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THE QUANTITATIVE DETERMINATION OF THE PURINE AND PYRIMIDINE NUCLEOTIDES OF RIBONUCLEIC ACID IN TISSUES

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" If thou shouldst succeed in mastering the management of the (four)elements and their combinations without introducing any impurity therein; then thou hast truly mastered what thou hast set out to master and make straight.

For an element is an entity that hath no impurities, and whosoever introduceth an impurity therein strayeth and falleth into error."

ABŪ BAKR IBN BASHRŪN

(9th CENTURY AD)

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INTRODUCTION

Early work in the field of nucleic acid chemistry, as reviewed by Jones(1) and Levene(2), showed that Pentose nucleic acid (RNA) is a compound of complex structure composed of nucleotide units.+

Only the nucleotides of two purines (adenine and guanine) and of two pyrimidines (cytosine and uracil) have been demonstrated in RNA. However the possibility of the presence of other nucleotides in trace amounts is not ruled out completely as yet (3).

The fundamentals of the structure of the individual nucleotides were established early in the twentieth century (2). The 3' position on the ribose was assigned for the attachment of phosphorus in nucleotides obtained by chemical hydrolysis (4,5), but such nucleotides have recently been proved to consist of a mixture of the 2' and 3' phosphoesters (6-9).

Though work done by Levene and his co-workers advanced our knowledge in the field of nucleic acid chemistry considerably, and more than any other individual work, his misleading tetranucleotide hypothesis, which assumed that RNA was built simply of one each of the four nucleotides (2,P.262) slowed development in the field for many years.

This tetranucleotide idea, according to Gulland (10,11), was based mainly on an erroneous molecular weight determination (12), also on the evidence submitted by Jones (1,P.20), that purines and pyrimidine nucleotides were present in equal quantities in yeast RNA; moreover it seemed to be well supported by titration studies.

+ A nucleotide molecule contains a hetrerocyclic base (a purine or a pyrimidine), a pentose (generally accepted to be ribose) and a phosphate group.

Other factors which influenced Levene's hypothesis may be:

1. The assumption that the RNA of pancreas was a compound of " higher order" and quite different from that of yeast (2,13,14). Jorpes (15) finding more purines than pyrimidines in pancreatic RNA, assigned a pentanucleotide structure to it.

2. The absence of quantitative methods for the determination of the pyrimidines and of simple procedures for the determination of the purines.

3. The use of commercial yeast nucleic acid for the studies where the ratios of the 4 nucleotides is close to one of each.

Refinement of the methods for the preparation of RNA and the determination of its molecular weight brought about the realization that RNA molecules were much larger than required by the tetranucleotide theory (16,17,18).

The idea of a " structural tetranucleotide " as previously suggested by Levene was therefore discarded, but due to lack of sufficient evidence to the contrary, was replaced by a " statistical tetranucleotide " hypothesis(10), which suggested that the ~~four nucleotides~~ occur in RNA in equimolecular ratios.

With the development and application of new micro techniques to the separation and analysis of the constituents of RNA, it was shown (19,20, 21) that the composition of RNA preparations from tissues or micro-organisms seemed to be in disagreement even with the statistical tetranucleotide hypothesis. These techniques are set out briefly below:

1. <u>PARTITION CHROMATOGRAPHY</u> Employing in most cases starch columns for the separation of the constituants (22-28). This method remained limited in application due to the development of paper chromatography which uses a far smaller amount of material and is more conveniant to use.

2. <u>PAPER CHROMATOGRAPHY</u> This excellent qualitative tool was unfortunately used to excess in the quantitative analysis of the constituents of RNA. Although most of the publications in this field (6;29-42) claim recoveries which are almost quantitative, we could not obtain with them reproducable results in the analysis of RNA. Paper chromatographic methods in general suffer from the disadvantage that they are not aplicable for the quantitative determinations of a constituent which occurs in relativly small amounts with other constituents. This is due to the fact that on chromatographing such a mixture the constituent found in large amounts usually trails and spoils the determination if the amount of the mixture chromatographed is increased so as [illegible] enough of the small component for quantitative work.

3. <u>ION EXCHANGE CHROMATOGRAPHY</u> An excellent tool for preparative and [illegible] quantitative work, was first applied to the derivatives of nucleic [illegible] acids by Smith and Wander (43) for the separation of xanthine from [illegible] guanine, but owes its fullest development to the work of W. Cohn (7,44-47). It has been applied also to the separation of isomeric nucleotides and polynucleotides as well as to quantitative analysis (48-52) with excellent and reproducable results. However, the method suffers from being tedious and does not lend itself readily to serial determinations.

4. <u>DIFFERENTIAL SPECTROPHOTOMETRY</u> This method depends on measuring the extinction coefficient of a mixture of compounds in solution at several wave lengths (usually two compounds at two wave lengths) and calculating from simultaneous equations the quantities of each. It has been applied succesfully (29,53-54) to the analysis of RNA. While this method avoids certain losses resulting from dephosphorelation of certain constituants, yet it can be applied only to relatively pure materials for maximum accuracy and presupposes the qualitative knowledge of the mixture to be analysed. Any ultraviolet absorbing impurities found in the final solutions would be estimated with the material under study. It is applicable on the micro scale, and can easily be run for a series of determinations, and is distinguished by rapidity of operation. This is the method chosen for use in this work in conjunction with a procedure for isolating the nucleotides quantitatively from tissues.

5. <u>OTHER METHODS</u> Including enzymatic degradation (55,56) and the radio-isotope dilution method (57); these methods require special techniques and are not yet well developed. Paper electrophoresis was recently applied to the analysis of RNA (58-60), but does not seem to offer any outstanding advantages in quantitative work over paper chromatography.

A limitation which applies to all of the fore-going methods is that they are applied only to isolated RNA preparations free from other tissue constituents, and it is improbable that such preparations are representative of the RNA as it actually exists in tissues (Native RNA).

It has been pointed out (8) that the molecular weight of the isolated RNA depends on the method of isolation, being higher the milder the treatment. It has also been shown (21,55) that the ratio of the four bases in RNA preparations varies with the method of isolation. The fact that only 20-40% of the RNA preparations could be extracted from tissues (or cultures in the case of microorganisms) by the milder methods available (40,61-67), may mean that the RNA thus prepared is not representative of the " native " RNA. Since the milder methods do not exclude enzyme action, the RNA prepared by them may be enzymatically degraded.

An attempt to avoid this error was made by Volkin and Cohn (64), however there seems to be no way of measuring the dependability of the method.

These considerations show that the composition of the " native " RNA has not been determined with certainty by the use of the available methods. Some attempts to remedy this situation by introducing minor modifications to the available techniques have been made (68-73), but they all suffer from interference due to contamination, (68-72) or non-quantitative recovery (71-73). A critique of these methods will be presented in Part 1.

The need for a quantitative and simple method of separating the nucleotides of RNA from tissues is therefore evident. Such a method would offer a means for the study of the composition of " native " RNA and would also be very useful in any quantitative studies of RNA metabolism using tracer technique and ~~in testing~~ the dependability of the methods of preparing RNA. The first part of this work deals with the development of such a method. The second part deals with a differential spectrophotometric method, for the quantitative determination of the nucleotides of RNA.

CHAPTER 1

THE SEPARATION OF RNA NUCLEOTIDES FROM TISSUES

Several attempts have been made to determine the nucleotides of ribonucleic acid as they occur in tissues without prior extraction of RNA.

Marshak and Vogel(70)determine the total purines and pyrimidines of both RNA and DNA together. This is a procedure which might be applicable to organisms which contain no DNA for the analysis of RNA,but is not applicable to tissues which contain both,except as a measure of the total purines and pyrimidines of both RNA and DNA.

Thomas(71)analyses yeast by hydrolysing with acid the TCA-extracted,lipid-extracted dried residues and assumes that all the nucleic acid in it is RNA. It is strange that his paper chromatograms did not reveal the interference of the degradation products of DNA.

Fairley et al(69)analyse the acid-soluble fraction of the alkaline hydrolysate of dried tissues by differential spectrophotometry. The interference of the partially split proteins in this case is too large to be ignored and according to our experience would produce errors of about 20% in the pyrimidine fractions.(See Fig.6)

Elson et al(68)use the same fraction as Fairley et al(69)for the analysis,but seperate the nucleotides by chromatography. The authors admit that there are interfering substances which are high enough in certain

cases to render the analysis inaccurate,but do not state the degree of interference. This makes it difficult to ascertain the reliability of the method.

