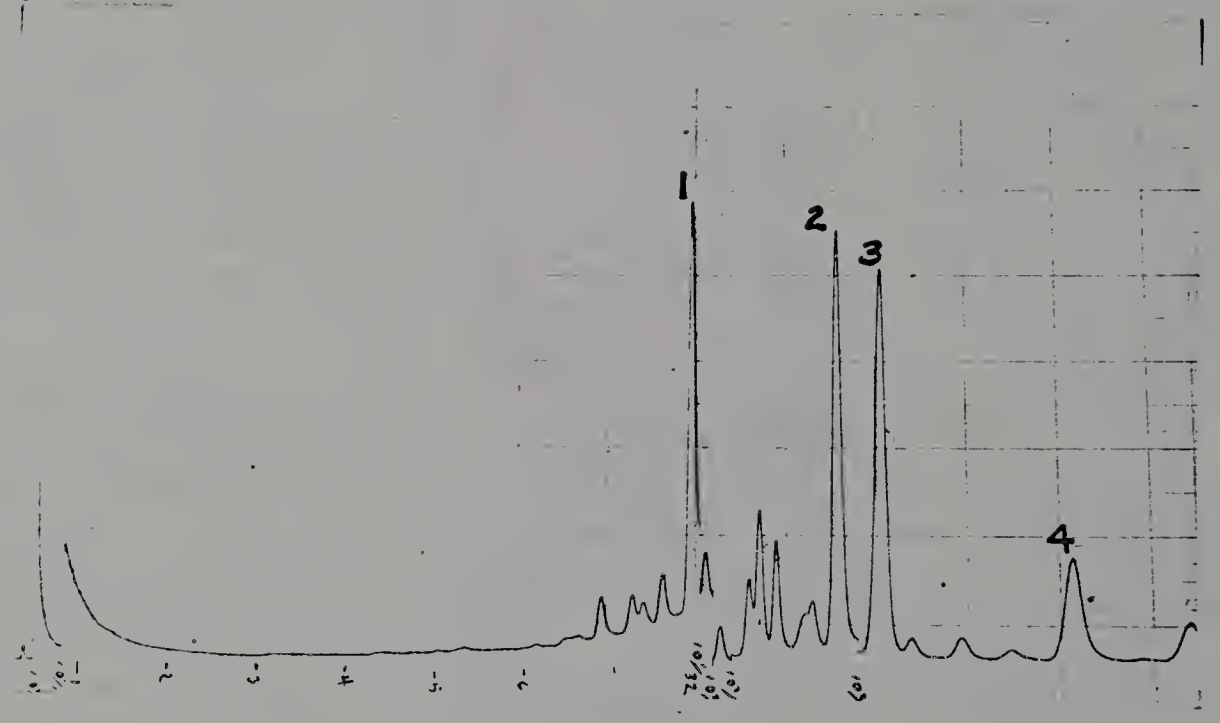
2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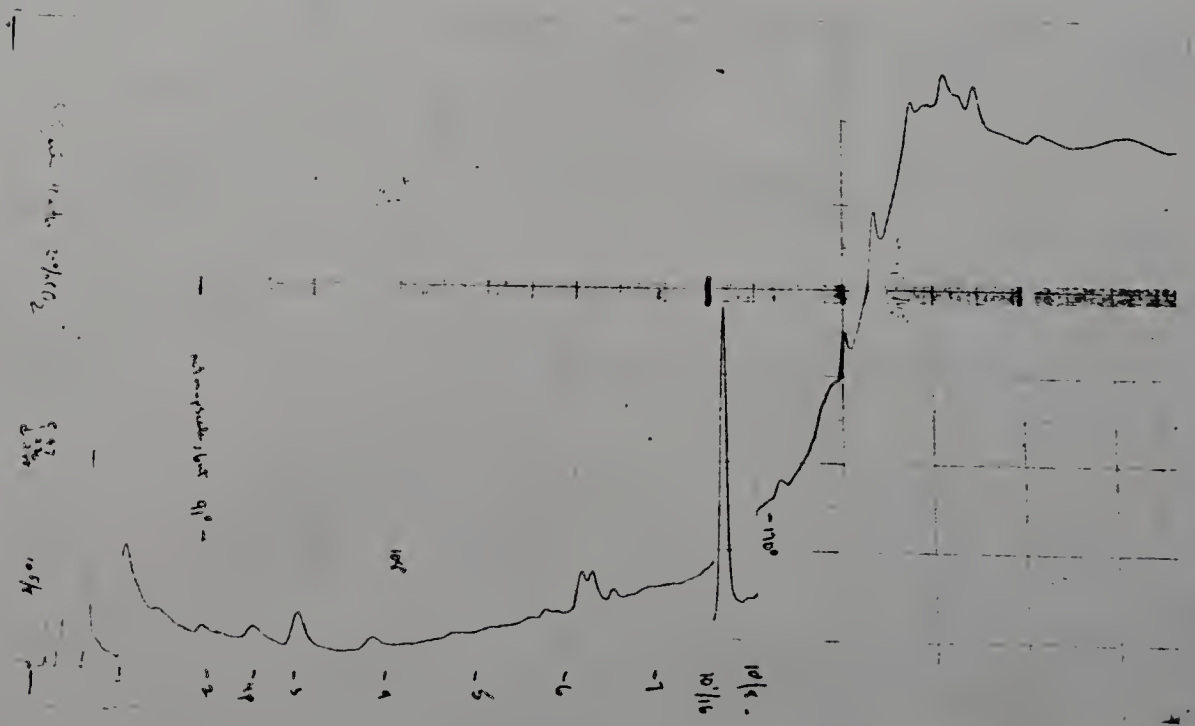


FIGURE 5

ACETONE-NO CLEAN-UP IV.A.5.d.

Chromatographic conditions: 15% DEGS on Diatoports-S; column oven temperature, programmed 83°-180°C, 4°C/min, 10 min post injection interval; injection port temperature, 190°C; flame detector temperature, 281°C; range 10^5 to 10^2; attenuation, 8. Peak 1, myristate; peak 2, palmitate derivative; peak 3, stearate.

