A COMPARISON OF METHODS FOR THE ISOLATION OF DEOXYRIBONUCLEIC ACID FROM SMALL AMOUNTS OF TISSUE

by

CATHERINE MEZEI

A Thesis Submitted in Partial Fulfilment of
The Requirements for the Degree of

M A S T E R OF S C I E N C E

in the Department
of
Biochemistry

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA
September, 1960

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Department of Biochemistry

The University of British Columbia,
Vancouver 8, Canada.

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ABSTRACT

The chemical and physico - chemical properties of deoxyribonucleic acid preparations isolated from small amounts of liver and intestinal mucosa of rat (1-10 g.) by five different procedures, have been compared. The first method (29), used for preparation of deoxyribonucleic acid was based on the separation of nuclei from tissue homogenates, followed by extraction and deproteinization of deoxyribonucleic acid with strong salt solutions. The second method (20, 31) consisted of the extraction anddeproteinization of nucleic acids by detergent solutions, and separation of ribonucleic acid and deoxyribonucleic acid by fractional precipitation with iso-propyl alcohol. In the third procedure crude deoxyribonucleic acid was isolated from nuclei according to the first method and the crude product was further purified according to the second procedure. The fourth method (32) was based on the disintegration of tissues by high frequency sonic oscillations, extraction of nucleoprotein from the nuclear fragments with strong salt solutions and deproteinization of deoxyribonucleic acid with chloroform - amyl alcohol mixtures. In the fifth method (36, 37) nucleic acids were extracted from tissues by hot, strong salt solutions, ribonucleic acid and deoxyribonucleic acid were separated by alkali treatment and deoxyribonucleic acid was precipitated with concentrated acid solutions. The advantages and shortcomings

of the different procedures with respect to yield, purity and macromolecular state of the isolated material have been discussed. An improved technique has been described for the elution of purine and pyrimidine bases from paper chromatograms.

ACKNOWLEDGMENTS.

The author wishes to express her sincere thanks and appreciation to Dr. S.H. Zbarsky for his continual advice and encouragement.

The personal assistance of the National Research Council in the form of a Bursary and Studentship is gratefully acknowledged.

Dr. M. E. Reichmann kindly performed the molecular weight determinations by light scattering measurements on some DNA preparations.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Historical Introduction:

Among the components of animal and plant cell the nucleic acids occupy a position of unusual interest because of their special position within the nucleus with their consequent relation to specific nuclear processes. In this respect deoxyribonucleic acid (DNA) the chief constituent of chromosomes plays a very important role in modern biochemistry. It is now generally realized that DNA is in all probability, chiefly responsible for determining the inheritable characteristics of bacterial viruses, bacteria and higher organisms (1) and that, therefore, the question of its structure and function as the genetic carrier must rank as one of the central problems of biology. In order to have a fair idea about the physico-chemical structure and physiological function of a naturally occuring compound, it is necessary to devise procedures by which that compound can be isolated from its natural sources in a state similar to that in which it occures in the living cell. In the following section the history of isolation of DNA is reviewed briefly.

In 1871 Friedrich Miescher announced the preparation of a material obtained from digest of pus cells. (2) He called the substance "nuclein". The isolation of this material was performed by treating pus cells with dilute hydrochloric acid for a period of weeks and the product was then shaken in a separatory funnel with ether. Part of the

solid material gathered in the interface between the ether and water and the second solid layer formed on the bottom of the separatory funnel. The latter consisted of practically pure nuclear material. A very similar substance resulted by digesting pus cells with artifical gastric juice. This substance had practically identical properties with those of the sediment obtained by the mechanical method. Miescher's studies on this substance convinced him that it was a complex phosphorous-containing acid of high molecular weight.

The discovery of Miescher's "nuclein" opened a new chapter in the history of biochemistry. A tremendous interest arose and several other scientists continued the investigations on nuclear materials of different origins. The term "nucleic acid" was not used nor was a convenient and general method described for its preparation until the pbulication of Altmann in 1889. (3). The newer methods of preparation of nucleic acids are practically all based on that developed by him.

In 1899 Neumann (4) published a new modification of the original Altmann method. In this publication the old traditional fear of using drastic methods for the separation of the nucleic acid from the protein was abandoned. Neumann's method essentially consisted of heating the minced organs in a 3% solution of sodium hydroxide and precipitation of the nucleic acids not as a sodium salt, which was water soluble, but as a water insoluble barium salt. Feulgen (5) and Levene (6,7) introduced

further drastic methods for the isolation of nucleic acids.

The chemical investigation of nucleic acid preparations from many biological sources has demonstrated that they resembled either yeast nucleic acid and contained a pentose, identical with D-ribose, or thymus nucleic acid and contained a deoxypentose, identical with 2-deoxy-D-ribose. Although it was once thought (8,9) that the pentose nucleic acids (RNA) were characteristic of plant tissues, whereas the deoxyribonucleic acid (DNA) were confined to animal cells, by 1924 an idea began to develop (10,11) that both animal and plant tissues contain DNA and RNA, the former being confined to cell nuclei, while RNA is present mainly in cytoplasm and nucleoli.

The traditional belief in the unusual lability of nucleic acids was revived in 1924. From then on scientists realized that the nucleic acids isolated by the drastic alkali extractions and acid precipitations were particularly degraded and denatured, and did not represent at all the natural state as they are present in cells. Methods were devised to avoid exposing the tissues to alkali or acid, and heat. All preparations were carried out in the cold. These preparative procedures for isolating DNA from mammalian tissues can be divided into five groups.

1. Extraction of tissues with strong salt solution and deproteinization of nucleoprotein by saturation with sodium

chloride. This method was developed by Signer and Schwander (12) using the necleoprotein preparation of Mirsky and Pollister (13-15). This procedure consists of:

a. mechanical mincing of the gland in molar NaCl with addition of an enzyme inhibitor (usually sodium citrate).

b. extraction of nucleoprotein into molar NaCl.

c. precipitation of the nucleoprotein by dilution to 0.15 M sodium chloride.

d. splitting of the nucleoprotein into protein and DNA by saturation with sodium chloride.

ê. removal of the protein portion by filtration.

f. precipitation of DNA by ethanol.

2. Extraction of tissues with strong salt solution, deproteinization with chloroform - amyl alcohol mixture. This method was essentially developed by Gulland, Jordan and Threlfall (16). The nucleoprotein is prepared by the method of Mirsky and Pollister (13-15). The resulting nucleoprotein is repeatedly treated with a mixture of amyl alcohol and chloroform, and the denatured protein is sedimented by centrifugation (17).

3. Extraction of tissues with water and deproteinization either by saturation with sodium chloride or by using detergents. This isolation procedure is based on the work of Crampton et al. (18).

4. Extraction of tissues with anionic detergents. This

method used to prepare DNA from calf thymus, was developed by Kay et al. (19-21). In this method the tissue is homogenized several times with ice-cold physiological saline, containing 0.01 M sodium citrate. The sediment after centrifugation is taken up in 1.5 M sodium-chloride - 0.01 M citrate solution and the proteins are precipitated by the addition of sodium xylene sulphonate. DNA is precipitated with 98% iso-propyl alcohol.

5. Liberation of DNA from tissues by the action of certain salt solutions and phenol. The isolation of DNA from mammalian tissues by the phenol method was developed by Kirby (21-23). DNA is freed from protein by the action of phenol and the salt solutions. Contaimnating RNA is removed by ribonuclease treatment. This method has been further improved by S. Kit (24) and G.P. Georgiev (25).

The above mentioned gentle methods have several disadvantages, which can be summarized as follows:

1. Most of them use rather large quantities of fresh tissue as a starting material for DNA.

2. They are time consuming and cumbersome.

3. Special precautions and modifications become necessary on application to a wide variety of tissues.

4. DNA isolated from the same tissue by different methods does not have exactly the same physical and chemical properties.

The aim of the present study was to overcome some of these difficulties, and find a method suitable for small quantities of tissue (1 - 10 g.) which gives relatively high yields of DNA in highly polymerized "native" form. In order to achieve this aim several preparative procedures were tried on two kinds of tissue, namely, liver and mucosa of small intestine of rat. These tissues were chosen because the former shows a high degree of metabolic activity with respect to nucleic acids and was particularly studied in this laboratory (26 - 28) whereas the latter has a low nuclear/cytoplasmic ratio, i.e. it is rich in RNA and other non-nuclear constituents (polysaccharides).

After the preparation of DNA from these tissues by different methods, the isolated material was characterized by chemical and physical means in order to compare the properties of different preparations and evaluate the best possible procedure for further investigations.

Four procedures seemed to be especially promising and applicable to relatively small quantities of tissues.

1. A rapid method for preparing polymerized DNA developed by Emanuel and Chaikoff (29). These investigators claimed that the procedure is specially favorable for application to tissues having low nuclear/cytoplasmic ratios. It is based on the removal of nuclei from the tissue by means of

controlled homogenization and subsequent separation of nuclei from extraneous cellular elements in the homogenate by absorption on Celite (diatomaceous earth). A rapid method for preparation of a homogenate with a high yield of nuclei is described by these workers (30). They used a hydraulic homogenizer for the controlled release of cellular components from various tissues. In the present study no such hydraulic homogenizer was applied, but a combined homogenization with a Teflon homogenizer and short disintegration with a Servall omni-mixer was attempted. The absorbed nuclei were then dispersed with a strong salt solution (NaBr) and their DNA was separated from its basic protein which adheres to the absorbing Celite. The sodium nucleate were filtered off or centrifuged from the protein - Celite mixture. Several salt saturation and precipitation steps were used for further purification.

2. The second procedure was based on the original work of Kay et al. (20) using the modifications applied by Stevens and Duggan (31). In this method the nucleic acids were extracted with sodium dodecyl sulphate and sodium xylene sulphonate solution containing some ethylene-diamine-tetra-acetate (Versene) in order to inhibit any deoxyribonuclease action. Their protein was removed by the detergent treatment combined with adjustment of the pH of the solution to somewhat lower values (pH 4.3). After readjusting the pH of the protein-free sodium nucleate solution RNA and DNA were separated by

fractional precipitation with iso-propyl alcohol.

3. The methods of Emanuel and Chaikoff and Kay et al. (29,20) were combined in this procedure. The nuclei were isolated according to Emanuel and Chaikoff, crude DNA was obtained by disrupting nuclei with salt saturation and this crude product was further purified by the previous detergent method.

4. The fourth procedure was based on the work of Zubay (32). In this method the tissues were disintegrated by high frequency sound, the nuclear fragments were separated by centrifugation and the nucleoprotein was extracted with strong salt solution (13-15). DNA was deproteinized according to the procedure of Sevag et al. (17).

Zubay applied his method to a number of mouse tissues (spleen, liver, lymphoma) using very small quantities of starting materials (1- 3 g.). Although several workers found (33-35) that ultrasound waves are capable of damaging DNA in solutions by causing the disruption of hydrogen bonds in the DNA molecule and thus degrading it into smaller fragments having lower molecular weight than the original native DNA, Zubay claimed that when DNA is in the form of nucleoprotein and in solution, it is well protected from this damaging effect. He furnished some experimental evidence, showing that DNA preparations by his method had very high intrinsic viscosities although he admitted that DNA preparations of viral origin lost their transforming activities.

In order to obtain a DNA preparation which presumably yields very degraded and denatured DNA and thereby enable the comparison of the properties of a degraded material with those of native ones the procedure of Bendich et al. (36) and Tyner et al. (37) was tried. This procedure is based on the extraction of tissues with hot (85°C) 10% sodium chloride for several hours, precipitation of nucleic acids with alcohol, separation of RNA from DNA by incubating the precipitate with 0.1 N NaOH, and finally reprecipitation of DNA from the basic mixture with concentrated HCl. Thus the procedure seemed to be drastic enough for yielding denatured DNA.

Characterization of DNA Preparations.

At present it is not known whether even a most carefully isolated DNA can in all respects be identical with the DNA as it existed in the living cell. Strictly speaking no compound, once it is isolated from the cell, can be considered as native. However the series of degradative changes to which it may be exposed, in the course of its isolation will usually be gradual, and while it may not yet be possible to define the perfect compound, the badly degraded one can be easily recognized. (38)

There are actually three main methods by which DNA can be characterized;

1. Chemical methods based upon the determination of DNA content, base composition, and nitrogen-phosphorous

contents of the preparation.

2. Physical methods by which the integrity of macromolecular state of DNA can be defined.

3. The determination of its biological activity (39). One biological activity which can be demonstrated in certain DNA preparations is their transforming activity. Unfortunately at present only a few bacterial DNAs lend themselves tothe assay for transforming activity.

During the course of this study chemical and physical methods were used to compare the properties of DNAs prepared by different procedures from the same source to decide whether the compound is in native, undegraded state.

1. The Chemical Characterization of the DNA Preparations.

The chemical composition of DNA is less likely to change on mild denaturation. A very good indication can be obtained, however, by chemical means about the degree of purity of such preparations. The investigator may encounter three very important impurities in DNA samples; the presence of RNA, proteins and polysaccharides. For example the presence of protein can be evaluated from the N/P ratio of the DNA preparation. Owing to the several structural regularities in all DNAs, the N and P contents are closely similar for different preparations from different sources. The N/P value calculated on the basis of Watson-Crick (40) model of DNA is 1.65. Any

higher value than this would surely indicate protein contamination. Therefore nitrogen and phosphorous determinations were carried out on the isolated DNA samples, using the micro Kjeldahl method (41) for N and the colorimetric procedure of Bartlett (42) for P estimations.

Similarly protein contamination can be demonstrated by the complete hydrolysis of samples followed by paper chromatography of the hydrolysate for free amino acids. Only qualitative identification of amino acids was attempted in this study (43,44).

One of the most important chemical characterization of DNA is the determination of its base composition. By the year 1930 it was definitely known that DNA on hydrolysis yielded phosphoric acid, a sugar (deoxyribose) and four nitrogenous bases namely; adenine, guanine, cytosine and thymine (38). The early work of Levene and Jones suggested that DNA prepared by extraction with alkali was composed of equivalent proportions of the four nucleotides derived from adenine, guanine, cytosine and thymine. When it was recognized that the nucleic acids could be obtained in the form of particles of extremely high molecular weight, the tetranucleotide hypothesis had to be modified.

The development of the methods of chromatography encouraged the application of similar techniques for the quantitative determination of the product formed on cleavage of the

nucleic acids. It was found that from hydrolysates of DNAs the purine and pyrimidine bases could be easily separated by paper chromatographic methods using different solvent systems (38).

Several procedures for hydrolysing DNA can be used. The most suitable for quantitative measurements appears to be the formic acid procedure originally developed by Vischer and Chargaff (45). A convenient solvent for separating the purine and pyrimidine bases on paper chromatograms was recommended by Wyatt (46). The description of an arrangement permitting the easy demonstration of the purine and pyrimidine spots and the application of a commercially available ultraviolet lamp (47) facilitated the performance of analyses. For quantitative estimation spots were cut out from the chromatograms and eluted by soaking them in a given volume of 0.1 N HCl. According to Chargaff (38) if this elution is allowed to proceed overnight at room temperature, with shaking at the beginning and at the end it is quantitative. It was found in this laboratory however, (48) that this elution technique is not necessarily quantitative, even with several (three-times) extractions of spots with aliquot portions of 0.1 N HCl. Therefore a new elution procedure was tried (49) and compared with the old technique. This method will be described and discussed later.

The application of the above mentioned paper

chromatographic method toDNA preparations of different cellular origin soon demonstrated significant chemical differences between these compounds (50 - 53). Thus the determination of base composition proved to be a useful mean to characterize DNA preparations specially with respect to the purity of such samples. One of the most troublesome inpurities in DNA is the presence of RNA as already mentioned above. The paper chromatographic analysis of DNA hydrolysates would surely indicate the presence of any such impurity.

Non-nuclear contaminants such as polysaccharides or salts could be also easily demonstrated by the determination of the DNA content of the samples. Most of these estimations are based however, on the determination of the deoxyribose content of the material or on spectophotometric estimations, compared with that of a "reliable" standard (38). No such reliable reference standard nucleic acid preparation is available at present, therefore direct DNA estimations were not performed during this study.

II. Physico-Chemical Characterization of DNA.

The criteria of integrity of a macromolecular substance of natural origin are not easy to define, but as regards to DNA certain features can be described.

1. It has a very high and, within the species uniform

molecular weight as shown by diffusion experiments (54 - 56), sedimentation in the ultracentrifuge (55, 56), and determination of viscosity and streaming birefringance. (57).

2. The character of monodispersity is lost even when the specimen is prepared under very mild conditions if partial enzymatic attack in the course of isolation is not avoided.

3. Both the value and the uniformity of the molecular weight are affected if the isolation is carried out under degradative conditions (58) or if the preparation is exposed subsequently to degradation by chemical or physical means.

4. It has an anomalous amphoteric behavior on titration.

5. The molecules posses a high and stable asymmetry. Solutions of undegraded DNA exhibit double refraction of flow (59) and very considerable viscosity.

6. Undegraded DNA shows a typical "hyperchromic" effect in solution.

The following two characteristic physico-chemical properties were studied during this experiment:

(a) <u>The Hyperchromic Effect of DNA Solutions</u>.

The depolymerization or denaturation of nucleic acids is associated with intensification of their absorption of ultraviolet light. In other words the extinction of intact

preparations is lower than would correspond to the sum of their constituent mononucleotides. This effect applies to RNA, DNA and synthetic polynucleotides. The intensification brought about by deoxyribonuclease on DNA was first described by Kunitz (60). Similar effects produced by acid, heat, alkali or the addition of salts have been studied in greatest detail by Thomas (61) and more recently by Schack (62). According to Chargaff et al. (38) the extinction of the maximum is almost constant for different DNA preparations. When the extinction at the maximum and at pH 7 is expressed as the atomic extinction coefficient with respect to phosphorous and designated as $\epsilon_{(p)}$ (63), preparations isolated cautiously from a large variety of sources will show surprisingly little divergence from the value of $\epsilon_{(p)} = 6600$. According to Chargaff (38) an $\epsilon_{(p)}$ value higher than about 7200 is a sign of denaturation of DNA sample.

(b) The Anomalous Viscosity of DNA Solution.

Physico-chemical studies on DNA are largely concerned with a number of properties associated with the native hydrogen bonded macromolecular state of the nucleate (40). The highly anomalous viscosity of DNA has been subject of considerable study. The striking effect of electrolytes (59,64) and of acid and alkali (59,54) in reducing the viscosity of DNA solutions provide important information with respect to its physico-chemical state. The destruction of the secondary structure

of DNA by heat, acid or alkalies at constant molecular weight produces a particle processing a greatly decreased (Newtonian) viscosity. In order to perform a measurement which is meaningful in terms of molecular properties the reduced viscosity $\frac{\eta spec}{c}$ (65)

where $\eta spec = \left(\frac{\eta}{\eta_0} - 1\right)$ equ.(1)

η = viscosity of solution

η_0 = viscosity of solvent

must be measured in solutions which are dilute enough to allow independent molecular motions and then extrapolated to zero concentration. Plots of $\frac{\eta spec}{c}$ versus c are generally linear in the low concentration range, and the extrapolated value is called the intrinsic viscosity.

$$[\eta] = \lim_{c \to 0} \frac{\eta spec}{c} = \lim_{c \to 0} \frac{1}{c} \ln\left(\frac{\eta}{\eta_0}\right) \quad \text{equ. (2)}$$

where c = number of grams of polymer in 100 ml. of solution.