Davidson et al(73),using paper electrophoresis report recoveries which are low and variable for the different nucleotides.

Boulengar et al(72)do not report recoveries,but according to our findings their technique of adsorption of the nucleotides on resin columns at pH 4-4.2 would result in large losses of nucleotides,especially cytidylic acid(See Fig.3 and (7)).

In order to seporate the nucleotides from tissues we adopted a slightly modified Schmidt and Thanhauser (74)procedure in conjunction with adsorption on ion exchange resins.

<u>Outline of Procedure.</u> The procedure consists of extraction of the acid-soluble compounds from the tissues by trichloroactic acid(TCA),and the lipids by lipid solvents; hydrolysis of the residue with alkali to convert the RNA into nucleotides; precipitation of most of the proteins with $HClO_4$; adsorption of the nucleotides quantitatively on anion exchange columns without the adsorption of interfering material; and lastly quantitative elution of the adsorbed nucleotides from the columns with sulphuric acid. Each step in the procedure will be discussed seporately.

<u>Extraction and Washing with Trichloroactic Acid.</u> Schmidt and Thanhauser(74)mince the tissues in 7 per cent TCA,filter,and wash the residues with 1 per cent TCA till the supernatent fluid is free from inorganic phosphate.

It was found that the supernates of 1 per cent TCA washings of tissues were colloidal; however those obtained with 2 per cent TCA were clear.

It is impractical to filter and wash adequately on the filter when the tissue sample is large, and very difficult to transfer the precipitate quantitatively from the filter paper, hence centrifugation was used instead. An experiment was designed to determine the number of washings needed to free the tissue from the TCA soluble material without causing the decomposition of the nucleic acids.

> 3.3 gms of dog's pancreas were frozen in solid CO_2-ether mixture, ground in a mortar and dropped into 20 ml. of ice cold 8 per cent TCA. The mixture was stirred mechanically in an ice bath for 30 minutes and centrifuged. The residue was washed twelve times by suspending it in 10 ml. of 2 per cent TCA, stirring mechanically for 10 minutes in an ice bath and centrifuging in the cold. The supernates of the washings which were clear were made N with respect to H_2SO_4 by the addition of 10 N H_2SO_4 and placed in a boiling water bath for 1 hour to hydrolyse completely any purine nucleotides. The optical densities of the solutions were determined at 260 and 280 mμ using 10 mm. cells and appropriate blanks.

Table 1 shows the results of the experiment. From these results it may be concluded that four to five washings with cold 2 per cent TCA remove essentially all of the acid-soluble nucleotides without evidence of any splitting of the RNA. It must be noted here that over-washing the tissue residues with acidic solutions may cause the splitting of the purines of the nucleic acids especially if the temperature is allowed to rise(74). Kerr and Seraidarian(75) in a similar experiment determined the adenine and guanine contents of the supernatents and arrived at the same conclusions regarding the number of

Table 1

Extraction of light-absorbing material by repeated washings with 2 per cent trichloroacetic acid.

Washing No.	OD260	OD280	OD280/OD260	% of 1-11	Remarks
1	7.57	3.63	0.48	72.0	20cc 8%TCA
2	2.69	1.31	0.50	14.7	10cc2%TCA
3	1.106	0.564	0.51	5.6	"
4	0.556	0.316	0.51	2.8	"
5	0.263	0.156	0.57	1.3	"
6	0.214	0.131	0.61	1.1	"
7	0.152	0.105	0.69	0.8	"
8	0.112	0.098	0.87	0.6	"
9	0.097	0.078	0.80	0.5	"
10	0.090	0.076	0.84	0.4	"
11	0.058	0.054	0.93	0.3	"
12	0.194	0.147	0.76		"
13	0.042	0.056	1.33		"

The results are also presented in Figure 1.

washings to be used.

<u>Lipid Extraction</u>. Prior to the extraction of the lipids the tissue residues from the TCA treatment are washed with ethanol in the cold until the supernate is free from brown color(5-6 washings). The residues are then washed 3-5 times with ethanol-ether mixture(3:1), once or twice with ether and finally dried and weighed. The residue thus obtained will be termed "dry tissue".

The Schmidt-Thanhauser procedure of refluxing with chloroform-methanol mixture was avoided whenever the absolute quantities of ribonucleotides in the tissue were determined,due to the difficulty of controlling quantitative conditions in such a procedure. Further, it has been proved(76) that the complete extraction of the lipid material from tissues is impractical and was therefore not attempted.

<u>Alkaline Hydrolysis</u>. Dilute alkali hydrolyses RNA yielding the mono-nucleotides quantitatively(77-79). Different conditions of alkaline hydrolysis have been used,N NaOH or KOH at room temperature or 37°for fifteen to twenty-four hours being the most common(74,78). Other methods employ concentrations of alkali as low as 0.3 N under the same conditions(51,72,80): 0.1 N alkali at 100° for 150 minutes(50); ammonia solutions in the autoclave (78); Ba (OH) 2 or Ba CO_3 with heat or cold(36,81).

The methods using autoclaving are tedious and require special set-ups. The use of NaOH,KOH,Ba $(OH)_2$, involves the disadvantage that these regents partially deaminate cytidylic acid(CyA) yielding uridylic acid(UrA) and ammonia (50,82,84).

A reagent which will accomplish the hydrolysis without deaminating the cytidylic acid is there-fore needed. Addition of ammonia to a KOH hydrolysis mixture was tested in the hope that the added ammonia would supress the deamination.

About 10 mgs. of CyA were dissolved in 20 ml. of N KOH. 5 ml. portions were added to each of the following:

1. 5 ml. N KOH, making a final solution of N KOH
2. 5 ml. water, making a final solution of 0.5 N KOH
3. 5 ml. 2N NH_4OH making a final solution of 0.5N KOH-N NH_4OH

Two ml. samples of each were added to 10 ml. of N HCl. The optical density was determined at 280mμ using the Beckman model DU spectrophotometer and 10mm cells. The remainder of the solutions of CyA were incubated for twenty hours at 25°C and then the dilution with HCl and determination of the optical density was repeated.

It is clear from the molecular extinction coefficients of CyA and UrA at 280mμ that if a millimolar solution of CyA were deaminated the optical density of the solution would diminish by 0.00848. The deamination in each case was determined from this change, with the results presented in Table 11.

Table 11

The deamination of CyA

Sample	Alkali Concentration	Change in OD 280mμ	mM CyA deaminated	Per cent deamination
1.	N KOH	.009	1.06	6.3
2.	0.5N KOH	.005	0.59	3.0
3.	0.5N KOH-N NH_4OH	.000	0.00	0.0

In a similar experiment CyA was incubated for 20 hours at 25°C. with(a) N NaOH and(b) with 0.5 N NaOH-N NH_4OH, and then chromatographed(37). The chromatogram revealed a spot for UrA in solution(a) but not in (b) (see Figure 2). Hence for the alkaline hydrolysis of RNA the 0.5 N KOH-N NH_4OH reagent was adopted.

<u>Precipitation of Proteins</u>. For the precipitation of proteins $HClO_4$ was chosen for three reasons:

(a) The solution can be freed from the precipitant later by means of KOH. Thus a low salt concentration is maintained in the final solution, whereas a high salt concentration would prevent the complete adsorption of the nucleotides by resin columns.

(b) Even if some of the perchlorate should remain in the final solution, its interference would not be great because of its low absorption in the ultraviolet(74,83).

(c) Precipitation of proteins with $HClO_4$, which is a strong acid, avoids the co-precipitation of nucleotides which, we have observed, occurs when the precipitation is performed at low acidity. The precipitant should, theoretically, produce a pH at which the proteins are positively charged while the nucleotides remain as anions, if pH of precipitation higher than pKs of nucleotides. (pH of precipitation should approach 0.7, the lowest pK for the four nucleotides of RNA(2)).