FIGURE 6

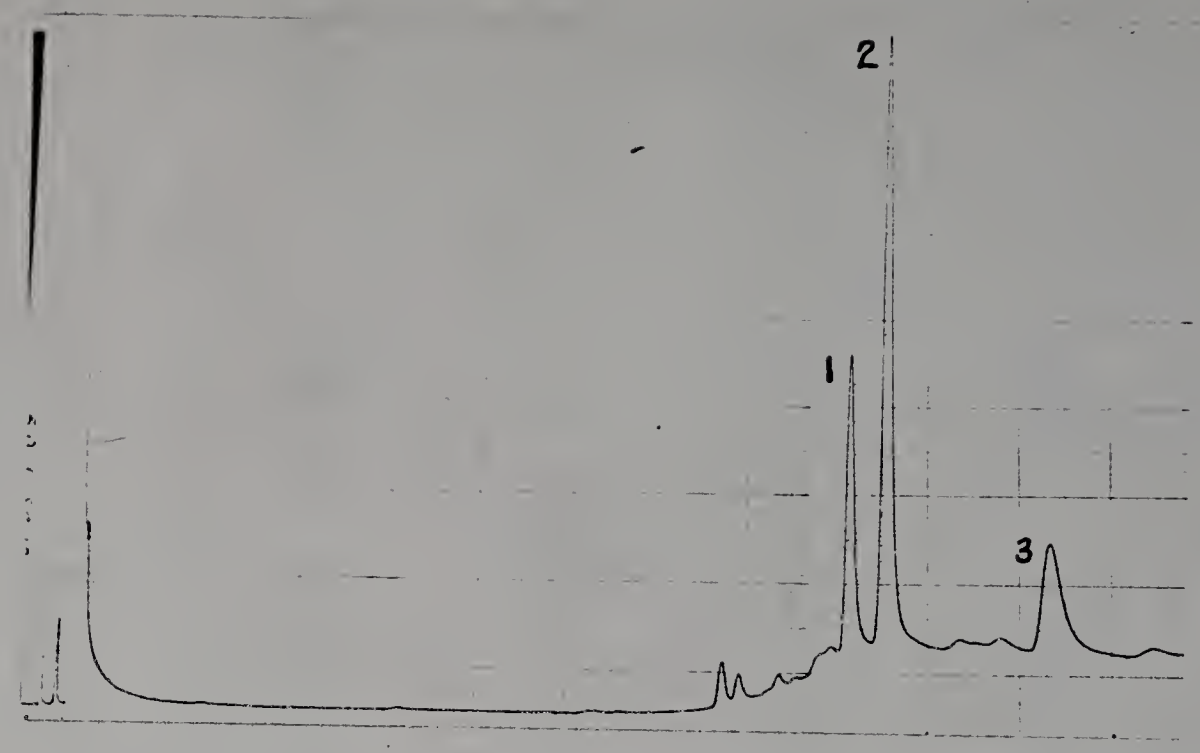
ACETONE AND WATER-HEXANE LIQUID-LIQUID EXTRACTION IV.A.5.e.

Chromatographic conditions: 6% DEGS, 3% OV-17 on Diatoports-S; column oven temperature, programmed 83°-180°C, 6°C/min, 10 min post injection interval; injection port temperature, 191°C; flame detector temperature, 282°C; range, 10^5 to 10^2; attenuation, 8.

FIGURE 7

HOT PERCHLORIC ACID-SILICIC ACID, ETHER ELUTION CHECK IV.A.5.f.

Chromatographic conditions: 6% DEGS, 3% OV-17 on Diatoports-S; column oven temperature, programmed, 70°-180°C, 6°C/min, 10 min post injection interval; injection port temperature, 180°C; flame detector temperature, 280°C; range, 10^5 to 10^2; attenuation 8. Peak 1, succinate; peak 2, glutarate.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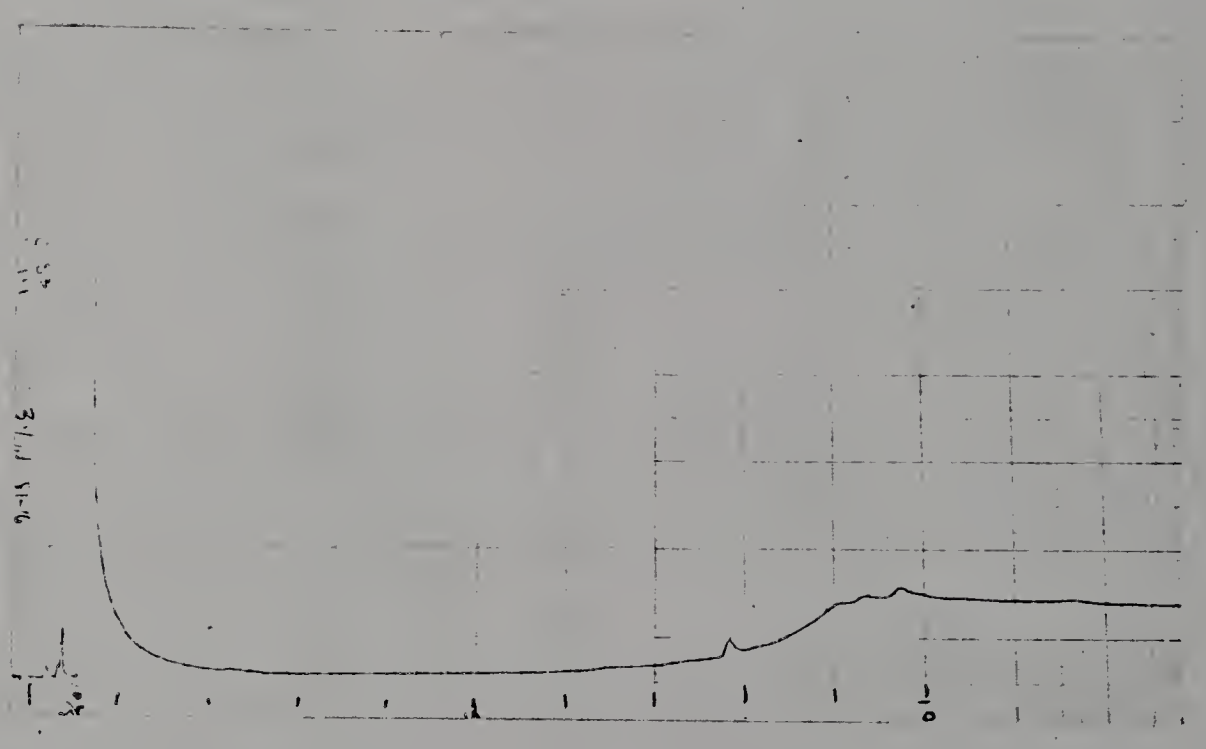
1
3



