ISOLATION AND CHARACTERIZATION OF THE DEOXYRIBONUCLEIC ACIDS OF DROSOPHILA MELANOGASTER

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Charles G. Mead

1960

This is to certify that the

thesis entitled

ISOLATION AND CHARACTERIZATION
OF THE DEOXYRIBONUCLEIC ACIDS
OF DROSOPHILA MELANOGASTER

presented by

Charles G. Mead

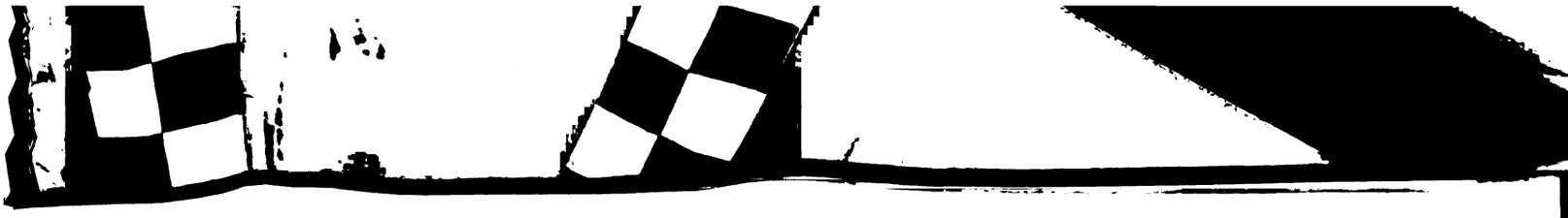
has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Genetics

Allen S. Fox
Major professor

Date November 22, 1960

O-169



ISOLATION AND CHARACTERIZATION OF THE DEOXYRIBONUCLEIC ACIDS OF DROSOPHILA MELANOGASTER

By

Charles G. Mead

AN ABSTRACT

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Agricultural Chemistry

1960

Approved Allen S. Fox

ABSTRACT

ISOLATION AND CHARACTERIZATION OF THE
DEOXYRIBONUCLEIC ACIDS OF DROSOPHILA MELANOGASTER

by Charles G. Mead

A procedure is described for the isolation of DNA from Drosophila melanogaster. An enzymatic deproteinization procedure is described which results in a product that is free from RNA and protein and not extensively denatured. Two types of DNA are observed upon precipitation of the isolated product with cold ethanol. One of these is fibrous in nature, typical of most DNA's, whereas the other is of a flocculent nature. The isolated DNA is found to be relatively non-viscous even in concentrated solutions. Since the DNA is not denatured to any large extent, this observation is interpreted to mean that Drosophila DNA has a relatively low molecular weight.

Perchloric acid and formic acid hydrolysates of the DNA contain the purines adenine and guanine, and the pyrimidines thymine, cytosine, and 5-methylcytosine. The 5-methylcytosine is characterized both chromatographically and spectrophotometrically. The exceptional pyrimidine, 5-methylcytosine, is not observed when the DNA is subjected to mild alkaline conditions prior to acid hydrolysis. It is suggested that the amino group at the 6 position of the pyrimidine ring of the 5-methylcytosine nucleoside or nucleotide is alkali labile, and that deamination under mild alkaline conditions results in the corresponding thymine derivative.

Charles G. Mead

Nucleoside preparations of Drosophila DNA hydrolysed with snake venom and subjected to paper chromatography exhibit two unexpected free pyrimidines. These are identified as uracil and thymine. It is suggested that these two pyrimidines result from the degradation of deoxy-5-methylcytidine under acid conditions.

Nucleotide preparations of Drosophila DNA hydrolysed with purified snake venom phosphodiesterase and subjected to paper chromatography exhibit the unexpected pyrimidine uracil. This pyrimidine also probably results from the degradation of deoxy-5-methylcytidylic acid under acid conditions.

Ion exchange chromatography of snake venom phosphodiesterase digests of Drosophila DNA result in the recovery of five UV. absorbing peaks. The second, third, fourth and fifth peaks eluted are identified as deoxycytidylic, deoxythymidylic, deoxyadenylic and deoxyguanylic acids respectively. The first peak eluted is probably deoxy-5-methylcytidylic acid but could not be identified as such by rechromatography on paper due to the small quantities of this compound recovered.

A quantitative difference in the molar content of 5-methylcytosine is demonstrated between the two types of ethanol-precipitated DNA's. The flocculent type is demonstrated to contain more 5-methylcytosine than the fibrous type.

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ACKNOWLEDGEMENTS

The author expresses his sincere appreciation to Dr. Allen S. Fox for his tireless efforts in guiding the work of this study. The personal interest of Dr. Jean Burnett is also gratefully acknowledged.

Acknowledgement is made to the National Institutes of Health for financial support as a Predoctoral Research Fellow.

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I. INTRODUCTION

A relationship between the genetic material and nucleic acids has been amply demonstrated in the last twenty years. The relationship of the genetic units of inheritance to the chemical structure of nucleic acids, however, has not been elucidated. The two major difficulties confronting this problem have been the lack of suitable methods for analyzing the structural differences in nucleic acids and the lack of genetically defined nucleic acid.

Since suitable techniques are rapidly becoming available, the need for a source of nucleic acid which could be genetically defined with accuracy has become imperative.

Of all organisms used in genetic studies, Drosophila melanogaster is the best known genetically. Numerous genetic markers are available which are distributed through its entire genome. Widely varying alterations in chromosomal structure are also available. Genetic backgrounds can be constructed which are identical genetically except for single genes (recons), segments of chromosomes, or whole chromosomes (X, Y, 4). Thus the DNA of Drosophila can be genetically defined with great accuracy and altered genetically with ease.

An attempt was therefore undertaken to isolate and characterize the DNA of Drosophila. Very few reports on the nucleic acids of Drosophila have been published. RNA has been isolated and characterized for purines and pyrimidines by Levenbook, et al. (1958). Recently Kirby

(1959) has isolated DNA from Drosophila. Guanine, adenine, thymine, and cytosine were identified in formic acid hydrolysates of this DNA. In addition, other components were present in the hydrolysates which were not identified.

II. MATERIALS AND METHODS

A. Culture and Collection Methods

Stocks of Oregon-R Drosophila melanogaster were grown in half pint milk bottles on standard corn meal-molasses-agar medium enriched with brewers yeast and seeded with living yeast. Flies were collected after a period of from two to three weeks. The collected flies were frozen, lyophilized, and stored in a freezer at -20°C.