It has been determined that 0.33 N $HClO_4$ is the optimal concentration for protein precipitation(84), hence this concentration was adopted in our procedure. It is important to perform the precipitation in ice-cold solution since acid solutions tend to split the purines from purine nuc-

leotides(84). A factor which should be accounted for here is the correction for the volume of the precipitated protein. Fiske and Subbarrow(86) recommend that the volume of the dry protein precipitate should be subtracted from the final volume for accurate quantitative work. Due to the difficulty in determining such volumes on the dry weight of the sample, known volumes of alkali hydrolysis mixture and $HClO_4$ are added to the dry TCA-extracted lipid-extracted tissues. The final volume is considered to be the volume of the two added solutions.

The Precipitation of the ClO_4^-. Since $KClO_4$ is only slightly soluble in water, excess KOH added to $HClO_4$ will produce a solution almost free of ClO_4 ions. The supernate from the protein precipitation is therefore treated with KOH to give a final concentration of about 0.2M.

That the precipitation of $KClO_4$ does not adsorb nucleotides significantly was determined experimentally by treating a KOH hydrolysate of RNA with $HClO_4$ and determining the optical density(OD) of the solution before and after the precipitation. The results showed that only 0.9 per cent of the nucleotides were adsorbed by the $KClO_4$.

Adsorption of Nucleotides on Resin Columns. It is clear from the work of Cohn(52) that nucleotides are adsorbed by resin columns in alkaline but not in acid solutions. Experiments were conducted to find the minimum pH at which nucleotides are adsorbed quantitatively, with the expectation that at that pH most of the interfering material would not adsorb due to the fact that the nucleotides are relatively strong acids by virtue of their phosphoric acid radical.

Conflicting results were obtained until it was found that reproduceable results were secured provided the Dowex-2 columns were first washed with a buffer solution at the pH at which the nucleotides were to be adsorbed.

The Dowex-2 anion exchange resin, after receiving the usual preliminary washings with acid and alkali, was again washed three times with the required buffer and once with water, then spread in a thin layer to dry and stored.

Figures 3 and 4 show the results of experiments on the adsorption of nucleotides, where the pH of the columns and solutions were adjusted by phosphate buffers. It is apparent that at a pH of 6-8 maximum adsorption occurred, hence the minimum value (6.0-6.1) was chosen. Since the selectivity of Dowex-2 to the $H_2PO_4^-$ and the CH_3COO radical is about equal * acetate buffer of pH 6.0-6.1 was adopted for our purposes, rather than phosphate, because it does not interfere with phosphate determination when these are needed.

Figures 5 and 6 show the seperation of RNA nucleotides in dry tissue by Cohn's method (47). Figure 5 gives the results of absorption from dilute alkali solutions, and Figure 6 that from buffer at pH 6.0 on a column washed with phosphate buffer at pH 6.0.

It is clear that when the "dry-tissue" hydrolysate is adsorbed at an alkaline pH certain non-nucleotide light-absorbing material adsorbs and is subsequently

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* The Dow company bulletin (86) shows a K of 0.34 for $H_2PO_4^-$ and 0.18 for CH_3COO^-. K is defined as:

$$K = \frac{\text{Conc ion in resin}}{\text{Conc Cl in sol.}} \times \frac{\text{Conc Cl in resin}}{\text{Conc ion in sol.}}$$

eluted by 0.01 N formate (Figure 5) and that such interfering material is not adsorbed at pH 6.0 (Figure 6).

In order to determine the minimum size of columns required to adsorb the nucleotides under the conditions used, 15 ml. portions of RNA alkaline hydrolysate containing 13.8 mgs. of RNA each were adjusted to pH 6.0 and passed through Dowex-2 phosphate columns of different heights and 0.5 sq.cm. cross section, the columns were washed with 200 ml. of water. The effluent solutions with the washings were acidified and their optical densities at 260 mμ were determined.

The results, presented in Figure 7, show that for a column with cross section of $0.5 cm^2$ a minimum height of 3.5 cm. is required (equivilent to 1 gm. of resin) for 14 mgs. of hydrolysed RNA (i.e., 0.25cm. per mg. RNA). For this work, 7-8cm. columns were used for quantities of about 30 mgs. of RNA hydrolysate.

Washing the Columns. It was at first thought that it might be possible to adsorb the nucleotides together with the interfering material from alkaline solution onto the resin column, and to elute only the latter but not the nucleotides. Cohn (87) suggested the use of 0.01 M $(NH_4)_2SO_4$ for this purpose. 0.005 M $(NH_4)_2SO_4$ at both pH 5.0 and 8.5 and 0.001 M $(NH_4)_2SO_4$ were tested on columns which had adsorbed an alkaline hydrolysate of RNA, but it was found that in each case the nucleotides were eluted. (See Figures 8,9,10). The use of such eluting solvents was therefore discarded. Since water does not elute any nucleotides (Figure 6), water alone was adopted for freeing the columns of non-adsorbed material.

Elution of the Nucleotides. After the nucleotides are adsorbed and the column is freed as far as possible from non-adsorbed material by washing with water, the nucleotides are eluted with H_2SO_4. Since in a later step the purines are precipitated with Ag^+, and the smaller the volume the more complete is the precipitation, the minimum quantity of H_2SO_4 must be used.

In recovery experiments it was found that 25 ml. of N H_2SO_4 applied directly to the columns eluted only 80 to 90 per cent of RNA nucleotides, but if the flow of the sulphuric acid was maintained at a slow rate by the Mariotte bottle principle (see Figure II) the recoveries rose to 96-99 per cent as measured by total phosphorus and optical density determinations. Similar recoveries were obtained if the flow was reduced by means of a stop-cock to one drop every one or two minutes, or if 10 ml. of N H_2SO_4 were passed several times through the column, collecting this each time in the same 25 ml. volumetric flask, and followed by 15 ml. of fresh N H_2SO_4 to wash the resevoir and column.

The Procedure. The complete procedure as finally adopted is as follows:

A weighed portion of the fresh tissue is frozen immediately after removal from the animal, ground in ice-cold 6 per cent TCA and washed on the centrifuge 5 times with ice-cold 2 per cent TCA. The residue is washed with 95 per cent ethanol until the extracts are colorless, then 3 times with ether-alcohol mixture to remove lipids, then once with ether, and the residue is finally dried and weighed. A weighed portion of the dry tissue estimated to

contain about 80 mgs. of RNA is placed in a dry test tube and exactly 10.0 ml. of 0.5 N KOH-N NH_4OH are added. The tube is stoppered and incubated at 25° for 16 to 20 hours. A few drops of N-octanol are added to the mixture to prevent foaming and the mixture is aerated with air saturated with water vapor for 30 minutes in order to free it from most of the ammonia. 10.0 ml. of ice-cold 1.2 N $HClO_4$ are then added. After thorough mixing the contents of the tube are transfered to a dry centrifuge tube and centrifuged at 2000 rpm or filtered in the cold. A 15.0 cc aliquot of the supernate(equivilent to 0.75 per cent of the original dry tissue)is pipetted into another centrifuge tube and 10.0 ml. of N KOH are added. After centrifuging off the precipitated $KClO_4$,a 20.0 ml. aliquot of the supernate(equivilent to 60 per cent of the dry tissue)is placed in a small beaker and adjusted to pH 6.0-6.1(glass electrode)by the addition of 10 per cent acetic acid. The solution and the washings of the electrode and beaker are transferred quantitatively to a 50 ml. volumetric flask,made to volume and mixed. Duplicate 20.0 ml. portions(equivilent to 24 per cent of the original dry tissue each)are passed through two columns (7-8 cm X 0.5 cm^2)of Dowex-2 previously washed with acetate buffer,pH 6.0. The columns are washed with 200 ml. water to remove non-adsorbed material,then 25 ml. of N H_2SO_4 are passed through the columns at a rate not exceeding one drop per minute. The effluents are collected in 25 ml. flasks, diluted to volume,and analyzed by the procedure described in the next section.

The Purity of the Separated Nucleotides. Two samples of beef pancreas dry tissue (equal to 0.24 gm. each) after being subjected to the above procedure were eluted in 25 ml. of N H_2SO_4. The eluent gave a negative Dische reaction(88), a negative Stumpf reaction(89) for DNA, and a negative ninhydrin test.