The intrinsic viscosity may be used as a qualitative measure of the molecular size and presumably of the molecular weight. Since the DNA molecule in solution is probably not a simple linear polymer but a strand composed of two polynucleotide chains, one would expect no simple relationship between the intrinsic viscosity and the extent of degradation. However, for a qualitative comparison of different preparations the intrinsic viscosity provides a sensitive measure of differences. Moreover since the viscosity is a monotonically decreasing function of the gradient, the $[\eta]$ measured at some higher more

experimentally accessible average gradient (ca.1000 sec.$^{-1}$) can also be utilized to qualitatively detect changes in molecular configuration (39).

One of the essential parameters which characterizes a high molecular weight polymer compound such as DNA is its molecular weight. The estimation of the latter is a difficult problem solving of which involves complicated apparatus such as for instance analytical ultracentrifuge, setups for determination of the coefficient of diffusion and the measurement of light scattering. In addition to these, for a series of high molecular weight compounds the molecular weight has been determined by means of utilization of the simple viscosimetric technique. The following equation expresses the relation between intrinsic viscosity and the molecular weight of the high polymer (65):

$$[\eta] = KM^{\alpha} \qquad \text{equ. (3)}$$

where M = molecular weight of high polymer.
The constants α and K depend upon the type of polymer, the solvent, and the temperature of the viscosity determination. The values of K and α are known for several synthetical polymers. Utilizing equation (3) Spitkovskii suggested a similar formula for the determination of the molecular weight of DNA (67):

$$[\eta] = 33.22 \times 10^{-4} M^{0.616} \qquad \text{equ.(4)}$$

The values for $\alpha = 0.616$ and $K = 33.22 \times 10^{-4}$ were derived by him from theoretical considerations. Using a series of data

of molecular weight and the corresponding characteristic viscosities found in the literature, Spitkovskii was able to prove the validity of his equation. A very satisfactory agreement was found between the data in literature and molecular weights, calculated according to equation (4). Thus his viscosimetric method seemed to be quite promising for obtaining estimations of the molecular weight of DNA preparations isolated during this study.

EXPERIMENTAL

The Preparation of DNA from Small Intestinal Mucosa and Liver of Rat by Different Procedures.

I. Isolation of DNA by the Method of Emanuel and Chaikoff (29).

Reagents: Celite (Johns-Manville Co.)
Saturated Sodium Bromide Solution
Potassium Arsenate Solution

A 0.2 M solution of potassium dihydrogen arsenate was prepared by dissolving 3.6 g. of potassium dihydrogen arsenate (technical grade) in 100 ml. distilled water and the pH was adjusted to 7 by titration with concentrated potassium hydroxide solution and this neutral solution was then diluted with water until its molarity was 0.014.

Homogenization Medium.

11.5 g. of solid potassium chloride was dissolved in 1000 ml. of 0.014 M potassium dihydrogen arsenate solution and the mixture was diluted to 2000 ml. This yielded a medium which was 0.007 M in arsenate ion and 0.038 M in chloride ion. The purpose of adding arsenate ion in the homogenization medium was to inhibit deoxyribonuclease action during isolation procedure.

Procedure:

The preparation of tissues:

Wistar rats weighing 200 g each, from the colony of British Columbia were used in the experiments. The animals were killed by blow on the head and decapitated. The liver was removed and frozen by dropping it into liquid nitrogen. The small intestine was removed and cut into 10 cm. segments which were flushed free of contents with the cold homogenization medium. The segments were split open, applied to a chilled glass plate. The mucosal epithelium was then scraped from the muscularis with the edge of microscope slide and placed in liquid nitrogen. Both tissues were stored at -15°C.

Procedure.

The tissues were homogenized with 40 ml. arsenate buffer for 10 minutes with a glass tissue grinder. This homogenizer consisted of a piston-type Teflon pestle and a grinding vessel of Pyrex brand glass, and will be referred hereafter as Teflon homogenizer. The homogenization was continued for another two minutes in a Servall Omni Mixer. The mixture was then filtered through a single layer of muslin cloth. The retained connective tissue was washed with 20 ml. of homogenization medium. The filtrates were collected in a beaker immersed in ice, 4.2 g. of dry Celite was added and the mixture was stirred at high speed for about 10 seconds with a magnetic stirrer. The mixture was poured into a 10 cm. cooled Buchner funnel containing a filter paper overlaid with 2.9 g. of Celite which had been suspended in water and

sucked very dry. The pad was washed with chilled homogenization medium until the filtrate was colorless, then another 100 ml. of this solution was sucked through the pad. As a rule a total of 300 ml. of solution suffices to remove non-nuclear materials. The Celite pad with its adsorbed nuclei was lifted out of the funnel and sufficient saturated sodium bromide solution (15-20 ml.) was added to it with stirring. During the stirring a small amount of solid sodium bromide was added to insure saturation of the mixture with the salt. The suspension was next filtered on a 5.5 cm. Buchner funnel and the filtrate was collected in a suction flask. Two sheets of Whatman No. 1 filter paper overlaid with 2mm. of Celite were used for filtration. To remove residual nucleic acids, the pad was washed 3 times with 4 ml. portion of chilled saturated sodium bromide solution. The very viscous clear filtrate was diluted then with one part water to five part of filtrate. While the solution was being swirled by hand, 2 or 3 volumes of 95% ethanol were added to it and the precipitated fibrous DNA was removed with a glass rod. The crude DNA was dissolved in 20 ml. of homogenization medium. The mixture was stirred until all of the nucleic acids dissolved. 0.5 g. Celite was added to the solution, followed by solid sodium bromide saturation. The mixture was centrifuged, the clear supernatant was diluted with one part of water to each five part of solution and DNA was precipitated with 2 or 3 volumes of ethanol (95%). The

fibrous DNA was washed several times with 75% ethanol then dried in vacuo at room temperature over phosphorous pentoxide. Dried DNA was then stored in a vacum dessicator at -15°C.

II. Isolation of DNA by the method of Kay et al. (20) modified by Stevens and Duggan (31).

Reagents:

Solution A. Arsenate buffer. (described on page 19)

Solution B. Sodium lauryl sulphate - Versene solution.

2 g. sodium dodecyl sulphate - 8 ml. 5 M Versene solution.

Solution C. Acetate buffer (pH 7.)

Anhydrous sodium acetate 12.3 g.

Acetic acid 0.4 ml.

Distilled water up to 500 ml. Adjust pH to 7.0.

Solution D. Acetate buffer - Versenate solution 0.01 M.

0.372 g. Versene in 100 ml. acetate buffer.

Solution E. Versene, potassium chloride, Sodium Xylene Sulphonate solution. (Nease Chem. Comp.)

10 m M Versene 0.372 g.

0.2 M potassium chloride 1.492 g.

12 % sodium xylene sulphonate 12.00 g.

distilled water up to 100 ml.

This solution should be kept in refrigerator.

Solution F. 0.05 M Versene solution

18.6 g. Versenate/1000 ml. distilled water.

Solution G. 0.01 M Versene solution.

3.72 g. Versene/ 1000 ml. distilled water.

Solution H. 80% w/v sodium xylene sulphonate solution.

Preparation of tissues:

Livers and intestinal mucosa were obtained as described previously from rats fed ad libitum.

Procedure:

The tissue was blended for two minutes in ten ml. of solution B. The resulting gel received a little toluene for preservative. The pH was adjusted to approximately 7.5 with ammonium hydroxide using pH paper. The gel was stored overnight at room temperature. Four volumes of chilled solution E was added, and after 30 second blending in ice bath the mixture was transferred to a beaker in an ice bath. The pH was adjusted to 4.3 with glacial acetic acid. The protein detergent residue was discarded, after centrifuging the mixture at 4500 RPM for 45 minutes at 0° C. The pH of the supernatant was adjusted to 7.0 with ammonium hydroxide solution, and was stirred during the addition of 90% iso-propyl alcohol. The precipitated nucleic acids were recovered by centrifugation. Crude nucleic acids were dissolved in 5-10 ml. of solution F and an equal volume of solution H was added. The mixture was stirred for an hour, and 4 volumes of solution G. were added with continous stirring. While the solution was chilled in an ice bath the pH was adjusted to 4.3 with glacial acetic acid.

The protein residue was centrifuged for 60 minutes at 4°C at 16500 RPM and the pH of the clear supernatant was then adjusted to pH 7.0 with ammonium hydroxide. An equal volume of 90% iso-propyl alcohol was added. The nucleic acid residue was recovered by centrifugation at 4°C. The residue was dissolved in 3 to 5 ml. of solution D and slowly iso-propyl alcohol was added to a final concentration of 30% (v/v). The precipitated RNA was discarded, after centrifugation. To the clear supernatant iso-propyl alcohol was added to a final concentration of 34 to 40% v/v. The precipitated DNA was recovered by centrifugation and the DNA precipitate was washed with 95% ethanol, then dried and stored as described on page 22

III. Isolation of DNA by the Combined Methods of Emanuel and Chaikoff (29) and Kay et al. (20,31).

Procedure:

It was felt, that a suitable procedure could be obtained by combining certain desirable features of the methods of Emanuel and Chaikoff and Kay et. al. (29,31). The procedure of Emanuel and Chaikoff was followed until the first precipitation of DNA was obtained, then crude DNA was deproteinized according to the method of Kay et al. and Stevens et al. (20,31)

IV. Isolation of DNA by the Method of Zubay (32).

Reagents:

Solution A. Arsenate buffer (described on page 19)

Solution B. Neutral saline - Versene solution

0.15 M sodium chloride 8.777 g.

0.01 M ethylenediamine tetraacetic acid Na. 3.72 g.

Distilled water up to 1000 ml.

Solution C. 0.15 M sodium chloride solution

8.77 g. NaCl/1000 ml. distilled water

Solution D. 3 M sodium chloride solution

175.35 g. sodium chloride/1000 ml. distilled water

Solution E. Chloroform - amyl alcohol mixture

Chloroform 3 parts

Amyl alcohol 1 part

Preparation of Tissues:

Six male rats were starved and their livers and intestinal mucosa were obtained and frozen as described on page 20 . These tissues were used for the isolation of DNA by procedures IV and V.

Procedure:

Starting material: 3 g. of intestinal mucosa and 6 g. of liver The tissue was homogenized shortly (10-20 strokes) with a Teflon homogenizer in a final total volume of about 60 ml. The samples of cell suspension were exposed to sonic irradiation

in a Raytheon sonic oscillator (9 k.cycles/sec) for 1.5 minute. The nuclear fragments obtained after disintegration of tissues were washed 6 - 8 times with 60 ml. solution B1 by alternate centrifugation for 8 minutes at 3500 RPM in a Servall refrigerated centrifuge. The washed nuclear fragments were resuspended in 45 ml. of cold solution C. in a Servall top-drive Omni Mixer and run at about 3000 rev/minute. 90 ml. of solution D was at once added and blending was continued for 20 minutes at 3°C and at a same low speed. The dissociated solution was then emulsified up to 8 times successively, with 30 - 45 ml. of Chloroform - amyl alcohol mixture, each emulsification was carried out in a vibratory shaker for 10 minutes and was then alternated with centrifugation for 10 minutes at 4500 RPM on Servall centrifuge. When no further interfacial film of protein was produced by emulsification, the solution was diluted with an equal volume of water and DNA precipitated by addition of equal volume of ethanol. The precipitated DNA was dissolved again in minimum amount of solution G and centrifuged if necessary to obtain a crystal clear solution. DNA was precipitated from the supernatant as previously. DNA was washed dried and stored as described on page 22 .

V. Isolation of DNA by the Methods of Bendich et al. (36) and Tyner et al. (37).

Reagents:

Solution A. 10% sodium chloride solution

Solution B. 5% sodium chloride solution

Solution C. Approximately 0.1 N sodium hydroxide solution

Solution D. Ether (anhydrous)

Procedure:

3 g. of fresh tissue was homogenized shortly (10-20 strokes) with a Teflon homogenizer, with 20 ml. of 10% sodium chloride solution. The tissues were extracted with the 20 ml. 10% sodium chloride solution for 6 hours at 85°C. The mixture was then centrifuged at 2500 RPM. and the residue was discarded. To the supernatant 3 volumes of 95% ethanol were added and the precipitated nucleic acids were left overnight in the fridge. The nucleic acids were centrifuged and the obtained precipitate was washed with 2 ml. of 95% ethanol then with 2 ml. ether, and dried in vacuo. The dry pellets were dissolved in 10 volumes of 5% sodium chloride and stirred for 10 to 15 minutes at 85°C until all mucleic acids went into solution. The solution was then centrifuged. To the supernatant 3 volumes of 95% ethanol were added and chilled it for couple hours. The nucleic acids were recovered by centrifugation and washed with 95% ethanol, 50:50 ethanol - ether mixture, then with ether. The residue was dried in vacuo at room temperature. The nucleic acid residue was weighed and 1 ml. of 0.1 N. sodium hydroxide

solution was added for each 10 mg of dry precipitate. The solution was then incubated at 37.5° for 20-22 hours. After incubation, the solution was neutralized with concentrated hydrochloric acid drop by drop until good precipitate formed. The mixture was chilled and left overnight in fridge. Next day the mixture was centrifuged and the DNA residue washed 2 times with 0.1 N hydrochloric acid, then with ethanol and 50:50 ethanol - ether mixture, and ether and finally dried in vacuo at room temperature.

The Chemical Characterization of DNA.

The determination of nitrogen content by the micro Kjeldahl procedure was performed according to ref. (41,68).

Standards:

Urea recrystallized (analytical grade) (Fischer. Sc. Co.)

Guanine hydrochloride (Nutritional Biochemicals Corp.) purified by ion exchange chromatography (69).

Deoxyribonucleic acid (California Corp. for Biochem. Res.)

For testing the nitrogen recoveries by this procedure urea, guanine hydrochloride and commercial DNA were used as nitrogen standards. The result of the analyses are shown in Table I.

The Determination of Phosphorous Content of DNA by the Method of Bartlett. (42).

TABLE I

Comparison of the Theoretical and Experimentally Determined Nitrogen Contents of Different Standards.

Name of Compound	Theoretical N%	Micro Kjeldahl N% found experimentally
Urea	46.67	46.58
"	"	46.59
Guanine hydrochloride	37.34	37.39
"	"	37.41
DNA commercial	15.2 (38)	14.99
"	"	15.09

Reagents:

Solution A. Phosphorous Standard: 1 μgP/ml.

2.1935 g potassium dihydrogen phosphate in 500 ml. distilled water. 1 ml. = 1 mg P

1 ml. of this solution was diluted to 1000 ml. and the resulting solution contained 1 μg P/ml.

Solution B. 10 N sulphuric acid solution.

Solution C. 5% ammonium molybdate solution

Solution D. Fiske - Subba-Row reagent.

0.5 g. of 1-amino-2-naphtol-4-sulphonic acid was dissolved with stirring in 200 ml. freshly prepared 15% sodium bisulphite solution, followed by the addition of 1 g. anhydrous sodium sulphite. The solution was filtered and stored in a dark bottle and freshly prepared weekly.

Adenosine 5' Phosphate (AMP) (Pabst. Fine Chemicals)

Thymidine 5' Phosphate (TMP) (Pabst. Fine Chemicals)

Procedure:

1 - 2 mg. previously dried DNA samples were digested in a Kjeldahl flask with 1 ml. concentrated sulphuric acid for 4-5 hours. The digest was transferred quantitatively into a 50 or 100 ml. volumetric flask and 3-5 ml. aliquots corresponding to 4-6 μg phosphorous were taken for phosphorous analyses. The aliquots of unknown were pipetted into a test tube, calibrated to 10 ml., 1 ml. 10 N sulphuric acid, 0.4 ml. 5% ammonium

TABLE II

The Determination of Phosphorous Content of Phenyl disodium Phosphate Using Different Hydrolysis Procedures.

Method of Hydrolysis	% P found experimentally	% P theoretical
1 ml 70% $HClO_4$	10.51	14.2
"	10.35	"
1 ml cc.H_2SO_4	13.8	"
"	13.89	"

TABLE III

Comparison of Theoretical and Experimentally Found Phosphorous Contents of Different Organic Standards.

Name of Compound	% P found experimentally	%P theoretical
AMP	8.86	8.92
"	8.8	"
TMP	8.62	8.59
"	8.31	"
Fructose-1,6-di phosphate	11.4	10.01
"	11.5	"
"	11.3	"

molybdate 0.4 ml. Fiske-Subba-Row reagent were added, in the order described and the solution was then made up to 10 ml. with distilled water. In order to obtain uniform readings it was found to be very important, to stir the solution after addition of each reagent. The solution was heated for 7 minutes at 100° C and the colour red at 830 mμ in a Beckman D.U. spectrophotometer, against a reagent blank.

The colour produced was proportional to the concentration of phosphorous up to 8 μg/10 ml. of the reaction mixture. A calibration curve was constructed (Fig. 1) which shows the concentration dependence of the colour produced for standard phosphorous solutions. The colour of the solutions was stable for at least 24 hours. To test the validity of the procedure, the phosphorous content of several organic substances were determined by this method. A comparison between the perchloric acid (70) and concentrated sulphuric acid hydrolysis of phosphorous containing substances was also performed. Table II summarizes the results of such experiments. It can be seen that sulphuric acid hydrolysis gave higher phosphorous values, and these values were actually closer to the theoretical ones.