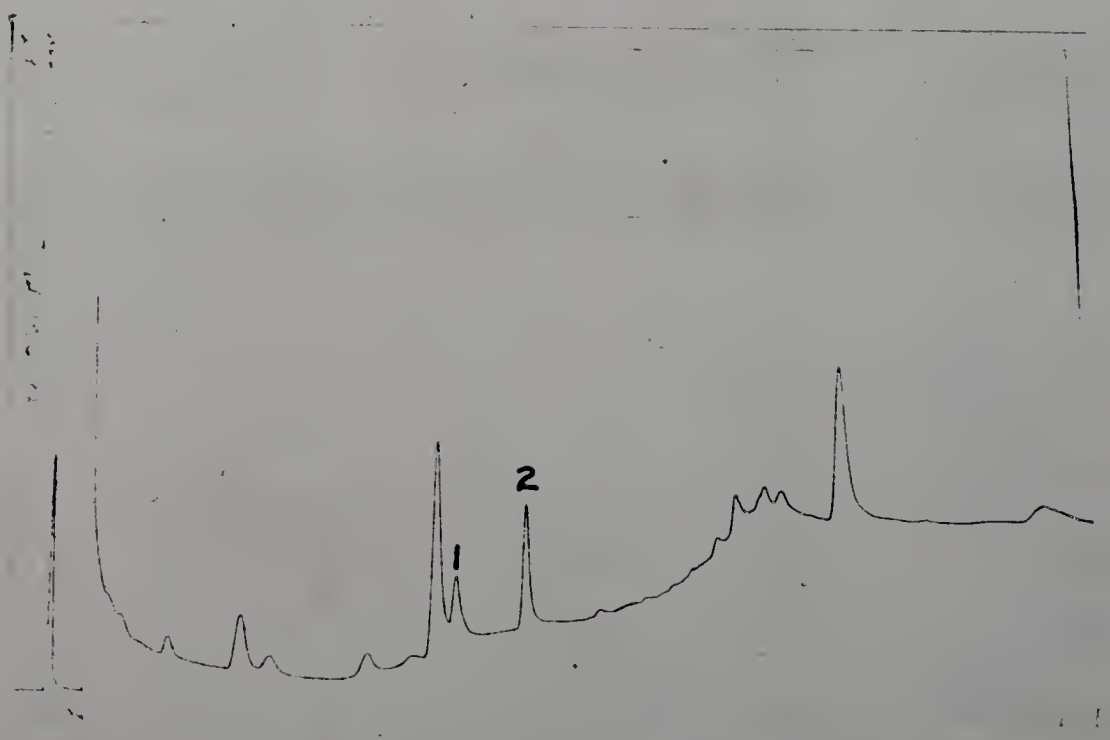
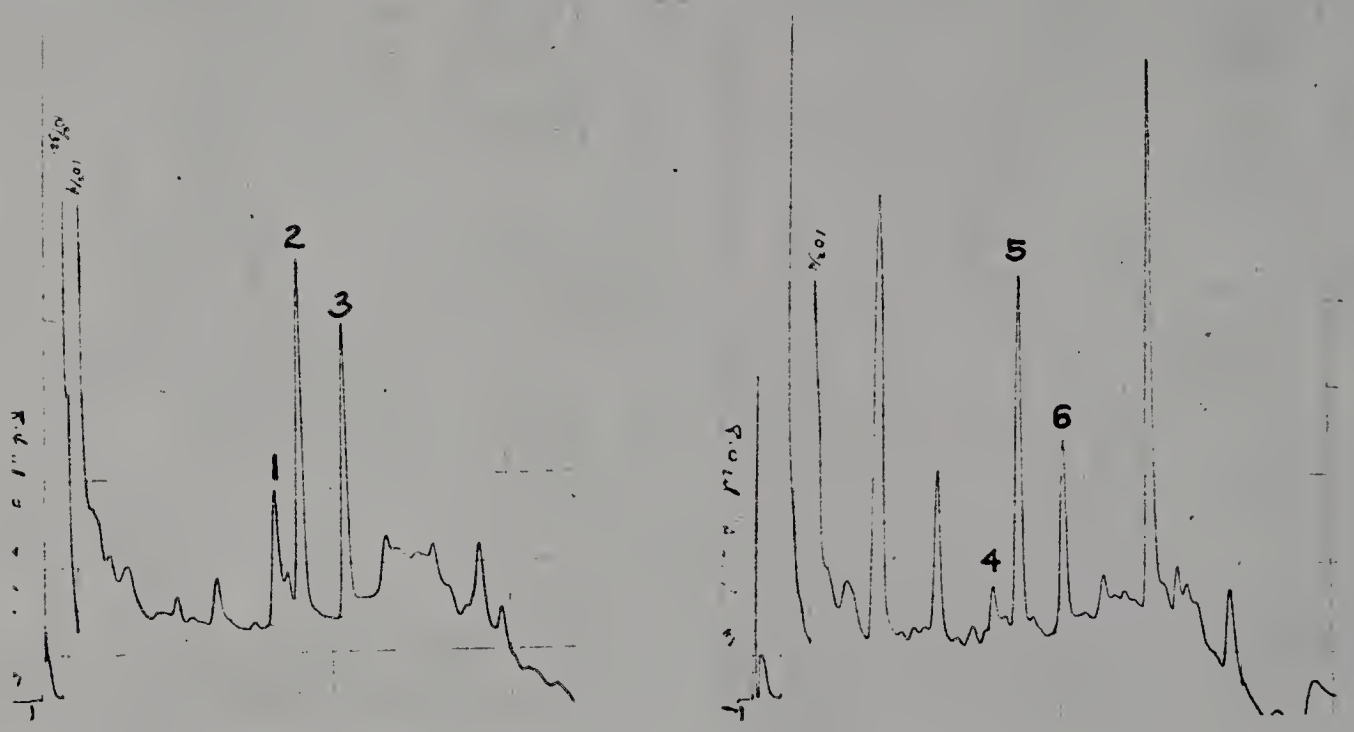

1
2

FIGURE 8

PERCHLORIC ACID-ANION EXCHANGE COMPARED TO SILICIC ACID PURIFICATION IV.A.5.g.*



* Chromatographic conditions: 6% DEGS, 3% OV-17 on Diatoports S; column oven temperature, programmed 80°- 180°C, 6°C/min; injection port temperature, 180°C; flame detector temperature, 275°C; range, 10^5 to 10^2; attenuation, 32 to 4. Peak 1, fumarate; peak 2, succinate; peak 3, glutarate; peak 4, fumarate; peak 5, succinate; peak 6, glutarate.

