A QUANTITATIVE STUDY OF THE NUCLEOTIDE COMPONENTS OF RIBONUCLEIC ACID OF CERTAIN MAMMALIAN TISSUES UNDER VARIOUS PHYSIOLOGICAL CONDITIONS

by

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A C K N O W L E D G E M E N T

I wish to express my gratitude to Dr. Stanley E. Kerr, Chairman of the department of Biological Chemistry, American University of Beirut, under whose supervision this work was carried out.

I also wish to extend my appreciation to the members of the department for valuable help, with special thanks to Dr. A. Khatchadourian for the preparation of animal tissue.

Divers weights are an abomination unto the Lord; and a false balance is not good.

Proverbs, XX,23.

T A B L E O F C O N T E N T S

I N T R O D U C T I O N

It is the aim of biochemical research to correlate the chemical properties of natural substances with their metabolic activity in the living organism. Of particular interest is the study of the properties and function of nucleic acids in the cell, and this was made possible after the development of more accurate analytical micro methods among which is the estimation of the individual components of desoxyribo- and ribo- nucleic acids (DNA and RNA), especially the pyrimidines.

According to the generally accepted Boivin-Vendrely findings the average content of DNA per nucleus in somatic cells of a given species and under normal conditions is constant (1). It seems that DNA is the least variable cell constituent. An important application of this fact is the use of DNA values as reference when the concentration of other cell components is studied.

Considerable isolated data have been reported on the nucleic acid content of tissues under various circumstances. The DNA_n [1] is not changed in adult rats fed protein-free diets or fasting, while RNA_c and the RNA : DNA

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1. The subscript n will be used to denote DNA or RNA value per nucleus, subscript c, the value per cell, and subscript g, the value per gramof tissue.

ratio decreases (2-7). The DNA_n increases, however, in young rats or at least in those exposed to prolonged fasting or protein deprivation (6). In pregnancy RNA_c increases up to the third week (8). In regenerating liver both DNA_n and RNA_c or RNA_g increase (4,9,10). Apparently in the regenerating liver of rats RNA_g is higher in animals on a protein-free diet than in those on a high protein diet (11). The nucleic acid content of chick heart fibroblast cultures, growing in tubes, has been studied at various time intervals (12,13). With apparent signs of growth, RNA per tube increases and is followed by a rise in DNA. In neoplastic tissue DNA_c and RNA_c are reported to increase (14,17). After pilocarpine administration (which stimulates secretion), Rabinovitch and his colleagues (18) found no statistically significant variation in RNA_g and DNA_g (also in the RNA : DNA ratio) in mouse pancreas,while other workers (19) found an increase in both RNA_g and DNA_g of dog's pancreas three to four hours after secretin administration. Davidson found no significant change in RNA_n and DNA_n as well as in the RNA : DNA ratio in alloxan diabetes (4), and an increase in cell number and RNA_c without alteration of DNA_c after insulin treatment (20,23). It is reported that DNA_g and DNA_n, the latter determined by the Feulgen reaction, increase after alloxan administration (21), and that it is due to alloxan per se and not to diabetes (22), because pancreatectomized diabetic rats did not have higher DNA_n values. The authors also suggest an increase in nuclear RNA because differences

in total nucleic acid of nuclei (as measured by absorption spectrophotometry) could not be accounted for by differences in DNA alone. However, this is not sufficiently direct evidence, and their ultraviolet absorption measurements may have been affected by the presence of proteins as well as by physical changes in the nucleic acids.

There is a tendency to describe DNA as "species specific" because its composition is found to be identical in different organs of the same species, showing sometimes variation in two different species (24,25).

Whether RNA is species specific or organ specific is difficult to judge from the published analyses. The findings of several authors show that RNA isolated from the livers of different species differs somewhat in composition (26,28), also that RNA isolated from various organs of the same animal varies in composition (27). However, divergent values are reported by different investigators (27,29,30) for the RNA obtained from the same source, and the variations one can find in the composition of RNA obtained from the same organ of the same species are as great as those in RNA composition from the same organ of different species; thus we cannot conclude from the data so far published whether RNA is organ specific or species specific. The only striking difference so far reported is that found in human liver RNA, which has a higher content of guanylic and cytidylic acids than the liver RNA of other

species (26,108).

The lack of agreement in published data pertaining to nucleic acid composition could, in part, be explained by the fact that in the experiments reported above, as well as in other instances (31-33), the nucleic acids have been isolated from the tissue before analysis with possibly incomplete recovery, and enzymatic degradation. In fact, it has been shown that the molecular weight of the isolated ribonucleic acids and the ratio of the four bases depend upon the method of isolation (34,35) and purification (30). The question is whether the isolated preparation can still be regarded as representative of the state in which it occured in the living cell. There are a few instances (36-41,111) where ribonucleotides were determined without prior isolation of RNA from the tissue, but no study was undertaken, using such methods, on the RNA composition under special physiological conditions. Further, the validity of the methods used by these workers (36-41) has been challenged (103,p.5). The method of Olmsted and Villee (111) seems to be very similar to ours; however, their results might not be quantitative due to losses of ribonucleotides from enzymatic and acid degradation during the preparation of the tissue because there was no mention that it was done in the cold. Also, while deproteinizing with Sevag's reagent losses may have occured by adsorption on the protein gel.

Studies on neoplastic tissue (from which DNA and RNA were isolated prior to analysis) revealed no change in DNA composition (24,42,43), while in RNA, some increase was reported in cytidylic and possibly in guanylic acid, as compared with adenylic acid (26,27). It was found that during growth and maturity RNA composition remains constant in the mitochondrial and microsomal fractions (28,111).

Data on turnover rates of nucleic acids reveal that under certain conditions there is a quantitative change in nucleic acid metabolism. It has been found that labeled formic acid and adenine are incorporated at different rates in the nucleic acids of various organs (44,51). In regenerating tissue and in growth the turnover rates of RNA and DNA, as determined by the incorporation of phosphorus P^{32} or N^{15}- labeled glycine, are increased (45-47). The turnover is also increased in pancreas stimulated to secretion by secretin (49) and in tumors, where it was found to be intermediate between that of regenerating and resting tissue (50, 46). In the recent work of Munro and his group (52,53), it is observed that on a high protein diet when the energy level of food in terms of fat and carbohydrate is raised [2], the content of RNA is increased without an increase in P^{32}

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2. Note that there are four groups of animals:
   I-A : high protein, low calorie intake
   I-B : " " , high " "
   II-A : low protein, low " "
   II-B : " " , high " "

incorporation; on low protein diet, when the energy intake is raised, total RNA is slightly changed, while P^{32} incorporation is tremendously increased. This has also been observed by Davidson (20) who notes (48,p.172) a quantitative metabolic relationship between RNA and energy metabolism.

It is obvious from the previous discussion that few studies have been made on the composition of tissue RNA under various physiological conditions, and each of these studies suffers from a radical defect, this being either the prior and incomplete isolation of nucleic acid with probable enzymatic decomposition, or inaccuracy of the method. Hence we have undertaken such a study using analytical methods (103) which do not require the prior isolation of RNA from the tissue. We limited ourselves to a comparison of liver RNA in normal and diabetic rats with the intention of continuing the next part of the experiment (i.e. the effect of insulin treatment) in the near future.

In diabetes the situation is ambiguous; it seems this is a generalized disease and that insulin is related not only to carbohydrate, but also to fat, protein, and phosphorus metabolism (54-56). We thought of studying diabetes because of the relationship of insulin to protein metabolism, which in turn seems to be related to the nucleic acids as shown by the above discussion on the

turnover rates of nucleic acids and by other evidence (57-60). In diabetes there is a decrease in the incorporation of C^{14} amino acids into glutathione, this being restored to normal only after addition of glucose and insulin (61). Insulin decreases the level of amino acids in blood in proportion to their amount in body protein and skeletal muscle (62). Insulin raises the incorporation of S^{35} methionine into muscle protein in diabetic animals (63). It also decreases blood and plasma levels as well as urinary excretion of ascorbic acid (64). The latter, therefore , goes to extravascular tissues , and it is known that it is concerned with the deposition of the intercellular "cement" and collagen (glycoproteins) and with dehydrogenation (for example the conversion of folic acid into the citrovorum factor which is essential in the biosynthesis of nucleic acid) (65). A relationship between insulin and purine metabolism was found long ago (66). In a breed of the Dalmatian dog, which excretes both allantoin and uric acid in measurable quantities, insulin increases the blood uric acid, but only if there is hypoglycemia; uric acid in urine is increased while allantoin is not changed. This is the type of evidence which suggests a possible correlation between the action of insulin and the metabolism of protein and possibly the nucleic acids as well.

Although the main objective of this study is RNA composition, we determined also the content of protein

(those values are lacking in Davidson's report (4)) and DNA, the latter serving as reference inasmuch as its content per cell remains constant.

We believe the results should be significant from two points of view:

1) They will show whether or not changes in RNA composition may occur under the specified conditions.

2) Even if no change is observed the results will be valuable in revealing the composition of the "native" RNA of rat liver.

M A T E R I A L S A N D M E T H O D S

I. Animals.

Four groups of female albino rats, of about the same age and weight, were used:

Group 1; normal untreated.
2; diabetic, untreated.
3; normal, insulin treated[3].
4; diabetic, insulin treated.