Davidson(59) reports that certain non-nucleotide phospho-esters appear in the acid-soluble fraction of the alkaline hydrolysate of dry tissues. These substances are eluted from the columns after the nucleotides(90). 0.305 gms. of dry tissue were subjected to the procedure described above but were eluted by the Cohn formate method(47). After the completion of the elution the column was washed with 100 ml. of N H_2SO_4. The optical density of the eluent was determined at 260mμ. The amount of this material expressed as OD_{260} X volume was calculated and found to represent only 2.6 per cent of the total nucleotides. The solution was neutralized and dried in vacuum, dissolved in a small volume and chromatographed on paper(37). The chromatogram showed the usual three nucleotide spots. It appears that interference from the compounds referred to by Davidson et al, is negligible in our procedure. (less than 2.6 per cent).

Recoveries. To ascertain the reliability of the method the recovery of known quantities of RNA from a mixture containing protein was made.

Dog plasma was used as the protein solution because it is known to be free from RNA and is probably more representative of tissue protein than egg white or casein.

The plasma was cooled and treated with 50 per cent TCA to get a final concentration of 7 per cent and centrifuged. The precipitate was washed several times with 2 per cent TCA as in section 1, then it was treated with alcohol three times and extracted three times with alcohol-ether mixture, washed with ether and dried.

For the recovery experiments, portions of the plasma protein of RNA were treated separately with 0.5 M KOH-M N H_4OH, then aliquots of the digests were pooled and the procedure run on the pooled sample. For comparison, an RNA aliquot unmixed with protein was also taken and diluted with H_2SO_4 to give a final acid concentration of about 1 N. The eluates from the columns and the diluted RNA "standard" were placed in a boiling water bath for one hour to complete the purine hydrolyses which would have started and to establish the equilibrium between the 2' and 3' forms of the pyrimidine nucleotides.

The "standard" RNA hydrolysate and column eluents were then appropriately diluted and read in the Beckman model DU spectrophotometer at 260 mμ.

Table III
Recovery of Nucleotides from Protein

Experiment	Column	% Recovery	% Recovery + 3 per cent*
49-397	1	95.4	98.4
	2	94.3	97.3
	3	95.4	98.4
	4	95.4	98.4
49-429	1	95.0	98.0
	2	94.4	97.4

It is clear from Table III that the nucleotides can be recovered almost quantitatively after being subjected to the procedure described.

* About 3 per cent of the RNA alkaline hydrolysate is not adsorbed by resin columns (see Figure 3,4,7). Probably this represents impurities in the RNA sample.

<u>Discussion</u>. The method described here is designed to measure the individual nucleotides of ribonucleic acid in tissues without prior extraction or purification.

Its major use would be in the determination of the nucleotide ratios, which are important in any study of the composition of the "native " RNAs. This procedure may also be used in determining whether enzymatic or other changes in composition occur during the preparation of RNA.

Results obtained by this procedure may be referred to a basis of either fresh tissue or "dry tissue". In certain cases, however, there may be an advantage in referring the quantities of RNA nucleotides to the desoxy-ribonucleic acid(DNA)as a base. In such a case a determination of DNA on the perchloric acid precipitate can be done with relative ease using the method of Schmidt and Thannhauser(74)or that of Schneider(92).

The ratio of DNA nucleotides, if also required, may be determined also in the perchloric acid precipitate, using the method of Marshak and Vogel(70).

A disadvantage of our procedure is found in the relatively large quantity of tissue required, i.e., a sample containing about a hundred mg. of RNA for duplicate analyses In many cases this would be a prohibitive factor. A micro modification would, therefore, be of great value. This will require further study of many factors(e.g., the solubility of silver purines) and techniques, which we hope to undertake in the near future.

Chapter 11

THE ANALYSIS OF THE NUCLEOTIDE MIXTURE

This chapter deals with a spectrophotometric method for the analysis of the nucleotides after their elution from the resin columns; it is also applicable to purified RNA samples.

The procedure is essentially that of Kerr, Seraidarian and Wargon(53) with minor modifications. It consists of the acid hydrolysis of the nucleotides, the precipitation of the purines with silver and the separation from the pyrimidine nucleotides, and the determination of the resulting two binary mixtures by differential spectrophotometric equations.

For simplicity the steps will be taken separately.

<u>Acid Hydrolysis.</u> It has already been demonstrated that hot dilute mineral acids hydrolyse RNA quantitatively liberating the purine bases and the pyrimidine nucleotides (93,94). The time required for the hydrolysis at 100°C in N H_2SO_4 was also established to be well within an hour(53). For our purposes 1 hour in a boiling water bath was always used.

Since the nucleotides are recovered from the tissues in a N H_2SO_4 solution(Chapter 1), it is necessary merely to place in a water bath for one hour at 100°C.

<u>Silver Precipitation of the Purines.</u> Kerr and Seraidarian(95) studied the optimal conditions to ensure the quantitative precipitation of the free purine bases without precipitating nucleotides, and found a pH of around

3 to be optimal. Such a pH can easily be attained if the acid hydrolysate is neutralized and then reacidified with acetic acid. This is recommended if the analysis is performed on purified RNA samples, but eluates from the Dowex-2 acetate columns already contain acetic acid. This was determined by titration to be about 0.1 N. Hence, when the analysis is performed on column eluates about 9/10 of the acidity whould be neutralized by the addition of 10 N NaOH.*

The silver purine precipitate is washed three times with 0.1 N acetic acid saturated with silver sulphate to prevent the resolution of the precipitated purines.

Spectrophatometric Measurements. The Beckman model DU spectrophatometer, and cells with 10 mm light path were used througnout.

It is necessary when using such an instrument to keep the conditions as constant as possible to get reproduceable results, especially since it is impractical to keep standard solutions and measure them with all unknowns.

A source of error that has been overlooked by workers in the nucleic acid field is due to different normal band-widths caused by different slit-width settings when taking measurements. An excellent discussion of this is presented by Mellon(96). For our purposes an effective band-width of 1 mμ was found convenient and was used throughout. This is

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* It is important to add concentrated NaOH solutions to keep the volume of the solution small, to ensure the complete precipitation of the purines.

done by setting the slit-width at the appropriate opening for the wavelength used *,and adjusting the sensitivity control to bring the galvanometer needle to zero.

Reference Standards. In order to formulate simultaneous equations for the estimation of a mixture of purine basis or of pyrimidine nucleotides,it is necessary to know the molecular extinction coefficients not of the pure compounds,but rather of these after they have been subjected to the treatment(e.g.,acid hydrolysis)encountered in the analytical procedure. For the purpose the four nucleotides of RNA were liberated by alkaline hydrolyses from yeast nucleic acid and separated on a large Dowex-2 column by Cohns formate method(41).

The purine nucleotides were hydrolysed by making the eluants N with respect to H_2SO_4 and maintaining the solution for one hour,at 100°C. The purines were then precipitated as the silver salts by the addition of silver sulphate. The precipitate was washed several times with 0.1 N H_2SO_4 saturated with silver sulphate. The purines were then extracted with N HCl and the precipitated silver chloride was centrifuged off.

The supernate properly diluted,was used as the stock standard. On it nitrogen was determined by the micro-Kjeldah procedure(97)and from it portions were taken for spectral absorption determinations in 0.1 N. From these results the molar extinction coefficients were calculated(see Figure 12 and Table 4.)

The pyrimidine nucleotides were precipitated as the barium salts(98),washed and dryed. A portion of the salt was treated with N H_2SO_4 at 100°C for 1 hour. The precipitated barium sulphate was centrifuged off and the supernate used for nitrogen(97)and phosphorus(86)determinations and also for the determination of the absorption spectra in 0.1 N acid. The nitrogen to phosphorus ratios were quite close to the theoretical being 3:1.008 for CyA and 2:1.026 for UrA. The molar extinction coefficients could be calculated from either with the same results(see Figure 13 and Table 5).

* The Beckman company bulletin No 89A provides the information.

Table IV

Molar Extinction Coefficients X 10^{-3} of Adenine(46.0 M)and Guanine(63.4 M)in 0.1 N HCl after heating 1 hour 100° with N H_2SO_4.