6. Esterification procedures.

b. 1) Effect of time and temperature on acid-methanol and diazomethane (Table 3, Experiment I). High temperatures drastically reduce the yield of succinate di-methyl ester when HCl-MeOH is the esterifying agent. Temperature does not effect the yield with H_2SO_4 drastically, but the yield at either 55° or 100°C is well below the possible maximum. Methanol in the presence of diazomethane reduces the yield of the succinate ester.

b. 2) Effect of time on diazomethane-methanol (Table 3, Experiment II). Increasing the incubation period from 3 to 90 minutes reduces the yield of the succinate ester by approximately 50%.

b. 3) Effect of evaporation temperatures on esters (Table 3, Experiment III). There is little loss of the succinate ester when evaporated *in vacuo* at 24°C. However, nearly all of the ester is lost at 65°C.

b. 4) Effect of time and temperature on diazomethylation (Table 4, Test I). Yields of succinate ester are increased with lower temperature but not with increased time. Fumarate ester is not synthesized except at the lowest temperature and time (Table 4, Test II). At -60°C the yield of the succinate ester increases with increasing time while that of the fumarate ester decreases. At 25°C the fumarate ester is produced only at the 1 minute reaction

TABLE 3

RESULTS OF SUCCINATE METHYLATION UNDER VARIOUS ESTERIFICATION CONDITIONS.

Sample	Esterification Reagent	Reaction Time	Reaction Temperature	Area Units* (Succinate)
Exp. I				
1	HCl-MeOH	10 min	100°C	2.3
2	" "	4 hrs	55°C	18.0
3	H_2SO_4-MeOH	10 min	100°C	12.6
4	" "	4 hrs	55°C	10.2
5	Diazomethane-MeOH	3 hrs	22°C	17.0
6	Diazomethane	3 hrs	22°C	21.3
Exp. II				
1	Diazomethane-MeOH	3 min	24°C	21.0
2	" "	90 min	24°C	12.5
Exp. III		Solvent Evaporation Temperature		
1	Diazomethane	24°C		16.9
2	"	65°C		0.9

*Chromatographic conditions: 15% DEGS on Diatoports-S; column temperature, 110°C; injection port temperature, 220°C; flame detector temperature, 280°C; range 10^4 to 10^2; attenuation, 8 to 4.

TABLE 4

RESULTS OF SUCCINATE AND FUMARATE METHYLATION WITH DIAZOMETHANE UNDER VARIOUS TIME AND TEMPERATURE CONDITIONS. (EXP. IV)

Sample	Reaction Time (min)	Reaction Temperature (°C)	Area Units (Succinate)	Area Units[a] (Fumarate)
Test I				
1	1	25	1.3	trace
2	8	25	1.2	0.0
3	1	-60	2.8	1.5
4	8	-60	2.1	0.0
Test II[b]				
1	1	25	1.0	1.7
2	2	25	off chart	0.0
3	4	25	2.8	0.0
4	8	25	2.5	0.0
5	1	-60	0.4	3.7
6	2	-60	1.1	2.5
7	4	-60	1.3	2.5
8	8	-60	1.6	1.8
9		BF_3-MeOH	1.2	1.4

[a] Chromatographic conditions: 3% DEGS, 3% OV-17 on Diatoports-S; column oven temperature, programmed 80° to 180°C at 6°C/min; injection port temperature, 180°C; flame detector temperature, 270°C; range, 10^2; attenuation 32 to 16.

[b] Only one chromatogram of sample 2. Samples 2, 3, and 4 have slightly elevated values due to solvent evaporation.

time. The BF_3:MeOH treatment served as a check and yielded nearly equal amounts of the two acids.

7. Organic acid analysis of manure. The procedure used does not give evidence of the presence of organic acids in spite of the large amount of manure (4 gm) used in the test. Fatty acid material is present (Figure 9). The manure contains three fatty acids in about the same proportions as the fly tissues (Figure 5), however, the large peak in Figure 9, palmitate ester, is not found in the fly (Figure 5). There is a large, lower boiling point compound in the fly of equal proportion. Experiments performed by Dr. L. J. Edwards on the organic acids in manure are presented in the discussion section.

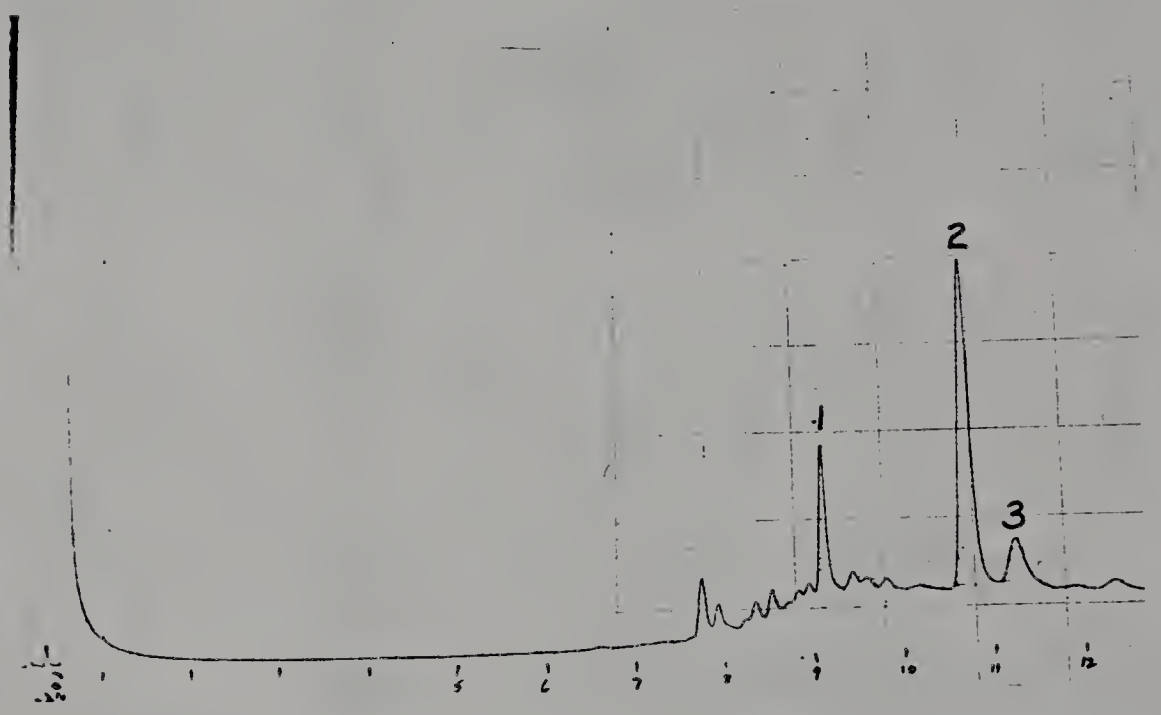
IV. B. Succinate Levels in the Face Fly

The results of the tests on the changes in the fresh body weight (Table 5) indicate that a severe decline in weight occurs in the dry environment, but not in the moist conditions employed in these tests. Adults fail to emerge from puparia subjected to the dry environment. It is also noted that the dry environment has no effect on the pupa if not begun until age P_4. However, pupae in a dry environment seem to take up water when transferred to a moist one.