B. Isolation of Deoxyribonucleic Acid

1. Extraction Procedure

Ten grams of lyophilized flies were homogenized with a Davis-Layne Duall tissue grinder in 300 ml. of saline-citrate solution (0.15 M NaCl and 0.015 M $Na_3C_6H_5O_7 \cdot 2H_2O$) at 0°C. The resulting homogenate was centrifuged at 10,000 x g. for 20 minutes at 4°C. The supernate was discarded and the residue re-homogenized in 300 ml. of saline-citrate solution. This process of homogenization and centrifugation was repeated four more times (a total of six homogenizations and centrifugations). After the final centrifugation the residue was homogenized in 300 ml. of 0.15 M NaCl at 0°C. Twenty-seven ml. of 5% Duponol C (sodium lauryl sulfate, E. I. Du Pont de Nemours and Co.) in 45% ethanol was added and the mixture stirred with a magnetic stirrer for 3 hours at room temperature. The mixture was then made 1 M with respect to NaCl, stirred an additional 10 minutes and

Centrifuged at 10,000 x g. for 20 minutes at 4°C. The resulting supernate was treated with an equal volume of cold 95% ethanol and placed in the cold for 1 hour to assure complete precipitation. The precipitate was collected by centrifugation at 10,000 x g. for 20 minutes at 4°C. and was then ready for deproteinization.

2. Deproteinization and RNA Removal Procedures

The deproteinization methods of Sevag et al. (1938) using amyl alcohol and chloroform and of Kirby (1957) using methoxyethanol were utilized and found to be effective. Due to the extreme lability of Drosophila DNA constituents, the following enzymatic deproteinization and RNA removal procedure was elaborated.

The ethanol-precipitated DNA from the isolation procedure was dissolved in 150 ml. of 0.15 M NaCl without mechanical agitation. 3.5 ml. of 5% Duponol C in 45% ethanol was added and the mixture stirred with a magnetic stirrer for 1 hour at room temperature. The mixture was made 1 M with NaCl, stirred 10 minutes, centrifuged at 10,000 x g. for 20 minutes at 4°C. and the supernate precipitated with an equal volume of cold 95% ethanol. The precipitate was collected by centrifugation at 10,000 x g. for 20 minutes at 4°C. and the whole procedure repeated once more. The resulting precipitate was then dissolved in 50 ml. of 0.15 M NaCl to which was added 10 mg. of trypsin (Worthington, 2X crystalline) in 1.0 ml. of water. The mixture was incubated at 37°C. for 3 hours, made 1 M with NaCl, centrifuged at

10,000 x g. for 20 minutes at 4°C., and the supernate precipitated with an equal volume of cold 95% ethanol. The precipitate was collected by centrifugation and redissolved in 50 ml. of 0.15 M NaCl to which was added 10 mg. of chymotrypsin (Worthington, 3X crystalline) in 1.0 ml. of water. The mixture was incubated for 3 hours at room temperature after which it was made 1 M with NaCl, centrifuged at 10,000 x g. for 20 minutes at 4°C. and the resulting supernate precipitated with an equal volume of cold 95% ethanol. The precipitate was collected by centrifugation and dissolved in 50 ml. of 0.1 M ammonium acetate-ammonium hydroxide buffer at pH 5.0 (0.1 M with respect to acetate). 10 mg. of ribonuclease (Worthington, 4X crystalline) in 1.0 ml. of water was added and the mixture incubated at 37°C for 3 hours. The mixture was then made 1 M with NaCl, centrifuged at 10,000 x g. for 20 minutes at 4°C. and the supernate precipitated with an equal volume of cold 95% ethanol. The precipitate was collected by centrifugation and dissolved in an appropriate solvent.

C. Hydrolysis Methods

1. Chemical Methods

a. Perchloric acid: DNA was hydrolysed in 0.1 ml. of 70% perchloric acid at 100°C. for 1 hour (Marshak and Vogel, 1951). The hydrolysate was neutralized with KOH, centrifuged in the cold, and the supernate chromatographed.

b. Formic acid: DNA was hydrolysed in 2.5 ml. of 90% formic acid at 175°C. for 30 minutes (Wyatt and Cohen, 1953) in a glass lined steel bomb. The contents were then evaporated to dryness, taken up in 0.1 ml. of 0.1 N HCl and chromatographed.

2. Enzymatic Methods

a. Deoxyribonuclease: DNA was dissolved in 0.2 M ammonium acetate-ammonium hydroxide buffer, pH 7.0, and assayed for total phosphorus (Table 1, No. 14). Magnesium ion was added so that the ratio of Mg^{++} to phosphorus was 3:1. Deoxyribonuclease (Worthington, 1X crystalline) was then added dissolved in water. The mixture was incubated at 37°C. until digestion was complete as judged by UV. absorption or titration with alkali (Table 1, No. 4 and 5).

b. Snake Venom Phosphodiesterase: 500 mg. of lyophilized Crotalus atrox venom (Ross Allen's Reptile Institute) was processed by the Razzel-Khorana (1959) modification of the Koerner-Sinsheimer (1957) method. The resulting preparation was chromatographed on DEAE cellulose by the method of Boman and Kaletta (1957). The phosphodiesterase was recovered in two peaks followed by a third peak of 5'nucleotidase. Only the first phosphodiesterase peak was used for enzymatic hydrolysis of DNA since the second peak was slightly contaminated with 5'nucleotidase.

A deoxyribonuclease limit digest was adjusted to pH 8.9 by the addition of 0.2 M ammonium acetate-ammonium hydroxide

buffer, pH 8.9. Phosphodiesterase was added and the mixture incubated at 37°C. until digestion was complete as judged by UV. absorption. The digest was then either applied directly to an ion-exchange column or adsorbed to charcoal and eluted for paper chromatography.

c. Whole Snake Venom: Whole venom was used to degrade DNase limit digests to free nucleosides. The digestion procedure used was identical to that used with the purified snake venom phosphodiesterase.

D. CHROMATOGRAPHIC METHODS

1. Column Chromatography

The method of Sinsheimer and Koerner (1951) was modified by using a gradient elution system (Bock and Ling, 1954) rather than the original stepwise elution system.