Table III summarizes the results obtained using different organic standards. These substances were hydrolysed with concentrated sulphuric acid. The experiments show, that the phosphorous recoveries in the case of nucleotides AMP. and

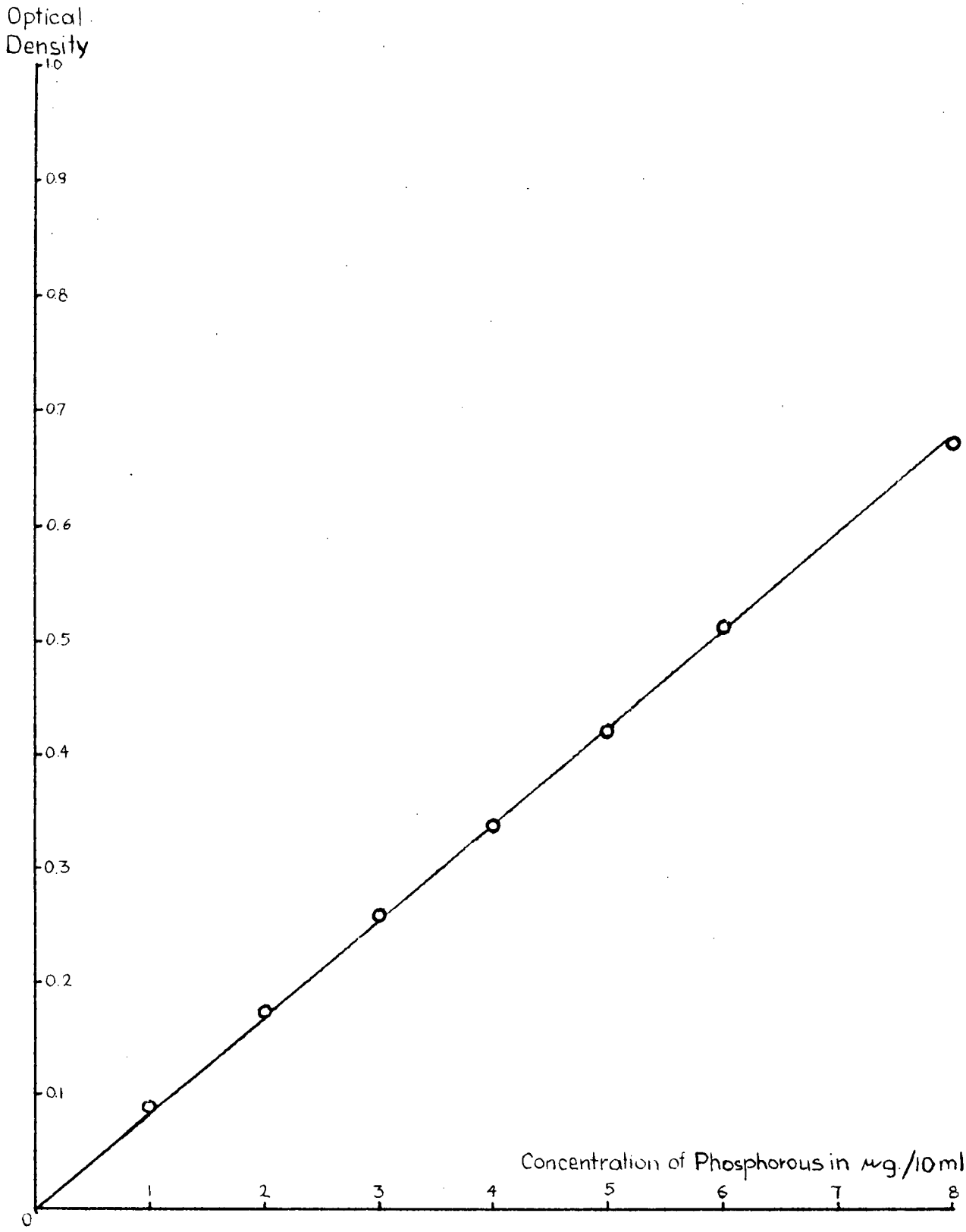


Fig. 1. Calibration curve for Phosphorous Determination.

TMP were very satisfactory. The analysis of fructose-1.6 diphosphate gave somewhat higher values than the theoretical one, however the purity of this sample was doubtful.

The Determination of Base Composition of DNA

Reagents:

Guanine hydrochloride (Nutritional Biochemical Corp.)
Purified by ionexchange chromatography (69).

Adenine (N.B.C.)
Purified by sublimation in vacuo at 220°C. (71)

Cytosine hydrochloride. (N.B.C.)
Purified by ion exchange chromatography. (69)

Thymine (N.B.C.)
Purified by recrystallization from hot water.

Uracil (N.B.C.)
Purified by recrystallization from hot water.

Wyatt's solvent.

Iso-propyl alcohol	170 ml.
Concentrated hydrochloric acid	41 ml.
Distilled water up to	250 ml.

Procedure:

I. Hydrolysis of DNA by concentrated formic acid solution (72).

1 - 4 mg. of DNA previously dried in vacuo over phosphorous pentoxide were accurately weighed on an analytical micro balance and placed in a pyrex glass tube. The tube was sealed and placed into the oven and heated for two hours at 161 - 163°C. At the end of two hours hydrolysis the sealed tubes were cooled in dry ice-ethanol mixture and opened cautiously, by melting the tips of sealed ends in flame. The contents of tubes were poured in 10 ml. beakers, cooled again in dry ice - ethanol mixture, and evaporated to dryness in vacuo over solid potassium hydroxide. The purine and pyrimidine bases were extracted with three successive portions of 2 ml. 0.1N.hydrochloric acid, followed by centrifugation to remove charred particles. The 0.1 N hydrochloric acid extracts were combined and evaporated in vacuo. The dry residue was then taken up with a known quantity of 0.01 N hydrochloric acid. Usually the final volume was made up to exactly 1.0 ml. in a volumetric flask.

II. Separation of Purine and Pyrimidine Bases by Paper Chromatography (46).

Procedure:

Strips of Whatman No. 1 paper were used. 50-200λ. of hydrolysates containing 50-300μg DNA were applied on chromatograms using calibrated micro-pipettes. The chromatograms were placed into an all glass chromatographic cabinet,

and some solvent was placed at the bottom of cabinet to get saturation with respect to the vapours of solvent. A descending technique was applied and the chromatograms were run about 32-36 hours. At the end of this period the solvent line almost reached the bottom of the paper. The paper strips were taken out from the cabinet, dried in air, and the purine and pyrimidine spots were outlined with the aid of untraviolet light (47). The $R_{(f)}$ values of the unknown spots were compared with the $R_{(f)}$ values of standards run on the same paper and at the same time, as the unknown samples.

The $R_{(f)}$ values of the purine and pyrimidine bases using the iso-propanol-hydrochloric acid solvent are summarized in Table IV. The values are compared with those found in ref. (38) Table IV, shows that the $R_{(f)}$ values obtained during this investigation are somewhat larger than in ref. (38). This may be due to the large temperature fluctuation in this laboratory, or the slight composition difference in solvent systems.

III. <u>The Quantitative Estimation of Purine and Pyrimidine Bases Separated by Paper Chromatography</u>.

For quantitative elution of the bases from the spots outlined under the ultraviolet light, two techniques were tried and the results compared. It was mentioned before on page 12 that the elution of spots with 0.1 N hydrochloric acid for 24

TABLE IV

The Comparison of R_f Values of Purine and Pyrimidine Bases in iso-propanol Hydrochloric acid Solvent.

Name of Compound	R_f Values found experimentally	R_f Values from ref. (38)
Adenine	0.34	0.32
Guanine	0.25	0.22
Cytosine	0.47	0.44
Uracil	0.69	0.66
Thymine	0.78	0.76

hours was not quantitative even if several extractions were performed. Therefore a new technique was tried, which was based on the personal communication of I. Csizmadia (49). The description of the two elution techniques is as follows:

Procedure I. (Extraction Method)

The paper spots were cut into very small pieces and placed in a test tube, 2 ml. of 0.1 N hydrochloric acid was added and the mixture allowed to stand overnight. Next day the test tube was shaken in a shaker for 30 minutes and after standing most of the 0.1 N hydrochloric acid extract was decanted. Another 2 ml. portion of 0.1 N hydrochloric acid was pipetted into the test tube and the previous procedure was repeated twice. The combined extracts were centrifuged and transferred into a small beaker. The contents of beaker were evaporated to dryness, and the residue was taken up in an exact volume of 0.1 N hydrochloric acid appropriate for the cells in which the extinctions were to be read. The ultraviolet absorption curves were recorded in a Beckman Automatic D.K. 2 spectrophotometer. To allow for ultraviolet absorbing substances in the paper, blanks were cut equal in area to the spots and at equal distances from the starting line, and were eluted and read at the same wavelenghts as the corresponding spots. The extinction coefficients used in estimating nucleic acid derivatives by the absorption at their maxima were

determined previously in this laboratory.

Procedure 2. (The column extraction method.)

Ultraviolet absorbing areas on paper chromatograms were cut into very fine pieces and these were placed intubes resembling Pasteur pipettes. The diameter of tubes were about 7 mm., and the ends were drawn out to form a veryfine and narrow outlet with an approximately 1 mm. diameter and 20 cm. lenght. The upper part of the tubes was widened in order to facilitate the packing. The glass wool was packed very tightly in the bottom of the wide part of the tube up to about 1 - 1.5 cm. height. The tubes were filled with the finely cut pieces of paper to form a fairly uniform and tight packing. Then another 2 cm. layer of tightly packed glass wool was placed on the top of the paper columns.

The tubes were placed on a stand which consisted of a piece of fairly thin wood containing several 3-4 mm. diameter wholes which allowed through the lower capillary end of tubes but kept the upper part in a straight and solid position. The end of the capillary outlets reached a 10 ml. small beaker, to collect the eluates. About 20-30 tubes were eluted at the same time by filling up the columns continuously with 0.1 N hydrochloric acid. The rate of eflux was about 0.16 ml./minute. For each column about 9 ml. of eluate were collected and theh the contents of the beakers were evaporated in vacuo over

sodium hydroxide pellets and concentrated sulphuric acid. The dry residues were taken up in an appropriate volume of 0.1 N hydrochloric acid, and the ultraviolet spectra were recorded as described in Procedure I on page 35 . Blank spots were eluted and read in the same manner as described above. To show the differences between procedure 1 and 2, a standard solution was made up containing known amounts of each of the nitrogenous bases. To construct a calibration curve known and increasing amounts of this stock solution were applied on paper strips in duplicates and the separation of bases were performed as described on page 35 . The spots were eluted with both procedures 1 and 2.

Blank absorption.

It was found that very high blank readings were obtained using both procedures. These blanks might obscure the optical density values of standards especially at the low concentration region. Table V. summarizes the blank readings for each of the bases obtained at their absorption maxima. It can be seen that higher blank values were obtained by the column extraction method. In order to determine the cause of the high blank absorption in case of procedure 2, a chromatographic column was packed with glass wool and eluted with 0.1 N hydrochloric acid. The ultraviolet examination of the eluate gave a curve that was characterized by a continous increase of absorption at decreasing wavelenghts. It was concluded that glass wool contained some unknown, ultraviolet absorbing material

TABLE V

The Optical Density Values of the Blanks of Nucleic Acid Bases, Obtained from Readings at their Absorption Maxima.

Name of Blank	Optical Density/1 ml. of solution by procedure 1.	Optical Density/1 ml. of solution by procedure 2.
Adenine	0.236	0.654
Guanine	0.266	0.622
Cytosine	0.167	0.450
Uracil	0.250	0.656
Thymine	0.221	0.646

TABLE VI

Comparison of % Recoveries of Purine and Pyrimidine Bases Using Procedure 1 and Procedure 2 for Elution.

Name of Compound	Applied on paper in M.	Found exp. by proc. 1 in M.	% Recovery	Found exp. by proc. 2 in M.	% Recovery
Adenine	1.85×10^{-2}	1.23×10^{-2}	66.6	1.22×10^{-2}	65.8
	3.70	2.35	63.5	5.15	139.3
	11.08	8.53	76.9	9.44	85.2
	14.78	12.71	86.0	11.88	80.4
	18.45	16.52	89.4	18.23	98.7
Uracil	2.17	1.03	47.5	.78	36.6
	8.67	5.96	68.7	3.92	45.2
	13.01	10.58	81.4	18.61	143.0
	17.34	17.25	99.5	16.19	93.4
	21.68	22.49	103.7	23.88	110.1
Cytosine	1.75	.95	54.5	2.12	121.1
	3.51	2.54	72.4	4.30	122.7
	7.02	5.54	79.0	8.03	114.5
	10.52	8.64	82.1	9.50	90.3
	14.03	13.75	97.9	12.08	86.1
	17.54	18.00	102.6	19.74	112.5
Thymine	1.71	1.63	95.7	1.62	94.7
	6.82	3.44	50.4	3.67	53.8
	10.23	4.93	48.2	8.63	84.3
	13.64	10.87	79.7	11.88	87.1
	17.05	13.53	79.3	19.85	116.4

Note: Unpurified glass wool was used for procedure 2 and unwashed paper for procedure 1 and 2.

(perhaps very finely powdered glass) and this substance caused the high blank readings in procedure 2. Because procedure 1 gives also fairly high blank readings, this part of the absorbance must be due entirely to some ultraviolet absorbing material in paper.

Table VI summerizes the recoveries of nitrogenous bases using procedure 1 and 2. The values for guanine were not included, because during the preparation of standard solution a decomposition of this base took place, which was shown by the distorted ultraviolet absorption curve obtained for this compound, after the separation of the bases of standard solution by paper chromatography. Comparing the results of table VI the following observations can be made:

1. There was a large deviation in recoveries of purine and pyrimidine bases using both procedures.

2. The average percentage recovery was 70-80% using procedure 1, therefore this method did not effect the quantitative extraction of purine and pyrimidine bases from paper.

3. In some cases even higher then 100% recoveries were obtained by using procedure 2. This was maybe due to the fact that the amount of glass wool used for packing was not the same in each cases, and depending on the quantity of glass wool used, different amount of ultraviolet absorbing material was eluted.

Because the column extraction method seemed to be

more promising with respect to the quantitative extraction of the nitrogenous bases, a new standardization curve was constructed, using washed paper for separation of the bases and purified glass wool for the elution technique. The purification procedures and the preparation of a new standard solution is described as follows:

Purification of the paper

Whatman No. 1 paper strips were washed with 0.1 N hydrochloric acid for 12 hours in a chromatographic cabinet. The papers were dried, and washings were repeated with distilled water and with Wyatt's solvent.

Purification of glass wool

A large all glass chromatographic column was packed tightly with glass wool (pyrex. lab. glassware). The amount of the glass wool was about 200 g. The column was eluted with about 4 liter of 0.1 N hydrochloric acid (eflux time about 4 ml. /minute) and then with 2 liter of distilled water. The elution was continued until no more ultraviolet absorbing material was present in the eluate at 240 mμ.

Preparation of standard stock solution:

A new standard stock solution was prepared from the purified purine and pyrimidine bases. Guanine was dissolved

first in 2 N hydrochloric acid and then transferred to the stock solution containing the other four bases in 0.01 N hydrochloric acid.

The results of procedure 2 using purified paper and glass wool are represented graphically in Figures (2 - 6). In these graphs the applied amounts of purine and pyrimidine bases were plotted versus the recovered amounts. In the case of procedure 2 fairly straight lines were obtained proving that the deviation from the theoretical values were the same in all concentration regions. In contrast, at the low concentration range the recoveries of procedure 1 were always lower, than those of procedure 2, and approached the theoretical line at higher concentrations. The reason for this could be that the obscuring effect of paper blank absorption was relatively smaller at higher concentrations.

Typical standard curves of purine and pyrimidine bases are shown in Figures 7 - 11 using procedure 2 with purified paper and glass wool.

It was concluded from these experiments;

1. The hydrolysis product of nucleic acids obtained after separation by paper chromatography can be eluted almost quantitatively from the paper using the column extraction method (procedure 2).

2. The general recoveries of the bases approached 90%,

Fig. 2.

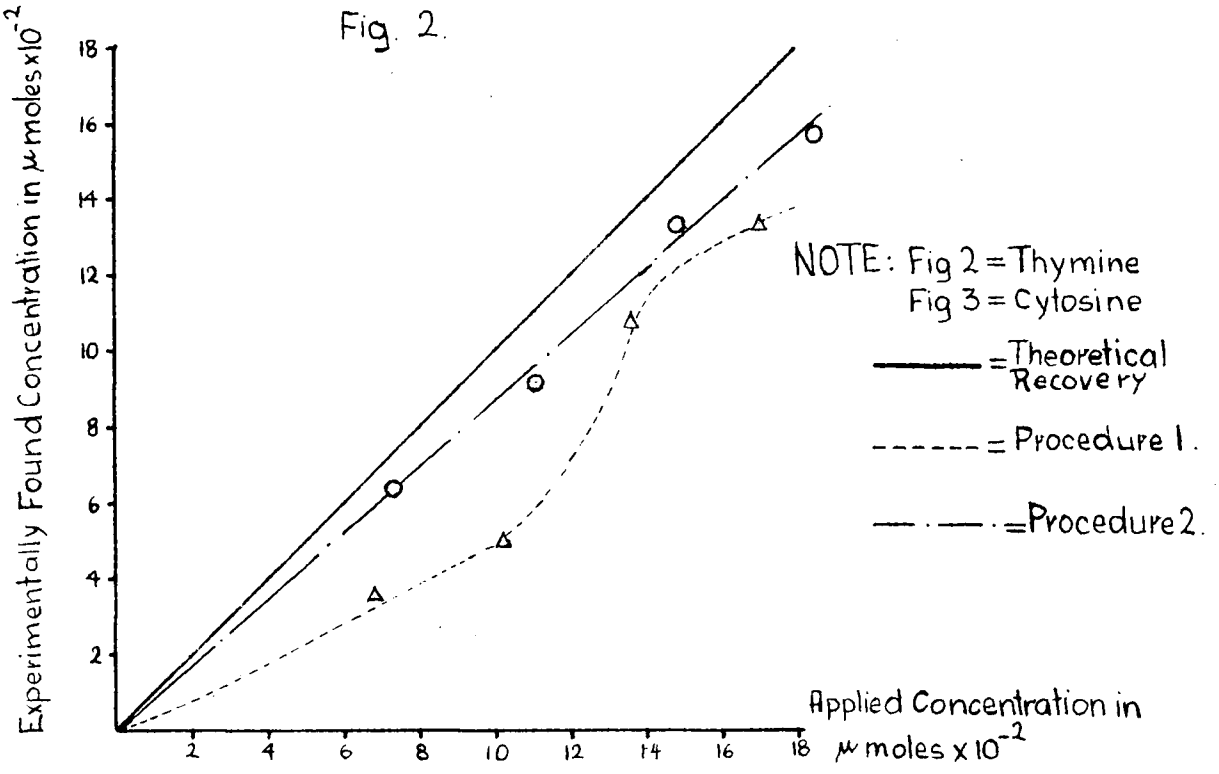


Fig. 3.