(all values at normal band-width of 1mμ)

λ mμ	Adenine	Guanine
220	4.55	4.36
225	3.45	3.57
230	2.68	4.60
235	3.57	7.31
240	5.31	9.20
245	7.58	10.[illegible]
250	9.03	11.19
255	11.68	10.11
260	12.43	8.25
262	12.60	7.74
265	12.39	7.40
270	10.70	7.38
275	7.38	7.25
276	6.74	7.22
280	4.72	6.66
284	2.20	5.80
285	1.72	5.58
290	0.35	4.01
295	0.07	2.21
300	0.11	0.88
310	0.08	0.06

Table V

Molecular Extinction Coefficiants X 10^{-3} of Cytidylic(69.2 M)and Uridylic(33.8 M)a cids in 0.1 N H_2SO_4 after heating 1 hour at 100°C with N H_2SO_4.

(all values at normal band-width 1mμ).

λ mμ	CyA	UrA
220	8.53	4.60
225	6.29	2.56
230	3.95	2.15
235	2.47	2.54
240	1.96	3.85
245	2.27	5.61
250	3.26	7.40
255	4.83	8.95
260	6.77	9.58
265	8.93	9.22
266.5	9.56	8.95
270	10 .96	7.76
275	12 .17	5.58
280	12.47	3.26
285	11.29	1.31
290	8.86	0.33
295	5.65	0.09
300	2.40	0.00
310	0.23	0.00

The treatment of the "standard" solutions thus prepared with N H_2SO_4 at 100°C for 1 hour serves to duplicate the conditions used in the procedure. This has the advantage that it accounts for changes occuring in the spectral curves due to deamination,dephosphorlation,etc;,which might occur during acid hydrolysis. It also serves in the case of the pyrimidine nucleotides to establish equilibrium in the mixture of the 2' and 3' isomers(50). This is of great importance since the absorption spectra of the two isomers are different(7).

It must be stressed that the values reported for molar extinction coefficients here are not the values for the pure compounds but rather for these compounds after being subjected to treatment with normal H_2SO_4 at 100°C. for 1 hour.

<u>Equations</u>. From the molar extinction coefficients the equations were calculated,according to the method of Kerr et al(53):

Adenine(μM per liter)=103.4 OD_{262} − 137.6 OD_{284}

Guanine " " " =224.5 OD_{284} − 39.25 OD_{262}

Sum of Purines" " " =$OD_{275.5}$X 139.8

Cytidylic acid" " " =98.6 OD_{280} − 32.9 OD_{260}

Uridylic Acid " " " =128 OD_{260} − 69.6 OD_{280}

Sum of Pyrimidine Nucleotides(μM per liter)=$OD_{265.5}$X109.7

<u>Procedure</u>. The procedure finally adopted is as follows: The sulphuric acid eluate from the column is diluted to 25 ml. and mixed well. Duplicate 10.0 ml. portions in 25 ml. centrifuge tubes are placed in a boiling water bath for 1 hour,after which 1 ml. of 9 N NaOH is

added, thus rendering the solution approximately 0.1 N with respect to acetic acid. The tube is then cooled in ice. 3 ml. of saturated silver sulphate solution and a pinch of solid powdered silver sulphate are added. The tube is left in ice for at least one hour for the silver purine precipitate to fluctuate, and then centrifuged sharply to pack the precipitated purines. The supernate is decanted into a 100 ml. volumetric flask. The precipitate is washed three times with 10 ml. portions of N/10 acetic acid saturated with silver sulphate, the washings being added to the first supernate in the volumetric flask. The washed precipitate is reserved for the determination of the purine.

To the combined supernatent fluid and washings, containing the pyrimidine nucleotides are added 25 ml. of N HCl to precipitate the excess silver, and the mixture is diluted to volume. This is reserved for determination of the pyrimidine nucleotides.

(1) Blanks. A Dowex-2 column containing no nucleotides was eluted with 25 ml. of N H_2SO_4, and this eluate was treated exactly as the eluate containing the hydrolyzed nucleotides in order to secure "blank" solutions for the spectrophotometric measurements of the purines and the pyrimidine nucleotides.

(2) Purines. The silver-purine precipitate is extracted 3 times for 10 minutes in a boiling water bath with 5 ml. portions of N HCl. The extracts are combined in a 100 ml. volumetric flask and made to volume. Measurements of optical density is made at 262, 275.5, and 284mμ.

Adenine and guanine concentrations are calculated by means of the equations above.

(3) Pyrimidine Nucleotides. A portion of the solution of pyrimidine nucleotides is freed of silver chloride by centrifugation, and the supernate is examined spectrophotometrically, the optical density being measured at 260, 265.5, and 280 mμ.

Uridylic acid and cytidylic acid concentrations are calculated by the use of the equations above.

Discussion. The principle of the method and its accuracy have well been established(53,54) and need no further discussion. A few points however need to be made clear.

Loring et al(54) object to the use of the pyrimidine nucleotides in the binary mixture for spectrophotometric determination(53), due to the fact that the 2' and 3' nucleotides have different absorption spectra. These authors therefore hydrolyze the nucleotides enzymatically to split them down to the nucleosides before the determination. We see no advantage in that step, since Loring et al(50) have proved that when any one of the isomers is heated with a mineral acid it forms an equilibrium mixture of the two isomers. Since our procedure entails heating for an hour with mineral acid, the pyrimidine nucleotides in the final solutions are in the form of those equilibrium mixtures. Our formulae for the calculation of the components of the binary mixtures were developed from spectral data determined on the equilibrium mixtures of the pyrimidine nucleotides. Hence we see no advantage in splitting off the phosphate group.

Loring et al(54) point out that even the purest RNA preparations contain aromatic amino acids which absorb ultra-violet light and suggest a tedious procedure for getting rid of them. Our solutions (the eluates from the columns) are shown to contain no amino acids in detectable amounts (See Section on Purity of Isolated Nucleotides in Chapter 1.)

SUMMARY

The methods of analysis of RNA have been reviewed and discussed. It is apparent that a method is needed for the assay of the nucleotides of RNA as it occurs in the original tissue, i.e., the "native" RNA. A method is devised, the first stages being based on the Schmidt-Thannhauser procedure(74) with minor modifications. The tissues are treated with trichloracetic acid to remove the acid-soluble fraction, and the lipids are extracted, and the dry residue thus prepared is treated with 0.5 N KOH - N N H_4OH. This shown to split the ribonucleic acid into mononucleotides without causing the deamination of cytidylic acid.

From this point the procedure is designed to get rid of light-absorbing material derived from tissue proteins, which interfere with all procedures so far used in nucleic acid analysis.

The proteins are then precipitated with $HClO_4$ and the excess ClO_4^- ions are precipitated with KOH. The supernate is adjusted to pH 6.0 and portions of it are passed through Dowex-2 columns which are washed previously with a buffer at pH 6.0. The nucleotides adsorb quantitatively, while very little interfering material adsorbs. The nucleotides are then eluted with sulphuric acid, the recovery of nucleotides in this treatment being about 98 per cent.

The sulphuric acid solution of the nucleotides is heated at 100° for 1 hour to hydrolyse the purine nucleotides to the free bases. The procedure from this point is

essentially that of Kerr et al(53),modified to improve the spectral data from which the simultaneous equations are derived. The spectral transmission curves of the purines and the pyrimidine nucleotides are recorded after these receive the treatment they undergo in the procedure. From these curves the simultaneous equations for estimating the individual components of the binary mixtures are recalculated.

The conditions for the various steps in the procedure are studied and discussed.

The method as a whole and its applications are discussed.

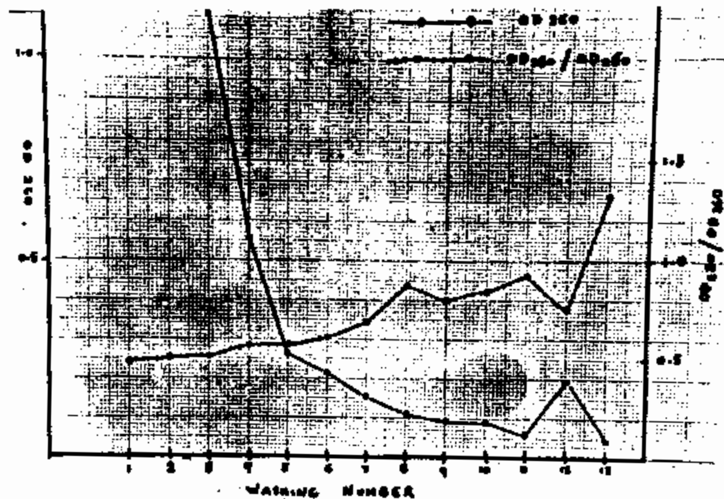


FIGURE 1

Washing frozen fresh tissue with TCA. The first washing is with 8 per cent. All subsequent washings with 2 per cent TCA.

