RIBONUCLEIC ACID

(Studies on ribonucleic acid in relation to cell structure).

By R.M.S. Smellie.

Thesis presented for the Degree of Doctor of Philosophy,
The University of Glasgow.

April, 1952.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Acknowledgments.

I should like to express my very sincere gratitude to Professor J.N. Davidson who provided the opportunity for carrying out this research, and without whose unfailing encouragement and advice it would not have been possible. Thanks are also due to Dr. G. Crosbie for permission to include unpublished results on the chromatographic analysis of ribonucleic acid, to Dr. I.M. Dawson for electron micrographs and to Mr. R. Logan for help in the cytoplasmic fractionation experiments. I am also grateful to Mr. D.R.S. Cameron and Miss R. Pevie for help with photography and to Mr. J.W. Sommerville for technical assistance.

I am moreover indebted to all members of the Biochemistry Department for many helpful discussions, in particular to Dr. S.C. Frazer, Dr. W.C. Hutchison, Dr. W.M. McIndoe and Dr. G.T. Mills.

Contents.

		Page
Part 1.	**Introduction.**	
1.1	Historical	1
1.2	Cell Fractionation	4
1.3	The Physical Properties of the Cytoplasmic Fractions	12
1.4	The Chemical Composition of the Cytoplasmic Fractions	16
1.5	The Pentose Nucleic Acid of the Cytoplasm	24
1.6	The Biological Activity of the Nucleic Acids	26
Part 2.	**Methods.**	
2.1	Animals	32
2.2	The Administration of Radioactive Phosphate	32
2.3	The Excision of Organs	33
2.4	Preparation of Homogenates	33
2.5	The Fractionation of Liver Tissue	34
2.6	Fractionation of Phosphorus Compounds in the Tissues	35
2.7	The Technique of Ionophoresis	39
2.8	Location of the Ribonucleotides	42
2.9	The Elution and Identification of the Spots	42
2.10	The Purification of Ribonucleic Acids	43
2.11	The Estimation of Phosphorus	44
2.12	The Assay of Radioactivity	45
2.13	Autoradiographs	46

Page

2.14 The Separation of very small Quantities of Organic and Inorganic Phosphates 46

Part 3. Results.

3.1 The Separation of the Pure Nucleotides 48

3.2 The Recovery of the Nucleotides after Ionophoresis 49

3.3 The Comparison of Analysis of a Nucleotide Mixture by Ionophoresis and by Chromatography 50

3.4 The Effect of Alkaline Incubation on the Nucleotides 51

3.5 The Hydrolysis of RNA by 0.3N-KOH 52

3.6 The Analysis of Specimens of Purified RNA 53

3.7 The Ionophoresis of the A_2S Fraction of Rat Liver 54

3.8 The Analysis of Fraction A_3S prepared from the Saline Extract of Dry Tissue Powder 56

3.9 Preliminary Experiments with ^{32}P 57

3.10 The Ionophoresis of the A_2S Fraction from the Livers of Rats which had received ^{32}P 59

3.11 Comparison of the Specific Activities of the Nucleotides from the Isolated RNA and the A_2S of whole Cytoplasm 64

3.12 The Elimination of the Concomitants 66

3.13 Results of Analysis of Cytoplasmic Fractions 68

3.14 The Specific Activities of the phosphorus containing Fractions from the Cytoplasmic Constituents 70

3.15 The Ionophoresis of the A_2S Fraction from the Cytoplasmic Constituents 70

3.16 Results obtained using the 0.25M-sucrose Procedure of Schneider (1948) 71

Page

3.17 The Composition of the RNA from the Cytoplasmic Fractions 75

3.18 The Relationship between Specific Activity and Time after Injection 76

3.19 The Specific Activities of the Nucleotides from the Mitochondrial Fraction obtained by different methods 80

3.20 The Determination of the True Specific Activities of all four Nucleotides, by Ionophoresis of the A_3S (procedure 3) from the three Cytoplasmic Fractions 83

Part 4. Discussion

4.1 Methods of Analysis of RNA 85

4.2 The Technique of Ionophoresis 92

4.3 The Analysis of the Schmidt and Thannhauser (1945) Ribonucleotide Fraction 95

4.4 The Use of Procedure 3 102

4.5 Cytoplasmic Fractionation Procedures 104

4.6 The Incorporation of Isotopes into the RNA of the Cytoplasmic Constituents 105

4.7 The Variation with Time of the Incorporation of Isotopes into the Ribonucleic Acids of the Cytoplasmic Fractions 112

Summary 117

Bibliography 120.