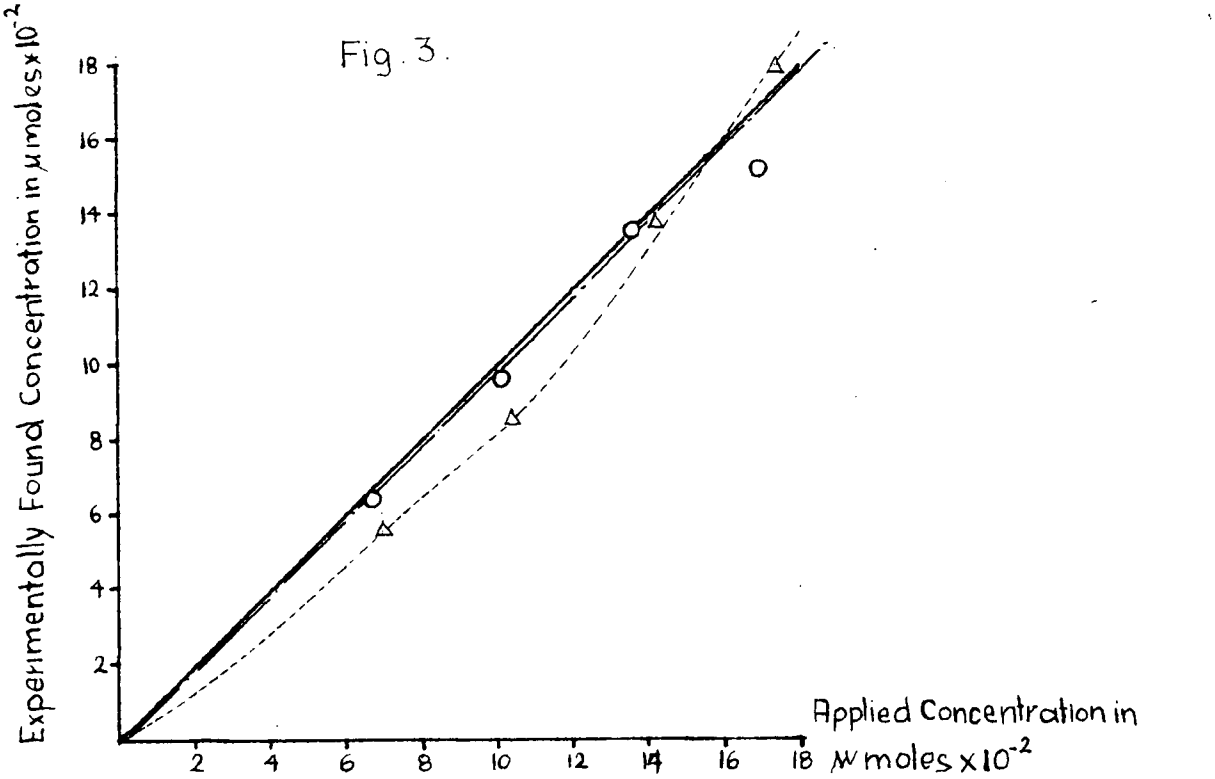


Fig. 2-3. The Comparison of Recoveries of Thymine and Cytosine from Paper Chromatograms Using Procedure 1 and 2 for Elution.

Fig. 4.

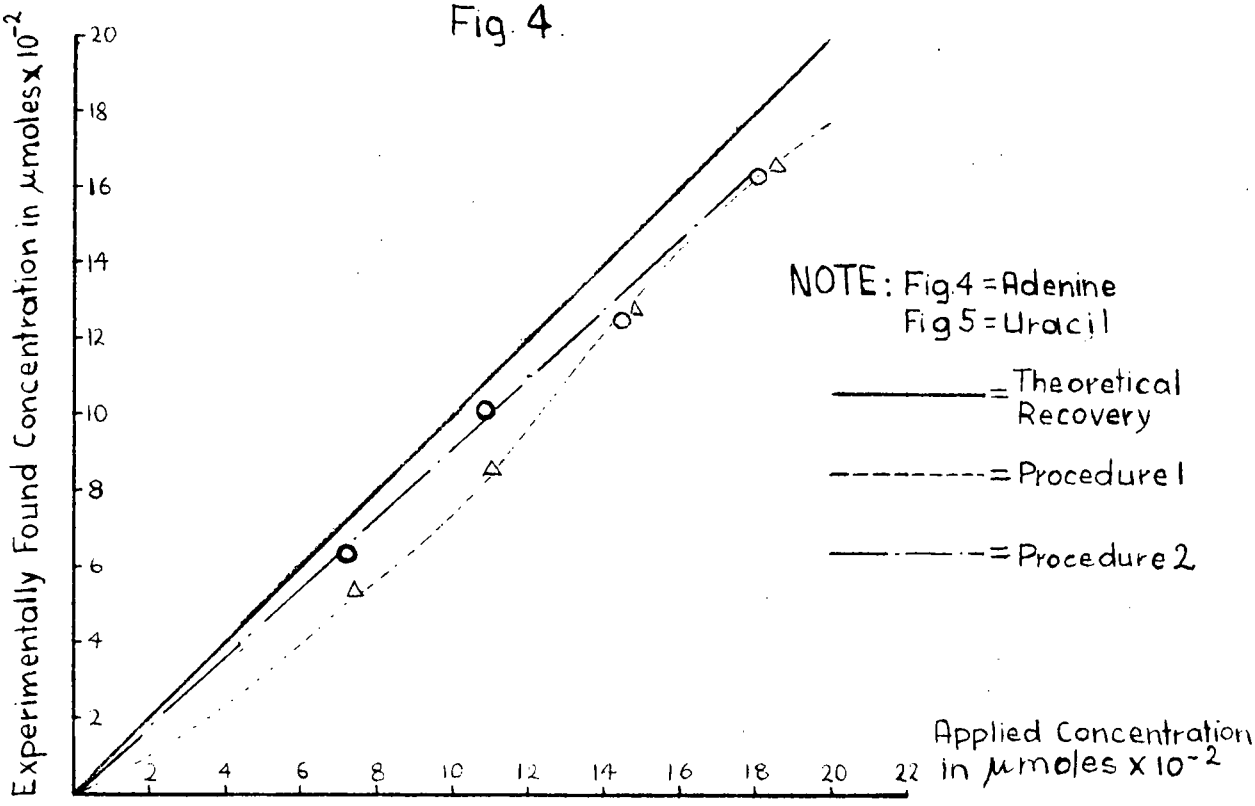


Fig. 5.

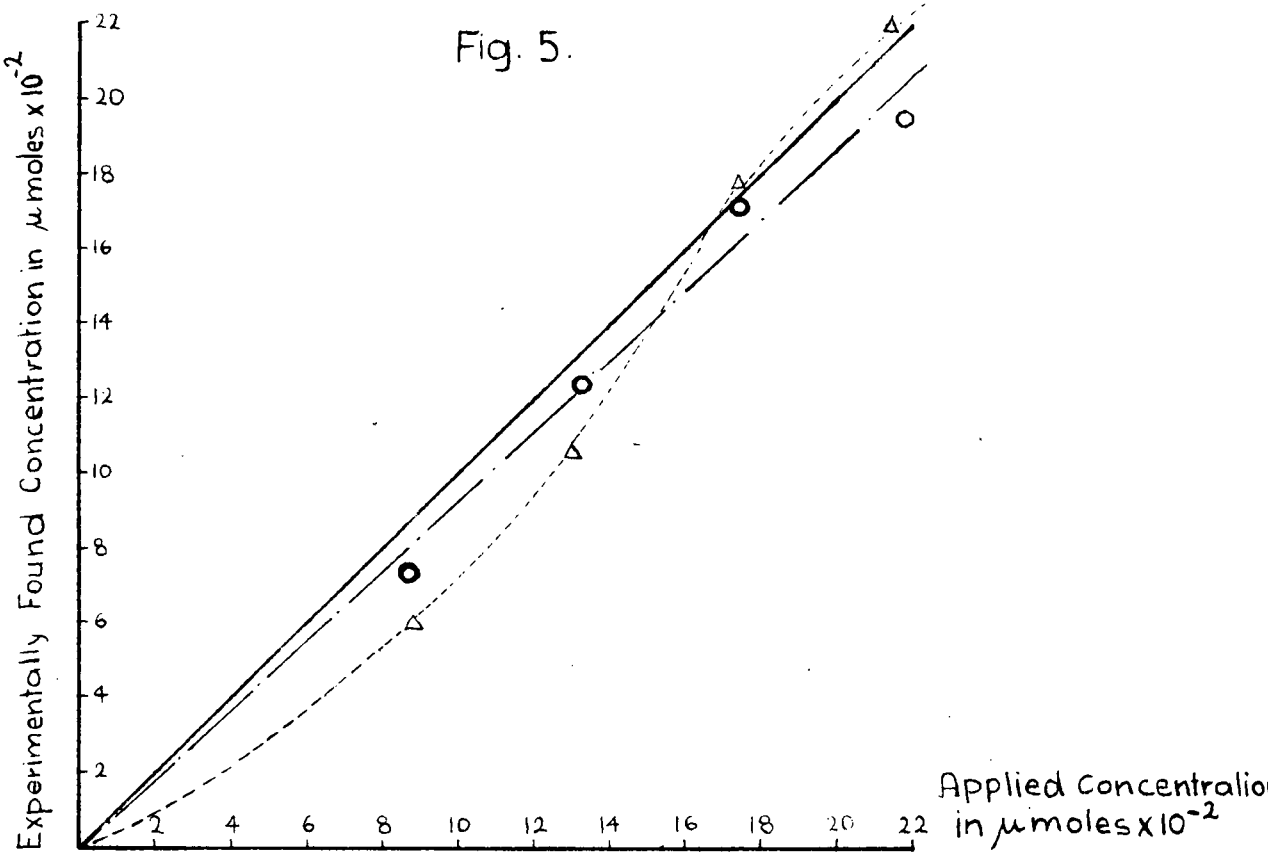


Fig: 4 and 5. The Comparison of Recoveries of Adenine and Uracil from Paper Chromatograms Using Procedure 1 and 2 for Elution.

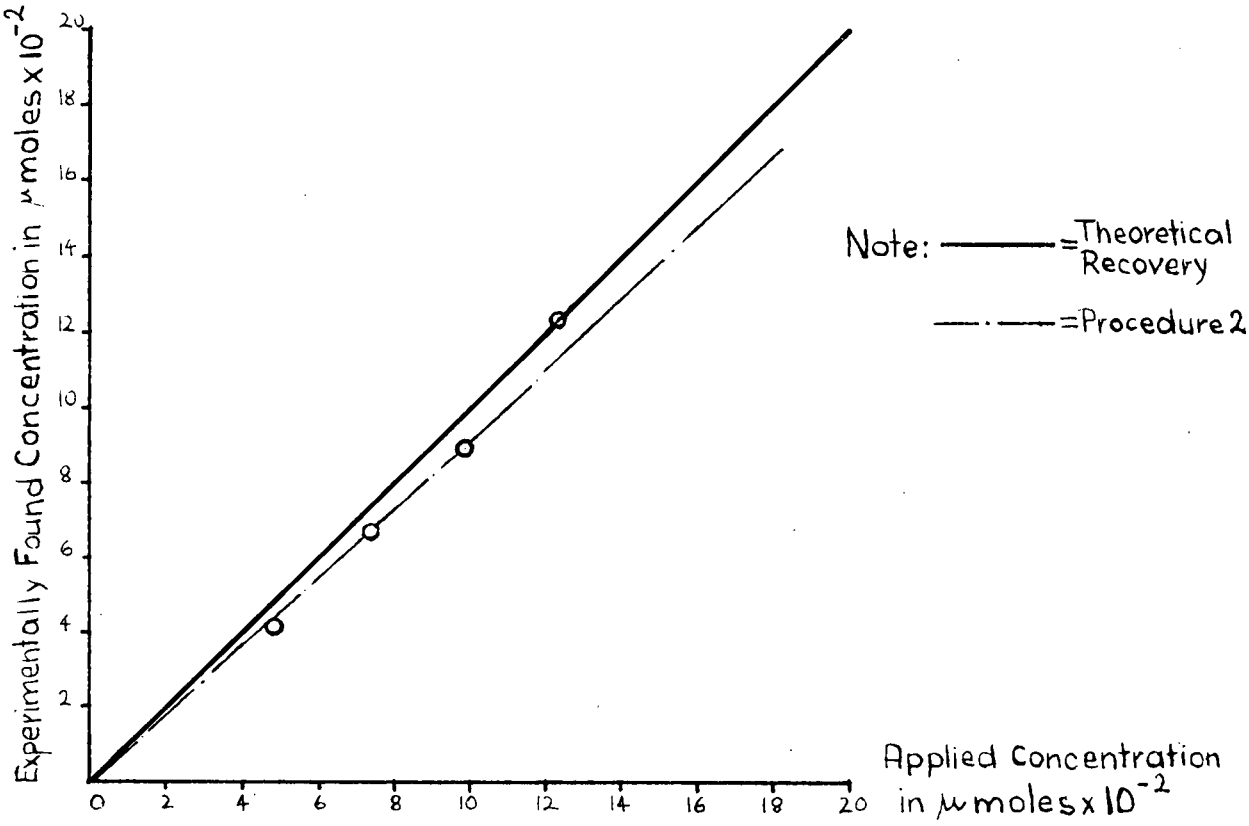
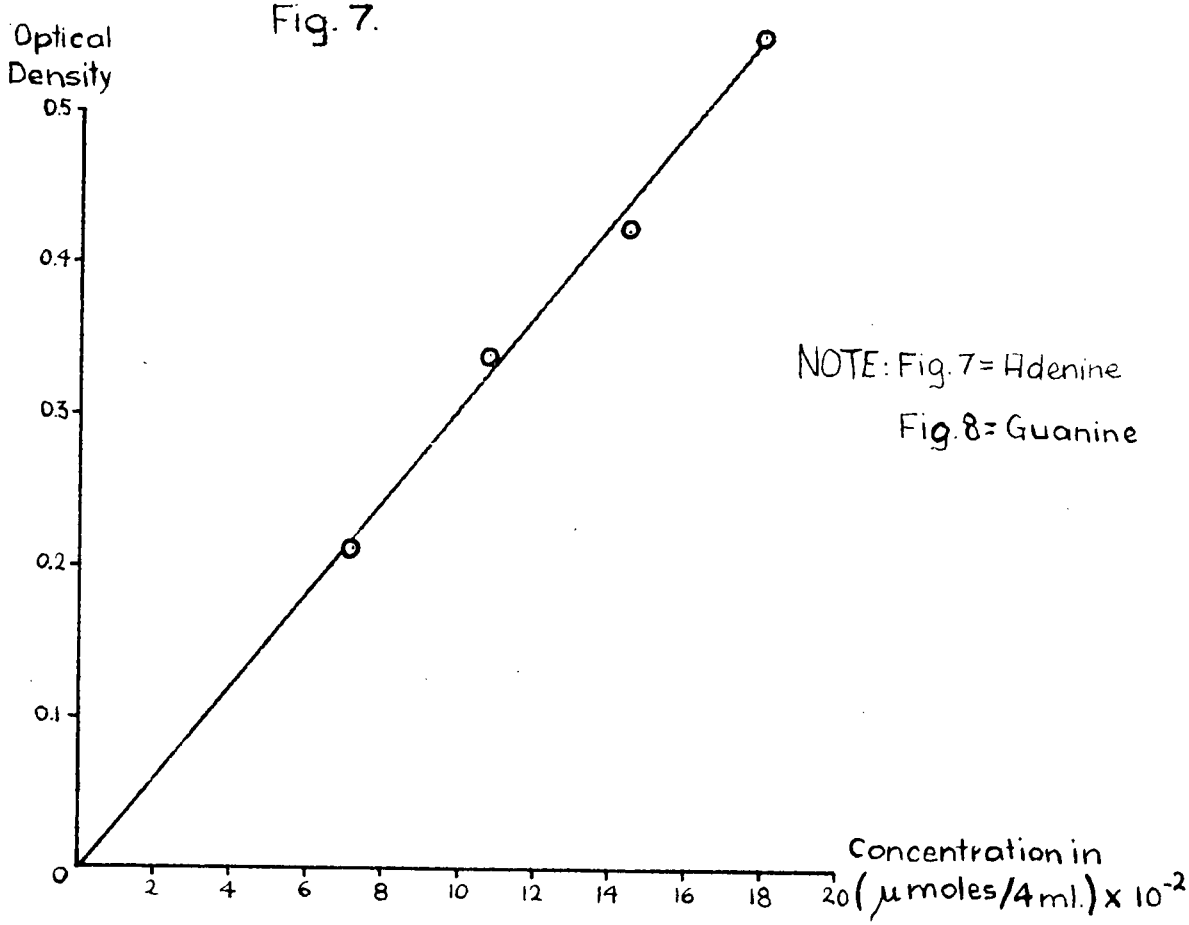


Fig. 6. The Comparison of Recoveries of Guanine from Paper Chromatograms Using Procedure 2 for Eluation.

Fig. 7.



NOTE: Fig. 7 = Adenine
Fig. 8 = Guanine

Fig. 8.

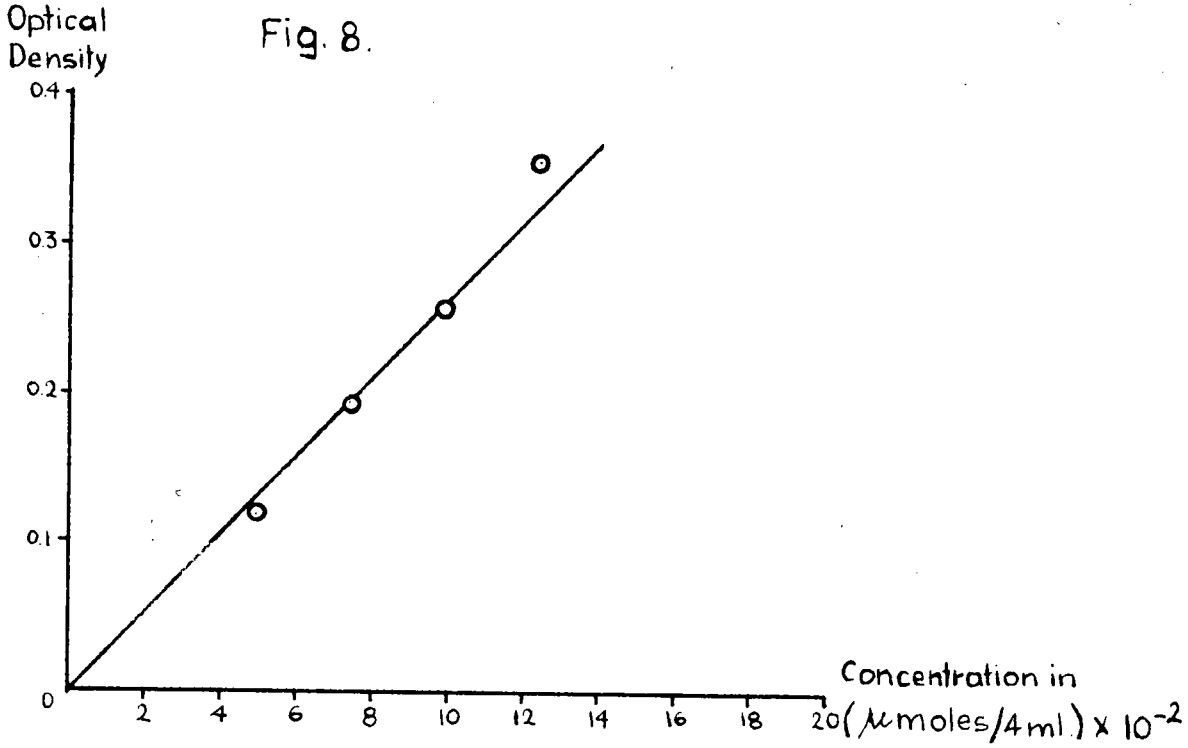
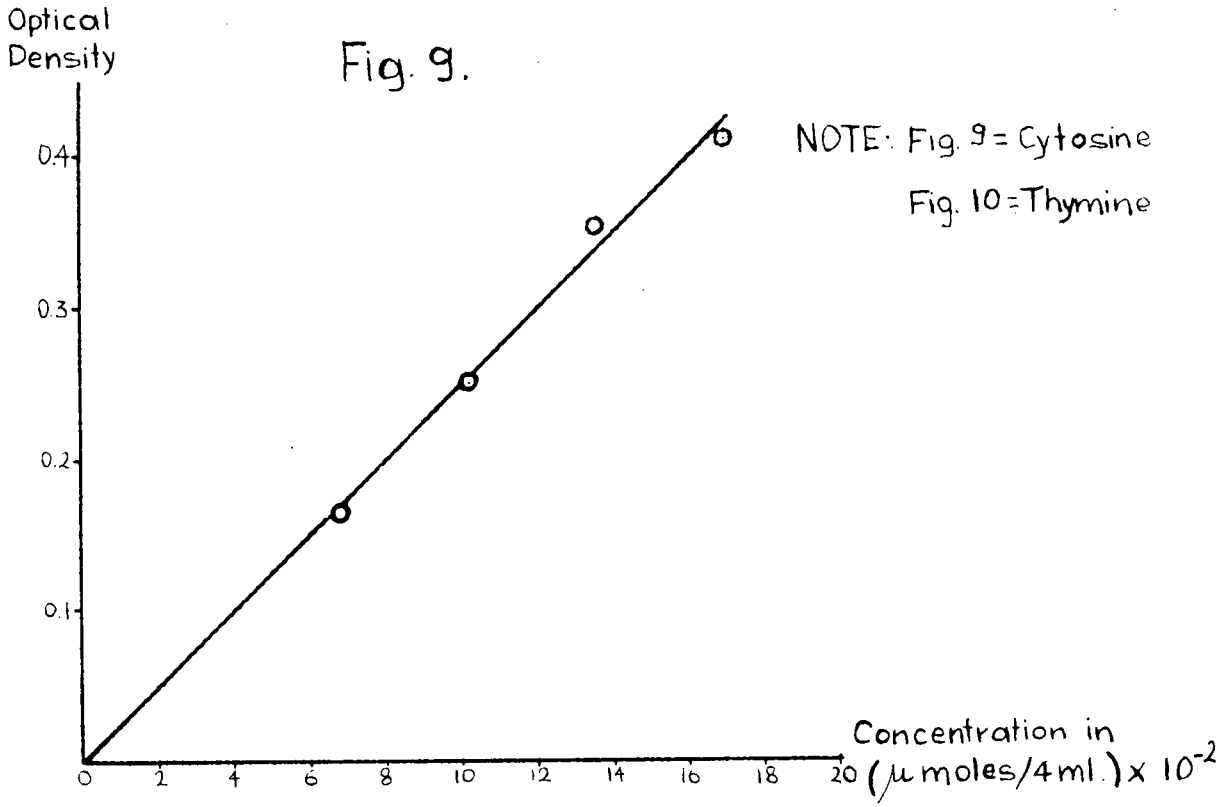


Fig. 7 and 8. Calibration Curve of Adenine and Guanine Using Procedure 2.