Note plateau maintained in Od 280/OD 260 in washings 1-6. (Data from Table 1)

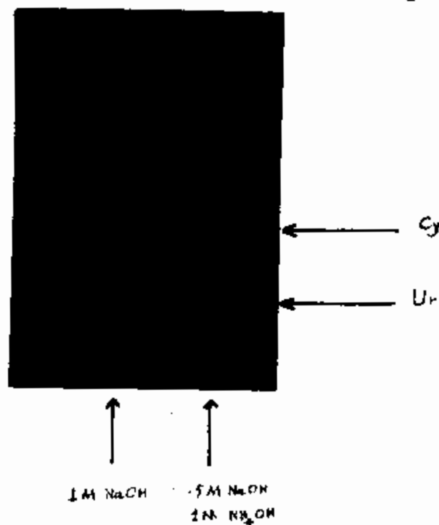
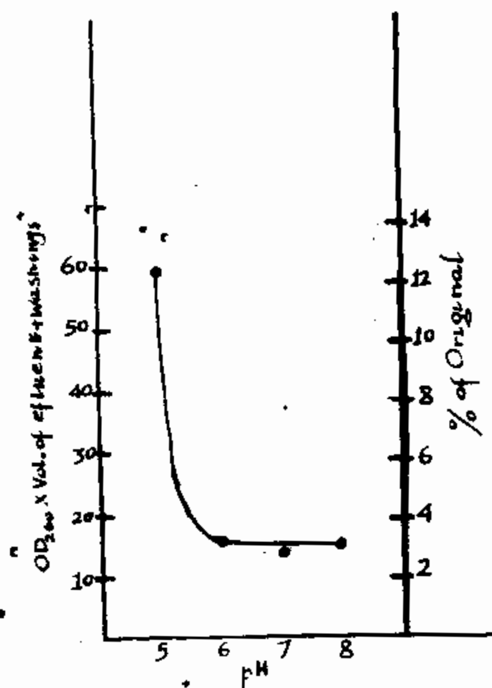


FIGURE 2

The ultraviolet direct print of a chromatogram of cytidylic acid treated with N NaOH and 0.5N NaOH-N NH_4OH overnight at room temperature. Notice the faint white spot before the cytidylic acid spot in the case of treatment with N NaOH.



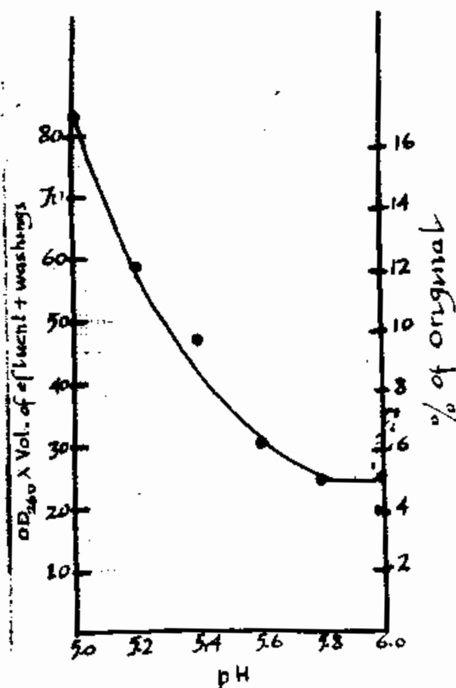


FIGURE 3 FIGURE 4

Adsorption of RNA hydrolysates onto Dowex-2 columns.

Columns: Dowex-2, 0.5 sq. cm X 5 cm;, phosphate form, washed with phosphate buffer at indicated pH.

Test Materials: 14 mgms. RNA alkali hydrolysate in 25 ml. adjusted to the appropriate pH by addition of H_3PO_4.

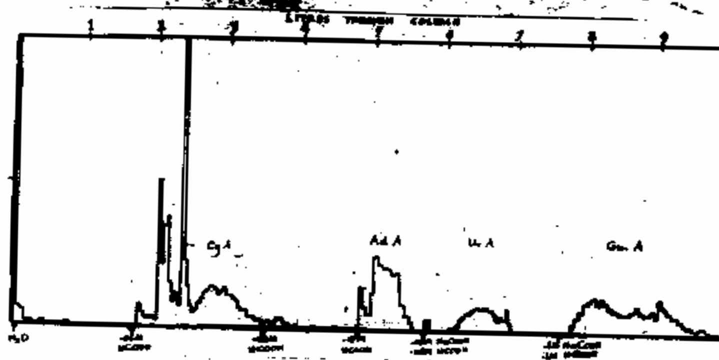


FIGURE 5

Cohns' separation of "dry-tissue" alkaline hydrolysate adsorbed onto the column from 0.2N NaOH solution. Notice the non-nucleotide interfering material which was eluted from the column just before the cytidylic acid. Compare with Fig. 6.

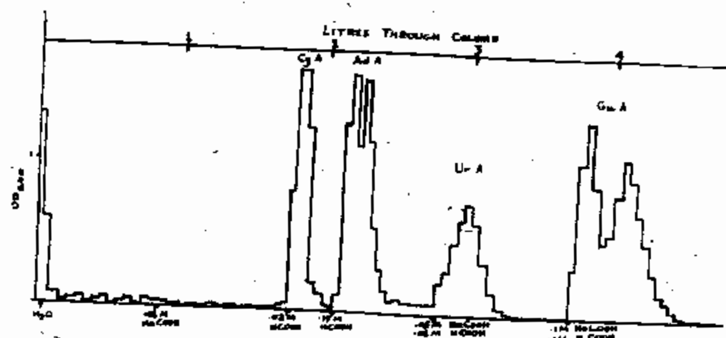


FIGURE 6

Cohns' separation on "dry-tissue" alkaline hydrolysate, adsorbed at pH 6.0 by a Dowex-2 column previously washed with phosphate buffer pH 6.0.

Notice the separation of the nucleotides from non-nucleotide light absorbing material.

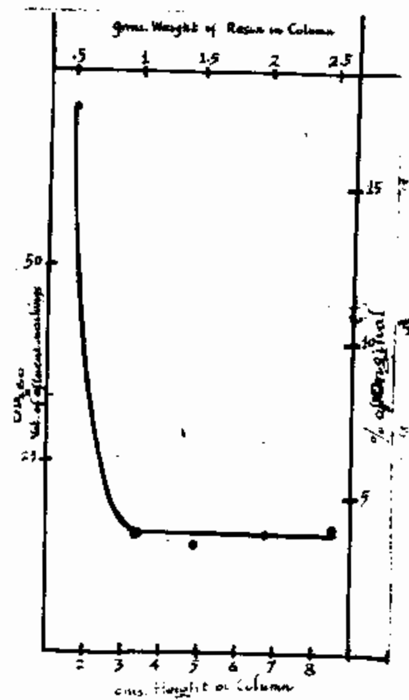


FIGURE 7

Determination of the minimum size column of Dowex-2 required to adsorb 14.8 mgms. of RNA alkaline hydrolysate.

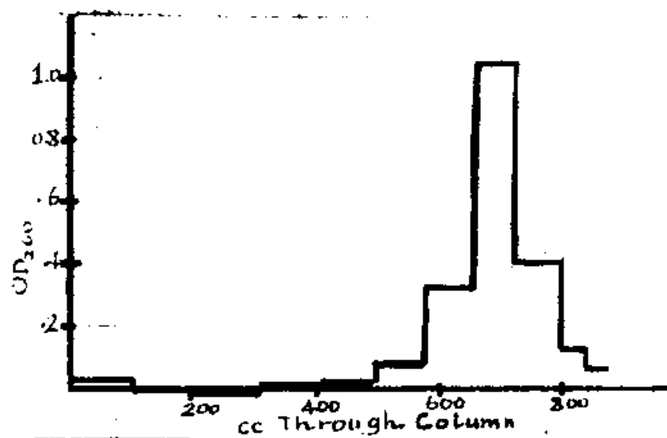


FIGURE 8

The elution of nucleotides from Dowex-2 columns by M/200 $(NH_4)_2SO_4$, pH 5.0.