PART I. - INTRODUCTION.

1.1 Historical.

One of the earliest uses of the word "Cell" in connection with biological materials is to be found in the description of the microscopical examination of a piece of cork published by Robert Hooke in 1665. In the years that followed, many workers repeated this observation on a variety of plant tissues; among them Grew and Malpighi observed within their sections, small cavities which they called utricles or vesicles. Mirbel in 1808 concluded that plants were composed of a "Membranous cellular tissue," and Lamarck that no body could have life if its parts were not cellular tissue, or were not formed by cellular tissue. It was not however until after the improved design of the compound microscope by Amici in 1827 that a more or less complete cell theory was put forward by Schlieden in 1838 for plants, and by Schwann in the following year for animals. The theory as stated by Schwann is as follows:- "The cells are organisms, and animals as well as plants are aggregates of these organisms, arranged in accordance with definite laws." It is pertinent to recall these words after more than a century, and to observe how closely they conform to our present day views on the subject.

Until the beginning of the 19th century only the existence of the cell was recognised and nothing was known as to what, if anything, it might contain. The discovery therefore by Brown in 1831, of the constant presence within the cell of what we now call the nucleus represented a considerable step forward. Rapid progress was made in the researches on the nucleus bringing to light the various phenomena associated with cell division, and the structures which it contained.

Studies on the cytoplasm (the substance surrounding the nucleus and bounded by the cell membrane) made less rapid progress, and it was not until Schultze in 1861 remarked on the particulate matter within the cytoplasm that any advance was made. The delay in recognising these particles may in part be attributed to the fact that the fixatives, which proved so suitable for studies of the nucleus, brought about coagulation of the cytoplasmic contents so as to obscure the particulate matter which was present. In view of this, the development by Altmann (1894) of the bichromate fixative which preserved the cytoplasm and made possible its examination must be regarded as of considerable importance. By this means large rod-shaped particles, which we now know as <u>mitochondria</u>, were observed as a constant constituent of the cytoplasm, as was the reticular apparatus --- the <u>Golgi Body</u>.

Furthermore, dark field illumination made visible numerous highly refringent bodies of much smaller dimensions which appear to correspond to what has since been called the ground substance of the cell or the microsomes. A combination of chemical and histochemical methods made it clear that mitochondria were distinct from secretory granules and glycogen particles, that they were comparatively little affected by lipid solvents, but that, in common with the ground substance, they exhibited a marked affinity for basic dyes (Berg, 1934). Several protein reagents gave positive reactions with both substances, but application of the Feulgen staining technique (Feulgen and Rossenbeck, 1924) indicated that no deoxyribonucleic acid (DNA) was present.

Though of very considerable value, the methods of the histologist are somewhat limited in application, in that they are not readily adapted to quantitative measurements. Even from the qualitative point of view they are open to considerable criticism, on the grounds that the frequently drastic preparatory treatment to which the tissues are subjected may bring about, within the cell, multiple changes which may or may not be recognised. It would therefore be of very great interest, if the internal environment of the cell could be studied by some other

means which would enable our present conceptions to be verified, and make possible a more thorough and detailed examination of the nature of the cellular contents and of the processes carried out therein.