Diabetes was induced by alloxan which has been widely used and shown to have a selective necrosing action on the islets of Langerhans (67,68). It probably combines with the sulfhydryl groups of proteins, inactivating essential enzymes (69-72). The blood sugar curve after alloxan shows first a hyperglycemic phase, then a transient hypoglycemia followed by persistent hyperglycemia (73-77). Some (78) have noted even a tetraphasic sugar curve with an initial short hypoglycemia. Many factors influence the response to alloxan (79-82), and the development of diabetes in this way is rather a tedious job. The susceptibility of animals and their mortality rate are found to vary tremendously from one experiment to the other.

In our experiments alloxan was administered as the hydrated form, the dosage being 175 mg per kilo of body

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3. In groups 3 and 4 liver tissue has not yet been prepared and analyzed. The discussion and results of the study of the first two groups only will be presented here.

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weight, after a fast of 48 to 60 hours, as suggested by Kass and Waisbren (83). It was injected hypodermically as a 2 percent aqueous solution. When injected intraperitoneally alloxan does not seem to be diabetogenic (84). The control animals (except III-1, III-2, and III-3) were injected with water. All rats were then given water ad libitum and fed a stock diet[4] composed of the following:

Panboiled wheat (burghul)	35 parts
Yellow corn meal	35
Skim milk powder	15
Casein	10
Cod liver oil	2
Sodium chloride	1
Yeast	2

Only those rats showing characteristic signs of diabetes over a period of at least two weeks, with blood sugar above 250 mg per 100 cc, were used.

II. Preparation of Tissue.

The rats were anesthetized by intraperitoneal injection of 70 mg of amytal per kilo of body weight. The abdomen was opened and the liver perfused carefully with cold 0.14 M saline after ligating the inferior vena cava above the renal vein, cutting the portal vein and canulating the inferior vena cava above the liver. The liver was then removed, freed from connective tissue, cut into

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4. The stock diet composition was suggested by Dr. W. Adolph, professor of nutrition in the Department of Biochemistry.

pieces, blotted with filter paper and weighed.

For the preparation of "dry tissue" (acid-washed, lipid-free), a modified Schmidt and Thannhauser procedure was applied (85). The wet tissue was frozen in solid carbon dioxide-ether mixture and ground in a mortar with solid CO_2 . It was transfered to a centrifuge tube containing 8 percent trichloroacetic acid (TCA) in an ice bath. It was stirred for about 20 minutes, then centrifuged. The residue was washed 4 times with cold 2 percent TCA in order to remove the acid-soluble nucleotides, then 3 times with 95 percent alcohol, or until the supernate no longer contained the brown (hemin ?) pigments, 3 times with hot alcohol-ether mixture (3:1) to remove lipids, and finally twice with ether. It was left to dry in air, weighed and bottled. The results of this preparation are given in Table I .

III. Determination of Proteins.

The alkali-solution of the dry tissue sample (prepared as described in section IV, below) was assayed for total proteins by the biuret reaction (86,87), standardized by the micro-Kjeldahl procedure (88,89). The calibration curve used for calculation is given in Figure 1.

It was found that nucleic acids do not interfere with the biuret reaction since the addition of ribonucleotides to a protein solution did not alter the color intensity.

TABLE I

Record of Liver Samples

Rats	Initial weight	Wt. when sacrificed	Wt. of fresh liver	Liver sample wet wt.	Liver sample dry wt.
Normal					
I-7	170 gr	155 gr	6.05 gr	5.35 gr	0.903 gr
I-8	240	250	9.25	9.05	1.468
I-9	200	200	8.0	7.9	1.250
III-1	-	270	11.0	10.5	0.959
III-2	-	240	6.0	5.8	1.430
III-3	-	230	8.0	7.7	1.428
Diabetic					
II-6	175	125	6.0	5.8	1.141
II-8	145	105	6.2	6.0	1.091
II-3	180	140	5.78	5.58	1.048*
II-5	170	110	3.9	3.8	0.819*
II-10	155	110	5.28	5.08	1.103*
IV-2 +	115	123	6.4	6.1	1.463
IV-5 +	135	152	5.4	5.3	1.098
IV-8 +	145	140	5.7	5.4	1.170

(*) These three samples were mixed for analysis.

(+) These rats were given insulin for a few days, and then allowed to develop the characteristic symptoms of diabetes.

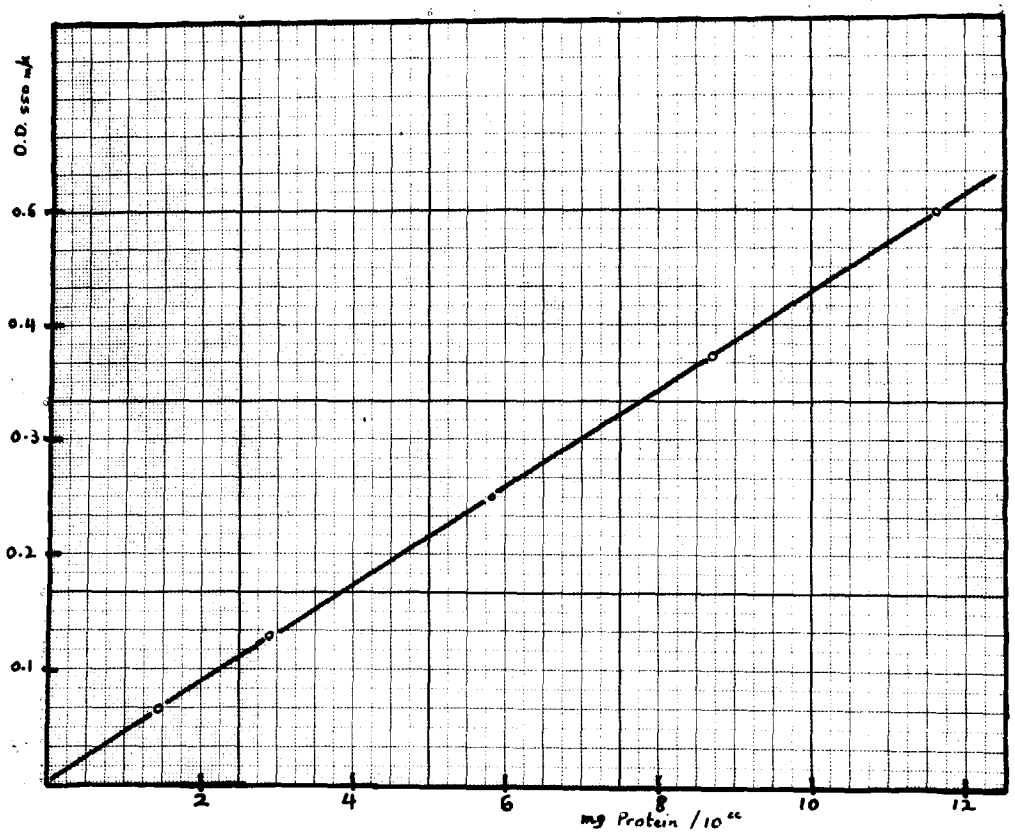

Fig. 1. Protein calibration curve for the biuret reaction

IV. Separation of RNA from DNA.

It is known that RNA is hydrolyzed in alkali quantitatively to mononucleotides soluble in dilute acid, while DNA is resistant to alkaline hydrolysis and may be reprecipitated by mineral acids (26,41,85,90-96).

It is also known that DNA is completely extractable from the protein precipitate with hot acids (97,98). On these two facts is based the method used for the separation of RNA from DNA.

The concentration of alkali used, as well as the temperature and the time of hydrolysis of RNA vary with different authors. The use of the following has been reported:

1 N	NaOH or KOH at	37°C	for	15-24	hours	(85,92,99).
0.5 N	" "	20-37°	"	18	"	(27,95).
0.3 N	" "	37°	"	18	"	(40,41,28).
0.1 N	" "	100°	"	150	minutes	(100).

Hydrolysis by ammonia solution in the autoclave (92), or at 45° for 8 days (95) have been recommended; also the use of barium hydroxide and barium carbonate (93,101).

It has been reported (102) that N NaOH or KOH at 37° C causes partial deamination of cytidylic acid. Khalidi (103,p.10) used a mixture containing 0.5 N KOH and N NH_4OH in order to prevent deamination. However, later experiments which we performed together with Khalidi did not confirm this effect of ammonia on deamination, as shown in the

TABLE II

The Deamination of Cytidylic Acid by Alkali,

Experimental Scheme.

Tube	Sample of cyt. acid	Alkali added	Final alkali normality
1	4 ml	4 ml of 2 N NaOH	1.0 N NaOH
2	4 ml	4 ml of 1 N NaOH	0.5 N NaOH
3	4 ml	2 ml of 2 N NaoH + 2 ml of 4 N NH_4OH	0.5 N NaOH; 1 N NH_4OH
4	4 ml	2 ml 2 N " + 2 ml 2 N "	0.5 N " 0.5 N "
5	4 ml	2 ml 2 N " + 2 ml 1 N "	0.5 N " 0.25N "
6	4 ml	2 ml 2 N " + 2 ml 0.1 N "	0.5 N " 0.025N "

following study.

The basis of this experiment was the calculation of the amount of deaminated cytidylic acid by measurement of the rise in ultraviolet absorption at 260 mμ, or the decrease at 280 mμ, after incubation with various alkali mixtures, because when cytidylic acid is deaminated, it gives rise to uridylic acid which absorbs more at 260 mμ and much less at 280 mμ. For this purpose a solution of cytidylic acid in N H_2SO_4 was heated for one hour (to give an equilibrium mixture of the "a" and "b" isomers), and then neutralized. Aliquots were pipetted into tubes as shown in Table II.