2. Paper Chromatography

Descending chromatography using Whatman No. 1 filter paper was used exclusively. Papers were routinely washed with 0.1 N HCl for 36 hours followed by distilled water for 24 hours and dried in air at room temperature. The following solvents were used:

a. n-butanol Saturated with Water in an Ammonia Atmosphere (Hotchkiss, 1948): n-butanol and distilled water in the ratio of 2:1 by volume were shaken in a separatory funnel for 10 minutes, and the two phases were allowed to separate during a period of 1 hour. The upper butanol phase was placed in the chromatographic trough after equilibration

of the chamber. n-Butanol, distilled water, and 28% ammonium hydroxide in the ratio of 1:1:0.18 by volume were placed in the bottom of the chamber and the system equilibrated for 1 hour before introduction of the solvent.

b. 65% Isopropanol Made 2N with HCl (Wyatt, 1951): 650 ml. of isopropanol and 167 ml. of concentrated HCl were mixed and water added to make 1 l. A portion of the solvent was placed in the bottom of the chamber and the system equilibrated for 1 hour, after which a second portion of the solvent was added to the chromatographic troughs.

c. Isobutyric Acid-Ammonia (Magasanik et al., 1950): Isobutyric acid, distilled water, and concentrated ammonium hydroxide were mixed in the ratio of 66:33:1 by volume. A portion of the mixture was placed in the bottom of the chamber and the system equilibrated for 1 hour, after which the solvent was added to the chromatographic troughs.

E. Purification of Nucleic Acid Components with Activated Charcoal

1. Preparation of Charcoal

Commercial activated charcoal (Norit A) was further purified by the method of Lipkin et al. (1954).

2. Adsorption and Elution Procedure

0.5 g. of purified charcoal was placed in a sintered glass funnel, washed with 50 ml. of distilled water, 50 ml. of eluting solvent

(73% ethanol, 24% water, 3% concentrated ammonium hydroxide; Bennett et al., 1955), and again with 50 ml. of distilled water. The pH of the mixture of purines, pyrimidines, nucleosides, or nucleotides to be adsorbed was adjusted to 5.0 and the mixture applied to the charcoal. The charcoal was washed with water after which the adsorbed compounds were eluted with the eluting solvent. Elution was continued until no more UV. absorbing compounds were eluted.

If the substances eluted were to be chromatographed, the eluate was evaporated to dryness on a rotary evaporator, the residue taken up in 15 ml. of distilled water, lyophilized, and redissolved in a small volume of an appropriate solvent.

F. Assay Methods

Assay methods used are contained in Table 1, page 10.

G. Other Materials and Methods

Nativeness of DNA was determined by the method of Hotchkiss (1957). This method consists of a standard procedure for the alkaline denaturation of DNA. Degree of denaturation produced by the alkali treatment is expressed as percent increase in absorption at 260 mμ.

All authentic compounds were obtained from the California Corporation for Biochemical Research.

All spectra were obtained on a Beckman DK2 spectrophotometer.

TABLE 1. Assay methods.

Enzyme or substance	Procedure no.	Reference
Trypsin	1	Schwert and Takenaka (1955)
Chymotrypsin	2	Schwert and Takenaka (1955)
Ribonuclease	3	Kunitz (1946)
Deoxyribonuclease	4	Kunitz (1950)
	5	Titration with NaOH
Phosphodiesterase	6	Koerner and Sinsheimer (1957)
5'nucleotidase	7	Sinsheimer and Koerner (1951)
Protein	8	Gornall et al. (1949)
	9	Lowry et al. (1951)
Pentose	10	Dische (1955)
Deoxypentose	11	Dische (1955)
	12	Stumpf (1947)
	13	Buchanan (1951)
Phosphorus	14	Allen (1940)
	15	Bandurski and Axelrod (1951)

III. RESULTS

A. Isolation Procedure

Preliminary analysis of lyophilized Drosophila by the Schmidt-Thannhauser-Schneider procedure (Schneider, 1946) revealed that the ratio of RNA to DNA in Drosophila was 3 or 4 to 1 and that lyophilized flies contained from 1 to 3 mg. of DNA per gram dry weight.

In early attempts to isolate DNA from Drosophila, several unsuccessful methods were used. The weak salt method of Crampton et al. (1950), the strong salt method of Signer and Schwander (1949, 1950), and the phenol method of Braun et al. (1957) all proved unsuccessful. In light of the experience of Daly et al. (1950) and Chargaff et al. (1952) where NaCl solutions of high molarity were necessary to extract spermatozoa nucleoprotein, it was thought that increasing the NaCl molarity of the extracting solvent might meet with success. The NaCl molarity was increased stepwise to 5 M without successfully extracting DNA from Drosophila. Due to the large content of RNA in extracts of Drosophila it was thought possible that the DNA was not being detected. A saline extract was fractionated into 8 fractions by precipitating the nucleic acids with from 1 to 6 volumes of cold ethanol. All fractions were found to contain RNA and none of the fractions contained DNA.

The isolation procedure which finally proved successful was modified from the method of Kay, Simmons and Dounce (1952). The

major modifications consisted of more extensive washing of the saline-citrate homogenized residue, in the avoidance of dehydration of the isolated DNA-protein, and in maintenance of the salt content at or above 0.15 M NaCl at all times. The latter two modifications were introduced to assure an undenatured product.

An example of the effectiveness of the isolation procedure in obtaining a purified product of DNA is given in Table 2. About 90% of the RNA is removed in the first six saline-citrate extractions leaving a residue which is highly enriched in DNA. Upon Duponol extraction, the DNA is apparently recovered quantitatively (Table 3).

The amyl alcohol-chloroform, methoxyethanol, and the enzymatic deproteinization procedures all resulted in a product which is free of RNA as determined by the orcinol method (Table 1, No. 10) and free of protein as determined by the biuret method (Table 1, No. 8).

When the first Duponol extract containing DNA was precipitated with cold ethanol or ethoxyethanol, a flocculent precipitate was formed. This contrasts with the fibrous precipitates formed upon similar precipitation of DNA's from most other sources. When this flocculent precipitate was redissolved in saline, even in highly concentrated solutions, it was far less viscous than comparable solutions of salmon sperm DNA. Upon deproteinization of this crude DNA preparation two types of ethanol-precipitable DNA's were observed.

TABLE 2. Composition of product at various stages of the DNA isolation procedure.