1.2 Cell Fractionation.

Miescher (1897) perhaps pointed the way to one approach when he studied the chemistry of isolated cell nuclei. His technique has since been vastly improved, and numerous workers have applied it to a study of the substances present within the nucleus, and of its metabolic processes (Dounce, 1950). The isolation of cytoplasmic constituents proved a more difficult task, one of the first attempts being that of Warburg (1913) who showed that the capacity of guinea pig liver to respire, was largely associated with the large granules sedimented by low speed centrifugation. He also suggested that these granules might be identical with those observed by microscopic examination. It was not however until some years later that Bensely and Hoerr (1934) succeeded in isolating mitochondria by differential centrifugation. Such studies were taken up by other workers, and Claude (1940), by a combination of long and short runs at a centrifugal force of 18000 g, succeeded in separating particles varying in diamter from 50mμ. to 200mμ. from homogenates in

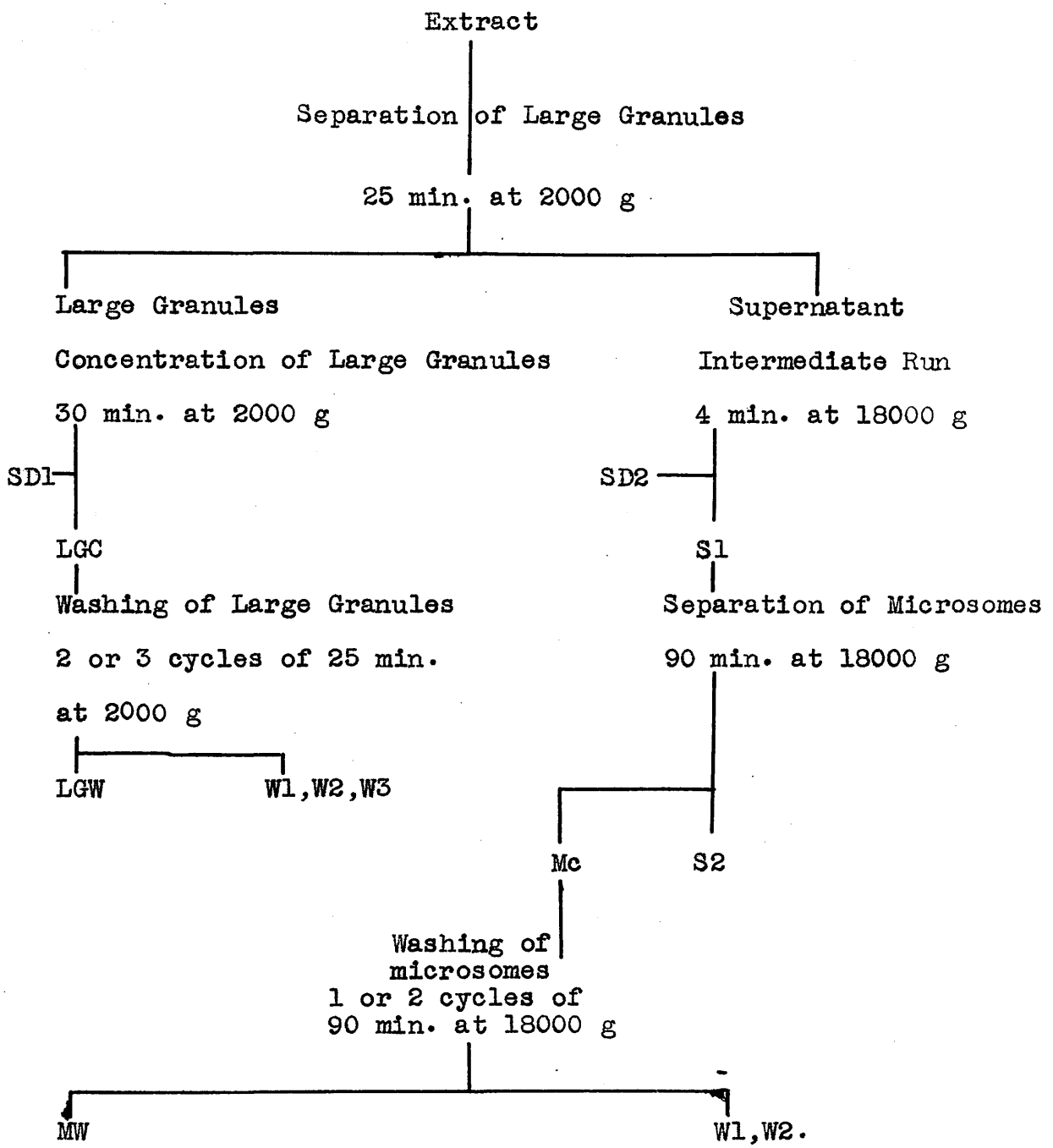
isotonic saline of normal and tumour tissue. Claude found that these granules were chemically similar irrespective of the tissue from which they were derived, and supposed them to be mitochondria. In the following year Claude (1941) showed that these particles tended to agglutinate in isotonic saline, and advocated the use of dilute phosphate buffer at pH 7.1 or "alkaline water " (0.0002N-NaOH) as the suspending medium. The particles obtained by centrifuging tissue homogenates in these media were similar in size to those obtained previously and these he called mitochondria. The mitochondria of Bensely and Hoerr (1934) were considerably larger than those obtained by Claude (1940, 1941) who maintained that these larger particles were secretory granules. In a later paper however Claude (1943a) renamed the smaller particles "microsomes", and agreed that the Bensely and Hoerr granules did contain mitochondria as well as secretory granules. Further studies by Claude (1944a, 1946) have confirmed this view, although he maintains that liver tissue is not the most suitable material with which to work since it contains a high proportion of secretory granules as well as mitochondria. Full details are given in these two papers of the methods of preparing the fractions, and since this is the procedure most commonly used for the isolation