Blank. In order to obtain significant measurable changes in the optical density readings a concentrated solution of cytidylic acid was used, and, as blank, a solution of high absorption at the wavelengths specified.

Immediately after the addition of alkali, before any deamination could take place, 3 ml of each mixture were pipetted into a second set of tubes, and these were acidified at once with HCl to prevent deamination and placed in ice. The alkaline solutions remaining in the first set of tubes were incubated overnight at 37°C. Then another 3 ml aliquot from each was pipetted into a third set of tubes (after deamination), and 5 ml of HCl were added. After proper dilution the optical densities of all solutions, before and after deamination, were then read at 260 mμ and 280 mμ. The results obtained were inexplicable.

Hence we omitted the use of NH_4OH and chose to hydrolyze RNA with 0.5 N KOH at 23°C for 18 hours, under which conditions deamination is minimal. Another reason for using 0.5 N alkali is that it minimizes nucleoside formation.

DNA and proteins were precipitated from the alkaline hydrolysate with perchloric acid (PCA) in the cold at a final concentration of 0.33 N which was reported to be the best (104). Khalidi (103,p.10) states that "the pH of precipitation should approach 0.7, the lowest pK

for the four nucleotides", because if the pH is higher the proteins will become positively charged and the nucleotides negatively charged; the latter will therefore be adsorbed by the protein precipitate. Experiments performed later to clarify this point proved that there is no adsorption up to pH 2.7 . Thus it may be that the adsorption predicted on a theoretical basis, does not actually occur unless the pH used is quite high (i.e. higher than 3) in order to give the nucleotides a strong negative charge.

The experiment consisted in mixing RNA alkaline hydrolysates with a solution of lipid-free, acid washed plasma proteins at different PCA concentrations, the perchlorate ion concentration being kept constant by the proper addition of $NaClO_4$. After centrifugation the optical densities at 260 mμ of the supernatant solutions were measured and corrected for proteins by means of a "protein blank". The ribonucleotide recovery in each case was then computed by reference to a standard composed of RNA alone, treated in the same way. The results of such an experiment are given in Table III.

V. Analysis of RNA.

An aliquot of the perchloric acid filtrate obtained after removal of DNA and protein was taken for ribose determination by the orcinol reaction (105) and for total phosphorus determination (112). However, the ribose values are of doubtful significance since the orcinol reaction is not specific and is altered by the presence of mucoprotein.

Another aliquot was taken for the determination of ribonucleotides by a micro modification of the method developed in this laboratory by Khalidi (103). The method involves adsorption of the nucleotides on Dowex-2 acetate

TABLE III

Adsorption of Ribonucleotides by Proteins as a Function of the pH of Precipitation

Tube	ml. RNA in 0.2N NaOH*	ml. Protein in 0.33 N NaOH	ml.N $HClO_4$	ml.N $NaClO_4$	pH	$O.D._{260}$	O.D.-Protein blank +	% total
1	2	2	4	0	0.44	0.621	0.592	103
2	2	2	3.5	0.5	0.53	0.631	0.602	105
3	2	2	3	1	0.61	0.594	0.565	98.5
4	2	2	2.5	1.5	0.76	0.619	0.590	103
5	2	2	2	2	0.95	0.617	0.588	102
6	2	2	1.5	2.5	1.30	0.591	0.562	98
7	2	2	1.2	2.8	1.92	0.620	0.591	103
8	2	2	1.1	2.9	2.67	0.620	0.591	103
9(standard)	2	2ml 0.33N NaOH	2	2		0.574	0.574	100
10(Protein Blank)	2 ml. 0.2N NaOH	2	2	2		0.029		

* The RNA was incubated in 0.5N NaOH at 25°C for 18 hours. The solution was then diluted to a final 0.2N NaOH.

\+ The protein blank is the $O.D._{260}$ of tube No. 10 i.e. 0.029. It was further proved by the biuret reaction that the protein concentration of the supernates was nearly the same in all samples.

TABLE IV

Recovery of Ribonucleotides from RNA- protein Mixtures

Column	O.D. units# placed on column	O.D. units recovered from column	percent recovery	percent recovery + 3% ++
1a*	145.8	128.5	88	91
2a	145.8	130.9	90	93
3a	145.8	131.2	90	93
1b**	145.8	133.2	91.5	94.5
2b	145.8	131.3	90	93
1c+	145.8	134.0	92	95
2c	145.8	132.0	91	94
3c	145.8	132.0	91	94
4c	174.5	157.0	90	93
5c	58.16	54.08	93	96

#. O.D. unit = O.D. reading x vol of solution.

* In series a elution was done by passing the H_2SO_4 10 times through the columns at room temperature.

**. In series b elution was done by blowing the resin out from the columns and heating it at 100°C with H_2SO_4 for 20 minutes.

\+ In series c elution was done at 70-80°C with the aid of electrically heated tapes.

++ 3% are added to the recovery because when pure RNA is passed through the column 3-5% of it are not adsorbed by the resin (alkaline hydrolysate) (103, P.17)

at pH 6.0-6.1, removal of interfering substances with water and elution of the nucleotides with N H_2SO_4 at 70-80°C, the temperature being maintained in the column by the use of an electrically heated tape. The eluates are then hydrolyzed in acid solution (N H_2SO_4) to yield purine bases and pyrimidine nucleotides (106,107). The purines are precipitated as the silver salts, then extracted with hot HCl. The filtrate containing pyrimidine nucleotides is freed of silver with HCl. The solutions containing the mixed purines and the pyrimidine nucleotides are analyzed spectrophotometrically with the aid of two simultaneous equations according to the general method of Kerr (108). The revised simultaneous equations developed by Khalidi (103) were adopted.

Recovery experiments on mixtures of RNA with pure protein in the same concentration found in tissues gave results which were almost as successful as those given by the "macro" method (103,p17), the recovery of nucleotides being 93-96 percent, (see table IV).

VI. Determination of DNA.

DNA was extracted from the DNA-protein precipitate with hot acid. Various authors have recommended the following: 5 percent TCA at 90°C for 15 minutes (98),
5 " OR 0.5N PCA at 70° for 20 minutes(97,109)
6 " PCA at 90° for 15 minutes (110),
N PCA at 80° for 30 minutes (111).

We adopted 0.5 N PCA at 100° for 20 minutes.

TABLE IV

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Column	O.D. units# placed on column	O.D. units recovered from column	percent recovery	percent recovery +3% ++
1a*	145.8	128.5	88	91
2a	145.8	130.9	90	93
3a	145.8	131.2	90	93
1b**	145.8	133.2	91.5	94.5
2b	145.8	131.3	90	93
1c+	145.8	134.0	92	95
2c	145.8	132.0	91	94
3c	145.8	132.0	91	94
4c	174.5	157.0	90	93
5c	58.16	54.88	93	96

(#) O.D. unit = O.D. reading x vol. of solution.

(*) In series (a) elution was done by passing the H_2SO_4 10 times through the columns at room temperature.

(**) In series (b) elution was done by blowing the resin out from the columns and heating it at 100°C with H_2SO_4 for 20 minutes.

(+) In series (c) elution was done at 70-80°C with the aid of electrically heated tapes.

(++) 3% are added to the recovery because when the alkaline hydrolysate of pure RNA is passed through the column 3-5% of it are not adsorbed by the resin (103,p.17).

The DNA extracts were assayed by three independent methods:

1) Total Phosphorus Determination, by the method of Fiske and SubbaRow (112).

2) Colorimetric Determination of Desoxyribose.

The diphenylamine (113,114), cysteine-H_2SO_4 (115), carbazole-H_2SO_4 (116) and the tryptophan-$HClO_4$ (117) reactions, are known to be non-specific and//or not sufficiently sensitive. The indole-HCl reaction described first by Dische (118)and developed later with some modifications by Cerriotti (119) proved unsuccessful in our hands. Interference with the color development may have been caused by the formation of phosgene in the purified chloroform used (120), so that further studies are necessary.

The cysteine-H_2SO_4 reaction as described by Brody (121) is attractive because of its high sensitivity. Only after many trials, using utmost care in the procedure described, could a straight line relationship be obtained between color intensity and DNA concentration (see Fig. 2).

Note: Brody states in his procedure that the concentrated H_2SO_4 (96%) should be chilled to at least - 10° before use. But H_2SO_4 of this concentration (95-98%), with a specific gravity of 1.84, freezes at 10.47°C [4], and indeed it was found to be solidified in the ice box at around 4°C. Possibly, the sulfuric acid used by Brody had absorbed moisture, thus becoming $H_2SO_4.2H_2O$ or H_2SO_4.

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4. cf. Handbook of Chemistry and Physics, 31st ed. 1949, p. 526.; Chemical Rubber publishing Co.