	Composition of preparation							
	DNA[1]		RNA[2]		Protein[3]		Other	
	mg.	%	mg.	%	mg.	%	mg.	%
Whole flies[4] (lyophilized)	2.0	0.2	20.0	2.0	500.0	50.0	478.0	47.8
Saline-citrate[4] extracted residue	2.0	1.7	2.0	1.7	53.0	45.0	60.6	51.6
Duponol extract (supernate)	2.0	45.0	0.45	10.0	2.0	45.0	----	----
Deproteinized DNA-1 (amyl-chloroform)	1.9	70.0	0.08	3.0	0.73	27.0	----	----
Deproteinized DNA-2 (methoxyethanol)	1.8	>99	0.00	0.0	<0.02	<1.0	----	----

Recovery of DNA = 80-90%

[1]Calculated from purine deoxyribose (Table 1, procedure 11).

[2]Calculated from purine ribose (Table 1, procedure 10).

[3]Calculated as mg. bovine serum albumin (Table 1, procedure 8).

[4]From Schmidt-Thannhauser-Schneider analysis.

TABLE 3. Recovery of DNA from 1.0 g. of lyophilized flies by the Duponol extraction procedure.[1]

Sample	uM purine deoxyribose[2]	DNA[3] mg.
Whole flies	1.92	1.29
Duponol extracted residue	0.00	0.00
Duponol extract	1.94	1.30

[1] By method of Schmidt-Thannhauser-Schneider.

[2] By diphenylamine (Table 1, No. 11).

[3] Assuming DNA = 20% purine deoxyribose.

One of these was fibrous and comparable to other DNA's, whereas the other remained as a flocculent precipitate. After exhaustive deproteinization the two types of DNA retained their differences.

Table 4 lists some additional properties of the final product of the DNA isolation procedure as compared with commercial highly polymerized salmon sperm DNA. The E(P) (Chargaff, 1955) of Drosophila DNA is comparable to most other DNA's. The relatively high percentage increase in UV. absorption at 260 mμ upon denaturation with alkali indicates that the Drosophila DNA isolated by this method is not extensively denatured.

B. Characterization

1. Purines and Pyrimidines

Upon paper chromatography of the perchloric acid hydrolysates of Drosophila DNA in isopropanol-HCl, five UV. absorbing spots were observed. These spots were identified chromatographically as adenine, guanine, thymine, cytosine and 5-methylcytosine. Table 5 lists the R_T's (Table 5, footnote 1) of the observed spots as compared with the corresponding authentic compounds. Table 6 lists the R_T's of the five UV. absorbing spots observed after hydrolysis of the DNA with formic acid. The UV. spectrum of the 5-methylcytosine spot obtained from these hydrolysates is compared with the spectrum of authentic 5-methylcytosine in Figure 1.

TABLE 4. Ultraviolet absorption of *DNA* preparations.

DNA	E(P)[1]	Δ O. D.$_{260}$[2]
Drosophila	6930	20%
Salmon sperm	6720	10%

[1]Expressed as atomic extinction coefficient with respect to phosphorus at 260 mμ and at pH 7. 0 (Chargaff, 1955).

[2]Percent increase in O. D. at 260 mμ after alkaline denaturation (Hotchkiss, 1957).

TABLE 5. R_T[1] of purines and pyrimidines from perchloric acid hydrolysates in the isopropanol-HCl solvent

Compound	Authentic	Drosophila
Guanine	.32	.35
Adenine	.46	.48
Cytosine	.59	.60
5-Methylcytosine	.72	.73
Thymine	1.00	1.00

[1] R_T = distance of spot from origin/distance of thymine from origin.

Values are means of duplicate runs.

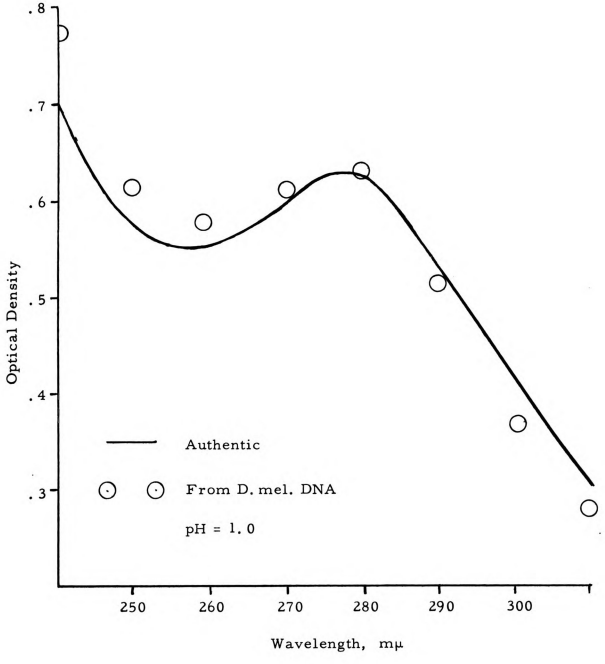
TABLE 6. R_T[1] of purines and pyrimidines from formic acid hydrolysates in the isopropanol-HCl solvent

Compound	R_T Authentic	R_T Drosophila
Guanine	.31	.31
Adenine	.45	.45
Cytosine	.61	.60
5-Methylcytosine	.69	.70
Thymine	1.00	1.00

[1] R_T = distance of spot from origin/distance of thymine from origin.

Values are means of duplicate runs.

FIGURE 1. Ultraviolet absorption spectrum of 5-methylcytosine.



The DNA preparation used in this latter experiment was shown to be slightly contaminated with RNA, and a third experiment was performed in an attempt to remove all contaminating RNA from the preparation. The DNA preparation was made 1 M with respect to KOH and incubated in a water bath at 37°C. for 12 hours. The mixture was then neutralized with perchloric acid, centrifuged in the cold to remove $KClO_4$, and the DNA precipitated from the supernate with an equal volume of cold ethanol. The supernate and the precipitate were hydrolyzed with $HClO_4$ and chromatographed on paper using the isopropanol-HCl solvent. Guanine, adenine, cytosine, and uracil were identified in the supernate fraction and guanine, adenine, cytosine, and thymine were identified in the precipitate fraction. 5-methylcytosine was notably absent.