of the cytoplasmic particles from saline homogenates, it is briefly described below.

Chilled rat livers were forced through a 1 mm. mesh masher to remove the main vessels and connective tissue. 60 g. to 80 g. of the resulting pulp were ground in a mortar for 5 minutes, and the solvent (0.85% NaCl adjusted to pH 9.5 by the addition of 2ml. of 0.1N-NaOH per litre) was added drop by drop until 20 to 30 ml. were intimately mixed with the tissue. The volume was finally adjusted to 5 times the weight of pulp taken, and the suspension centrifuged at 1500 g for 3 minutes. The sediment was discarded and the process repeated twice, the resulting supernatant being known as a liver extract. This extract was then submitted to fractionation according to the scheme illustrated in Fig.1.

Figure 1.

The Fractionation of Cytoplasmic Particles in 0.85% Saline (Claude, 1946).



About the same time, Schneider (1946a, b) made use of a similar technique, dispersing the tissue by means of a glass homogeniser as described by Potter and Elvehjem (1936). While Claude and Schneider believed essentially in the existence of two types of granules, large and small, Chantrenne (1947) maintained that the cytoplasmic granules could be separated into 5 different fractions depending upon the nature of the suspending medium and the centrifugal fields applied.

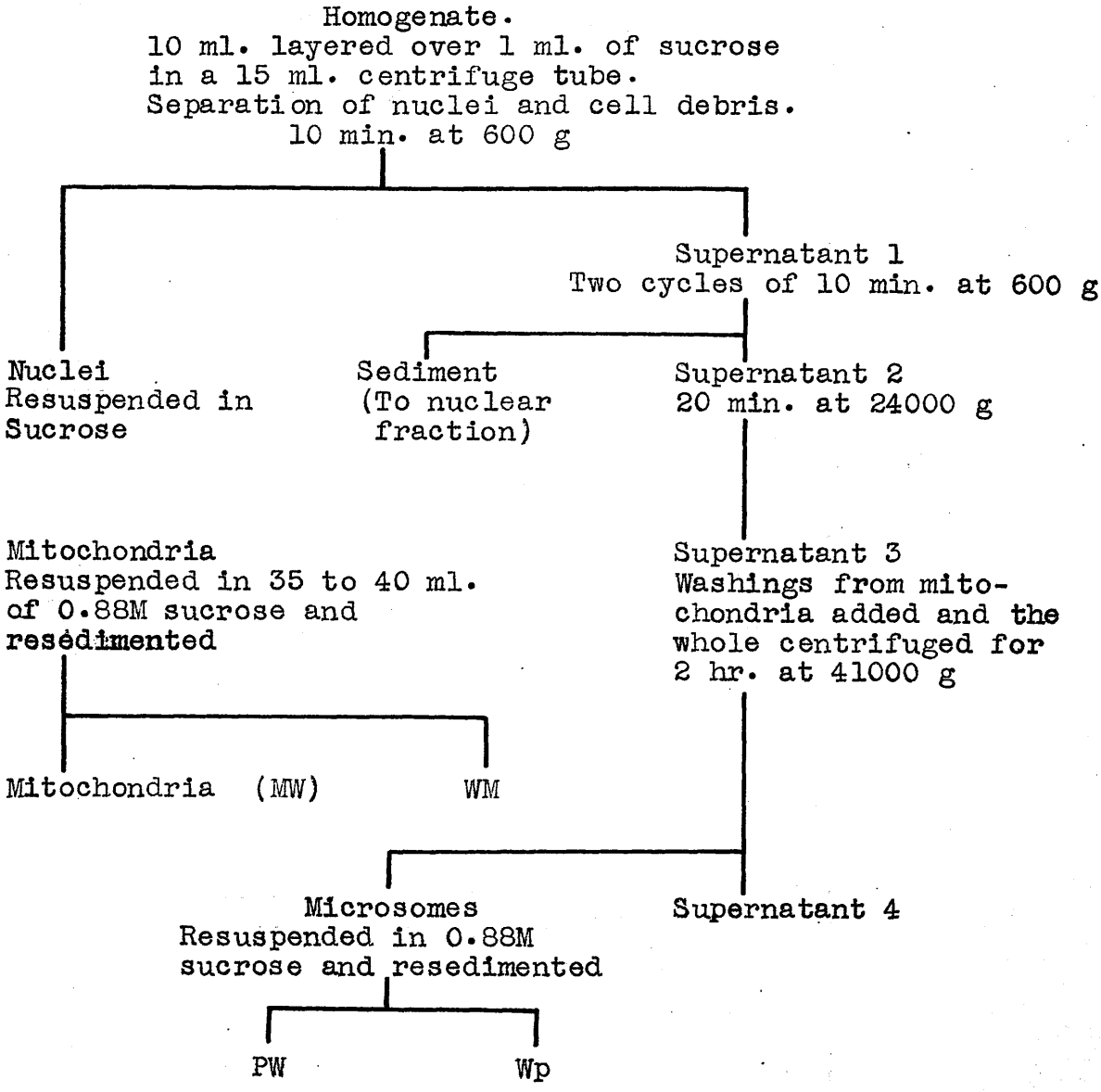