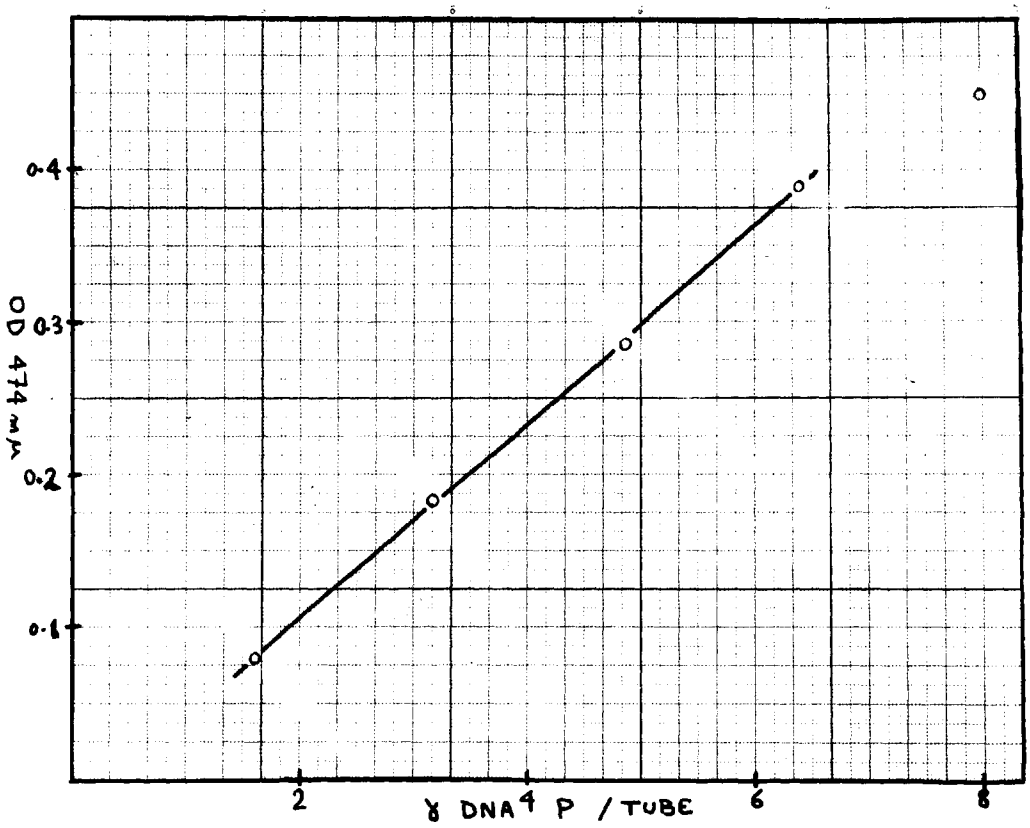

Fig. 2. Calibration curve for the cysteine-H_2SO_4 reaction

4 H_2O with a much lower freezing point. Hence, we used as reagent a mixture of concentrated H_2SO_4 and water in the ratio of 10 : 1 and found this to be successful.

An important disadvantage in Brody's method of determining DNA is interference from proteins. The perchloric acid extracts contain not only the DNA but also much protein or its degradation products which inhibit color development. This effect has not yet been studied quantitatively. We made use of Brody's procedure realizing its limitations, and hope to make the necessary corrections later.

A new micro method for the estimation of DNA has lately been published (122), but it was not possible to apply it due to shortage of time.

3) Ultraviolet Absorption.

Due to the presence of significant amounts of non-nucleotide light-absorbing material in the DNA fractions, a correction was indispensable.

For this, dry tissue powder of rat liver was prepared by the procedure designed above in section II, and nucleic acids were extracted with hot PCA as mentioned previously. The first four extracts (representing nucleic acids plus some protein) were discarded; the 5th, 6th, and 7th extracts were kept separately. These are believed to be fairly good representatives of the proteins or protein degradation products contaminating the DNA fractions. The ultraviolet spectrum of the 5th extract

was measured between 240 and 310 mμ (see Fig. 3). It will be noted that light absorption is the same at 260 and 286.5 mμ, also at 250 and 290 mμ. The 6th and 7th extracts were also found to absorb equally at the wavelengths mentioned above. Hence, DNA was measured by its absorption at 260 mμ corrected by the O.D. value at 286.5 mμ, or at 250 mμ corrected by $O.D._{290}$. Expressing this mathematically,

$$\frac{O.D._{260} - O.D._{286.5}}{\epsilon_{260} - \epsilon_{286.5}}$$ moles DNA phosphorus per liter,

where ϵ . molar extinction coefficient, in terms of P.

Reference Standard for DNA Determination. A DNA sample prepared from sperm (Nutritional Biochemicals Cor.) was used as standard. It was freed from RNA by three successive incubations in 0.5 N KOH followed by precipitation each time with PCA at 0.33 M, and washing once with 0.3 N $HClO_4$. It was finally dissolved in N KCl with the aid of KOH at pH 6.5-7 and shaken with an amyl alcohol-chloroform mixture (1 : 2.5) in order to remove traces of protein as described by Sevag (123). Shaking was continued until there was no gel at the interface of the organic and aqueous layers. Finally, the DNA was precipitated with 95 percent ethyl alcohol, washed 3 times with alcohol, twice with ether, and dried in vacuo over P_2O_5 at 70°C.

A sample of the purified nucleic acid was dissolved in 0.5 N $HClO_4$ and heated for 20 minutes in a boiling

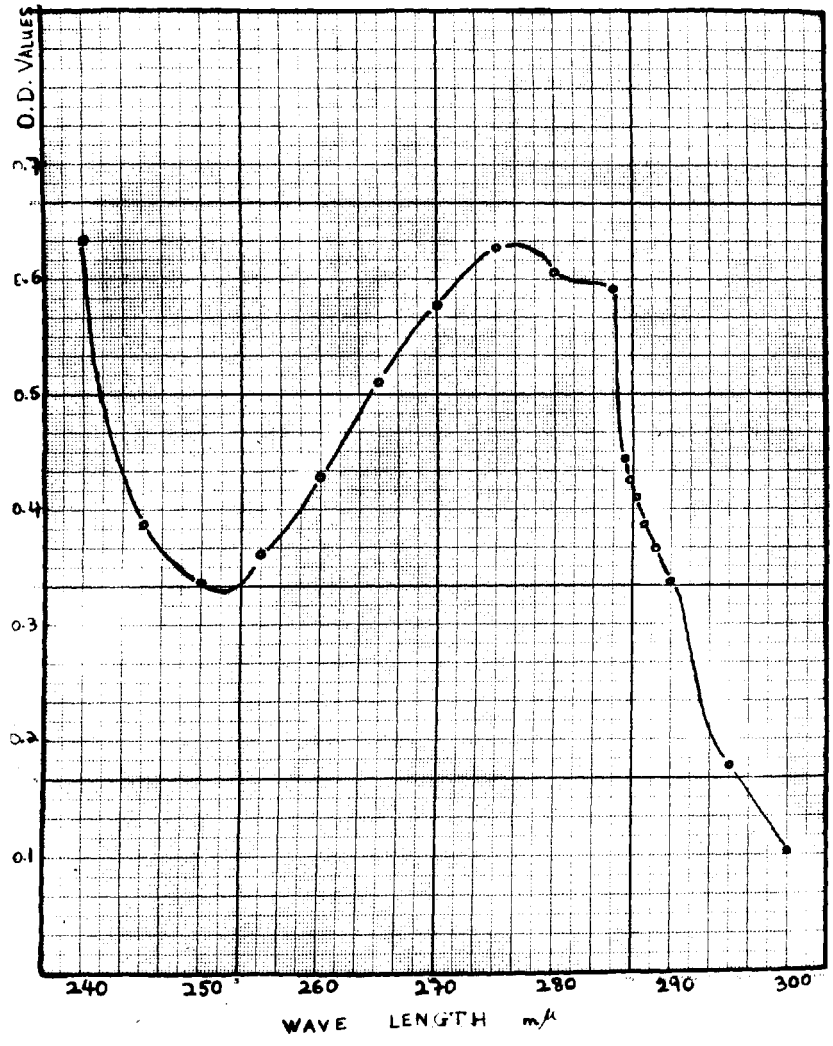

Fig. 3. Absorption Spectrum of proteins extracted with 0.5 N $HClO_4$ at 100°C.

water bath, in the same way as the tissue specimens. The phosphorus content of this solution was determined accurately (112). The cysteine-H_2SO_4 calibration curve as well as the ultraviolet absorption were determined on the same solution, all values being expressed per mg of DNA phosphorus (DNA P) (see Fig 2 for the cysteine-H_2SO_4 curve). The extinction coefficient for acid hydrolyzed DNA per milli-Mole of phosphorus at several wavelengths are given in Table V.

TABLE V

Extinction Coefficients of DNA P in 0.1 N $HClO_4$ after heating 1 hour at 100° with 0.5 N $HClO_4$

Wavelength (mμ)	Millimolar Extinction Coefficient
260	9.527
286.5	4.236
	Difference 5.291
250	8.083
290	3.073
	Difference 5.010

Discussion on DNA Determination. The DNA P values given by the three methods described above do not fully agree.

The values obtained by the cysteine-H_2SO_4 reaction are lower than those obtained by phosphorus or ultraviolet

absorption measurements because there is, as mentioned previously, interference from proteins.

We regard values obtained by ultraviolet absorption as the most reliable since the phosphorus determination is subject to contamination by phospholipids or unknown phosphorus compounds. Moreover, it is observed that the amounts of DNA P computed from the total P determination are always higher than those obtained by spectrophotometry. It is hoped that the latter will agree with values obtained by the cysteine-H_2SO_4 reaction after correcting for the proteins.

PROCEDURE

Alkaline Hydrolysis.

The dry tissue powder, prepared as described in section I, is incubated for 18 hours at 25°C in N KOH in the ratio of 20 ml per gram[5].

Protein Analysis.

A 5 ml aliquot of the alkaline hydrolysate is diluted to 50 ml and used for protein determination by the biuret reaction.

RNA Analysis.

Duplicate or triplicate 5 ml aliquots of the alkaline hydrolysate are pipetted into 15 ml centrifuge tubes. The DNA and proteins are precipitated by the addition of 1 ml of 4.48 N $HClO_4$ in the cold. After thorough mixing and centrifugation, 1 ml of the supernatant solution is diluted to 25 ml and used for ribose and phosphorus determination.