In a fourth experiment, the two types of ethanol-precipitable DNA's observed in the isolation procedure, one fibrous and the other flocculent, were analyzed for their 5-methylcytosine contents. A partially deproteinized preparation of DNA was precipitated with cold ethanol and the fibrous fraction collected by winding the fibers on a glass rod. The fraction of the DNA which could not be collected in this manner was considered flocculent DNA. Each preparation was then hydrolyzed with perchloric acid and chromatographed on paper using the isopropanol-HCl solvent. Although both preparations were still contaminated with some RNA as indicated by the presence of slight amounts of uracil in the hydrolysates, a definite quantitative difference

in the pyrimidine composition of the two types of DNA was demonstrated by comparing the molar ratios of 5-methylcytosine to thymine. These ratios were obtained by elution of the spots and calculation of the molar concentration of each compound from its respective UV. molecular extinction coefficient. These data are contained in Table 7.

2. Nucleosides

A deoxyribonuclease limit digest of Drosophila DNA was digested further with Crotalus adamanteus snake venom for 5 hours at 37°C. The rate of digestion was followed by the increase in UV. absorption at 260 mμ and the digestion was continued until no further increase in absorption could be detected. The hydrolysate was then adsorbed to charcoal, washed with water and eluted. The eluate was evaporated, the residue taken up in 0.1 N HCl and subjected to paper chromatography using the n-butanol-ammonia solvent. Six UV. absorbing spots were observed in the developed chromatogram. Each of these six spots was eluted and rechromatographed on paper using the isopropanol-HCl solvent and the n-butanol-ammonia solvent. One of the original spots was resolved into two spots in both solvents making a total of seven components. Three of these components contained deoxyribose as determined by the cystein-HCl procedure (Table 1, No. 13). The seven spots were identified as deoxyguanosine, deoxycytidine, deoxythymidine, xanthine, hypoxanthine, thymine, and uracil. Table 8 lists the chromatographic and spectral constants of these compounds.

TABLE 7. 5-Methylcytosine:thymine ratios in fibrous and flocculent DNA.

DNA	Hydrolysate	5MC mμM	T mμM	$\frac{5MC}{T}$
Fibrous	1	24.5	142.	.172
Fibrous	1	13.3	88.	.151
Fibrous	2	18.9	103.	.183
Fibrous	3	20.4	134.	.152
				$\bar{X}$ = .165 ± .008
Flocculent	4	24.5	114.	.215
Flocculent	4	34.2	157.	.218
Flocculent	5	32.6	135.	.242
Flocculent	5	26.6	101.	.267
Flocculent	6	31.6	136.	.233
Flocculent	6	31.2	136.	.230
				$\bar{X}$ = .234 ± .007

s.e. = $\frac{6}{\sqrt{n}}$

TABLE 8. Chromatographic and spectral constants of compounds isolated from snake venom digests of DNA.

Compound	R_T[1] n-butanol-NH_3		R_F isopropanol-HCl		λ max mμ	
	Authentic	D. mel.	Authentic	D. mel	Authentic	D. mel
Deoxyguanosine	.27	.27	.24	.26	255	---
Deoxyadenosine	1.12	none	.36	none	258	---
Deoxythymidine	.84	.84	.83	.84	267	266
Deoxycytidine	.68	.68	.58	.58	280	278
Xanthine	.15	.16	.26	.28	267	264
Hypoxanthine	.38	.38	.33	.31	248	247
Thymine	1.00	1.00	.79	.78	264	---
Uracil	.55	.56	.73	.74	259	260

[1] R_T = distance of spot from origin/distance to thymine from origin.

Values are means of duplicate runs.

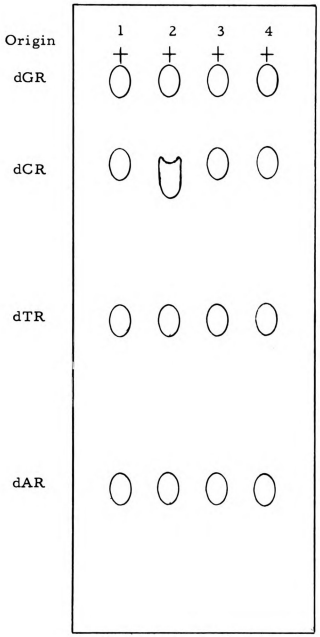
It was found later that only four components were recoverable from identical hydrolysates if the residue remaining after evaporation of the charcoal eluate was dissolved in water rather than 0.1 N HCl. These components exhibited R_f's identical with those of authentic deoxyguanosine, deoxyadenosine, deoxythymidine and deoxycytidine. The only peculiarity observed was that the spot exhibiting an R_f identical to that of authentic deoxycytidine was elongated and crescent-shaped (Figure 2).

It seems probable that the deoxyadenosine, deoxyguanosine and deoxy-5-methylcytidine were degraded during the spotting of the purified hydrolysates in 0.1 N HCl. Drastic enough conditions may have been attained since a hair dryer was routinely used in the spotting of chromatograms. Hypoxanthine could have been derived from deoxyadenosine, xanthine from deoxyguanosine, and the thymine and uracil from deoxy-5-methylcytidine. The latter would require demethylation, deamination, and degradation of deoxy-5-methylcytidine to the free pyrimidines.

3. Nucleotides

A deoxyribonuclease limit digest of Drosophila DNA was digested further with purified snake venom phosphodiesterase and either applied directly to a Dowex-1 acetate column or purified by adsorption to charcoal followed by elution and paper chromatography.

FIGURE 2. Chromatogram of nucleosides (snake venom hydrolysates).



1 = authentic nucleosides

2 = D. mel. DNA nucleosides

3 = salmon sperm DNA nucleosides

4 = authentic nucleosides

Solvent: n-butanol-ammonia

Column chromatography of the phosphodiesterase digest yielded five UV. absorbing peaks (Figure 3). The second, third, fourth and fifth peaks were identified as deoxycytidylic, deoxythymidylic, deoxyadenylic and deoxyguanylic acids respectively by rechromatography of the concentrated fractions on paper using the isobutyric-ammonia solvent. The fractions were also shown to contain deoxyribose and phosphorus (Table 1, No. 12 and 14). The first peak eluted was in the area in which deoxy-5-methylcytidylic acid would be expected, but was recovered in such a minute quantity that it could not be identified as such by rechromatography on paper. The molar compositions of these peaks as calculated from their respective molecular extinction coefficients at 260 mμ are given in Table 9. The ratios of deoxyadenylic to deoxythymidylic and deoxyguanylic to deoxycytidylic plus deoxy-5-methylcytidylic are both approximately 1:1.