The mitochondria isolated by the methods so far described differed morphologically and histochemically from those present in the intact cell. The isolated material consisted of spherical particles which did not stain with Janus Green, while the mitochondria of the cell were rod-shaped, and stained readily with low concentrations of Janus Green. Moreover, the isolation procedure was complicated by the tendency of the mitochondria to agglutinate in isotonic saline and to be disrupted in water. These difficulties led to the development of a new technique by Hogeboom, Schneider and Pallade (1947, 1948) in which the tissue was homogenised in 0.88M-sucrose, in place of the saline or alkaline water, and the homogenate subsequently submitted to differential centrifugation. With such a medium, of course, the centrifugal fields employed had to

be much greater, owing to the increased density and viscosity of the strong sucrose solution. Homogenates prepared in this way contained an abundance of rod-shaped elements about 300 mμ. to 500 mμ. in diameter and from 1.0 to 5.0 μ. in length which stained readily with very low concentrations of Janus Green. There was no staining of these particles with neutral red which is recognised as a stain for secretory granules. The mitochondria isolated from such homogenates stained readily with Janus Green and were free from neutral red staining matter. The secretory granules migrated centripetally during centrifugation and this has been confirmed by Pallade and Claude (1949). The complete method of separation is described briefly below:-

Rat liver tissue was forced through a 1 mm. mesh screen, and the resulting pulp placed on ice. 5 g. of the pulp were then homogenised in the apparatus of Potter and Elvehjem (1936) with 50 ml. of 0.88M-sucrose, and fractionated according to the scheme shown in Fig.2.