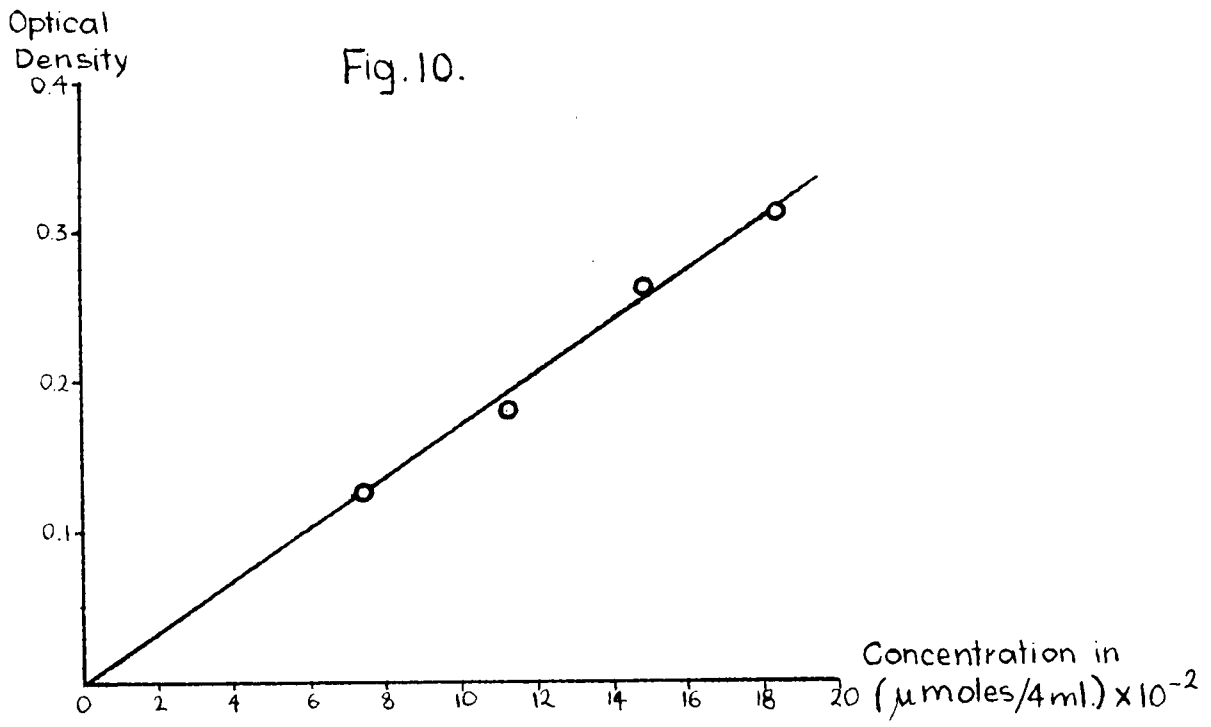


Fig. 9 and 10. Calibration Curve of Cytosine and Thymine Using Procedure 2.

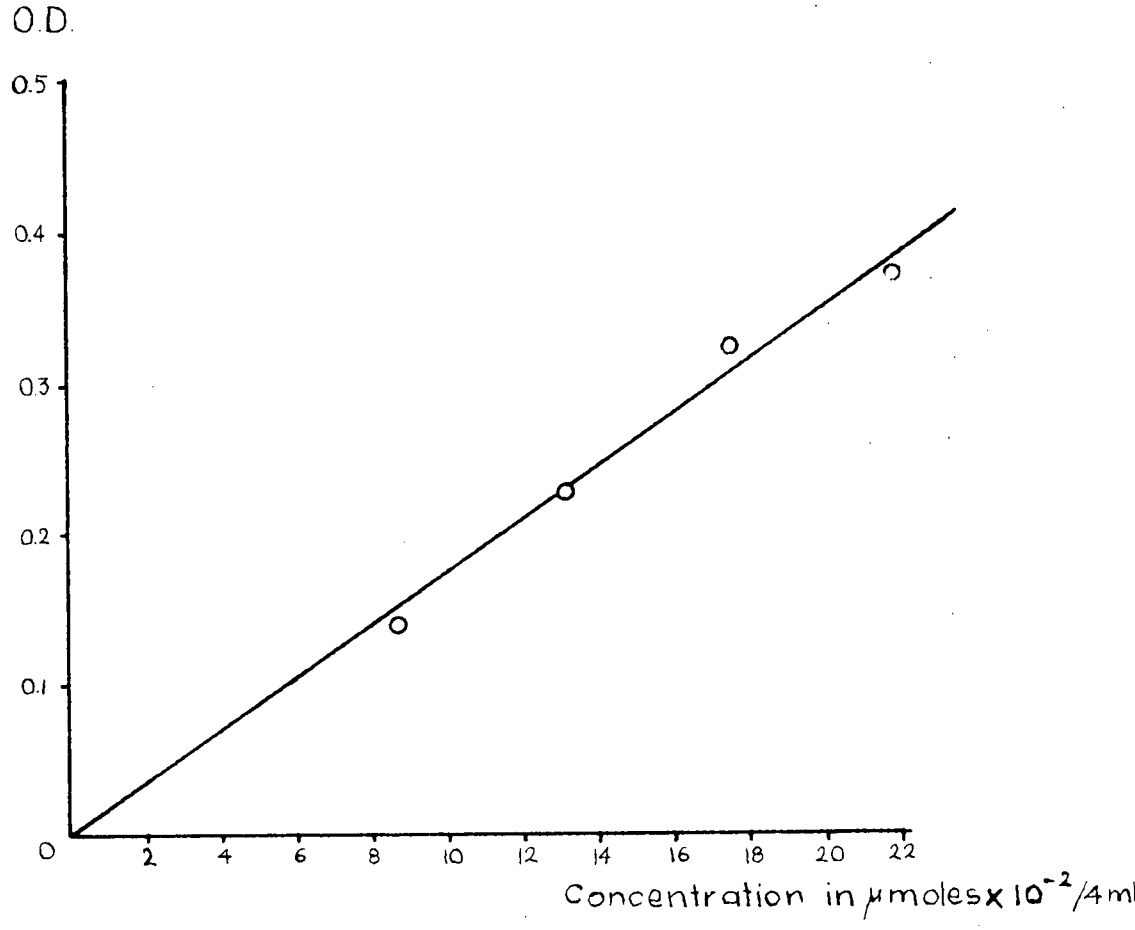


Fig. 11 Calibration Curve of Uracil Using Procedure 2.

except of thymine where recoveries averaged 86%.

3. This method proved to be very satisfactory provided that both paper and the glass wool used during the procedure were purified as described above. By these purification procedures blank readings were reduced considerably and did not obscure the evaluation of unknowns, even when working in the very low concentration range (4 - 6 x 10^{-2} μM/4 ml.)

4. The procedure was time saving and more effective as compared with procedure 1.

The Detection of Amino Acid Contamination of DNA Hydrolysates by Paper Chromatography.

Reagents:

A. Amino acid standards: Glycine, Alanine, Serine, Cysteine, Cystine, Threonine, Methionine, Valine, Leucine, iso-Leucine, Aspartic acid, Glutamic acid, Lysine, Arginine, Phenylalanine, Tyrosine, Tryptophan, Proline and Histidine. (Nutritional Biochemicals Corp.)

B. Butanol-acetic acid solvent.

To 500 ml. of a freshly shaken mixture of equal volumes of water and n-butanol is added 60 ml. of glacial acetic acid. After the layers separated the upper layer is used as the moving phase. An aliquot of the lower layer (25 - 100 ml.) is placed in the chromatogram chamber.

C. Ninhydrin reagent.

0.25% w/v Ninhydrin in acetone.

Procedure:

The formic acid hydrolysates of DNA samples were applied on large Whatman No. 1 paper sheets (58 x 48 cm). The chromatograms were run for about 24 hours, then air dried and sprayed with ninhydrin reagent and heated at 65°C for 30 minutes. The spots were identified with the aid of standard amino acids, run at the same time and on the same paper. The bands were outlined in pencil as fading of the colour took place after few days.

The Physical Characterization of DNA

1. The Determination of ϵ(p) values of DNA Preparations.

Reagents:

0.02 M sodium chloride solution.

1.169 g. sodium chloride/1000 ml. distilled water.

Procedure:

Accurately weighed DNA samples (about 1 mg.) were transferred into a 25 ml. volumetric flask. The samples were dissolved in 0.02 M sodium chloride solution, by allowing them to stand overnight at 0°C. When all the DNA was dissolved,

the volume of solution was made up to exactly 25 ml. The ultraviolet absorption spectra of the solutions were then recorded with a Beckman D.K. 2 spectrophotometer. From the optical density value at maximum absorption (usually around 257.5 mμ) the ϵ(p) value of the sample was calculated according to equation:

$$\epsilon(p) = \frac{\text{O D. at max.}\lambda}{C \times d} = \frac{\text{O D} \times 30.98}{\text{P cc in g./l} \times 1}$$

where ϵ(p) = the atomic extinction coefficient with respect to phosphorous.

O D = Optical Density of the solution

C = Phosphorous concentration of the solution in moles/liter

d = Internal cell lenght in cm.

2. <u>The Determination of Intrinsic Viscosity of DNA Preparations</u>

<u>Reagent</u>:

0.2 M sodium chloride solution

11.69 g. sodium chloride/1000 ml. distilled water.

<u>Apparatus</u>:

Ostwald type of capillary viscometer.

<u>Procedure:</u>

Accurately weighed DNA samples were transferred into a 25 ml. volumetric flask, and dissolved in 0.2 M sodium chloride

solution by allowing them to stand overnight at 0°C and by occasional inversion of the volumetric flasks at room temperature for 4 -5 hours. When the contents of the flasks seemed to be clear, the solutions were centrifuged at 2000 RPM for 5 minutes. The clear supernatant was used as a stock solution. The stock solution and three different dilutions of it were used for viscosimetric measurements. The flow time of these solutions were determined as follows:

The clear and dry viscometer was clamped vertically in the thermostat bath (25°C) in such a position that it could be viewed easily, and 4 ml. of the solution was added from a pipette. A dust free rubber tube loosely plugged with cotton was attached to the smaller tube and the liquid was drawn up into the enlarged bulb and above the upper mark. The liquid was then allowed to flow down through the capillary and the stop watch was started when the meniscus passed the upper mark and stopped when it passed the lower mark. Two or three check determinations on the time of outflow were made. The flow time for the solvent (0.2 M sodium chloride) was similarly determined. Since it was found that the densities of the dilute DNA solutions were not significantly different from that of the solvent, the density terms were not used for the calculation of relative viscosities.

The relative viscosities $\frac{\eta}{\eta_0}$ were calculated from equation.

$$\frac{\eta}{\eta_0} = \frac{d \times t}{d_0 \times t_0} \qquad \text{equ. (5)}$$

where η . η_0 = viscosities of solution and solvent respectively

because $d \simeq d_0$

t . t_0 = time of outflow of solution and solvent respectively

$$\frac{\eta}{\eta_0} = \frac{t}{t_0}$$

d . d_0 = densities of solution and solvent respectively

The intrinsic viscosities were determined graphically as described on page 16 , and calculated from the following equation (6):

$$[\eta] = \frac{\alpha [\eta]_2 - [\eta]_1}{\alpha - 1} \qquad \text{equ. (6)}$$

where $\alpha = \frac{C_1}{C_2}$

$[\eta]_1$ = viscosity at concentration C_1

$[\eta]_2$ = viscosity at concentration C_2

RESULTS AND DISCUSSION

In defining the properties of the DNA of a given tissue it is important that the isolation be as quantitative as possible, that degradation and impurities be reduced to minimum. Losses in preparation might yield a product whose properties are not representative of the whole, while degradation by such agents as acids, alkali, enzymes and heat might destroy some of the unique properties that would distinguish that particular substance. The presence of impurities in the isolated DNA would also change its physical-chemical structure and composition.

Tables VII and VIII summarize the yields of DNA from liver and small intestinal mucosa of rat obtained by the five different procedures used during this study. On inspection the data on Tables VII and VIII one can conclude:

1. The yield of DNA was in all cases higher from intestinal mucosa, than from liver. This fact is in accordance with the findings that the nuclear/cytoplasmic ratio is much higher in intestinal mucosa than in liver. (38)

2. In both tissues the highest yields were obtained by the detergent method (20). It is now almost generally accepted that the procedure of Kay et al. gives the highest yield among other methods (38, 73).

3. In case of small intestinal mucosa somewhat similar yields were obtained by the following three procedures

TABLE VII

Yields of DNA from liver of Rat Isolated by Different Methods.

Method of preparation	Sample No.	Yield of DNA in mg/10 g of fresh tissue	Average yield of DNA in mg/10 g of fresh tissue
Procedure I. Emanuel and Chaikoff (29)	A-1	4.9	
	A-2	5.7	
"	A-3	7.6	
"	A-4	6.-	5.7
"	A-5	6.5	
"	A-6	5.-	
"	A-7	5.2	
"	A-11	4.5	
Procedure II. Detergent method of Kay et al (20)	A-16	23.9	
	A-17	24.5	24.2
Procedure III. Emanuel and Chaikoff + detergent method.	A-13	7.-	
	A-21	13.-	10.-
Procedure IV. sonication method(32)	C-3	15.-	
Procedure V. hot 10% NaCl extraction method (36,37)	B-3	5.-	

TABLE VIII

Yields of DNA from Small Intestinal Mucosa of Rat Isolated by Different Methods.

Method of preparation	Sample No.	Yield of DNA in mg/10 g of fresh tissue	Average yield of DNA in mg/10 g of fresh tissue
Procedure I. Emanuel and Chaikoff (29)	A-8	13.6	
"	A-9	10.-	13.9
	A-10	18.-	
Procedure II. detergent method of Kay et al (20)	A-15	82.2*	
"	A-18	65.8	65.8**
	A-20	65.9	
Procedure III. Emanuel and Chaikoff + detergent method	A-12	13.3	
"	A-14	10.-	12.2
	A-19	13.5	
Procedure IV.	C-1***	77.-	
sonication method (32)	C-2***	26.3	
Procedure IV. hot 10% NACl extraction method (36, 37)	B-1	13.3	
	B-2	12.6	12.9

*Note: Preparations A - 18 and A - 20 were deproteinized three times with detergent solution, preparation A - 15 only two times.

**The yield of preparation A - 15 was not used for calculations of average yield by procedure II.

***Preparation C - 1 was deproteinized by shaking with chloroform-amyl alcohol mixture five times and preparation C - 2was deproteinized by shaking with chloroform - amyl alcohol mixture seven times.

Emanuel and Chaikoff (Procedure I), the Emanuel and Chaikoff method combined with the detergent method of Kay et al. (Procedure III) and the hot 10% Sodium chloride extraction method (Procedure V). All of these procedures are characterized by a very considerable loss of DNA, taking the average DNA content of intestinal mucosa as 129 mg/10 g. of fresh tissue (38).

4. With liver tissue quite similar yields were obtained by Procedure I and V. Somewhat higher yields resulted by Procedure III. The best yields were obtained with Procedure II, especially if one considers the values given for liver tissue in ref. (38). In this reference the DNA content of rat liver is given as 24 mg./10 g. of fresh tissue. If one considers that appreciable losses might occur specially working with small amount of tissue, the yield of Procedure II. seems to be quite unrealistic. It is very unlikely that any biochemical isolation process should give 100% yield. Only one preparation was performed according to Procedure IV therefore no general conclusion can be formed about the efficiency of recovery of DNA from liver tissue by this method. The yield of 15 mg./10 g. fresh tissue seems to be quite promising comparing it that lower values of procedures I, III, and V.

Considering the possibilities, where the losses of DNA may occur during different isolation procedures, it is

not surprising that appreciable amount of DNAs were lost with Procedures I, and III, using both intestinal and liver tissues. It was expected, that Procedure I would give the lowest possible yield, because no such "controlled homogenization" was performed as described in the original publication (30). Emanuel and Chaikoff used a hydraulic homogenizer in their study for the controlled release of muclei from other cellular elements. These workers claimed that much higher yields of DNAs were obtained by using the hydraulic homogenizer (30). The amount of DNA obtained from 10 g. of fresh rat liver tissue was 22 mg. compared with that of 10 mg. using the Teflon homogenizer alone. The average yields in Table VII are even lower 5.7 mg./10 g. of tissue. The appreciable loss of DNA maybe due to the fact that considerable part of nuclei were damaged during the homogenization with the Teflon homogenizer and blending with the Servall Omni Mixer. Once nuclei were damaged and their nucleoprotein content released, they no longer were retained by the negatively charged diatomaceous earth. As a matter of fact Zamenhof (74) advises avoiding the use of any stainless steel dissintegrator because according to him, minute traces of rust (Fe ion) would cause rapid degradation, and high speed mixing can effect the breakage of macromolecules into shorter fragments.