Paper chromatography of the phosphodiesterase digests in the isobutyric acid-ammonia solvent resulted in five UV. absorbing spots. This was in contrast to a phosphodiesterase digest of salmon sperm DNA which was treated identically, and exhibited only four UV. absorbing spots on chromatography. The observed chromatographic constants are listed in Table 10.

FIGURE 3. Ion-exchange column chromatography of 5'deoxyribonucleotides.

Sample: Snake venom phosphodiesterase digest of Drosophila melanogaster DNA.

Column: 1 x 20 cm., Dowex 1-X8 resin, acetate form.

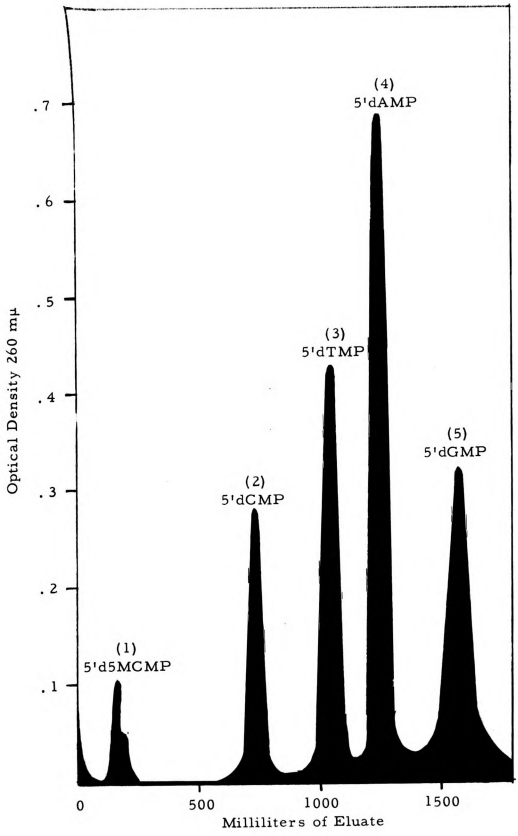
Elution system: Gradient elution system in which the molarity of an ammonium acetate-ammonium hydroxide buffer, pH 4.3, is increased at an increasing rate from 0 M to 1 M.

Elution rate: 1.12 ml. per minute with a 45 inch head.

Abbreviations: 5'dCMP = 5'deoxycytidylic acid, 5'dTMP = 5'deoxythymidylic acid, 5'dAMP = 5'deoxyadenylic acid, 5'dGMP = 5'deoxyguanylic acid, 5'd5MCMP = 5'deoxy-5-methylcytidylic acid.[1]

[1] Identity tentative. See text.

FIGURE 3. Ion-exchange column chromatography of 5'deoxyribonucleotides.



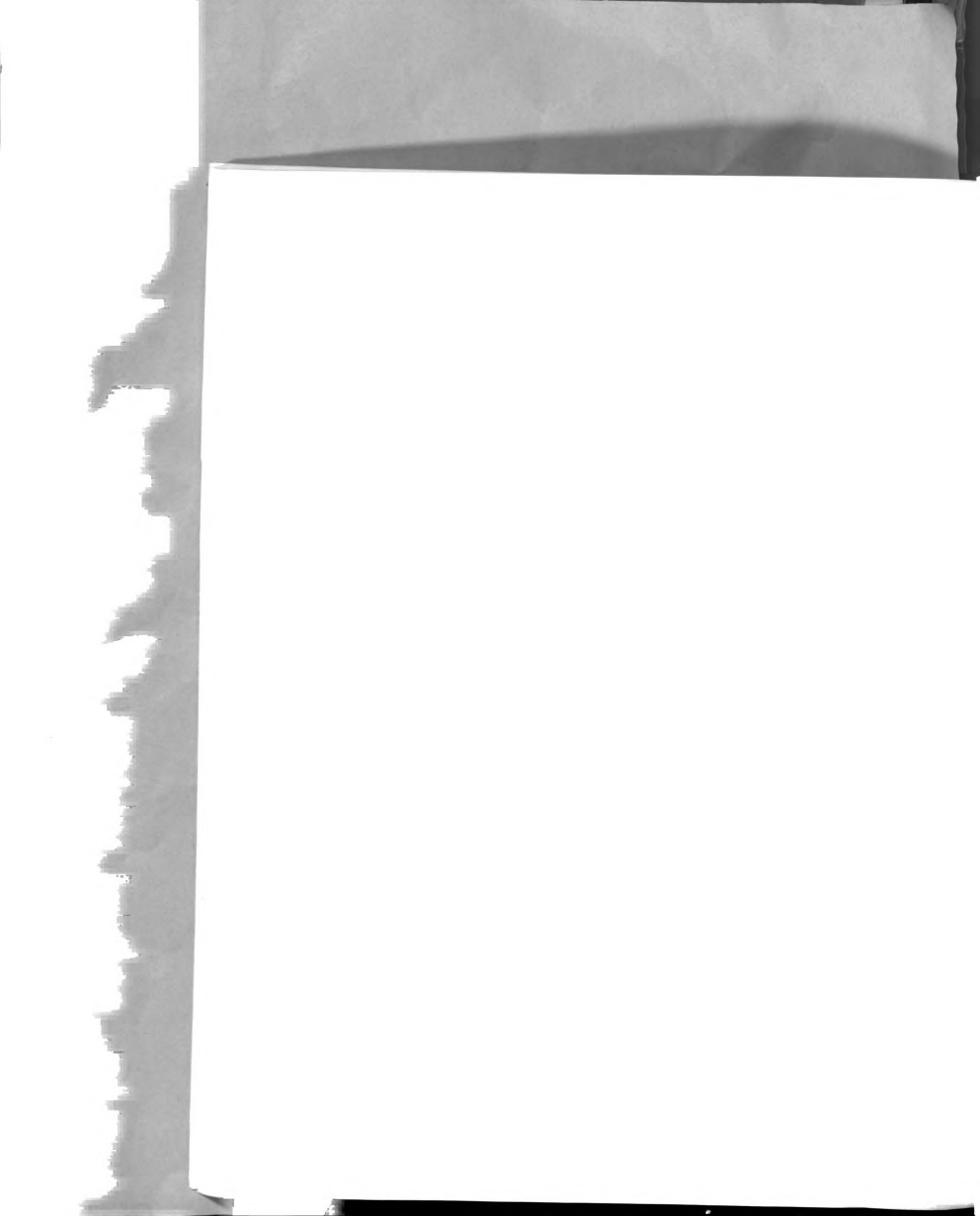


TABLE 9. Molar composition of nucleotides recovered by ion-exchange chromatography from phosphodiesterase digests of Drosophila DNA.