Although the data for the succinate concentration in the various stages of the fly are reported on a fresh weight

FIGURE 9

RESULTS OF GAS CHROMATOGRAPHIC ANALYSIS OF MANURE IV.A.7.*



* Chromatographic conditions: 6% DEGS on diatoports-S; column oven temperature, programmed 83°-180°C, 4°C/min; injection port temperature, 191°C; flame detector temperature, 282°C; range, 10^5 to 10^2; attenuation, 8. Peak 1, myristate; peak 2, palmitate; peak 3, stearate.

TABLE 5

VARIATION IN THE BODY WEIGHT OF THE PUPAL STAGE OF THE FACE FLY UNDER A HUMID OR DRY ENVIRONMENT (MG/PUPA).

Sample	Age	P_1	P_2	P_3	P_4	P_5	P_6	Adults Emerging Out of 25
Dry								
	1	31.1	11.5	10.8	10.7	10.8	10.8	0
	2	32.2	12.8	12.0	11.9	11.9	11.8	0
	av	31.2	12.2	11.4	11.3	11.3	11.3	
Humid								
	1	30.6	28.0	27.4	27.2	27.1	27.1	21
	2	29.4	24.4	24.2	24.0	23.9	23.7	21
	av	30.0	26.2	25.8	25.6	25.5	25.4	
Dry-humid[a]								
	1	31.9	12.0	11.1	11.1	13.4	13.5	0
	2	31.6	12.0	11.3	11.1	14.3	14.7	0
	av	31.7	12.0	11.2	11.1	13.9	14.1	
Humid-dry[b]								
	1	30.9	27.6	26.9	26.7	25.8	25.2	21
	2	32.5	29.1	28.6	28.6	27.7	27.3	21
	av	31.7	28.3	27.7	27.6	26.8	26.3	

[a] After weighing on P_4 the pupae were transferred to a humid environment from a dry environment.

[b] After weighing on P_4 the pupae were transferred to a dry environment from a humid environment.

TABLE 6

FRESH AND DRY WEIGHT OF THE PUPAL STAGE
OF THE FACE FLY (MG/INDIVIDUAL).

Age / Sample	L_6	P_1	P_2	P_3	P_4	P_5	P_6	P_7	A_1
Fresh weight	43.2	38.6	37.5	36.0	35.9	35.3	35.5	35.8	28.1
Dry weight	13.9	13.3	13.5	13.1	13.2	12.6	13.3	12.9	6.7
Fresh weight[a/]		31.7	30.6	29.1	29.0	28.4	28.6	28.9	
Dry weight[b/]		8.0	8.2	7.8	7.9	7.3	8.0	7.6	

a/ These values are the fresh weight of the pupae minus the fresh weight of the pupal case from A_1, 6.9 mg.

b/ These values are the dry weight of the pupae minus the dry weight of the pupal case from A_1, 5.3 mg.

basis, the data in Table 6 will allow readers to make a reasonable calculations based on the dry weight.

Experiment I. From a low of 57 µg/gm in P_1 the succinate level more than triples to 179 µg/gm in P_2. From there a gradual decline occurs over the remainder of the pupal stage to about 120 µg/gm. The dip to 91 µg/gm and the rise to 141 µg/gm on days P_6 and P_7, respectively, are correlated with the time for the pharate adult (Table 1). The level rises to 143 µg/gm in the adult stage (Table 7, p. 45).

Experiment II. The rise from P_1 to P_2 and the gradual decline after P_2 seen in Experiment I is confirmed (Table 7, p. 45).

Experiment III. The mixed adults reveal a gradual rise from age A_1 to A_3 followed by a decline to A_6. The initial adult value of 131 µg/gm agrees closely with that in Experiment I, 142 µg/gm (Table 8, p. 46).

Experiment IV. In both the males and females there is a rise from A_1 to A_3. After this time there is considerable fluctuation in the values. The value for A_1 is considerably below that of the previous experiments, being 81 and 92 for the males and females, respectively (Table 8, p. 46).

Experiment V. The gradual decline during the pupal stage and the gradual rise during the early days of the adult stage seen in the other experiments is also seen here. The larval values for L_3 and L_5 are nearly equal to the adult values for A_1 (Table 9, p. 47).

TABLE 7

SUCCINATE CONCENTRATIONS IN PUPAL* AND ADULT STAGES OF THE FACE FLY (UG/GM FRESH WEIGHT).

Sample \ Age	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9	A_1
Exp. I										
1	56	190	117	140	136	116	142	117	150	122 137
2	58	183 151	166 133	108	93	66	139	113	93	154
av	57	179	133	124	115	91	141	115	122	142
Exp. II										
1	194 230 193 190	316	266	226 242 210			218 235	192		
2	125	237	193	150			142	205		
av	163	277	230	188			184	199		

* These calculations include the additional weight of the pupal case.

TABLE 8

SUCCINATE CONCENTRATIONS IN ADULT STAGES OF THE FACE FLY (UG/GM FRESH WEIGHT).

Age / Sample	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8
Exp. III								
1	156	225	300	148	150	153		
2	106	67	142	183	126	132		
av	131	146	221	166	138	143		
Exp. IV								
1 (male)	81	119	140	128	163		131	119
2 (female)	80	86	111	79	86		149	133
	103			80				
av	92			80				

TABLE 9

SUCCINATE CONCENTRATIONS IN LARVAL, PUPAL*, AND ADULT STAGES OF THE FACE FLY (UG/GM FRESH WEIGHT).

Age	L_3	L_5	P_1	P_3	P_5	A_1	A_4	A_8	A_1	A_4	A_8
						Male			Female		
Sample											
Exp. V											
1	57	50	145	113	97	48	88	85	61	73	58
	62	46	140			45					
			158								
2						111	81	52	87	84	53	82	51
3						106	99	50	70		46	101	
av	60	48	148	110	92	50	82	85	53	85	55

* These calculations include the additional weight of the pupal case.