However in this experiment it was necessary to use short blending periods (one minute) at low speed with the

Servall Omni Mixer specially for preparations of rat liver homogenate. If the Teflon homogenizer were used alone incomplete homogenization were resulted and this prolonged the time required for isolation of nuclei, because the diatomaceous earth was obstructed by cellular material and the filtration rate was thereby slowed down. Another effect of the incomplete homogenization would be the contamination of DNA with cytoplasmic RNA and other extraneous non-nuclear materials.

It was found quite surprising that Procedure V did not give higher yields. Usually quite large amounts of nucleic acids were precipitated first, but most of the DNA seemed to be lost during the several purification processes. The combination of Procedures I and II did not show any appreciable improvement of yields in case of intestinal preparations; some increase was found with liver tissues.

In the preparation of biochemical substances it is usually necessary to make a choice between a high yield and a pure, high quality product. Pure quality may result because of change in the natural structure of the material caused by some drastic treatment during the preparation. If on the other hand a mild method is used, and a large amount of substance is obtained, this may contain considerable amount of impurity.

In order to conclude which procedure gives highly

polymerized pure product the properties of the different DNA samples were compared by physical-and chemical means.

Nitrogen and Phosphorous Content of DNA Preparations.

The nitrogen and phosphorous contents and the N/P ratios are shown in Table IX. Before making any conclusions about the results demonstrated in Table IX it has to be mentioned that the analytical data obtained for N content of DNA samples are somewhat doubtful and have to be interpreted very cautiously. Four DNA preparations were sent to Dr. Manser who performed nitrogen analyses according to the combustion method of Dumas. Table X compares the percentage of nitrogen in DNA samples determined by the combustion and the micro-Kjeldal procedures. It can be seen that the micro-Kjeldal determination gives considerably lower values for N content. This finding is in accordance with Chargaff's opinion (38) that the result of the Kjeldal determination is lower than that of Dumas'.

However this concept cannot be accepted entirely, because very good agreement was found between the theoretical and experimental N values of several standards used for testing the reliability of Kjeldal procedure. These findings are demonstrated in Table 1 page 29 . (experimental part).

In comparing the average nitrogen and phosphorous contents of DNA samples shown in Table IX the following general

TABLE IX

The Nitrogen and Phosphorous Contents of DNA Preparations Isolated by Different Procedures.

Method of Preparation	Source of tissue	Sample No.	N%	P%	Atomic N/P	N/P
Proc.I(29)	Liver	A-3	10.23	4.99	4.53	2.05
"	"	A-4	10.64	5.25	4.48	2.03
"	"	A-5	11.08	5.73	4.28	1.93
"	"	A-6	12.85	5.61	5.07	2.4
"	"	A-7	12.63	5.67	4.93	2.23
"	"	A-17	12.64	6.04	4.63	2.09
			avg. 11.67	avg. 5.54		avg. 2.12
Proc.II(20)	Liver	A-16	10.32	6.45	3.54	1.6
"	"	A-17	11.7	6.73	3.84	1.74
			avg. 11.01	avg. 6.59		avg. 1.67
Proc.III	"	A-13	12.35	6.8	4.02	1.82
"	"	A-21	12.61	8.37	3.33	1.51
			avg. 12.48	avg. 7.58		avg. 1.66
Proc.IV(32)	"	C-3	12.15	8.56	3.14	1.42
Proc.V(36,37)	"	B-3	13.77	8.36	3.64	1.65
Proc.I	Intestinal mucosa	A-8	13.28	7.47	3.93	1.78
"	"	A-9	12.92	6.28	4.57	2.07
"	"	A-10	11.92	6.73	3.92	1.77
			avg. 12.7	avg. 6.82		avg. 1.87
Proc.II	"	A-15	11.78	6.2	4.2	1.9
"	"	A-18	11.23	6.6	3.76	1.7
"	"	A-20 ×	9.76	5.66		1.73
			avg. 11.5	avg. 6.4		avg. 1.77
Proc.III	"	A-12	12.24	7.39	3.66	1.66
"	"	A-14	12.69	7.98	3.52	1.59
"	"	A-19	11.61	7.55	3.4	1.54
			avg. 12.18	avg. 7.64		avg. 1.59
Proc.IV	"	C-1	8.68	6.22	3.09	1.4
"	"	C-2	11.38	7.32	3.44	1.56
Proc.V	"	B-1	15.7	9.49	3.66	1.65
"	"	B-2	15.15	8.9	3.76	1.7
			avg. 15.42	avg. 9.19		avg. 1.67

Note: × Preparation A-20 was not used for the calculation of average values.

TABLE X

The Comparison of the Nitrogen Contents of DNA Preparations Determined by the Dumas and Kjeldahl Procedures.

Sample No.	% N by Dumas' method	%N by Kjeldahl's method
A - 13	13.24	12.35
A - 15	13.80	11.78
A - 17	13.64	11.70
A - 18	12.65	11.23

observations are noticeable:

1. Procedure I gives the highest N/P rations for both liver and intestinal preparations. As it was mentioned in the introduction N/P values higher than 1.65 would indicate protein contamination in DNA. Procedure I uses salt saturation for breaking the linkages between protein and DNA. The high N/P values, 2.36 for liver and 1.87 for intestinal DNAs would indicate that this deproteinization procedure is not completely satisfactory.

Frick (73) in his critical study discussed the possibility that separation of protein and nucleic acid by saturated sodium chloride may be dependent upon autolysis which has already been carried out by cellular enzymes. At low temperatures (0°C) these enzymes have little effect. If however, the nucleoprotein is allowed to stand in contact with the extraction liquid for a longer time and at a higher temperature, the protein and the nucleic acid can be more easily separated. In the case of Procedure I the nucleoprotein is in contact with the saturated sodium chloride solution only for a short time and at a low temperature, therefore, the chance for autolysis by enzymes is greatly reduced.

2. Procedures II, III, and V give values which are very close to the theorethical N/P ratio (1.65). However, this

does not necessary mean that the samples are completely free from protein contamination, especially if one considers the low N values obtained by the micro-Kjeldal determination. For instance if the N values of samples A-13, A-15, A-17, A-18 given by the Dumas' procedure were used for the calculation of N/P ratios, much higher values would be obtained.

3. DNA preparations of liver and intestinal mucosa isolated by Procedure IV have surprisingly low N/P ratios. These values are lower than the theorethical one. Two explanations may be possible: a, The percentage of nitrogen found by the micro Kjeldal procedure is low. b, The presence of a phosphorous containing non-nucleic acid impurity in DNA samples.

4. The nitrogen and phosphorous contents isolated by Procedures I, II, III, and IV are in all cases lower than the generally accepted values (38). This may be due to the presence of some inert impurity (polysaccharides, inorganic salts) in these samples.

5. The best analytical values for phosphorous and nitrogen contents of liver and intestinal DNAs were obtained by using Procedure V. This method is a very drastic one, and surely cleaves off impurities from DNA preparations.

In summary, the presence of protein and other impurities in DNA samples were indicated in some cases by the high N/P ratios and the generally low values of phosphorous and

nitrogen contents.

The Detection of Protein Impurities in DNA Preparations by Paper Chromatography

To get some better and more definite information about protein impurities in DNA samples, some qualitative amino acid demonstrations were performed in DNA hydrolyzates, as described on page 52 (experimental part) Table XI shows the results of these investigations. Unfortunately Table XI does not give a complete picture about the composition of protein impurities in all DNA preparations. However some generalizations can be made on the basis of experimental findings in Table XI.

1. The presence of definite protein contamination was shown in a number of DNA preparations obtained from liver and intestinal mucosa using Procedures I, II, III. Thus neither salt saturation nor detergent treatment could effect the complete removal of protein from DNA preparations. According to Kit (86) the dissotiation of DNA from lipoprotein by anionic detergents is slow without the hydrolytic assistance of either mitochondrial deoxyribonuclease or heat. In Procedures II and III the action of both heat and deoxyribonuclease were excluded, because all steps were performed in cold and in the presence of the enzyme inhibitor, Versene.

2. All DNA preparations having protein contamination yield aspartic acid and leucine on hydrolysis. The presence of cysteine

or cystine and glutamic acid were also demonstrated in the majority of cases. The fact that acidic amino acids predominate in the hydrolysates of DNAs support the findings of Butler (75) that the contaminating protein in DNA preparations is not a basic histone.

3. Glycine was found in all DNA samples tested for amino acids. This amino acid may arise from the destruction of some purine bases during hydrolysis. The results in Table XI have to be interpreted very cautiously because only one dimension chromatographic technique was used for separation of amino acids from DNA hydrolysates. However in generally one can conclude that majority of DNA samples tested were contaminated by protein.

The Nitrogenous Base Composition of DNA Preparations.

The results of a comparative study of the composition of many preparations show that the compsoition of DNA is characteristic of these species from which it is derived, but within the limits of present analytical methods the DNA of different tissues of the same species have the same overall composition (38). Chargaff points out however, that this result does not rule out the possibility that there may be differences in the sequence of arrangement of the nucleotides among nucleates of the same overall compositon.

A remarkable correlation emerges from the analyses

TABLE XI

Comparison of Amino Acid Contents of DNA Hydrolysates.

Method of Prep.	Source of tissue	Sample No.	Alanine	Aspart acid	Asparagine	Cysteine or Cystine	Clutamic acid	Glycine	Leucine	Methi-onine	Valine
Proc. II(20)	Liver	A-16	-	-	-	-	+	-	-	-	-
"	"	A-17	+	+	+	+	+	+	+	-	+
Proc. III(29, 20)	"	A-13	+	+	-	+	+	+	+	-	-
Proc. I(29)	Intestinal mucosa	A-9	-	+	-	+	-	+	+	-	-
Proc. II(20)	"	A-15	+	+	-	+	+	+	+	+	-
"	"	A-18	+	+	+	+	+	+	+	-	+
Proc. III (20,29)	"	A-12	+	+	+	+	+	+	+	-	-

of a large number of different DNAs (53, 76-78): the adenine/thymine and guanine/cytosine mole ratios are, in the great majority of cases, equal to one, within the error of analyses. Much of the analytical data on viral (79) and bacterial (80) DNAs maintain this correlation.

When the composition of many specimens of DNA from different cellular sources is compared, a very striking feature emerges, that two principal groups can be distinguised, namely the adenine-thymine type (A.,T.) in which adenine and thymine predominate, and the guanine-cytosine type (G., C.) in which guanine and cytosine are the major constituents (38). All total DNA preparations from animal sources described up to this time, belong to the A.T. type. The G.C. type has been encountered in several micro-organisms and viruses.

The nitrogenous base composition of several DNA preparations are shown in Table XII. Unfortunately not all DNA samples isolated were characterized by this method. A few observations can be made on inspections the data in Table XII:

1. In case of liver preparations Procedure I yielded DNA samples, whose base composition was in agreement with those reported in literature. (53, 76-78). The DNA of rat liver is A.T. type as indicated by the ratios of adenine + thymine/guanine + cytosine. The ratios of adenine/thymine and guanine/cytosine are close to unity.

DNA samples A-16 and A-17 isolated by the detergent method were very heavily contaminated by RNA as indicated by the presence of uracil and by the preponderance of guanine and cytosine. Their adenine + thymine/guanine + cytosine ratios are less than unity, which is not characteristic for mammalian tissues. The presence of contaminating RNA in DNA preparations obtained by procedure II can be explained by the fact, that no previous washings of the tissue homogenates with 0.15 M sodium chloride solutions were performed, thus both RNA and DNA were extracted and precipitated from the tissues. The fractional precipitation of RNA with iso-propyl alcohol did not effect the complete separation of the two nucleic acids.

The preparations obtained by Procedure III were characterized by an adenine + thymine/ guanine+cytosine ratio greater than unity. However the numerical value of this ratio (1.13) is lower than those obtained for samples isolated by Procedure I (1.24-1.29). According to Chargaff (38) a good DNA preparation is characterized by adenine/thymine and guanine/ cytosine ratios of 1 $\pm$ 0.05. This requirement was not fulfilled with preparations A-13, and A-21 where the guanine/ cytosine ratios were greater than 1.05. This finding indicated a preponderance of guanine in the samples mentioned.

Procedure IV yielded a DNA preparation which showed the characteristic features of mammalian DNAs. However the

mole percent of guanine was also slightly higher than in samples A-1 and A-2. Considerable RNA contamination was found in sample C-3 indicated by the presence of 3.96 mole percent uracil.

2. The base compositions of DNAs of small intestinal mucosa isolated by Procedure II and III are generally characterized by the presence of heavy uracil contamination. This finding indicates a considerable amount of RNA impurity in the preparations. According to Chargaff (38) the analytical results on preparations, containing more than three percent of this contaminant, "command little confidence" and this was the case with regard to preparations A-15, A-18, A-14 and A-19. None of these preparations showed the regularities described by Chargaff(38).

Only sample A-20 yielded the characteristic features of pure DNA samples. No uracil contamination was found in this case. On comparing the base composition of this preparation with those of "good" DNA preparations of liver (A-2, A-1 and C-3), no significant difference can be observed, which is in agfeement with the findings reported in literature (38).

3. The general base recovery was quite low in almost all of the cases, which would indicate the presence of some inert impurity in DNA preparations.

TABLE XII

Distribution of Purines and Pyrimidines in DNA

Proportion in Moles of Nitrogeneous Constituents/100 g atoms of Phosphorous.

Method of prep.	Source of tissue	Sample No.	A	G	C	T	U	$\frac{A+T}{G+C}$	$\frac{A}{T}$	$\frac{G}{C}$	% Rec.
Proc. I. (29)	Liver	A-1	28.45	21.56	23.1	26.88	-	1.24	1.05	0.93	67.1
	"	A-2	28.27	21.23	22.53	27.98	-	1.29	1.01	0.94	74.9
Proc.II (20)	"	A-16	19.87	25.73	26.61	20.61	7.18	0.96	0.97	0.97	81.-
	"	A-17	28.35	25.57	20.66	17.70	7.72	0.99	1.6	1.24	71.3
Proc.III (29,20)	"	A-13	26.88	24.98	21.87	26.27	-	1.13	1.02	1.14	76.0
	"	A-21	26.74	26.55	20.38	26.31	-	1.13	1.01	1.3	74.3
Proc. IV (32)	"	C-3	27.95	22.01	19.13	26.97	3.96	1.33	1.04	1.15	75.5
Proc. II	int. mucosa	A-15	24.18	20.63	25.08	23.99	6.12	1.05	1.01	0.93	98.9
(20)	"	A-18	19.66	22.28	22.02	19.97	6.07	0.89	0.98	1.01	99.5
"	"	A-20	23.23	21.66	20.96	29.13	-	1.46	0.95	1.03	63.2
Proc.III (20,29)	"	A-14	23.17	24.2	21.84	30.78	-	1.17	0.77	1.1	85.6
	"	A-19	26.5	22.92	13.8	20.24	16.51	1.27	1.31	1.66	92.7

Note: The Data in table XII are average values obtained from duplicate analyses. The proportions of nitrogenous bases are corrected for hundred percent recovery. The following abbreviations were used: Adenine (A) Guanine (G) Cytosine(C) Thymine (T) Uracil (U).

It should be mentioned that all DNA hydrolysates were chromatographed in duplicate and eluted by the two different methods as described in experimental part page 39 . However, in most of the cases, unwashed paper was used for paper chromatography which greatly obscured the results, specially when very small amount of DNA samples were applied on paper chromatograms. To overcome these difficulties a few DNA samples were re-hydrolysed and chromatographed on washed papers and eluted according to Procedure 2 (Experimental part page 39). A significant increase in percentage recoveries were found with these samples as compared with the percentage recoveries using the elution method of Chargaff (38). These findings are summarized in Table XIII. The same results were obtained for the base composition of RNA contaminated samples by using Procedures 1 or 2 for elution the nitrogenous constituents from paper chromatograms.

The Characterization of DNA by Ultraviolet Absorption.

As it was mentioned in the introduction, a great deal of information concerning the intact macromulecular state of DNA can be gained by measuring the extinction of its solution in ultraviolet light. Both nucleic acids and their purine and pyrimidine derivatives absorb strongly ultraviolet light in the neighbourhood of 260 mμ. However, it was found that nucleic acids show anomalously low extinctions (81,82). This absorption anomaly probable denotes some considerable degree

TABLE XIII

The Difference between the % of Recovery of Nitrogenous Bases from DNA Hydrolysates Using the Elution Technique of Chargaff (38) and the Column Chromatographic Elution Method.

Sample No.	% Recovery by proc. 1*	% Recovery by proc. 2*	% Increase by using proc. II.
A-14	76.1	85.6	9.5
A-16	69.8	81.0	11.2
A-17	59.8	71.3	11.5
A-18	87.5	99.5	12.0
A-21	61.8	74.3	12.5
			aver. % 11.3 increase

of intermolecular organization. A direct correlation has been shown to exist between the number of hydrogen-bonds joining the bases of a pair of DNA chains and the magnitude of the "hyperchromic" effect (83). Rupture of the hydrogen bonds due to ionization of the basic groups due to thermal denaturation, or to transfer to a medium of low ionic strenght is associated with considerable enhancement of absorption in the ultraviolet, whereas a certain degree of depolymerization involving shortening of the chains, but without the rupture of interchain hydrogen bonds, appears not to give this effect. Kunitz (60) observed that depolymerization of DNA at pH 5. by deoxyribonuclease was accompanied by ultimate increase in absorption at 260 mμ of nearly 30%, and increases of similar order were reported by other investigators (62,82). The findings that high molecular weight DNA can undergo a denaturation change by the actions of heat, acid, alkali and deoxyribonuclease, and this change is manifested as about 33% increase of its ultraviolet absorption became the basis for estimation the degree of denaturation of DNA samples.

According to Chargaff et al. (63) the atomic extinction coefficients with respect to phosphorous $\epsilon(p)$ of DNA preparation is a good indication of its state of degradation. Table XIV summarizes these values for the different DNA preparations investigated during this study. The data in Table XIV show that:

1. Definitely high ($\epsilon_{(P)}$) values (above 7200) were obtained by using Procedure I, II and V. for preparing DNA from liver tissues. In the case of intestinal mucosa only Procedure V. yields DNA with an $\epsilon_{(P)}$ higher than 7200. According to Chargaff (38) an $\epsilon_{(P)}$ value higher than 7200 is considered as a sign of denaturation of DNA. It is not surprising that Procedure V. which involves hot 10% sodium choride extraction of tissue, and exposes nucleic acids to the action of alkali and acid, gives high $\epsilon_{(P)}$ values for both liver and intestinal DNA preparations.

2. The $\epsilon_{(P)}$ values of DNA samples from intestinal mucosa isolated by Procedures I., II., III. and IV. are lower, ranging from 6300 to 6900. Similarly lower values were obtained for liver DNAs using Procedure III.