Peak no.	Nucleotide	O. D. 260 mμ units	uM	
1	Deoxy-5-methylcytidylic[1]	2. 66	. 79	2. 42
2	Deoxycytidylic	10. 37	1. 63	
3	Deoxythymidylic	23. 38	2. 78	
4	Deoxyadenylic	40. 21	2. 76	
5	Deoxyguanylic	28. 91	2. 47	

[1]Tentative identification. See text.

TABLE 10. R_{fl}[1] of nucleotides derived from DNA.

Nucleotide	Salmon sperm	Drosophila
Deoxyguanylic	. 45	. 45
Deoxythymidylic	. 55	. 55
Deoxycytidylic	. 65	. 67
Deoxyadenylic	. 80	. 81
(Uracil)		1. 10

[1]R_{fl} = distance of spot from origin/distance of second front from origin.

Solvent: Isobutyric acid-ammonia.

Values are the means of duplicate runs.

The four spots resulting from chromatography of the salmon sperm DNA digest, and four of the five spots from the Drosophila DNA digest, were demonstrated to contain deoxyribose and phosphorus (Table 1, No. 13 and 15). The fifth spot from the Drosophila DNA digest contained neither phosphorus nor deoxyribose.

The four phosphorus and deoxyribose containing substances were demonstrated to be identical chromatographically with authentic samples of deoxyguanylic, deoxyadenylic, deoxythymidylic and deoxycytidylic acids using the isobutyric acid-ammonia solvent. The chromatographically separated spots were eluted and their UV. absorption spectra determined. The four phosphorus and deoxyribose containing substances were shown to have spectra consistent with those of the same authentic samples. The fifth spot obtained from the Drosophila DNA digest exhibited a spectrum with a peak at 263 mμ and a 280 mμ/ 260 mμ ratio of 0. 31 which is consistent with the spectral properties of uracil.

It seems likely that the deoxy-5-methylcytidylic acid was degraded to free uracil during the preparatory stages before paper chromatography.

IV. DISCUSSION

Adult Drosophila melanogaster flies are not a rich source of DNA as compared with other tissues. This disadvantage is however overcome by the fact that Drosophila DNA can be genetically defined with great accuracy and manipulated genetically with ease.

The isolation procedure finally adopted results in a relatively undenatured DNA which is free from RNA. The insolubility of the DNA in water, saline or strong salt homogenates of flies is an unexplained observation. This property may be due to a strong adsorption of the DNA to the insoluble chitin in the homogenates or to the unsuccessful fragmentation of nuclei during homogenization. This property makes possible a nearly quantitative separation of RNA from the insoluble DNA by exhaustive extraction of the insoluble residue with saline and subsequent treatment of the residue with Duponol to liberate the DNA.

The observation that the isolated DNA is not viscous even in concentrated solutions would at first suggest that it was partially degraded. The relatively large increase in UV. absorption upon denaturation with alkali indicates, however, that the final product is not extensively denatured. It is therefore likely that the low viscosity of the product is due to its relatively low molecular weight as compared to salmon sperm DNA.

The flocculent nature of Drosophila DNA upon precipitation with cold ethanol is not understood. It is of interest that in the only other report of the isolation of Drosophila DNA (Kirby, 1959) the final product was also of the flocculent type when precipitated with cold ethanol. In Kirby's report, several DNA's were isolated by the p-aminosalicylate-phenol method. All of the DNA's isolated were fibrous when precipitated with ethanol except two. These two were those of Drosophila and wheat germ. It should be noted that of the DNA's examined to date, 5-methylcytosine occurs in extensive amounts only in these same two DNA's.

The observation that 5-methylcytosine is present in acid hydrolysates of Drosophila DNA, but not in acid hydrolysates which had been previously treated with alkali, was at first puzzling. However, Wyatt (1951) has reported an increase in thymine and a decrease in 5-methylcytosine in the DNA of beef spleen and herring sperm, when treated with dilute alkali under mild conditions before hydrolysis with acid to purines and pyrimidines. Amos and Korn (1958) reported the quantitative deamination of 5-methylcytidylic acid to thymidylic acid by treatment with 1 N KOH at 37°C. for 16 to 24 hours. The free pyrimidine 5-methylcytosine, however, is not altered by such alkali treatment. It thus seems likely that the amino group at the 6 position of the pyrimidine ring of deoxy-5-methylcytidylic acid is alkali labile, and that deamination takes place under mild alkaline conditions resulting in thymidylic acid.

The appearance of free thymine and uracil in the snake venom hydrolysates of Drosophila DNA which were subject to acid conditions during application to filter paper for chromatography was entirely unexpected. It appears that the enzymatically produced nucleoside of 5-methylcytosine had been partially demethylated at the 5 position of the pyrimidine ring, completely deaminated at the 6 position, and degraded to the level of free pyrimidine. The conditions of this experiment were fairly drastic, for deoxyadenosine was completely degraded to hypoxanthine and deoxyguanosine was partially degraded to xanthine. Even under these conditions, however, the extensive degradation of deoxy-5-methylcytidine would not be expected. Nevertheless, Cohen (1953) has reported the extreme lability of deoxy-5-hydroxymethylcytidine, which when stored in the freezer for 1 month was degraded to the corresponding free pyrimidine to the extent of 30-40%. The lability of the carbon to carbon bond at the 5 position of the pyrimidine ring in 5 methylated derivatives suggests substitution on the 5-methyl group (Bendich, 1955). This, however, is unlikely in this case, in that most 5-methyl substituted derivatives are extremely labile even in the form of the corresponding pyrimidine, and are cleaved to small carbon units even by the action of hot water.

The appearance of the four UV. absorbing spots in snake venom hydrolysates of Drosophila DNA, when the hydrolysate is subject to milk conditions, suggests that the degradation products observed

under more drastic conditions are not the result of enzyme activities present in the snake venom.