Figure 2.

The Fractionation of Cytoplasmic Particles in 0.88 M-Sucrose. (Hogeboom et al. 1948).

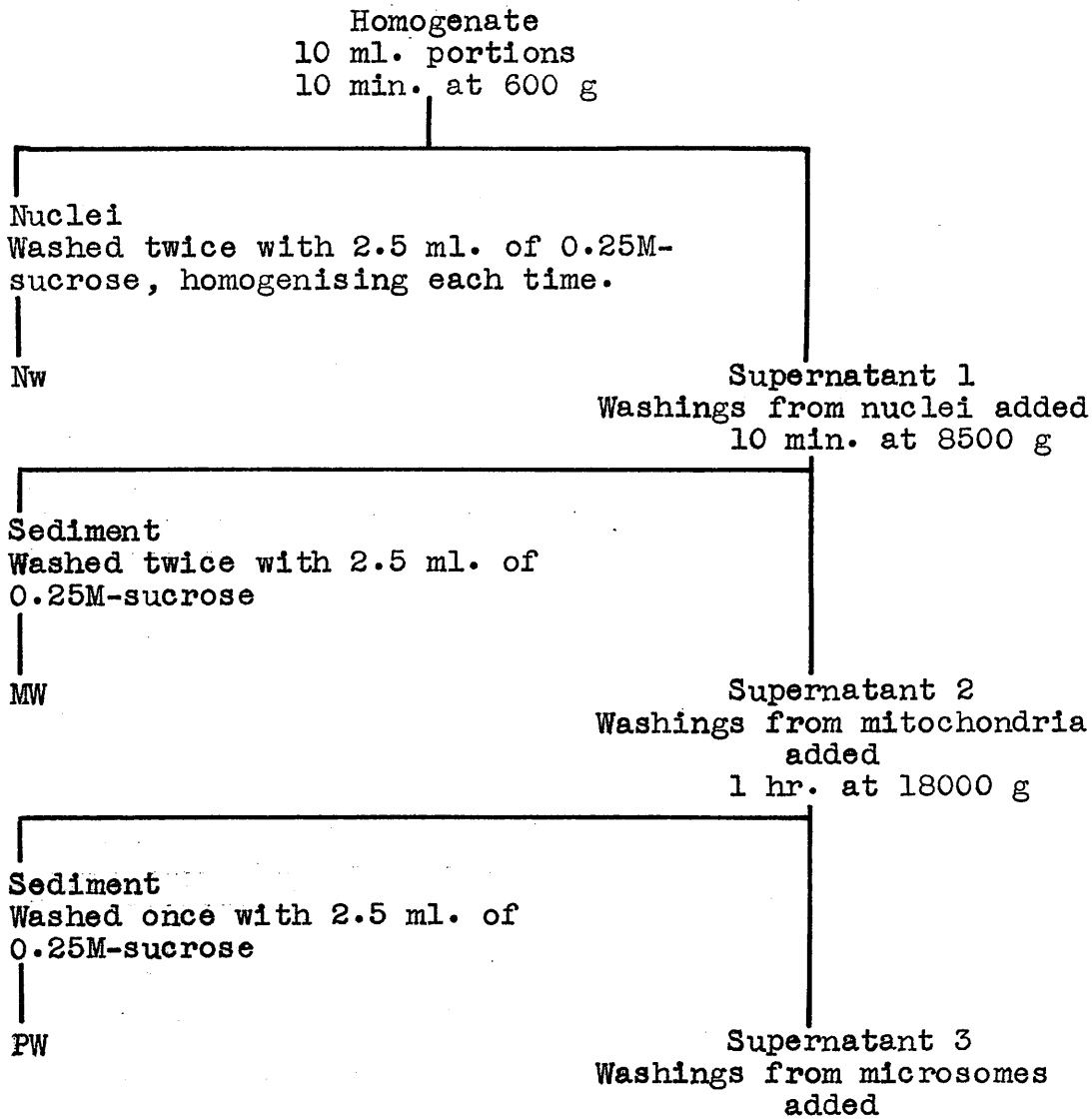


Two major difficulties arise from the technique of Hogeboom et al. (1948), the first being the extremely high centrifugal force required to sediment both mitochondria and microsomes, and the second being inhibition by the high concentration of sucrose, of some of the enzyme systems in the fractions which it is desired to study (Lehninger and Kennedy, 1948; Schneider, 1948). Hogeboom et al. (1948) however showed that mitochondria could be isolated in isotonic (0.25M-) sucrose solution; these were not morphologically identical with those isolated at the higher concentration, nor did they stain with Janus Green, but they showed no tendency to agglutinate or to be contaminated with microsomes. At the same time these particles resembled those obtained from saline homogenates, at least in so far as the activities of the various enzyme systems were concerned (Schneider and Hogeboom, 1950a), and Schneider (1948) has suggested that the use of 0.25M-sucrose is to be preferred, at least for enzyme studies.

The procedure adopted by Schneider with 0.25M-sucrose was similar to that of Hogeboom et al. (1948) in respect of the homogenisation, and the actual fractionation scheme is illustrated in Fig.3.

Figure 3.

The Fractionation of Cytoplasmic Particles in 0.25M-Sucrose.
(Schneider 1948)



Various modifications have been suggested for the isolation of the cytoplasmic particles from sucrose. Thus Kennedy and Lehninger (1949) added potassium chloride to the extract after the removal of nuclei and cell debris, in order to agglutinate the mitochondria and render them more easily sedimented at low centrifugal fields, but this procedure has met with disapproval on the grounds that the salt may tend to agglutinate the microsomes as well. Cunningham, Griffin and Luck (1950) fractionated liver tissue in a medium composed of 0.88M-sucrose, 0.14M-sodium chloride and 0.01M-phosphate buffer, but their results suggest that there has been some contamination of the various fractions. In a recent paper, Muntwyler, Seifter and Harkness (1950) have carried out histological examination of the fractions, and have concluded that an initial centrifugal field of 200 g. is quite adequate for the removal of all nuclei and cell debris from a homogenate in 0.25M-sucrose.