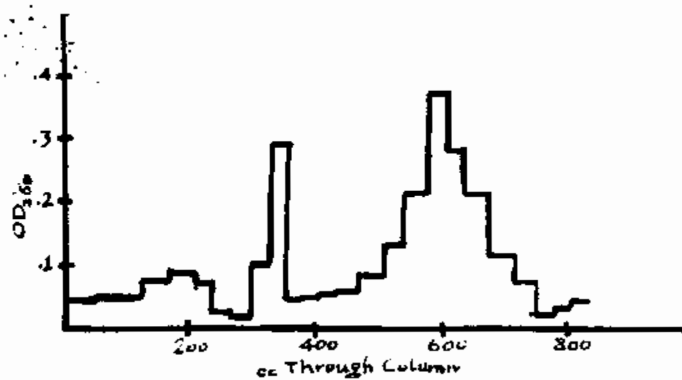


FIGURE 9

The elution of nucleotides from Dowex-2 columns by M/200 $(NH_4)_2SO_4$, pH adjusted to 8.5 by NH_4OH.

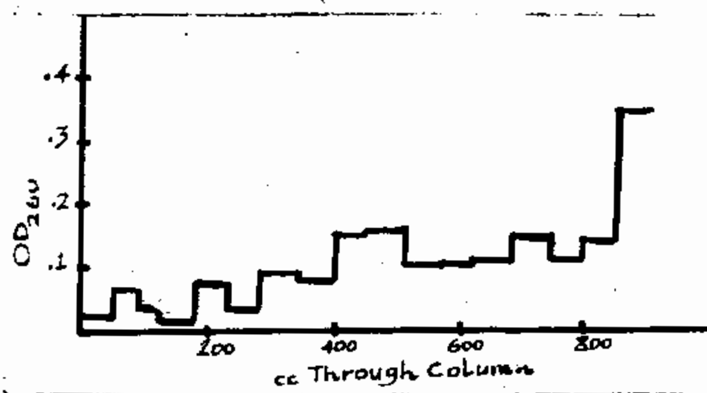


FIGURE 10

The elution of the nucleotides from Dowex-2 columns by M/1000 $(NH_4)_2SO_4$.

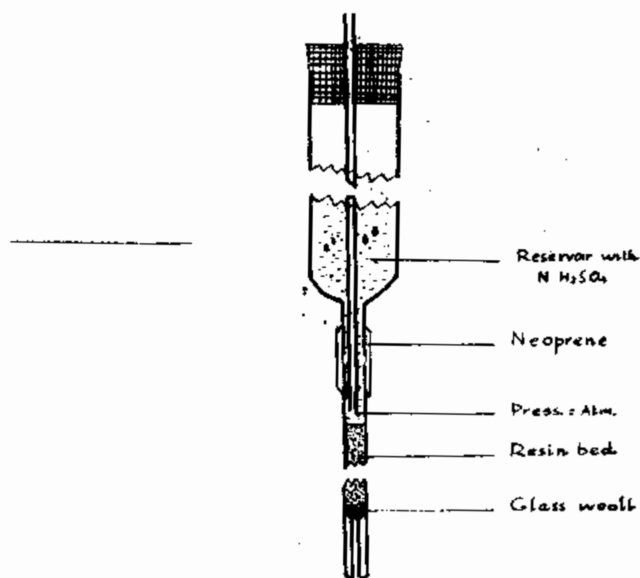


FIGURE 11

The use of the Marriott Bottle principle

for the elution of nucleotides from resin columns.

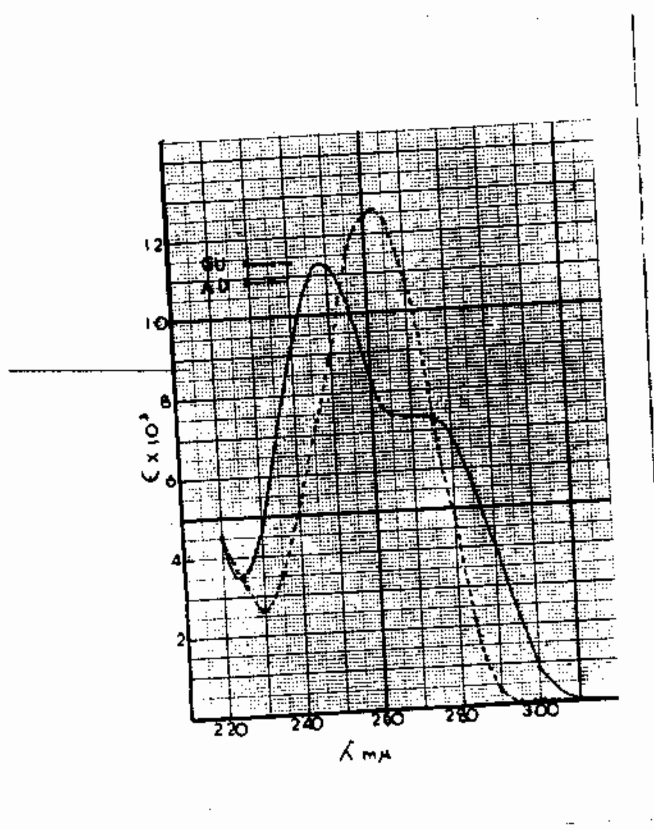


FIGURE 12

The ultraviolet absorbtion spectra of Adenine and Guanine(data from Table 4)

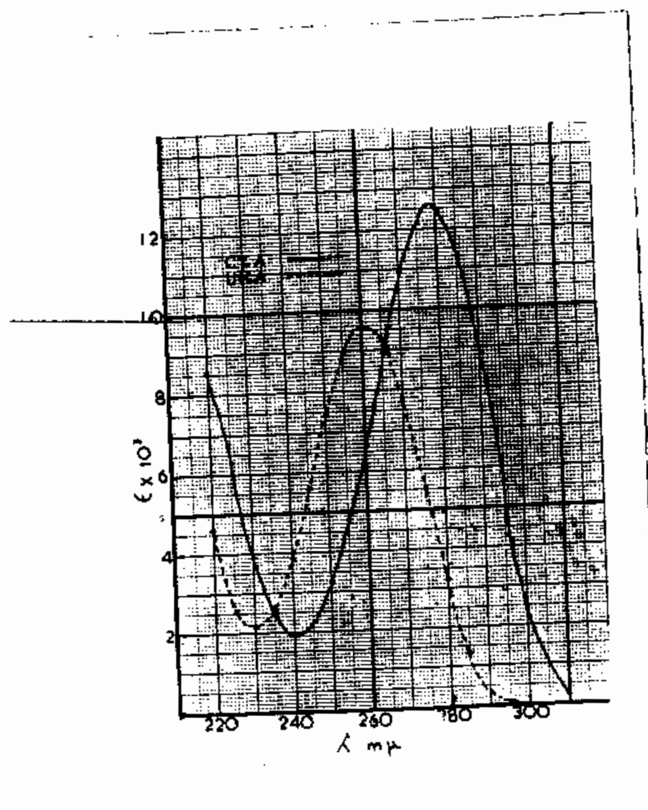


FIGURE 13

The ultraviolet absorbtion spectra of CyA and UrA(data from Table 5)

BIBLIOGRAPHY

(1) Jones,W. "Nucleic Acids", Longman Green & Co. NY,(1920)2-Ed

(2) Levene,P.A. Bass,L.W. "Nucleic Acids", Chem.Cat Co.,NY(1931)

(3) Gulland,J.M. Cold Spring Harbor Symp Quant Biol, 1295(1947)

(4) Levene,P.A. Tipson,R.S., J.Biol.Chem. 93,623(1931)

(5) Levene,P.A. Harris,I.F. J. Biol.Chem. 101,484(1933)

(6) Carter,C.E.,J.Am.Chem.Soc.,72,1466(1950)

(7) Cohn,W.E.,J. Am.Chem.Soc.,72,1471(1950)

(8) Loring,H.S.,Luthy,N.G. J. Am.Chem.Soc.,73,4215(1951)

(9) Brown,D.M.,Todd,A.R.,J.Chem.Soc.,(1952),44

(10) Gulland,J.M.,J.Chem.Soc.,(1944),208

(11) Gulland,J.M.,Barker,GR,Jordan,D.O.,An.Rev.Biochem,14,175 (1945),

(12) Myrback,K.,Jorpes,E. Z.Phys.Chem.,237,159(1935)