Another 3 ml aliquot of the supernatant solution is added to 2 ml of N KOH in order to precipitate the excess perchlorate and the mixture is filtered through sin-

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5. A small residue always remains insoluble in alkali. After separating it by centrifugation, washing twice with 0.5 N KOH, then extracting at 100°C for one hour in N KOH (in which it still was insoluble), it was found that the extract contained negligible amounts of nucleic acids and proteins, and hence, the residue was discarded.

thered glass and washed out quantitatively with the minimum amount of water. The pH is then adjusted to 6.0-6.1 (glass electrode) by the addition of acetic acid ; the electrodes are rinsed with water, and the solution is transferred quantitatively to the Dowex-2 columns (7-8 cm by 0.2 cm^2).

The resin is prepared by shaking Dowex-2 with 2 N alkali, then with 2 N H_2SO_4 until it is chloride free, and finally with 0.2 M acetate buffer at pH 6.0-6.1 (4 times).

The columns are washed with about 100 ml of distilled water to get rid of interfering substances. They are eluted with 6-7 ml of N H_2SO_4 at 70-80°C, heating being accomplished by electrically heated tapes wrapped around the columns. The H_2SO_4 is passed in successive 3, 1, 1, and 1 ml portions, and the resin stirred throughout elution by means of a finely drawn glass rod. Stirring is necessary because the resin which has taken up some bicarbonate (from distilled water, etc...), gives it up on the addition of H_2SO_4 , thus forming gas bubbles which prevent uniform contact of the resin and the sulfuric acid. Recoveries of nucleotides from the resin without stirring were about 20 percent lower than the others.

The eluates,received in 15 ml conical centrifuge tubes are hydrolyzed at 100°C for one hour to liberate the purine bases. Sodium hydroxide (0.3 ml of 9 N) is

added to render the solution approximately 0.1 N with respect to sulfuric acid. After cooling in ice, 1.5 ml of saturated silver sulfate are added. After stirring, the precipitate of silver-purines is left overnight to flocculate and is then centrifuged. The supernate containing the pyrimidine nucleotides is decanted into a 100 ml volumetric flask and the precipitate is washed twice with 0.1 N H_2SO_4 saturated with silver sulfate, the washings being added to the supernate in the volumetric flask. Excess silver is precipitated by the addition of 10 ml of N HCl, and the mixture is made up to volume and centrifuged in order to separate the silver chloride.

The silver-purine precipitate is extracted 3 times with 5 ml portions of N HCl at 100°C for 10 minutes. The extracts are combined in a 100 ml volumetric flask and made up to volume.

The optical density of the solutions containing the pyrimidine nucleotides is measured at 260, 265.5, and 280 mμ, and the optical density of the solutions containing the purines is measured at 262, 275.5, and 284 mμ. For this purpose the Beckman, model DU, spectrophotometer with 10 mm quartz cells was used.

The concentration of individual purines and pyrimidines are calculated from the following equations (103):

Adenine (μM per liter) $\quad 103.4\ O.D._{262} - 137.6\ O.D._{284}$

Guanine " " " $\quad 224.5\ O.D._{284} - 39.25\ O.D._{262}$

Sum of purines (μM per liter) 139.8 $O.D._{275.5}$

Cytidylic acid " " 98.6 $O.D._{280}$ - 32.9 $O.D._{260}$

Uridylic acid " " 128 $O.D._{260}$ - 69.6 $O.D._{280}$

DNA Analysis.

The protein-DNA precipitate obtained by acidification of the tissue alkaline hydrolysate is washed once with 0.3 N $HClO_4$. It is then redissolved in 5 ml of 0.5 N NaOH, reprecipitated with 1 ml of 4.48 N $HClO_4$, and washed with 0.3 N $HClO_4$ in order to effect complete removal of the ribonucleotides. The supernatant solutions and washings are discarded. The residue is extracted 3 times with 0.5 N $HClO_4$ in a boiling water bath for 20 minutes. The extracts are combined in a 25 ml volumetric flask and made up to volume. Later, aliquots of this final solution are taken for the cysteine-H_2SO_4 reaction, for total phosphorus, and for ultraviolet absorption measurements (the latter were always done at a final 0.1 N acid concentration).

Corrections Used.

When the dry tissue is dissolved in alkali, there is obviously an increase in volume; since this solution is sampled, allowance should be made for this increase in the calculations.

In order to measure the magnitude of this increase in volume, 1 gr of dry beef pancreas tissue (weighed accurately) was dissolved in 20 ml of 0.5 N NaOH in a 25 ml

volumetric flask. From a buret 0.5 N NaOH was added to bring the solution to the mark; 4.2 ml were required. Therefore, the final volume of the alkaline hydrolysate was 20.8 ml. Hence, there is an increase in volume of 0.8 ml for every gram of dry tissue (the ratio of dry tissue to alkali used being always 1 gr per 20 ml)[6]. Therefore, the actual final volume of the alkaline hydrolysate equals ml 0.5 N KOH added, plus 0.8 x gr of dry tissue analyzed. Also, when proteins are precipitated, the volume of the precipitate should be accounted for in the calculations. Fiske and SubbaRow (112) recommend that for accurate quantitative work, the volume of the dry protein precipitate should be subtracted from the final volume; for example, in this case 5 ml of the alkaline hydrolysate are added to 1 ml of $HClO_4$ to precipitate the proteins and DNA; the final volume of the supernate is not 6 ml but 6 ml minus the volume of proteins contained in these 5 ml (the amount of proteins in the 5 ml being calculated and 1 gr of protein being considered as occupying a volume of 1 ml).

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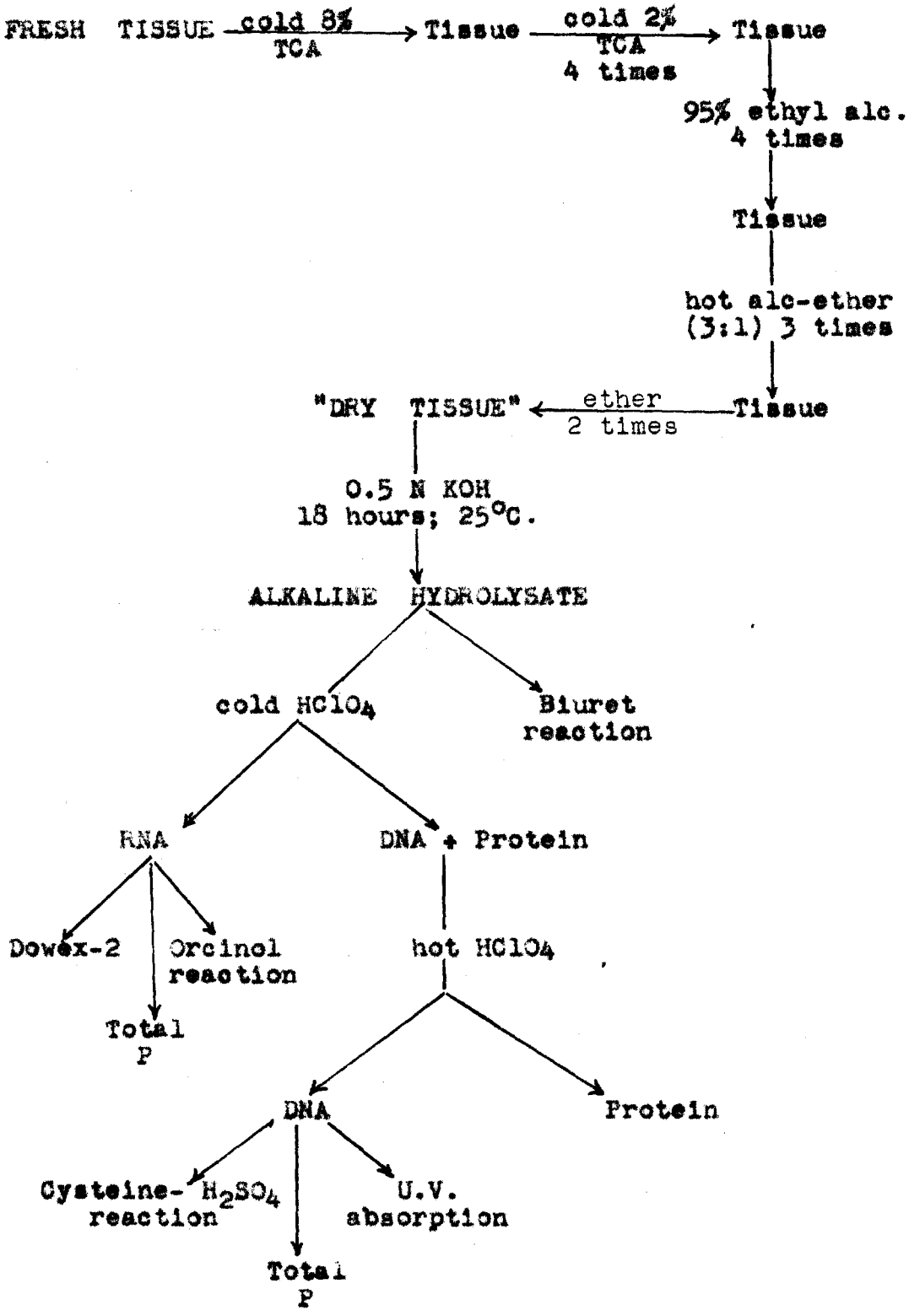
6. This correction is in close agreement with that suggested by Fiske and SubbaRow (112) (1 gr of dry tissue contains roughly 0.8 gr of protein).