The peculiar shape and elongation of the UV. -absorbing spot corresponding in R_f to authentic deoxycytidine suggests that this spot also contained deoxy-5-methylcytidine. Although no authentic deoxy-5-methylcytidine was available for co-chromatography in this solvent, all published R_f's of deoxy-5-methylcytidine are identical or very similar to the R_f's of deoxycytidine.

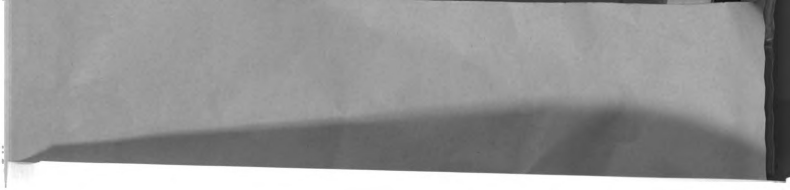
The first peak eluted on column chromatography of the nucleotides of Drosophila DNA is probably deoxy-5-methylcytidylic acid. Although this peak was not identified by rechromatography, its position in the elution pattern is in the region where deoxy-5-methylcytidylic acid would be expected according to the data of Hurst et al. (1953) and Sinsheimer and Koerner (1951).

The appearance of the fifth UV. -absorbing spot on chromatography of the phosphodiesterase digests of Drosophila DNA on paper was also unexpected. This spot has been identified tentatively as uracil which probably arises as a degradation product of deoxy-5-methylcytidylic acid. The degradation of this nucleotide to the free pyrimidine, and its deamination and demethylation, must again be attributed to the acid conditions to which it is subjected before chromatography.

The validity of these suggestions, that the unexpected products observed in hydrolysates subjected to drastic conditions arise

from the nucleotide and/or nucleoside of 5-methyl cytosine, will depend on subsequent examination of these compounds in the future.

The Watson-Crick model of DNA structure does not place restrictions on the substitution of 5-methylcytosine for cytosine in the polynucleotide chains making up the double helix. It is probable, however, that such substitutions do have structural consequences. 5-methylcytosine does not seem to take the place of cytosine at random in DNA's analyzed thus far. There is a remarkable constancy of 5-methylcytosine content in the DNA of a given species, and large differences in 5-methylcytosine content between species. The difference in the 5-methylcytosine content of the fibrous and flocculent type DNA's from Drosophila suggests that there are types of DNA molecules containing more 5-methylcytosine than others. Similar evidence as to the non-random distribution of 5-methylcytosine in the DNA within a species has been reported by Chargaff (1955), where calf thymus DNA was fractionated by solubility methods and the ratio of 5-methylcytosine to thymine was found to vary widely between different fractions. One gains the impression that there are DNA molecules within a given preparation which contain widely varying 5-methylcytosine contents. It is very possible that, as in the case of phage DNA where 5-hydroxymethylcytosine completely replaces cytosine, there are molecules in the DNA's of higher organisms in which the cytosine is entirely replaced by 5-methylcytosine.



The existence of 5-methylcytosine in the DNA of Drosophila makes possible a variety of experiments which might lead toward the elucidation of the relationship between the genetic units and the chemistry of DNA. We now have a label in Drosophila DNA which can be identified with ease and there are indications that this label is not randomly distributed among DNA molecules. An attempt to identify these labeled DNA's with specific genetic units could yield profitable results.

V. SUMMARY

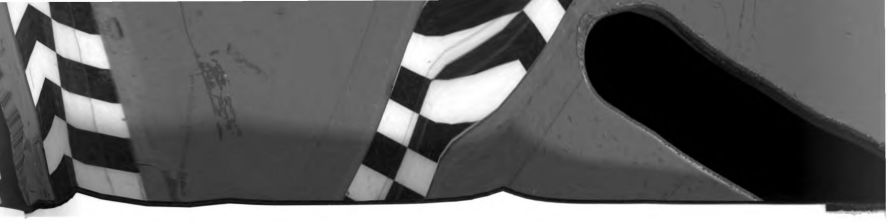
1. A procedure is described for the isolation of DNA from Drosophila melanogaster. An enzymatic deproteinization procedure is described which results in a product that is free of RNA and protein and not extensively denatured.

2. Two types of DNA are observed upon precipitation of the isolated product with cold ethanol. One of these is fibrous in nature, typical of most DNA's, whereas the other is of a flocculent nature.

3. The isolated DNA is found to be relatively non-viscous even in concentrated solutions. Since the DNA is not denatured to any large extent, this observation is interpreted to mean that Drosophila DNA has a relatively low molecular weight.

4. Perchloric acid and formic acid hydrolysates of the DNA contain the purines adenine and guanine, and the pyrimidines thymine, cytosine, and 5-methylcytosine. The 5-methylcytosine is characterized both chromatographically and spectrophotometrically.

5. The pyrimidine, 5-methylcytosine, is not observed when the DNA is subjected to mild alkaline conditions prior to acid hydrolysis. It is suggested that the amino group at the 6 position of the pyrimidine ring of the 5-methylcytosine nucleoside or nucleotide is alkali labile, and that deamination under mild alkaline conditions results in the corresponding thymine derivative.



6. Nucleoside preparations of Drosophila DNA hydrolysed with snake venom and subjected to paper chromatography exhibit two unexpected free pyrimidines. These are identified as uracil and thymine. It is suggested that these two pyrimidines result from the degradation of deoxy-5-methylcytidine under acid conditions.

7. Nucleotide preparations of Drosophila DNA hydrolysed with purified snake venom phosphodiesterase and subjected to paper chromatography exhibit the unexpected pyrimidine uracil. This pyrimidine also probably results from the degradation of deoxy-5-methylcytidylic acid when under acid conditions.

8. Ion exchange chromatography of snake venom phosphodiesterase digests of Drosophila DNA result in the recovery of five UV. absorbing peaks. The second, third, fourth and fifth peaks eluted are identified as deoxycytidylic, deoxythymidylic, deoxyadenylic and deoxyguanylic acids respectively. The first peak eluted is probably deoxy-5-methylcytidylic acid but could not be identified as such by rechromatography on paper due to the small quantities of this compound recovered.

9. A quantitative difference in the molar content of 5-methylcytosine is demonstrated between the two types of ethanol-precipitated DNA's. The flocculent type is demonstrated to contain more 5-methylcytosine than the fibrous type. Consequences of this observation are discussed.

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