(13) Hammarsten,E.J.Biol.Chem,43,243(19[illegible])

(14) Hammarsten,E.,Hammarsten,G. J.Biol.Chem.68,215(1928)

(15) Jorpes,E. Biochem.J. 28,2102(1934)

(16) Loring,H.S,J.Biol.Chem.128,Soc.Proc.33,61(1939)

(17) Cohn,W.E.,Stanley,W.M. J.Biol.Chem.144,589(1942)

(18) Fletcher,W.E. Gulland,J.M.,Jordan,D.O.,Dibben,H.E. J.Chem.Soc.(1944)30

(19) Loring,H.S.,Carpenter,F.H.,Roll,P.M.J.Biol.Chem.169, 601(1947)

(20) Visher,E.,Chargaff,E. J.Biol.Chem.,176,715(1948)

(21) Kerr,S.E.,Seraidarian,K.,Wargon,M.J.Biol.Chem.181, 773(1949)

(22) Pinker,J.F.,Brown,G.B.J.Biol.Chem.,173,585(1948)

(23) Edman,P.,Hammarsten,E.,Richard,P. J.Biol.Chem. 178,395(1949)

(24) Daly,M.M.,Musky,A.E. J.Biol.Chem.,179,981(1949)

(25) Richard,P. J.Biol.Chem.,179,763(1949)

(26) Vischer,E.,Chargaff,E. Fed.Proc.,7,197(1948)

(27) Plaut,G.W.E.,Kuly,J.A.,Lardy,H.A., J.Biol.Chem.184,
243(1950)

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

(29) Hotchkiss,R.D. J.Biol.Chem. 175,315(1948)

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

(31) Vischer,E.,Chargaff,E. Fed.Proc. 7,197(1948)

(32) Markham,R.,Smith,J.D. Nature,250,216(1949)

(33) Markham,R.,Smith,J.D. Biochem.J. 45,294(1949)

(34) Vischer,E.,Magasnik,B.,Chargaff,E. Fed.Proc.8,263(1949)

(35) Chargaff,E.,Magasnik,B.,Doniger,R.,Visher,E.J.Am.Chem;
Soc. 71,1513(1949)

(36) Smith,J.D.,Markham,R. Biochem.J. 46,509(1950)

(37) Magasnik,B.,Vischer,E.,Doniger,R.,Elson,D.,Chargaff,E.
J.Biol.Chem. 186,37(1950)

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

(39) Markham,R.,Smith,J.D. Biochem.J. 49,401(1951)

(40) Chargaff,E.,Magasanik,B,Vischer,E.,Green,C.,Doniger,R.,
Elson,D. J.Biol.Chem. 186,51(1950)

(41) Smith,J.D.,Markham,R., Biochem.J. 46,509(1950)

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

(43) Smith,S.,Wander,S.E. J.Am.Chem.Soc. 70,3719(1948)

(44) Carter,C.E.,Cohn,W.E.,Fed.Proc. 8,190(1948)

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

(46) Cohn,W.E. J.Am.Chem.Soc. 71,2275(1949)

(47) Cohn,W.E. J.Cell Comp Biol. 38,Sup. 1,2(1951)

(48) Roll,P.M. (unpublished)

(49) Cohn,W.E.,Carter,C.E. J.Am.Chem.Soc. 72,2606(1950)

(50) Loring,H.S.,Bortner,H.W.,Levy,L.W.,Hammell,M.L.
J.Biol.Chem. 196,807(1952)

(51) Volkin,E.,Carter,C.E. J.Am.Chem.Soc. 73,1516(1951)

(52) Cohn,W.E. J.Am.Chem.Soc. 72,1471(1950)

(53) Kerr,S.E.,Saraidarian,K.,Wargon,M. J.Biol.Chem. [illegible],761,
(1949)

(54) Loring,H.S.,Fairley,J.L.,Botner,H.W.,Seagun,H.L. J.Biol.
Chem. 197,809(1952)

(55) Kalckar,H.M. J.Biol.Chem. 167,429(1947)

(56) Loring,H.S.,Ordway,G.L.,Pierce,J.G. J.Biol.Chem. 176,
1123(1948)

(57) Abrahms,R. Arch.Biochem. 30,44(1951)

(58) Smellie,R.M.S.,Davidson,J.N. Biochem.J. 49,XV(1951)

(59) Davidson,J.N.,Smellie,R.M.S. Biochem.J. 52,594(1952)

(60) Markham,R.,Smith,J.D. Biochem.J. 52,552(1952)

(61) Kerr,S.E.,Saraidarian,K. J.Biol.Chem. 180,1203(1949)

(62) Barnum,C.P.,Nash,C.W.,Jennings,E.,Nygaard,O.,Vermund,H.,
Arch.Biochem. 25,376(1950)

(63) Grinnan,E.C.,Mosher,W.A. J.Biol.Chem. 191,719(1951)

(64) Volkin,E.,Carter,C.E. J.Am.Chem.Soc. 73,1516(1951)

(65) Barker,J.E.,Allen,F.W. J.Biol.Chem. 183,641(1950)

(66) Markham,R.,Mathews,R.E.F.,Smith,K.M. Nature 162,88(1948)

(67) Stacey,M. Sym.Soc.Exp.Biol. 1,86(1948)

(68) Elson,D.,Chargaff,E. Phos.Met. 2,329(1952)

(69) Fairley,J.L.,Seagram,H.L.,Loring,H.S. Fed.Proc. 9,169(1950)

(70) Marshak,A.,Vogel,H. J.Biol.Chem. 189,597(1951)

(71) Thomas,R. Biochem.Biophys.Acta. 8,71(1952)

(72) Boulanger,P.,Montreuil,J. Biochem.Biophys.Acta. 9,619,(1953)

(73) Davidson,J.N.,Smellie,R.M.S. Biochem.J. 52,599(1952)

(74) Schmidt, ,Thannhauser, J.Biol.Chem. 161,83(1945)

(75) Kerr,S.E.,Saraidarian,K. (unpublished data)

(76) Folch,J.,Le Baron,F.N. Fed.Proc. 10,183(1951)

(77) Jones,W. J.Biol.Chem. 25,93(1916)

(78) Cavelary,L.E. J.Biol.Chem. 77,489(1928)

(79) Steadie,W.C. Z.Phys.Chem. 120,295(1922)

(80) Davidson,J.N.,Smellie,R.M.S. Biochem.J. 52,599(1952)

(81) Loring,H.S.,Roll,P.M.,Pierce,J.G. J.Biol.Chem. 174,729 (1948)

(82) Kerr,S.E. Unpublished Observations (1945)

(83) Ogur,M.,Rosen,G. Arch.Biochem. 25,263(1950)

(84) Busch,H.,Hurbert,Potter J.Biol.Chem. 196,717(1952)

(85) Yamagawa,M. J.Biol.Chem. 43,339(1920)

(86) Fiske,C.H.,Subarrow,Y. J.Biol.Chem. 66,375(1925)

(87) The Dow Chemical Co.,"Dow Anion Exchange Resins",P.4, Midland,Michigan

(88) Cohn,W. (Private Communication with Prof. S.E. Kerr)

(89) Sevag,M.G.,Smolens,J.,Lackman,D.B. J.Biol.Chem. 134, 723(1940)

(90) Stumpf,P.K. J.Biol.Chem. 169,367(1947)

(91) Davidson,J.N.,Smellie,R.M.S.,(Private Communication with Author)

(92) Schneider,W.C. J.Biol.Chem. 161,293(1945)

(93) Levene,P.A. Berchemges 44,1027(1911)

(94) Jorpes,E. Acta.Med.Scand. 68,503(1928)

(95) Kerr,S.E.,Saraidarian,M. J.Biol.Chem. 159,211(1945)

(96) Mellon,M.G."Analytical Absorbtion Spectrescopy",John Willey and Sons Inc. N.Y.(1950)

(97) Ma,T.S.,Zuazaga,G. Ind.Eng.Chem.Anal.Ed. 14,280(1942)

(98) Levene,P.A.,La Forge,F.B. Ber.Chem.Ges. 44,1027(1911)