3. For two DNA preparations, one obtained from liver and the other from intestinal mucosa, very low values of $\epsilon_{(P)}$ (5687, 4413 respectively) were found by using Procedure IV. However, sample C-2 isolated by the same Procedure and from intestinal mucosa, shows an $\epsilon_{(P)}$ value which is in very good agreement with the average value reported by Chargaff (38). Perhaps the reason for the above mentioned very low values, is, that those samples (C-1 and C-3) were not properly purified, and may be they contained some inert phosphorous containing material, which does not absorb ultraviolet light at the region of 260 mμ.

TABLE XIV

The Ultraviolet Extinction Coefficient of DNA Preparations Expressed as $\epsilon_{(P)}$ Values.

Method of Preparation	Source of Tissue	Sample Number	$\epsilon_{(P)}$ at 257mμ
Procedure I. (29)	Liver		
"	"	A-4	7553
"	"	A-5	7748
"	"	A-6	7540
			avg. 7613
Procedure II.(20)	"	A-16	7540
"	"	A-17	6997
			avg. 7268
Procedure III(29,20)	"	A-13	6128
"	"	A-21	6386
			avg. 6257
Procedure IV. (32)	"	C-3	5687
Procedure V. (36,37)	"	B-3	7570
Procedure I.	Intestinal mucosa	A-8	5948
"	"	A-9	6477
"	"	A-10	6560
			avg. 6328
Procedure II.	"	A-15	6789
"	"	A-18	7234
"	"	A-20	6737
			avg. 6920
Procedure III.	"	A-12	6536
"	"	A-14	6371
"	"	A-19	7646
			avg. 6851
Procedure IV.	"	C-1	4413
"	"	C-2	6624
Procedure V.	"	B-1	7533
"	"	B-2	7895
			avg. 7714

With the same reasoning the apparently good values obtained for intestinal samples using Procedures I, II, III, IV, and for liver samples Procedure III, cannot be taken as evidence for the intactness of the secondary structure of these preparations, especially if one considers the nitrogen and phosphorous contents of the samples. It has to be recalled (Table IX) that the percentages of nitrogen and phosphorous of DNA preparations obtained by all procedures except method V were much lower, than those reported in the literature (38) therefore the samples must contain some impurities.

Although it was observed by Chargaff et al. (84) that the extinction of nucleoprotein is not less than that of free nucleic acid, and the $\epsilon_{(P)}$ values remained close to 6500 the presence of some phosphorous containing impurity may obscure the $\epsilon_{(P)}$values in DNA preparations.

Keeping in mind the above discussed limitation of Chargaff's $\epsilon_{(P)}$value, it is now realized that a truer picture would have been gained about the state of denaturation of DNA samples by the application of the simple test described in reference (38) vol.I. page. 526. A similar and valuable procedure for the estimation of DNA was described by Schack (62).

The Viscosity of DNA Preparations.

Important information can be obtained about the size and shape of macromolecules by the application of simple viscosimetric measurements. Unfortunately only a limited number of samples could be characterized by this method in this study. Graphs 12 - 15 show the determination of $[\eta]$ of different DNA preparations by plotting $\frac{\eta sp}{c}$ versus concentration. Table XV summarizes the intrinsic viscosities of some DNA preparations. The molecular weights were estimated from equation (4) using the values determined experimentally. A few general statements can be made evaluating the data in Table XV:

1. All intrinsic viscosities were very low.
2. Highest intrinsic viscosity values were obtained with DNA preparations C-1, C-2, C-3. isolated by the sonication method of Zubay (32).
3. Because of the low $[\eta]$ values the molecular weights of DNAs calculated from equation (4) were very low.
4. No measurable viscosities were found for preparations of DNAs isolated by the hot 10% sodium chloride extraction method.

C.F. Thomas reports some $[\eta]$ values for DNAs isolated by the procedures of Schwander and Signer (12) and by the detergent method of Kay et al. (20). These values range from 48 to 57 dl./g. Reichmann et al. (85) performed also some

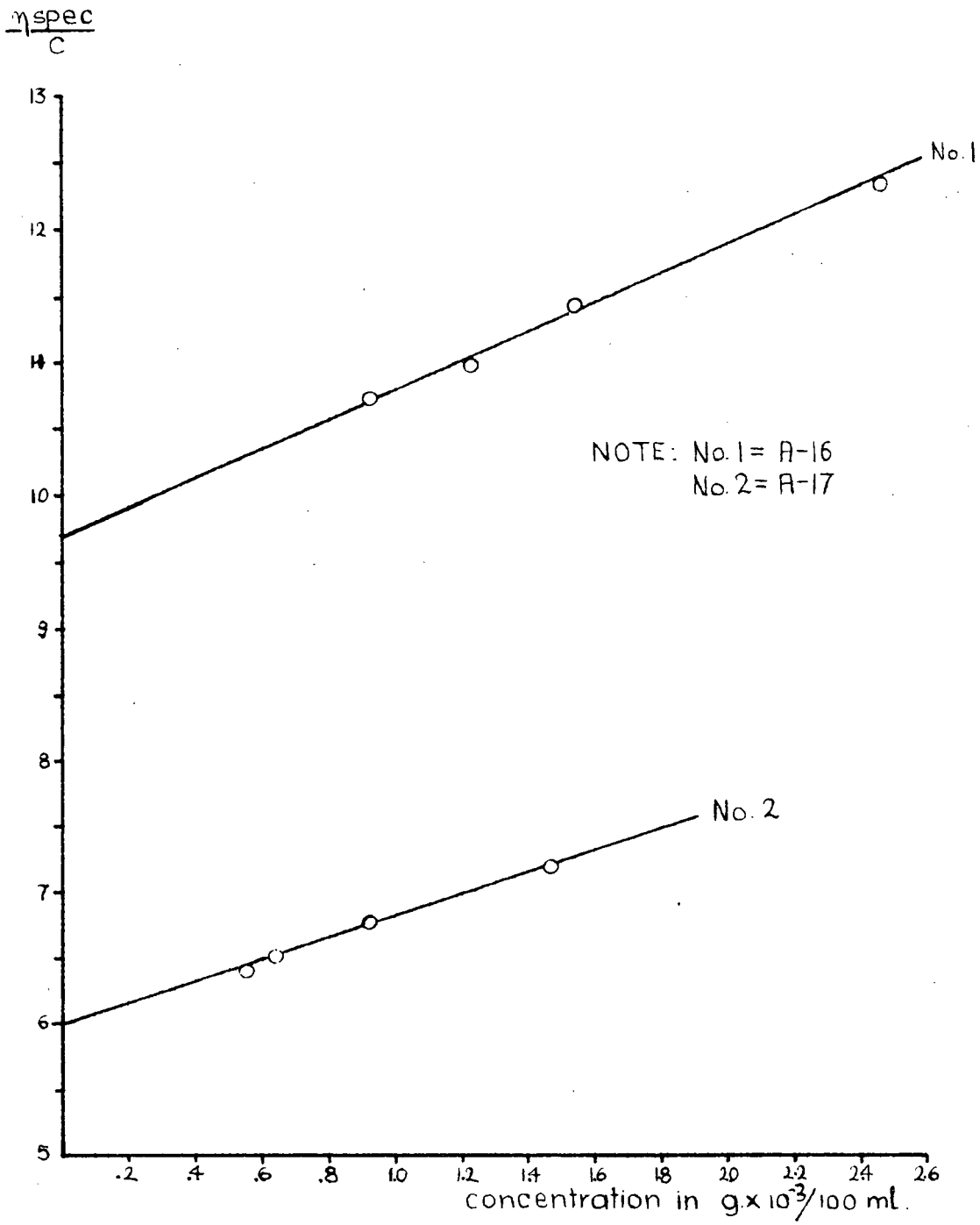


Fig. 12. Plot of the Reduced Viscosity ($\frac{\eta spec.}{c}$) Versus Concentration (g/100 ml.) of DNA Samples A-16 and A-17.

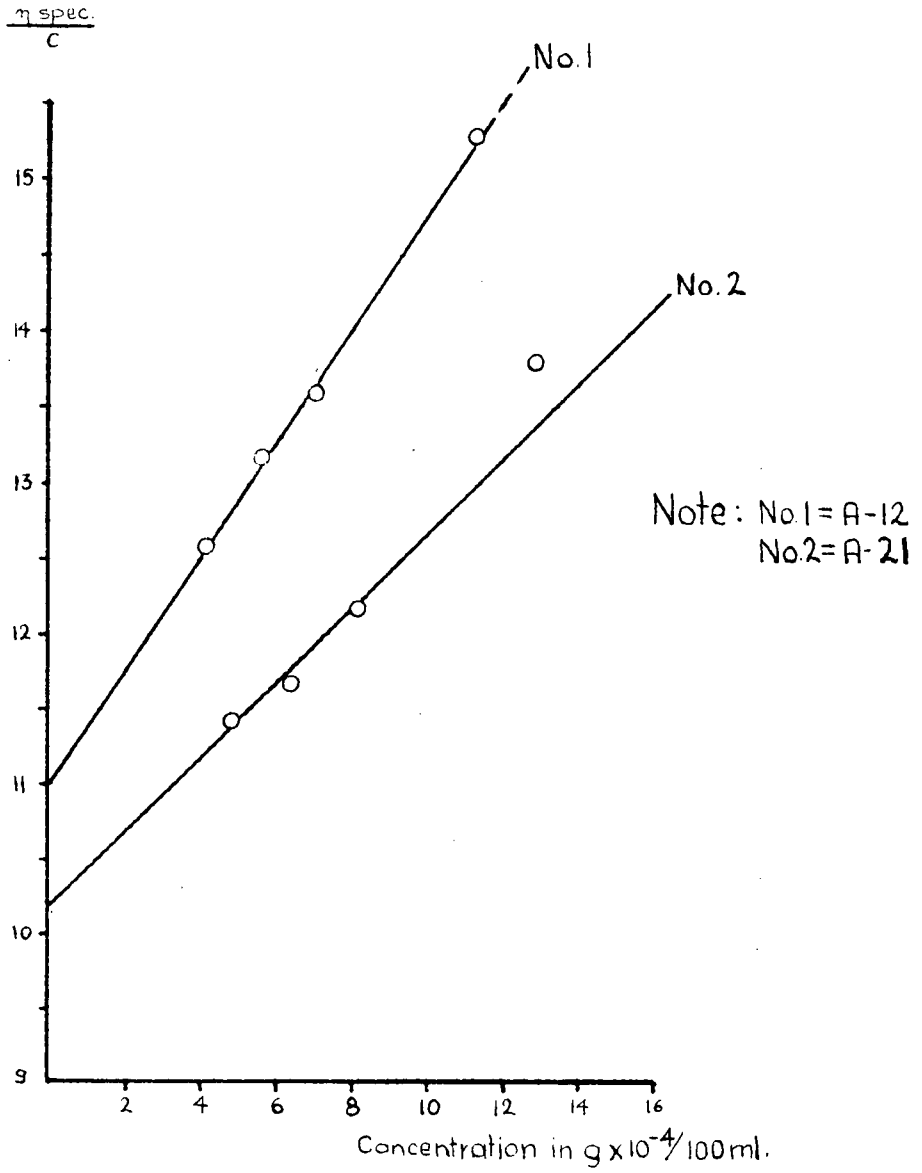


Fig. 13. Plot of the Reduced Viscosity ($\frac{\eta spec}{C}$) Versus Concentration (g/100 ml) of DNA Samples A-12 and A-21.

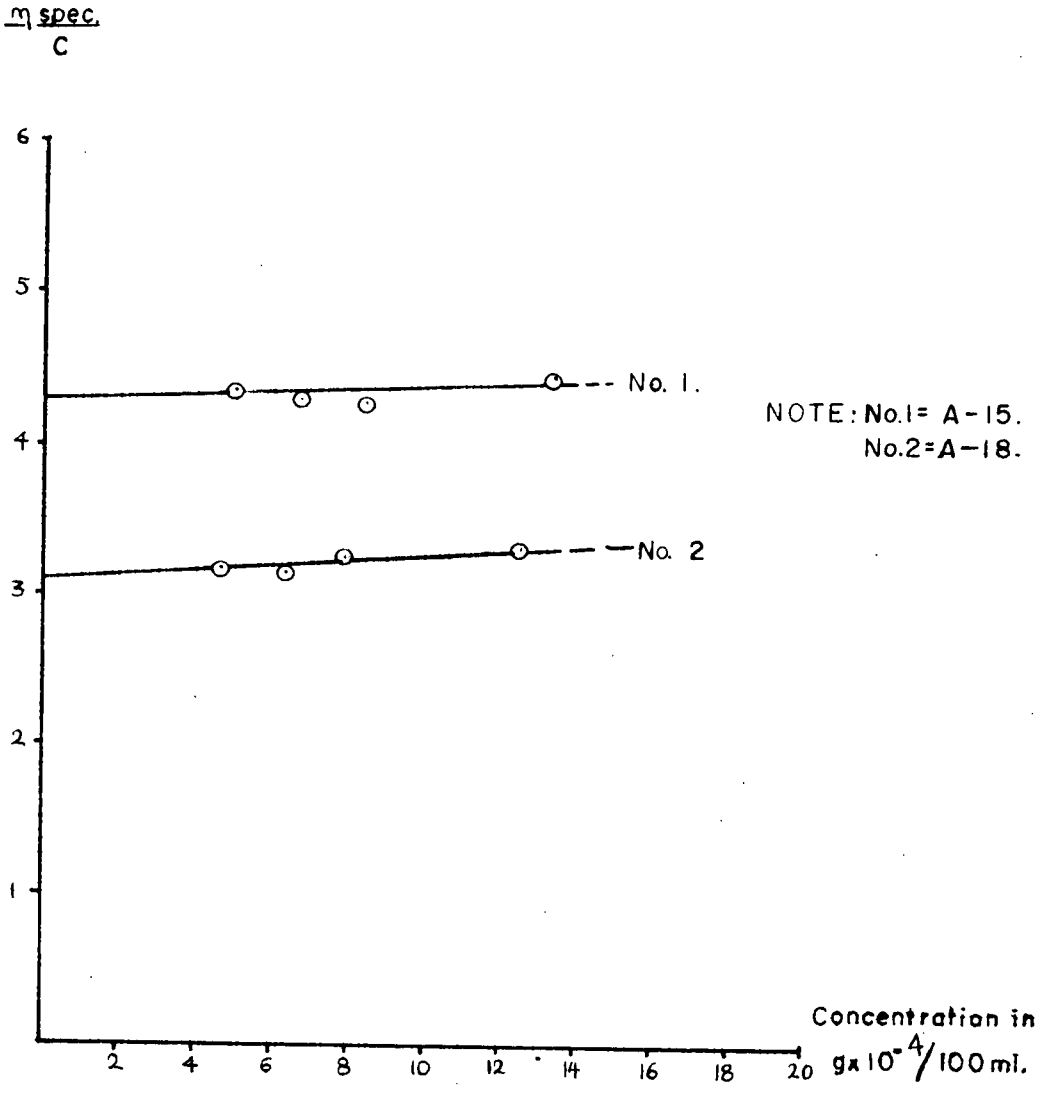


Fig.14. Plot of the Reduced Viscosity ($\frac{\eta spec.}{C}$) Versus Concentration (g/100 ml.) of DNA Samples A-15 and A-18.

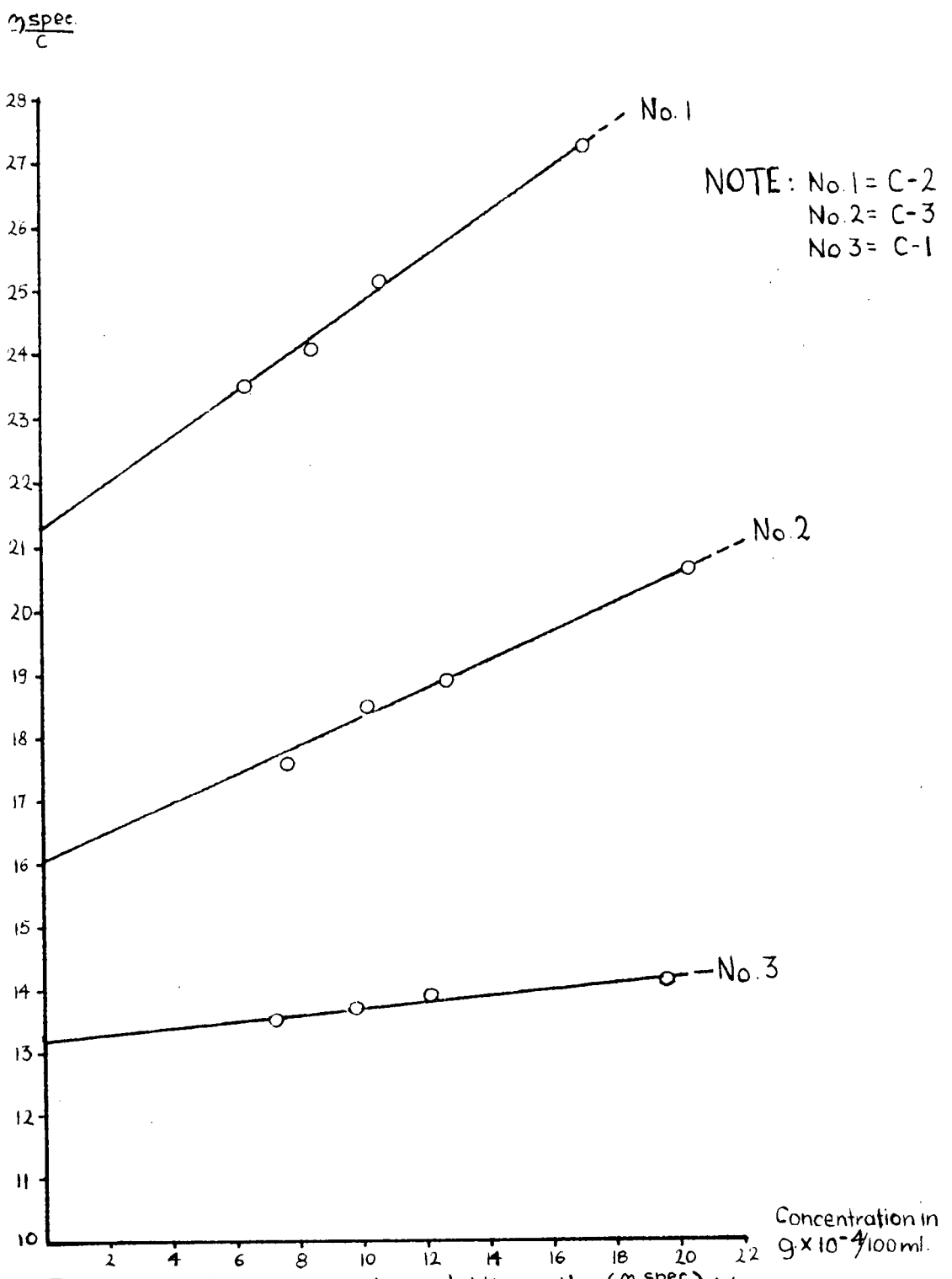


Fig. 15. Plot of the Reduced Viscosity ($\frac{\eta spec.}{C}$) Versus Concentration (g/100 ml.) of DNA Samles C-1, C-2, C-3.

viscosity measurements on supposedly high molecular weight samples, and their values were in the neighbourhood of $[\eta] = 50$. Just recently S. Kit (86) reported similar $[\eta]$ values for liver DNAs isolated by the modification of Kirby (22,23) procedure. Comparing the data of Table XV with the above mentioned values, one would conclude that all preparations isolated during this study are degraded. However this is not necessarily the case. On inspection of the articles published by the above mentioned investigators, one can see that all their measurements were made on a series of low velocity gradients, and the values were extrapolated to zero gradient.