Schneider and Hogeboom (1950a) have described a further procedure for the fractionation of cytoplasmic particles in 0.88M- and 0.25M-sucrose solutions, which differs from their earlier methods mainly in the somewhat stronger centrifugal fields employed in the sedimentation, and particularly in the washing of the fractions.

From all this work there emerge three main types of procedure for the fractionation of the cytoplasmic contents.

1. Fractionation of homogenates in isotonic saline or "alkaline water" (Claude, 1946).

2. Fractionation of homogenates in 0.25M-sucrose (Schneider, 1948).

3. Fractionation of homogenates in 0.88M-sucrose (Hogeboom et al., 1948).

Each of these procedures has much to commend it, and the choice of the one to be used must depend on the object of the particular experiment. Thus fractionation in saline is eminently suitable for subsequent enzyme assay, and the particles are obtained without the use of excessive centrifugal forces. On the other hand, the recognised tendency for both mitochondria and microsomes to agglutinate in this medium make the nature of the final product uncertain, and the mitochondria obtained are not morphologically identical with those in the intact cell. The use of isotonic sucrose overcomes the difficulty of agglutination, but in order to obtain the same degree of sedimentation of the microsomes as is obtained with saline, higher centrifugal fields must be employed. The concentration of sucrose used does not appear to affect the enzyme studies, but once again the mitochondria differ

morphologically from those in the intact cell. Finally the use of hypertonic sucrose raises some difficulties in the assay of enzyme activities owing to partial inhibition. Moreover, the centrifugal fields which must be employed in order to sediment the microsomes are so large as to render the technique impossible in many laboratories. The mitochondria which are obtained by this method, however, resemble those in the whole cell both in shape and dimensions and also in their characteristic affinity for Janus Green.