Schematic Diagram of the Procedure.



RESULTS AND DISCUSSION

The results of the analyses performed on normal and diabetic rat liver are given in Table VI. They include the content of proteins, of DNA and RNA in terms of phosphorus, and the content of individual ribonucleotides, all values being expressed per 100 grams of fresh tissue.

Protein and Nucleic Acid Content.

It is noticed that the ribonucleic acid values as determined by total phosphorus do not agree with those obtained by adding the values of the four nucleotides; the former are always higher than the latter. This is also the case with desoxyribonucleic acid. The discrepancy may be explained by contamination with phospholipids (since the tissue was not refluxed with methanol-chloroform as recommended by Schmidt and Thannhauser (85), and possibly with other phosphorus compounds. In fact, it has been shown by Kerr (124), and by Folch and Le Baron (125) that prolonged refluxing with chloroform-methanol failed to remove all of the undetermined phosphorus compounds from the RNA fractions. It has also been reported (41, 126) that in the acid-soluble fraction of the tissue alkaline hydrolysate, certain non-nucleotide phosphorus compounds are present and that a glycerylphosphate protein fraction contaminates the DNA fraction, according to the Schmidt and Thannhauser technique.

TABLE VI

Protein and Nucleic Acid Content of Normal and Diabetic Rat Liver

(all values are expressed per 100 gr of fresh tissue)

Animal	Protein (grams)	DNA P (µM)		RNA P (µM)		Ribonucleotides (µM)				Ribose (µ M)
		by total P	by U.V. absorp.	by total P	by U.V. absorp*	Ad.	Gu.	Cy.	Ur.	
Normal										
I-7	13.1	687	670	2680	2280	400	670	714	493	1300
I-8	12.7	552	478	2620	1890	332	572	578	407	8690
I-9	12.6	690	662	2560	1980	386	517	665	412	1410
III-1	7.0	483	430	1500	1010	179	287	333	213	596
III-2	18.7	1030	919	4290	2790	550	865	863	508	1550
III-3	14.2	779	696	3700	2500	454	738	851	479	1480
Diabetic										
II-6	16.0	781	668	3350	2450	452	776	732	498	1270
II-8	15.0	736	527	3140	2310	428	688	716	479	1220
II-3,5,10	15.9	792	672	3250	2350	465	698	707	477	1390
IIV-2	17.9	618	504	3180	2210	410	721	654	427	1260
IV-5	16.0	811	650	2790	2100	394	667	630	411	1390
IV-8	16.1	787	618	2970	2100	385	665	643	411	1190

(*). This value is obtained by adding the values for the 4 nucleotides.

Table VI shows that in the case of RNA, the value obtained by phosphorus determination is 18 to 50 percent higher than that computed from the sum of the ribonucleotides, in both normal and diabetic tissue. However, in the case of DNA, while the values given by total phosphorus and ultraviolet absorption measurements agree with each other within 2.5 to 12 percent in the normal tissue, yet, in diabetic tissue the difference amounts to 17-27 percent. The significance of this cannot be understood until the nature of the contaminating phosphorus compounds is known.

Ribose, as determined by the orcinol method, amounts always to about half the phosphorus value, since only the purine nucleotides give the orcinol reaction, and our results show that this ratio approaches 1 : 1 . However, the ribose value is somewhat inaccurate for the reasons specified above.

The results given in Table VI on the composition of rat liver are in fair agreement with those obtained by Davidson (4) for normal rats, and within the range of limits computed by Davidson (48 p.81) from data reported by various authors.

Values expressed per unit weight of tissue have little significance, however, because there are many fallacies to consider (e.g. the estimation of tissue weight depends upon the water and phospholipid content, and these are not constant in all cases). In order to be able to

compare the values of tissue analysis in different rats, one way of overcoming this difficulty is to express all values per unit of DNA since, according to the findings of Vendrely (1), the average DNA_c is constant in somatic cells

It was reported (21,22), as mentioned in the Introduction, that after alloxan administration DNA_n increases. If this were the case, then the values of RNA and protein relative to DNA should tend to be lower in alloxanized rats, and there is no such indication in our results.

The values expressed per micromole of DNA are given in Table VII. An illustration of the difference between the conventional method of expressing results per unit weight of tissue and that of expressing them per unit DNA is obtained from a comparison of Tables VI and VII. Normal rat I-8 has much lower values of protein and RNA P per 100 grams of fresh tissue than normal rat III-2. Yet, when results are referred to DNA, the values for rat I-8 become higher than those of rat III-2.

Table VII shows that there is a great scatter between the values of protein and RNA within one group of rats, especially the normal group, Moreover , the differences between the normal rats themselves are as great as those between normal and diabetic. These differences cannot be accounted for by some inaccuracy of the method above; they may reveal individual variations. We conclude,

TABLE VII

Protein : DNA, and RNA : DNA ratios in normal and diabetic rat liver

Animal	DNA μM P /100 gr fresh tissue	Protein mg/μM DNA P	RNA, μM P/μM DNA P	
			by P determ.	by U.V. absorp.
Normal				
I-7	670	19.6	3.97	3.39
I-8	478	26.6	5.47	3.98
I-9	662	19.1	3.88	2.99
III-1	430	16.4	3.49	2.36
III-2	919	20.4	4.67	3.04
III-3	695	20.5	5.32	3.60
Average		20.4 (±3.07)*	4.47 (±0.744)	3.23 (±0.512)
Diabetic				
II-6	668	23.9	5.01	3.68
II-8	627	23.9	5.02	3.92
II-3,5,10	672	23.6	4.84	3.50
IV-2	505	35.5	6.29	4.38
IV-5	650	24.6	4.29	3.24
IV-8	618	26.2	4.81	3.41
Average		26.3 (±4.21)	5.04 (±0.607)	3.69 (±0.376)

* This represents the standard deviation as calculated from the equation $\sigma = \sqrt{\frac{\Sigma x^2}{n}}$

therefore, that alloxan diabetes causes no change from the normal in the content of protein and RNA in rat liver.

However, there is an obvious correlation between protein content and RNA content. It is shown graphically in Figure 4. This again points towards the possible functional interrelationship between protein and RNA syntheses, which has already been observed by several authors.

RNA Composition.

The ratios of the four ribonucleotides are given in Table VIII a. They differ slightly from those reported

TABLE VIII b

Comparison of Nucleotide Ratios Reported by Various Authors

	Ad.	Gu.	Cy.	Ur.	Pur./Pyr.
Volkin & Carter (27)	10	18.3	18.9	8.5	1.04
Davidson (28), (	10	17.5	13.6	8.1	1.3
(					
two methods. (	10	17.6	14.3	10.8	1.1
Our values, (average, normal).	10	15.8	17.5	11.2	0.9

by Volkin and Carter (27) on RNA isolated from rat liver (see Table VIII b). Our values for uridylic acid are higher, and for guanylic acid, lower than theirs. The higher values of uridylic acid cannot be explained on the basis of greater deamination of cytidylic acid, since Volkin

TABLE VIII a

The Ratio of the Four Ribonucleotides in Normal and Diabetic Rat Liver

Animal	Adenine	Guanine	Cytidine	Uridine	Pur./Pyr.
Normal					
I-7	1.00	1.64	1.78	1.23	0.87
I-8	1.00	1.72	1.74	1.22	0.92
I-9	1.00	1.34	1.72	1.07	0.84
III-1	1.00	1.60	1.86	1.19	0.85
III-2	1.00	1.57	1.57	0.93	1.03
III-3	1.00	1.63	1.83	1.05	0.91
Average	1.00	1.58 (±0.12)*	1.75 (±0.095)	1.12 (±0.11)	0.90 (±0.063)
Diabetic					
II-6	1.00	1.72	1.62	1.10	1.00
II-8	1.00	1.61	1.67	1.12	0.94
II-3,5,10	1.00	1.50	1.52	1.02	0.98
IV-2	1.00	1.76	1.59	1.04	1.05
IV-5	1.00	1.69	1.60	1.04	1.02
IV-8	1.00	1.73	1.67	1.07	1.00
Average	1.00	1.67 (±0.089)	1.61 (±0.055)	1.07 (±0.032)	1.00 (±0.032)

* Standard deviation (see Table VII)