VI. DISCUSSION

It became apparent very early in the study that the techniques employed by other workers for the extraction of organic acids from plant tissues would not be satisfactory for the extraction of organic acids from insect tissues. The high levels of fatty acids in the fly tissues for the most part probably masked the organic acids. Thus, while the perchloric acid technique may not yet be the best procedure for obtaining the most complete extraction of the acids, it is possible to obtain consistent results with it.

The same is true for the esterification procedure using diazomethane. It is demonstrated in this thesis, as well as by other workers, that the technique is extremely dependent on time and temperature. Such variability has not been reported in the literature for BF_3:MeOH, and the constant conditions of time and temperature used throughout this work assured constant esterification.

A great source of variation results from the nutrition of the flies. It is not possible to feed each batch of flies on identical media. The manure is highly variable in terms of water content, solids, color, and odor. The great differences seen in the succinate concentrations between experiments further confirms the dietary irregularity.

The manure itself is considered as a source of organic

acids which might have some effect on the results for the larvae since a large portion of the larva is occupied by a manure-filled gut. Subsequent to my experiments, Dr. L. J. Edwards used my procedures established for the face fly and ion exchange chromatography for clean-up to determine the levels of organic acids in manure. This is analyzed in Table 10.

TABLE 10

EFFECT OF MANURE IN GUT ON
THE SUCCINATE VALUES OF
FACE FLY LARVAE

Source of Succinate	Concentration
larvae (L_5 from Table 9)	48 µg/g
manure	15.9 µg/g
larval gut*	4 µg/g
Affecting levels	8%

*/ Twenty-five percent is the assumed part by weight but is likely to be a high approximation because glutarate, the internal standard, is also present at a level of 21.4 µg/g in manure. During tests to determine a feasible internal standard, there are no peaks at this elution time in the chromatograms of the face fly extracts.

The precise value in determining the succinate

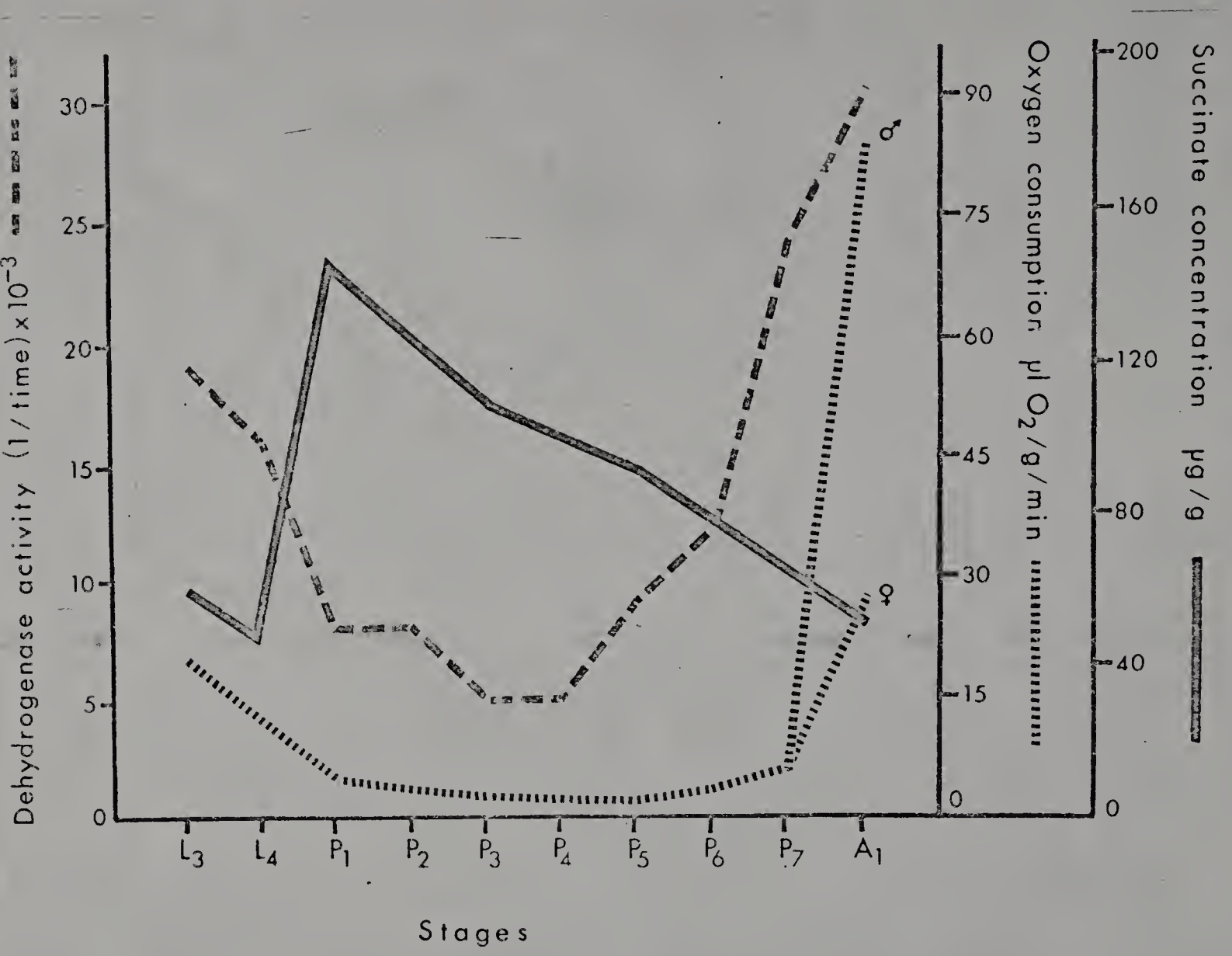
quantities in animals in terms of its physiological significance is not yet clear. Some obvious possibilities are the detection of succinate pools; its significance in terms of ion balance; correlation with enzyme activity, physiological state, behavior, and age; and further elucidation of the role of succinate in metabolism through comparative study.

A comparison of succinate concentration found in this study with the oxygen consumption (Guerra and Cochran, 1970) and succinic dehydrogenase activity (Rousell, 1967) (Figure 10) reveals some apparent correlations that bear further study. Succinate concentration seems to be inversely correlated with both oxygen consumption and succinate dehydrogenase activity, and in the pupal stage is best correlated with the latter. A gradual decline in succinate is accompanied by a gradual rise in succinate dehydrogenase activity. In fact, the correlation between these two factors is better than the correlation of either one of them with oxygen consumption, which remains essentially unchanged over the entire pupal period.