Up to recently no standard values were given in the literature for the viscosity of carefully prepared specimens. This was due to the fact that investigators did not realise the gradient dependence of DNA solutions. Greenstein et al. (64) studied in detail the nature of viscosity of calf thymus preparations. They concluded that the viscosity of thymonucleate was a function of velocity gradient, in other words solutions of this substance possessed anomalous or structural viscosities This structural viscosity is due to a high degree of molecular asymmetry of sodium nucleate. The pressure time product for solutions of DNA are not independent of the applied pressure except for either very dilute solutions of DNA or for very high pressures. Since the present theoretical status of shear independence allows interpretation of the intrinsic viscosity only when Brownian motion is overhelming, viscosity measurements

TABLE XV

Intrinsic Viscosities and Estimated Molecular Weights of Different DNA Preparations.

Method of Preparation	Source of Tissue	Sample No.	from graphs in dl./g.	in dl./g. calculated from equ.(6)	Molecular weights estimated according to equ.(4)
Proc. II. (20,31)	Liver	A-16	9.7	9.6	424,400
"	"	A-17	6.0	5.8	194,500
Proc. III. (20,29)	"	A-13	-	4.0	100,700
"	"	A-21	10.2	9.6	460,500
Proc. IV.(32)	"	C-3	16.1	16.4	966,400
Proc. II	Small intest. mucosa	A-15	4.3	4.2	108,100
"	"	A-18	3.1	3.2	66,000
"	"	A-20	-	8.6	349,000
Proc. III.	"	A-12	11.0	11.0	520,300
Proc. IV.	"	C-1	13.2	13.2	700,000
"	"	C-2	21.3	21.7	1,526,000
Proc. V.	"	B-2	not measurable		

must be performed over a range of shears and then extrapolated to zero shear. Thus it is necessary to make viscosity measurements in very dilute solutions and at very low rates of shear, and this condition present experimental difficulties.

In this study none of these difficulties were overcome, using only a simple Ostwald type of viscosimeter. The recent application of the Coette viscometers(87) greatly facilitates viscosimetric measurements.

Knowing the fact that the ordinary Ostwald viscometers give lower values for $[\eta]$, the values summarized in Table XV do not necessary mean that all preparations were badly denatured. According to Reichmann et al. (85) the ratio of $[\eta]$ at zero gradient and at 1000 $sec.^{-1}$ is 2.5. Most of the simple Ostwald viscometers have an average velocity gradient around 1000 sec^{-1} therefore multiplying the data in Table XV by this factor would give much higher $[\eta]$ values.

However for a qualitative comparison of different preparations the intrinsic viscosities obtained by the Ostwald apparatus provide some measure of differences between the DNA preparations. The values obtained for DNA samples isolated by Procedures II and III, are too low, even multiplying them with the factor of 2.5. The change of physico-chemical properties of these DNA preparations may be due to four important factors:

a. During the course of deproteinization DNA preparations of Procedure II and III were exposed to somewhat lower pH values (4.3). It was mentioned before (54, 59) that acide or alkalies may cause the rupture of hydrogen bondings in DNA molecules.

b. The presence of contaminating protein may influence the viscosity of their solutions. It was demonstrated by several investigators (88,89) that as the protein content of DNA increased the characteristic viscosity of its solution decreased, having a sharp drop of approximately half of the original viscosity value, when protein content reached about 10%.

c. The effect of storage of DNA preparations in vacuo over phosphorous pentoxide may cause some deformation in the macromolecular state of the samples. Zamenhof (90,91) demonstrated that storing over phosphorous pentoxide in vacuo of the transforming principles of Hemophilus influenzae resulted in 80% inactivation. Parallel experiments with calf thymus DNA also resulted the decrease of viscosity of its solutions. Nothing is known about the nature of changes accompanying the dehydratation of DNA, but it seems probably that breaking of a few labile bonds, such as hydrogen bonds, takes place during the storage of DNA preparations in vacuo.

d. The presence of heavy RNA contamination may influence the molecular weights and viscosity properties of these preparations. Sample B-2 prepared according to Procedure V does not yield any measurable viscosity, showing that the secondary

structure of DNA is completely destroyed by this drastic method. An interesting observation should be mentioned here: the effect of starvation of animals on the DNA preparation. All DNA specimens isolated by Procedure II and III, were obtained from non-starved animals. In most of these cases the macroscopic appearance of DNA was non fibrous. Thus starvation seemed to be influence the macromolecular state of DNA. This phenomenon cannot be explained at present, but it would be very interesting to speculate that it is connected with the high metabolic activity of intestinal mucosa, or with the level of glycogen in liver. The best values were obtained by the sonication procedure. This is quite surprising because several investigators found that ultra-sound waves damage the hydrogen bondings in DNA molecules. It has to be mentioned that the sonicator used in this experiment works only at the region of 9 k.cycles/sec. which is far from the ultra-sound range (above 16 k.cycles/sec.)

The interpretation of the intrinsic viscosity in terms of size and shape requires that the value at zero gradient be known, therefore the molecular weights estimated from the apparent $[\eta]$ of DNA samples are not reliable. Dr. Reichmann performed a molecular weight determination by light scattering measurement on DNA sample A-20. The molecular weight of this preparation was found to be 600,000 which was somewhat higher than the value given in Table XV.

SUMMARY

1. Deoxyribonucleic acid (DNA) has been isolated from small amounts of liver and intestinal mucosa of rat (1-10 g.) by the following procedures;

a. The first method (29) consisted of the removal of nuclei from the tissue by means of controlled homogenization, and subsequent deparation of nuclei from extraneous cellular elements by adsorbtion on diatomaceous earth. DNA was deproteinized by salt saturation.

b. In the second procedure (20,31) the nucleic acids were extracted and deproteinized by detergent solutions. Ribonucleic acid (RNA) and DNA were separated by fractional precipitation with iso-propyl alcohol.

c. In the third procedure crude DNA was obtained according to the first method and the crude product was further purified by the detergent treatment of the second procedure.

d. The fourth method (32) was based on the disintegration of tissues by high frequency sonic oscillations, separation of nuclear fragments by centrifugation, extraction of nucleoprotein with strong salt solution, and deproteinization with chloroform-amyl alcohol mixtures.

e. In the fifth method (36,37) nucleic acids were extracted from tissues by hot 10% sodium chloride solutions, RNA and DNA were separated by incubating the mixture with 0.1 N sodium hydroxyde, and DNA was precipitated from the basic solution by neutralization with concentrated

hydrochloric acid.

2. The chemical and physico - chemical properties of DNA preparations isolated during this study, have been compared. On the basis of experimental findings the procedures used for preparing DNA have been evaluated and discussed.
3. The method of Emanuel and Chaikoff (29) appeared to be very satisfactory in three respects:
   a. DNA preparations could be accomplished in nine hours.
   b. DNA was never exposed to heat, acid alkali, or low ionic strength.
   c. The possibility of RNA contamination was greatly reduced by the previous isolation of nuclei from homogenates.

   However, some difficulties have not been overcome by this procedure. First all DNA samples were heavily contaminated with protein. Second, some sign of denaturation have been demonstrated in liver preparations and third, very low yields of DNA were obtained both from liver and intestinal tissues. Because the actual quantities of DNAs were very small, the purification and handling of these samples were difficult.
4. Best yields of DNA have been obtained both from liver and intestinal mucosa by the detergent method of Kay et al. (30) and Stevens et al. (31). The preparations contained considerable protein and RNA impurities, indicated by

qualitative amino-acid tests and by the determination of the base composition. Some macromolecular damage was observed by using viscosity measurements, and in the case of DNA of liver tissue, the high values obtained, also indicated some degree of denaturation.

5. The combination of the methods of Emanuel and Chaikoff (29) and Kay et al. (20) have resulted in some improvements over the Emanuel and Chaikoff procedure, indicated by the general decrease of protein contaminations and slightly higher yields of DNAs from liver preparations. Although the deproteinization of DNA by detergent appeared to be more effective, than the salt saturation alone, the considerable losses of DNA during the preparation, the complete removing of impurities and avoiding degradation of samples have not been overcome by this procedure.
6. DNA preparations isolated by the method of Bendich et al. (36) and Tyner et al. (37) have been characterized by a high degree of chemical purity, however the secondary macromolecular structure of DNA was completely destroyed by the drastic heat and acid - alkali treatments.
7. The procedure of Zubay (32) have been appeared the most promising for obtaining DNA from small amount of tissue, provided that the procedure is improved by further purifications. This method had the following advantages:

a. DNA preparations could be accomplished in ten hours.

b. The yields of DNA were quite high.

c. Contaminating RNA was greatly reduced by the previous washings of nuclear fragments with physiological saline solutions.

d. The secondary structure of DNA was not effected appreciably by the high frequency sonic oscillations shown by the relatively high $[\eta]$ values and low ultraviolet extinction coefficients. The presence of some impurities were indicated by the low nitrogen and phosphorous contents of these preparations.

8. An improved technique has been described for the elution of purine and pyrimidine bases from paper chromatograms. Generally 10% increase in recoveries of nitrogenous bases have been found by this procedure compared to the extraction methods used previously.

BIBLIOGRAPHY

1. Sinsheimer, R.L., Science 125, 1123 (1957).
2. Miescher, F., Hoppe-Seyler's Med. Chem. Unters. 441 (1871).
3. Altmann, R., Arch. Anat. Physiol., Physiol. Abt. 524 (1889).
4. Neumann, A., Arch. Anat. Physiol. 552 (1899).
5. Feulgen, R., Z. Physiol. Chem. 90, 261 (1914)
6. Levene, P.A., Z. Physiol. Chem. 45, 370 (1905)
7. Levene, P.A., J. Biol. Chem. 53, 441 (1922)
8. Jones, W., "Nucleic Acids - Their Chemical Properties and Physiological Conduct." 2nd Ed. Longmans Green & Co., London, (1920).
9. Levene, P.A., Bass, L.W., "Nucleic Acids" Chemical Catalog Comp., New York, (1931).
10. Jones, W., Perkins, M.E., J. Biol. Chem. 62, 290 (1924-25).
11. Jorpes, E., Acta Med. Scand. 68, 253 (1928).
12. Signer, R., Schwander, H., Helv. Chim. Acta 33, 1521 (1950)
13. Mirsky, A.E., and Pollister, A.W., J. Gen. Physiol. 30, 117 (1946).
14. Pollister, A.W., Mirsky, A.E., J. Gen. Physiol. 30, 101 (1946).
15. Mirsky, A.E., and Pollister, A.W., Proc. Natl. Acad. Sci. U.S. 28, 344 (1942).
16. Gulland, J.M., Jordan, D.O., Threlfall, G.J., J. Chem. Soc. 1129, (1947).
17. Sevag, M.G., Lackman, D.B., Smolens, J., J. Biol. Chem. 124, 425 (1938).
18. Grampton, C.F., Lipshitz, R., Chargaff, E., J. Biol. Chem. 206, 499 (1954).
19. Marko, A.M., Butler, G.C., J. Biol. Chem. 190, 165 (1951).

20. Kay, E.R.M., Simmons, N.S., Dounce, A. A., J. Am. Chem. Soc. 74, 1724 (1952)

21. Simmons, N.S., Chavos, S., Orbach, H.K., Federations Proc. 11, 396 (1952).

22. Kirby, K.S., Biochem. J. 66, 495 (1957).

23. Kirby, K.S., Biochim. et Biophys. Acta 36, 117 (1956)

24. Kit, S., J. Biol. Chem. 235, 1756 (1960)

25. Georgiev, G.P., Biochemistry U.S.S.R. 24, 443 (1959)

26. Paterson, R.P.P., and Zbarsky, S.H., Canadian J. of Biochem. and Physiol. 36, 755 (1958)

27. Nixon, J.C., Zbarsky, S.H., Canadian J. Biochem. and Physiol. 37, 1405 (1959).

28. Zbarsky, S.H., Hori, A., and Findlay, B.S., Canadian J. Biochem. and Physiol. 36, 1185 (1958).

29. Emanuel, C.F., Chaikoff, I.L., Biochim. et Biophys. Acta 24, 261 (1957).

30. Emanuel, C.F., Chaikoff, I.L., Biochim. et Biophys. Acta 24, 254 (1957).

31. Stevens, V.L., and Duggan, E.L., J. Am. Chem. Soc. 79, 5703 (1957).

32. Zubay, G., Biochim et Biophys. Acta 33, 244 (1959).

33. Goldstein, G., and Stern, K.G., J. Polymer. Science, V, 687 (1950).

34. Guild, R.J., Defilippes, H., Biochim et Biophys. Acta 26, 241 (1957).

35. Oster, J., Gen. Physiol., 31, 89 (1947).

36. Bendich, A., Russell, P.J. Jr., Brown, G.B., J. Biol. Chem. 230, 305 (1953)

37. Tyner, E.P., Heidelberger, C., and LePage, G.A., Cancer Research, 13, 186 (1953).

38. Chargaff, E., Davidson, J.N., "The Nucleic Acids" Acad. Press. Inc., New York, (1955).

39. Colowick, S.P., Kaplan, N.O., "Methods in Enzymology" vol. III. 703, Acad. Press Inc., New York, (1957).

40. Watson, J.D., Crick, F.H.C., Nature, 171, 737 (1953).

41. Hawk, P.B., Oser, B.L., Summerson, W.H., "Practical Physiological Chemistry" 13th Ed. McGraw. Hill Co. New York (1947).

42. Bartlet, G.R., J. Biol. Chem., 234, 466 (1959).

43. Lederer, M., Lederer, E., "Chromatography" 2nd Ed. Elsevier Pub. Co. New York, (1957).

44. Phillips, D.M.P., Biochim. et Biophys Acta, 3, 341 (1949).

45. Wischer, E., Chargaff, E.J. Biol. Chem., 176, 715 (1948).

46. Wyatt, G.R., Biochem. J., 48, 584 (1951).

47. Chargaff, E., Magasanik, B., Doniger, R., Vischer, E., J. Am. Chem. Soc. 71, 1513 (1949).

48. Personal Communication from Dr. S.H. Zbarsky.

49. Personal Communication from I. Csizmadia.

50. Vischer, E., Chargaff, E., J. Biol. Chem., 168, 781 (1947).

51. Vischer, E., Chargaff, E., J. Biol. Chem., 176, 703 (1948).

52. Hochkiss, R.D., J. Biol. Chem., 175, 315 (1948).

53. Chargaff, E., Vischer, E., Doniger, R., Green, C., Misani, F., J. Biol. Chem. 177, 405 (1949).

54. Vilbrandt, C.F., Tennent, H.G., J. Am. Chem. Soc., 65, 1806 (1943).

55. Cecil, R., Ogston, A.G., J. Chem. Soc., 1382 (1948).

56. Kahler, H., J. Phys. and Colloid. Chem. 52, 676 (1948).

57. Signer, R., Caspersson, T., Hammarsten, E., Nature., 141, 122 (1938).

58. Tennent, H.G., Vilbrandt, C.F., J. Am. Chem. Soc., 65, 424 (1943).

59. Creeth, J.M., Gulland, J.M., Jordan, D.O., J. Chem. Soc., 1141 (1947).

60. Kunitz, M., J. Gen. Physiol., 33, 363 (1950).

61. Thomas, R., Biochim. et Biophys. Acta, 14, 231 (1954).

62. Schack, J., J. Biol. Chem., 233, 677 (1958).

63. Chargaff, E., Zamenhof, S., J. Biol Chem. 173, 327 (1948).

64. Greenstein, J.P., Jenrett, W.V., J. Natl. Cancer. Inst. 1, 77 (1940).

65. Daniels, F., Mathews, J.H., Williams, J.W., Bender, P., Alberty, R.A., "Experimental Physical Chemistry" Mc.Graw-Hill. Book. Co. New York, (1956).

66. Kraemer, G., Ind. Eng. Chem., 30, 1200 (1938).

67. Spitkovskii, D.M., Biophysics U.S.S.R. 1, 319 (1956).

68. Pregl, F., "Quantitative Organic Microanalysis" 5th ed. Julius Grant. London (1951).

69. Cohn, W.E., Science 109, 377 (1949).

70. King, J., Biochem J. 26, 292 (1932).

71. Paterson, A.R.P.,: The Metabolism of 2 - C^{14}-Adenine in the Adult Male Rat. Master of Art Thesis, U.B.C. (1952).

72. Jervell, K.F., Diniz, C.R., Mueller, G.C., Arch. Biochem. and Biophys. 78, 157 (1958).

73. Frick, G., Biochim et Biophys. Acta, 13, 374 (1954).

74. Zamenhof S., "Biochemical Preparations" vol. VI. John Wiley & Sons Inc. New York, (1958).

75. Butler, J.A.V., Phillips, D.M., and Shooter, K.V., Arch. Biochem. and Biophys. 71, 423 (1957).

76. Woodhouse, D.L., Biochem. J. 56, 349 (1954).

77. Chargaff, E., Lipshitz, R., J. Am. Chem. Soc., 75, 3658 (1953).

78. Chargaff, E., Lipshitz, R., Green, C., Hodes, M.E., J. Biol. Chem. 192, 223 (1951).

79. Cohen, S.S., Advances in Virus. Res. 3, 1 (1953).

80. Vischer, E., J. Biol. Chem., 177, 429 (1949).

81. Oster, G., and Grimsson, H., Arch. Biochem. and Biophys., 24, 119 (1949).

82. Tsuboi, K.K., Biochim. et Biophys. Acta, 6, 202, (1950).

83. Spirin, S., Gavrilova, L.P., Belozerskii, A.N., Biochemistry U.S.S.R. 24, 556 (1959).

84. Magasanik, B., and Chargaff, E., Biochim. et Biophys. Acta, 7, 396 (1951).

85. Reichmann, M.E., Rice, S.A., Thomas C.A., Doty, P., J. Am. Chem. Soc., 76, 3047 (1954).

86. Kit. S., Arch. Biochem. and Biophys. 87, 318 (1960).

87. Pouyet, J., J. Chim. Phys. 48, 90 (1951).

88. Tongur, V.S., Diskina, B.S., Spitkovskii, D.M., Biochemistry, U.S.S.R. 22, 879 (1957).

89. Diskina, B.S., Spitkovskii, D.M., Tongur, V.S., Biochemistry, U.S.S.R. 23, 360 (1958).

90. Zamenhof, S., Alexander, H.E., and Leidy, G.C., J. Experimental Med. 98, 373 (1953).

91. Zamenhof, S., Griboff, G., Marullo, N., Biochim. et Biophys. Acta, 13, 459 (1954).