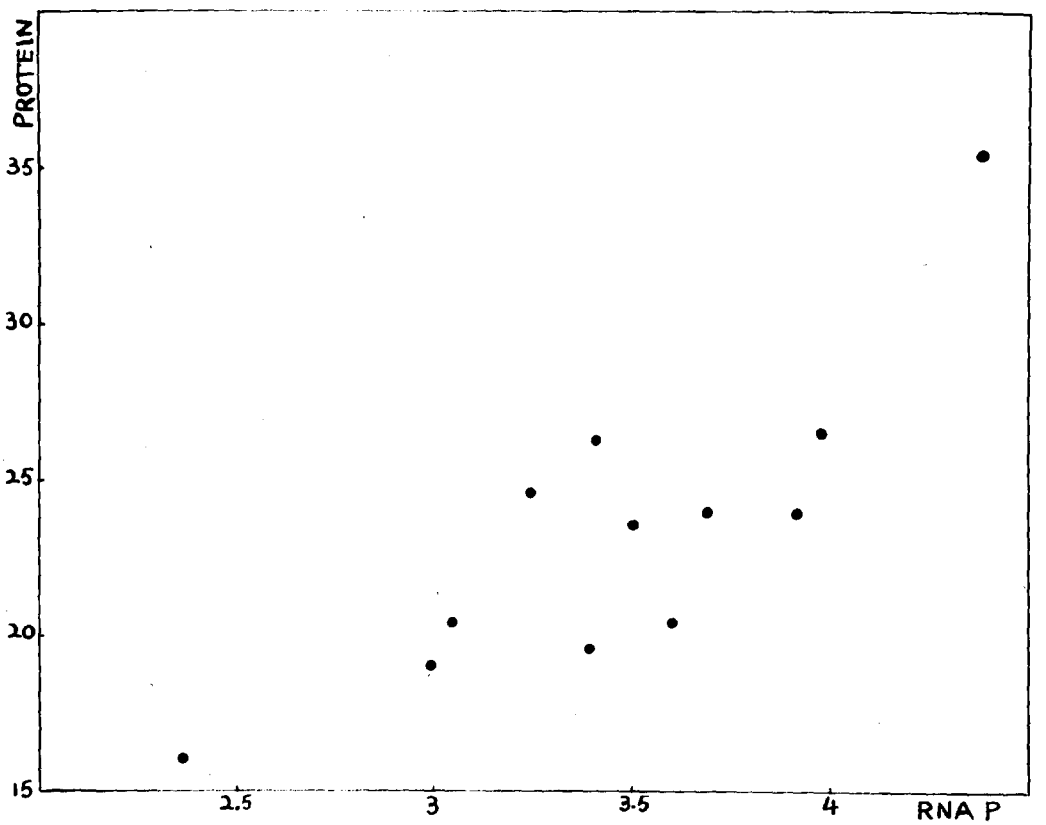


Fig. 4. Scatter diagram correlating protein and RNA content in rat liver. The values are those of columns 3 and 5 in Table VII.

and Carter used 0.5 N alkali for the hydrolysis of RNA (as we did) and our higher uridylic acid values are not accompanied by a similar decrease in cytidylic acid.

Our results differ also from those of Davidson et al. (28) in that we found a higher proportion of cytidylic, and a somewhat lower proportion of guanylic acid. The ratio of purine to pyrimidine reported by these two groups of workers was always higher than the one we found. The difference is possibly due to some enzymatic degradation during their isolation of RNA. The action of ribonuclease on RNA causes characteristically greater losses of pyrimidines and adenine, thus giving for the degraded product high ratios of guanine : adenine, and purine : pyrimidine.

Table VIIIa reveals that rat liver RNA is composed of approximately equal amounts of guanylic and cytidylic acids, and of adenylic and uridylic acids, the first pair predominating by 60 to 75 percent.

No significant difference between the values in normal and diabetic rat liver is seen in the four components of RNA, the differences between the means ranging from 5 to 8 percent.

However, a careful study of the purine : pyrimidine ratios reveals a 10 percent increase in the diabetic animals (14 percent if rat N° III-2 is eliminated). This increase may be significant though it is not striking.

It should be interesting to extend the same type

of study to regenerating and embryonic tissue vs. resting and adult tissue; and to secreting or neoplastic vs. normal tissue, trying to find out whether a change in the content and turnover rate of RNA under these circumstances is accompanied by a change in its composition.

A study of the ribonucleotide distribution in the spleen of leukemic mice was started but discontinued when it was found that in spleen, unlike pancreas or liver, the RNA fraction was contaminated by significant amounts of products which absorb ultraviolet light at 260 mμ and give all colorimetric tests specific for desoxyribose. Further investigation is needed to clarify the reason for this difference and to adapt the method to spleen tissue.

S U M M A R Y

A micromethod was developed for the determination of the individual components of ribonucleic acid without isolating it from the tissue. It was applied to the study of purine and pyrimidine ribonucleotides of normal and diabetic rat liver.

The average ratios of adenine : guanine : cytidine : uridine, with adenine taken as 1, were found to be 1 : 1.58 : 1.75 : 1.12 for normal rats, with a purine : pyrimidine ratio of 1 : 0.9, and 1 : 1.67 : 1.61 : 1.07 for diabetic rats, with a purine : pyrimidine ratio of 1 : 1.07.

No significant variation between normal and diabetic liver was observed in the ratio of the four nucleotides.

In the same tissues, no change was detected in the protein and ribonucleic acid content expressed per unit of desoxyribonucleic acid. However, a correlation between protein and ribonucleic acid content was obvious.

B I B L I O G R A P H Y

1. VENDRELY, R., and VENDRELY, C., Experientia, 4; 435 (1948), and 5: 327 (1949).
2. HARRISON, M. F., Biochem. J., 55: 204 (1953).
3. DAVIDSON, J. N., Cold Spring Harbor Symposium Quant. Biol., 12: 50 (1947).
4. THOMSON, R. Y., HEAGY, F. C., HUTCHISON, W. C., and DAVIDSON, J. N. , Biochem. J., 53: 460 (1953).
5. CAMPBELL, R. M., and KOSTERLITZ, H. W., Science, 115: 84: (1952).
6. ELY, J. O., and ROSS, M. H., Science, 114: 70 (1951).
7. LECOMTE, C., and DESMUL, A., Compt. Rend., 1400 (1952).
8. CAMPBELL, R. M., and KOSTERLITZ, H. W., J. Endocrinol., 6: 171 (1949).
9. NOVIKOFF, A. B., and POTTER, V. R., J. Biol. Chem., 173: 223 (1948).
10. PRICE, J. M., and LAIRD, A. J., Cancer Res. 10: 650 (1950).
11. DRABKIN, D. L., J. Bio. Chem., 171: 395 (1947).
12. DAVIDSON, J. N., LESLIE, I., and WAYMOUTH, C., Biochem. J., 44: 5 (1949).
13. DAVIDSON, J. N., and LESLIE, I., Exptl. Cell Res., 2: 366 (1951), cf. 48 p. 93 .
14. MENTEN, M. L., WILLMS, M., and WRIGHT, W. D., Cancer

Res., 13: 729 (1953).

15. BADER, S., Proc. Soc. Exptl. Biol. Med., 82: 312 (1953)

16. WEYMOUTH, P., DELFEL, N. E., DOELL, R. J., STEINBOCK, H. L., and KAPLAN, H. S., J. Natl. Cancer Inst., 15: 981 (1955).

17. LEVY, H., DAVIDSON, H. M., REINHART, R. W., and SCHADE, A. L., Cancer Res. 13: 716 (1953).

18. RABINOVITCH, M., VALERI, V., ROTHSCHILD, H. A., CAMARA, S., SESSO, A., and JUNQUEIRA, L. C. U., J. Biol. Chem., 198: 815 (1952).

19. GUBERNIEV, M. A., KORYREV, I. G., Chem. Abstr., 44: 1183a (1950).

20. DAVIDSON, J. N., private communication.

21. DIERMEIER, H. F., DI STEFANO, H. S., TEPPERMAN, J., and BASS, A. D., Proc. Soc. Exptl. Biol. Med., 77: 769 (1951).

22. BASS, A. D., DIERMEIER, H. F., DI STEFANO, H. S., CAFRUNY, E. J., J. Pharmacol. Exptl. Therap. 107: 478 (1953).

23. LESLIE, I., and DAVIDSON, J. N., Biochem. J. 49: xli 91951).

24. CHARGAFF, E., and LIPSHITZ, R., J. Am. Chem. Soc., 75: 3658 (1953).

25. WYATT, G. R., Biochem. J., 48:584 (1951).

26. CHARGAFF, E., MAGASANIK, B., VISCHER, E., GREEN, C., DONIGER, R., and ELSON, D., J. Biol. Chem., 186:

51, (1950).

27. VOLKIN, E., and CARTER, C. E., J. Am. Chem. Soc.., 73: 1516 (1951).

28. CROSBIE, G. W., SMELLIE, R. M. S., and DAVIDSON, J. N., Biochem. J., 54: 287 (1953).

29. BACHER, J. E., and ALLEN, F. W., J. Biol. Chem., 183: 641 (1950).

30. KERR, S. E., SERAIDARIAN, K., and WARGON, M., J. Biol. Chem., 181: 773 (1949).

31. CHARGAFF, E., VISCHER, E., DONIGER, R., GREEN, C., and MISANI, F., J. Biol. Chem., 177: 405 (1949).

32. VISCHER, E., ZAMENHOF, S., and CHARGAFF, E., J. Biol. Chem., 177: 429 (1949).

33. VISCHER, E., and CHARGAFF, E., J. Biol. Chem., 176: 715 (1948).

34. LORING, H. S., and LUTHY, N. G., J. Am. Chem. Soc., 73: 4215 (1951).

35. KALCKAR, H. M., J. Biol. Chem., 167: 429 (1947).

36. ELSON, D., and CHARGAFF, E., Phosphorus Metabolism, 2: 329 (1952).

37. FAIRLEY, J. L., SEAGRAM, H. L., and LORING, H. S., Federation Proc., 9: 169 (1950).

38. MARSHAK, A., and VOGEL, H., J. Biol. Chem., 189: 597 (1951).

39. THOMAS, R., Biochem. Biophys. Acta, 8: 71 (1952)

40. BOULANGER, P., and MONTREUIL, J., Biochem. Biophys.

Acta, 9: 619 (1953).

41. DAVIDSON, J. N., and SMELLIE, R. M. S., Biochem. J., 52: 599 (1952).

42. ASIMOV, I., and SIMON, R. R., Federation Proc. 12: 172 (1953).

43. WOODHOUSE, D. L., Biochem. J., 56: 349 (1954).

44. DROCHMANS, P., MARRIAN, D. H., and BROWN, G. B., Arch. Biochem., Biophys., 39: 310 (1952).

45. BERGSTRAND, A., ELIASON, N. A., HAMMARSTEN, E., NORBERG, B., REICHARD, P., and UBISH, H. V., Cold Spring Harbor Symp. Quant. Biol., 13: 22 (1948).