Whether the succinate concentrations found in this study are high enough to serve as a significant pool or reserve during periods of high metabolic demand is analyzed in Table 11. Making the assumption, unrealistic as it is,

FIGURE 10

COMPARISON OF SUCCINATE CONCENTRATION, OXYGEN CONSUMPTION, AND SUCCINATE DEHYDROGENASE ACTIVITY IN THE FACE FLY*.



* Succinate concentrations from Table 9; succinate dehydrogenase activity from Rousell (1967); oxygen consumption from Guerra and Cochran (1970). Succinate dehydrogenase activity is expressed as 1/time in minutes for 90% decolorization of methylene blue.

TABLE 11

CALCULATED RATE FOR COMPLETE LOSS OF SUCCINATE THROUGH THE ELECTRON TRANSPORT CHAIN IN ADULT MALES OF THE FACE FLY.

Oxygen consumption of adult males, A_1*.	85 µl/g/min = 5.1 µl/ mg/hr
uM equivalent under ideal gas law.	7.6 µM oxygen,O/g/min
Succinate concentration of adult males, A_1, from Table 7.	50 µg/g
uM equivalent.	0.42 µM/g
Under assumption, molar ratio of succinate to oxygen, O, during transport.	1:1
Time for complete loss of succinate through the electron transport chain.	0.055 min (3.3 sec)

* Guerra and Cochran, 1970.

that succinate is the only route to the electron transport, we obtain a time for the disappearance of succinate where succinate is not being replaced by other enzymatic reactions. Since the respiratory value is for the fly at rest, and the succinate would disappear in 3.3 seconds, this does not seem to represent a significant energy reserve.

Very little information on the succinate concentrations in animals is readily available. Rat tissues have the following in µg/gm: liver, 260; kidney, 71; muscle, 118; spleen, brain, thymus, lung, heart, and blood, 0 (Webb, 1966). The horse bot fly, Gasterophilus intestinalis, has 240 mg/100 ml of blood (Spector, 1956). The plasma of human blood has 0.5 mg/100 ml (Altman and Dittmer, 1961). The values for vertebrate tissues are in reasonably good agreement with the face fly values. The bot fly value converts to 2400 µg/gm and might be correlated with its unique environment in the stomach of a horse where the carbon dioxide concentration may reach 75%. Analyses of other insects were made in the Research Methods Course at the University of Massachusetts in Fall, 1972 under the direction of Dr. L. J. Edwards. These are compared in Table 12 with the face fly from Table 9.

These values are all based upon one analysis without an internal standard. In general, there does seem to be a

positive correlation between the activity of the insect and its succinate concentration. These data should prompt further comparative study to verify this hypothesis. Also, it would not be unscientific to hypothesize a correlation between succinate and mitochondrial content.

TABLE 12

SUCCINATE CONCENTRATION IN SEVERAL INSECT SPECIES

Species	Stage	Succinate Concentration (µg/gm)
Musca autumnalis	adult	85
Phormia regina	larva	300
	pupa	110
	adult	1695
Musca domestica	adult	5018
Apis mellifera	worker	6680
Gromphadorhina portentosa	adult	49
Blaberous discoidalis	adult	933
Tuberolachnus salignus	apterae	50

In order to make this kind of study complete, an examination of all the major Krebs cycle components is necessary. From the work of this thesis and literature reports it is obvious that no one procedure will enable a simultaneous determination of all the components. The ideal procedures

are possibly the use of perchloric acid extraction, silicic acid and/or ion-exchange purification, trimethylsilyl ester esterification (Dalgliesh, et al., 1966) and analysis by gas chromatography combined with mass spectrometry.

The procedure outlined in this thesis appears to be basically sound for the quantitative determination of succinate in the face fly. Other species might require more or less concentration of the final analysis sample. Analysis of malate appears to offer no particular difficulty but may be facilitated by an increase in the pH of the extraction medium.

Citrate is a large molecule and is further increased in size by methylation. This characteristic causes longer retention time during analysis and, subsequently, a drop in the accuracy of citrate analysis. A possible solution might be the use of a slightly less polar liquid stationary phase rather than trying to increase the temperature of the program. As indicated earlier, this would decrease the loss of some of the other sample components, as well as, citrate due to thermal decomposition.

Fumarate with its electrophore group has given difficulty in analysis due to the formation of pyrazole derivatives when using diazomethylation for esterification. The resulting ring derivatives are very hard to chromatograph

using the common liquid stationary phases. Simmonds et al. (1967) studying the Krebs cycle keto acids during esterification found that oxalacetate formed a fumarate derivative retaining the same electrophore group. He suggests exploiting the use of electron absorption detectors on the gas chromatograph which are suited to detecting double bonds. Fumarate, esterified with a less rigorous reagent, could also be examined with the same detector and with little change in the tissue extraction procedure.

Simmonds' study indicates the need of investigating the derivatives formed no matter what esterifying reagent is used. Any work designed to quantitate the levels of oxalacetate, alpha-ketoglutarate and pyruvate will require the combined quantitation of two or more peaks for each compound.

VII. CONCLUSIONS

A. An organic acid analysis technique has been developed for the quantitation of succinate from the face fly, Musca autumnalis De Geer. The procedure includes: extraction with a strong protein-precipitating acid, perchloric acid; sample purification with ether elution of the organic acid components off silicic acid; methyl esterification to the more volatile form with boron-trifluoride in methanol; and gas chromatographic analysis with glutarate used as an internal standard.

B. During the major stages of face fly development, succinate occurs at levels of 48-60 µg/gm fresh weight during the larval period, 92-277 µg/gm fresh weight during the pupal period and 53-149 µg/gm fresh weight for the adult female, and 50-163 µg/gm fresh weight for the adult male.

C. Succinate concentration in the face fly shows a slight correlation with the major stages during metamorphic development. Also, succinate concentrations show an inversely related correlation with the succinic dehydrogenase enzyme and respiratory activity found during the pupal stage.

VIII. REFERENCES

Ackman, R. G., B. A. Bannerman, and F. A. Vandenheuvel. 1960. Decomposition of short-chain dicarboxylic acid esters during separation on polyester gas chromatography media. Anal. Chem. 32: 1209.

Agrell, I. P. S. 1949. Localization of some hydrogen-activating enzymes in insects during metamorphosis. Nature 164: 1039-1040.

Alcock, N. W. 1965. A simple procedure for the extraction and esterification of some organic acids. Anal. Biochem. 11: 335-341.

_____. 1969. Separation of citric acid cycle and related compounds by gas chromatography. In: Methods in Enzymology (Ed. by J. M. Lowenstein), XIII. Academic Press, New York. 397-415.

Aldrich Chem. Co. 1968. Technical information D2800-0. Milwaukee, Wisconsin.

Altman, P. L. and D. S. Dittmer (Eds.). 1961. Blood and other body fluids. Fed. Am. Soc. Exper. Biol. Washington, D. C. 87 p.

Barbato, P. C., G. R. Umbreit, and R. J. Leibrand. 1966. Internal standard technique for quantitative gas chromatographic analysis. Applications Lab Report 1005. Hewlett-Packard, Avondale, Pennsylvania.

Burchfield, H. P. and E. E. Storrs. 1962. Biochemical Applications of Gas Chromatography. Academic Press, New York. 588-591.

Crompton, M. and L. M. Birt. 1969. Changes in the in vitro activity of glycolytic enzymes during metamorphosis of the blowfly, Lucilia cuprina. J. Insect Physiol. 15: 1119-1127

_____. and S. E. Polakis. 1969. The labelling of tissue components during the pharate adult life of Lucilia cuprina, after injection of U-^{14}C-glucose. J. Insect Physiol. 15: 1323-1329.

Dalgliesh, C. E., E. C. Horning, M. G. Horning, K. L. Knox, and K. Yarger. 1966. A gas-liquid-chromatographic procedure for separating a wide range of metabolites occurring in urine or tissue extracts. Biochem. J. 101: 792-810.

Diamantis, W. 1962. Activities of respiratory enzymes during the metamorphosis of the Mediterranean flour moth, Ephestia kuhniella Zeller. J. New York Entomol. Soc. 70: 68-78.

Edwards, L. J. 1971. Carbon dioxide anesthesia of the face fly, Musca autumnalis De Geer: effects of temperature and oxygen on knockdown rate. Comp. Biochem. Physiol. 38: 509-516.

Estes, F. L. and R. C. Bachmann. 1966. Gas chromatographic determination pyruvic and lactic acids and Krebs cycle components. Anal. Chem. 38: 1178-1182.

Fraenkel, G. and G. Bhaskaran. 1973. Pupariation and pupation in cyclorrhaphous flies (Diptera): terminology and interpretation. Ann. Entomol. Soc. Am. 66: 418-422.

Guerra, A. A. and D. G. Cochran. 1970. Respiration during the life cycle of the face fly. J. Econ. Entomol. 63: 918-921.

Hinton, H. E. 1946. Concealed phases in the metamorphosis of insects. Nature 157: 552-553.

Jenkin, P. M. 1965. A comparative view of the hormonal control of molting and ecdysis in arthropoda. Gen. Comp. Endocrinol. 5: 688-689.

_____. and H. E. Hinton. 1966. Apolysis in arthropod moulting cycles. Nature 211: 871.

Kesner, L. and E. Muntwyler. 1969. Separation of citric acid cycle and related components by partition column chromatography. In: Methods in Enzymology (Ed. by J. M. Lowenstein), XIII. Academic Press, New York. 415-425.

Kuksis, A. and P. Prioreschi. 1967. Isolation of Krebs cycle acids from tissues for gas chromatography. Anal. Biochem. 19: 468-480.

_____. and P. Vishwakarma. 1963. Simultaneous determination of Krebs cycle acids by gas-liquid chromatography. Can. J. Biochem. Physiol. 41: 2353-2362.

Ludwig, D. and M. C. Barsa. 1956. Variations in the activity of the succinoxidase system during development of the mealworm, Tenebrio molitor L. Ann. Entomol. Soc. Am. 49: 103-104.

_____. and _____. 1959. Activities of the respiratory enzymes during the metamorphosis of the house fly, Musca domestica L. J. New York Entomol. Soc. 67: 151-156.

McKeown, G. G. and S. I. Read. 1965. Esterification and gas chromatography of some acids of the tricarboxylic acid cycle. Anal. Chem. 37: 1780.

Myers, W. F. and K. Y. Huang, 1969. Thin layer chromatography of citric acid cycle components. In: Methods in Enzymology (Ed. by J. M. Lowenstein), XIII. Academic Press, New York. 431-434.

Rousell, P. G. 1967. Activities of respiratory enzymes during the metamorphosis of the face fly, Musca autumnalis De Geer. J. New York Entomol. Soc. 75: 119-125.

Rumsey, T. S. and C. H. Noller. 1966. A study of the quantitative measurement of certain metabolic acids by gas-liquid chromatography. J. Chromatog. 24: 325-334.

_____., _____, J. C. Burns, D. Kalb, C. L. Rhykerd, and D. L. Hill. 1964. Gas chromatographic resolution of pyruvic, lactic, glyoxylic, malonic, fumaric, malic, alpha-ketoglutaric, and citric acids in forage and rumen fluid. J. Dairy Sci. 47: 1418-1421.

_____., _____, C. L. Rhykerd, and J. C. Burns. 1966. Measurement of certain metabolic organic acids in forage, silage, and ruminal fluid by gas-liquid chromatography. J. Dairy Sci. 50: 214-219.

Sacktor, B. 1951. Some aspects of respiratory metabolism during metamorphosis of normal and DDT-resistant house flies, Musca domestica L. Biol. Bull. 100: 229-243.

Salminen, K. and P. Koivistoinen. 1967. Quantitative gas chromatography of nonvolatile organic acids: evaluation of the internal standard method. Acta Chem. Scand. 21: 1495-1500.

Simmonds, P. G., B. C. Pettitt, and A. Zlatkis. 1967. Esterification, identification, and gas chromatographic analysis of Krebs cycle keto acids. Anal. Chem. 39: 163-167.

Spector, W. S. 1956. Handbook of Biological Data. Saunders, Philadelphia. 67 p.

Von Korff, R. W. 1969. Ion-exchange chromatography of citric acid cycle components and related compounds. In: Methods in Enzymology (Ed. by J. M. Lowenstein), XIII, Academic Press, New York. 425-430.

Wang, C. M. 1964. Laboratory observations of the life history and habits of the face fly, Musca autumnalis (Diptera: Muscidae). Ann. Entomol. Soc. Am. 57: 563-569.

Webb, J. L. 1966. Enzyme and Metabolic Inhibitors. Vol. II. Academic Press, New York. 102 p.

Wigglesworth, V. B. 1965. The Principles of Insect Physiology. Dutton, New York. 573-574.