46. BRUES, A. M., TRACY, M. M., COHN, W. E., J. Biol. Chem., 155: 619 (1944).

47. HAMMARSTEN, E., "Isotopes in Biochemistry", Ciba Foundation Conference, p.203, London: Churchill (1951), see ref. 48 p.163.

48. DAVIDSON, J. N., "The Biochemistry of the Nucleic Acids". pl 163., New York: J. Wiley (1953).

49. GUBERNIEV, M. A., and IL'LINA, L. I., Chem. Abstr., 44: 8453e (1950).

50. GREENSTEIN, J. P.,"Biochemistry of Cancer", New York: Academic Press, 2nd ed. (1954).

51. BRACHET, J., Symp. Soc. Exptl. Biol., 1: 207 (1947), and Cold Spring Harbor Symp. Quant. Biol., 12: 18 (1947).

52. MIKRAMANAYAKE, T. W., HEAGY, F. C., and MUNRO, H. N., Biochem. Biophys. Acta, 11: 566 (1953).

53. MUNRO, H. N., NAISMITH, D. J., and WIKRAMANAYAKE, T.W., Biochem., J. 54: 198 (1953).

54. STADIE, W. C., Physiol. Rev. 34: 52 (1954).

55. HAUGAARD, N., and MARSH, J. B., "The Action of Insulin" C. T. THomas, publishers, Springfield Illinois (1953)

56. VILLEE, C. A. New England J. Med. 251:67 (1954).

57. CASPERSON, T., "Cell Growth and Cell Functions", New York: Norton (1950).

58. CASPERSON, T., Symp. Soc. Exptl. Biol., 1: 127 (1947).

59. BRACHET, J., Ann. N. Y. Acad. Sci., 50: 861 (1950).

60. GALE, E. F. , and FOLKES, J. P., Nature 173: 1223 (1954)

61. KRAHL, M. E., J. Biol. Chem., 200: 99 (1953).

62. LOTSPEICH, N. D., J. Biol. Chem., 179: 175 (1949).

63. FORKER, L. L., CHAIKOFF, I. L., ENTENMAN, C., and TRAVER, H., J. Biol. Chem., 188: 37 (1951).

64. RALLI, E. P., and SHERRY, S., J. Clin. Invest. 27: 217 (1948).

65. WEST, E. S., and TODD, W. R., "Textbook of Biochemistry" p.771, New York: Macmillan Co., 2nd ed. (1955).

66. CHAIKOFF, I. L., and LARSON, P. S., J. Biol. Chem., 109: 85 (1935).

67. GOLDNER, M. G., Bull. N. Y. Acad. Med., 21: 44 (1945).

68. JOSLIN, E. P., New England J. Med. 230: 425 (1944).

69. CONN, J. W., LOUIS, L. H., and WHEELER, C. E., J. Lab. Clin. Med., 33: 651 (1948).

70. PATTERSON, J. W., LAZAROW, A., and LEVEY, S., J. Biol. Chem., 177: 197 (1949).

71. PATTERSONE, J. W., and LAZAROW, A., J. Biol. Chem., 186: 141 (1950).

72. BANERJEE, S., DEB, C., and BELAVADY, B., J. Biol. Chem., 195: 271 (1952).

73. JACOBS, H. R., Proc. Soc. Exptl. Biol. Med. 37: 407 (1937).

74. HARD, W. L., and CARR, C. J., Proc. Soc. Exptl., Biol. Med., 55: 214 (1944).

75. DUNN, J. S., and Mc LETCHIE, N. G. B., Lancet, 2: 384 (1943).

76. DUNN, J. S., DUFFY, E., GILMOUR, M. K., KIRKPATRICK, J., and Mc LETCHIE, N. G. B., J. Physiol., 103: 233 (1944).

77. BAILEY, C. C., and BAILEY, O. T., J. Am. Med. Assoc., 122: 1165 (1943).

78. SHIPLEY, E. G., and BEYER, K. H., Endocrinology, 40: 154 (1947).

79. LUKENS, F. D. W., Physiol. Rev., 28: 304 (1948).

80. RODRIGUEZ, R. R., and KREHL, W. A., AM. J. Physiol., 169: 295 (1952).

81. BHATTACHARYA, G., Science, 120: 841 (1954).

82. NATH, M. C., GADGIL, J. S., and HATWALNE, V. G., Biochem. J. 53: 481 (1953).

83. KASS, E. H., and WAISBREN, B. A., Proc. Soc. Exptl. Biol. Med., 60: 303 (1945).

84. KENNEDY, W. B., and LUKENS, F. D. W., Proc. Soc. Exptl., Biol. Med., 57: 143 (1944).

85. SCHMIDT, G., and THANNHAUSER, S. J., J. Biol. Chem., 161: 83 (1945).

86. KINGSLEY, G. R., J. Biol. Chem., 131: 197 (1939), and J. Lab. Clin. Med., 27: 840 (1942).

87. KERR, S. E., "Laboratory Manual of Biological Chemistry" p. 90, Beirut: Dar Al Ahad, 6th ed. (1953).

88. MA, T. S., and ZUAZAGA, G., Ind. Eng. Chem. Anal. Ed., 14: 280 (1942).

89. KERR, S. E., and BLISH, E. M., J. Biol. Chem., 98: 193 (1932).

90. LEVENE, P. A., J. Biol. Chem., 40: 415 (1919), and 55: 9 (1923).

91. JONES, W., and PERKINS, M. E., J. Biol. Chem., 62: 557 (1925).

92. CALVERY, L. E., J. Biol. Chem., 77: 489 (1928).

93. LORING, H. S., ROLL, P. M., and PIERCE, J. G., J. Biol. Chem., 174: 729 (1948).

94. MARKHAM, R., and SMITH, J. E., Biochem. J., 49: 401 (1951)

95. MONTREUIL, J., and BOULANGER, P., Bull. Soc. Chim. Biol. 33: 784, and 791 (1951).

96. STEUDEL, H., and PEISER, E., Z. Physiol. Chem., 120: 292 (1922).

97. OGUR, M., and ROSEN, G., Arch. Biochem. Biophys., 25: 262 (1950).

98. SCHNEIDER, W. C., J. Biol. Chem., 161: 293 (1945).

99. MARSHAK, A., J. Biol. Chem., 189: 607 (1951).

100. LORING, H. S., BORTMER, H. W., LEVY, L. W., and HAMMELL, M. L., J. Biol. Chem., 196: 807 (1952).

101. SMITH, J. D., and MARKHAM, R., Biochem. J., 46: 509 (1950).

102. MARRIAN, D. H., SPICER, V. L., BALIS, M. E., and BROWN, G. B., J. Biol. Chem., 189: 533 (1951).

103. KHALIDI, U. A. S., Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science; American University, Beirut (1954)

104. BUSCH, H., HURLBERT, R. B., and POTTER, V. R., J. Biol. Chem., 196: 717 (1952).

105. KERR, S. E., and SERAIDARIAN, K., J. Biol. Chem., 159: 211 (1945).

106. LEVENE, P. A., Ber. Chem. Ges., 44: 1027 (1911).

107. JORPES, E., Acta Med. Scand., 68: 503 (1928).

108. KERR, S. E., SERAIDARIAN, K., and WARGON, M., J. Biol. Chem., 181: 761 (1949).

109. OGUR, M., MINCKLER, S., LINDEGREN, G., and LINDEGREN, C. C., Archiv. Biochem. Biophys., 40: 175 (1952).

110. SCHNEIDER, W. C., HOGEBOOM, G. H., and ROSS, H. E., J. Natl. Cancer Inst., 10: 977 (1950).

111. OLMSTEAD, P. S., and VILLEE, C. A., J. Biol. Chem., 212: 179 (1955).

112. FISKE, C. H., and SUBBAROW, Y., J. Biol. Chem., 66: 375 (1925).

113. DISCHE, Z., Microchemie, 8: 4 (1930), cf. CHARGAFF, E.,

DAVIDSON, J. N., "The Nucleic Acids", v. 1, p.287 Academic Press, Inc., New York (1955).

114. SEIBERT, F. B., J. Biol. Chem., 133: 593 (1940).

115. STUMPF, P., J. Biol. Chem., 169: 367 (1947).

116. GURIN, S., and HOOD, D. B., J. Biol. Chem., 139: 775 (1941).

117. COHEN, S. S., J. Biol. Chem., 167: 691 (1945).

118. DISCHE, Z., Biochem. Z., 204: 431 (1929).

119. CERRIOTTI, G., J. Biol. Chem., 198: 297 (1952).

120. CERRIOTTI, G., Private communication.

121. BRODY, S., Acta Chem. Scand., 7: 502 (1953).

122. WEBB, J. M., and LEVY, H. B., J. Biol. Chem., 213: 107 (1955).

123. SEVAG, M. G., LACKMAN, D. B., and SMOLENS, J., J. Biol. Chem., 124: 425 (1938).

124. KERR, S. E., Personal communication.

125. FOLCH, J., and LE BARON, F. N., Fed. Proc., 10: 183 (1951).

126. Phosphorus Metabolism., v. 2, ed. Mc Elroy W. D., and Glass, B., John Hopkins Press, Baltimore (1952), see discussion p. 200-202.