1.3 The Physical Properties of the Cytoplasmic Particles.

Mitochondria:-

About 30 to 35% of the total nitrogen of rat and mouse liver can be accounted for in the form of mitochondria (Schneider & Hogeboom, 1951) which, as has been shown by Hogeboom et al. (1948), are rod-shaped particles of 300mμ. in diameter and 1.0 to 1.5μ. in length staining readily with low concentrations of Janus Green. Isolation in 0.88M-sucrose yields particles similar in size, shape and staining properties (Hogeboom et al., 1948; Harman, 1950a), while mitochondria obtained from isotonic saline or 0.25M-sucrose homogenates are spherical bodies of 0.5 to 1.0μ. in diameter.

The similarity between these particles and osmotic systems has been commented upon by Claude (1943b, 1944a, 1946), who has described the swelling and final bursting of the granule when placed in distilled water, and the release of small particles similar in size to the microsomes. This phenomenon, in conjunction with some inconclusive electron microscope studies by Claude and Fullam (1945), and the observation that the mitochondria show no tendency to coalesce on centrifugation for prolonged periods, has led Claude (1947) to conclude that a continuous membrane surrounds the mitochondrion, a view which is supported by Dalton, Kahler, Kelly, Lloyd & Striebich (1949) and Schneider and Hogeboom (1951). Harman (1950a) however, after a careful study of the processes by which the mitochondrion in 0.88M-sucrose solution changes from a rod-shaped body to a sphere on dilution, and on the non-selective penetration of radioactive sodium and potassium has concluded that the phenomena exhibited are quite compatible with a fibrous gel type of structure.

The effect of pH changes upon suspensions of the mitochondria in isotonic saline has been studied by Claude (1946), who has observed that they remained dispersed over the comparatively narrow range of pH7 to pH10. Between pH 3.5 and pH7 the granules agglutinate, and in strongly

acid or alkaline solution they are found to disintegrate.

Microsomes:-

Rather less is known of the physical characteristics of the microsomes, partly due to the fact that they are not visible as particles using the light microscope. They are obtained as a pink or amber coloured jelly on high speed centrifugation of tissue extracts after the removal of the mitochondria, and on dispersion are found to range from 100 to 200 mμ in diameter (Claude, 1947), and to account for about 15 to 20% of the cell mass. Some of the properties of the pigment associated with the microsomes have been studied by Bensley (1947).

With regard to pH changes, Claude (1946) has shown the behaviour of the microsomes to be similar to that of the mitochondria, in that they are dispersed between pH 7 and pH 10, but agglutinate in slightly acid solutions and disintegrate in strong acid or alkali.

The relationship between the mitochondria and microsomes:-

There has been considerable discussion concerning the interrelationship of the mitochondria and microsomes in view of Claude's (1944a, 1946) finding that the mitochondria are disrupted in water releasing smaller particles. It is possible that the microsomes are artifacts produced as a result of the fractionation procedure, but the questi-

is difficult to solve since the microsomes are too small to be visible in the light microscope. Claude (1943b) and Claude and Fullam (1946) from studies on centrifugation of intact cells, and also from electron micrographs of liver sections, believe that the ground substance of the intact cell is particulate in nature, and that the microsomes as isolated exhibit many of the properties of this material. Lagerstedt (1949) however has found in most of the normal liver cells which he has examined, large particles about 6-7 μ. in length and 2-3 μ. wide which he terms Basophilic Cytoplasmic Inclusions (BCI). These BCI are composed of ribonucleoprotein, and account for most of the ribonucleic acid (RNA) of the cytoplasm. In view of this finding, he concludes that the microsomes are in fact breakdown products of the BCI released by the fractionation process. The BCI are distinct from mitochondria which are also found in the cytoplasm. Lagerstedt states that the BCI disappear on starvation or on maintaining the animals on a low protein diet for four to five days, but if the microsomes are derived from the breakdown of BCI, a contradiction arises, since Muntwyler et al. (1950) have examined the microsomes of animals maintained on a severely restricted protein diet, and find that although there is some diminution in the size of the fraction, it by no means disappears. It

would therefore appear most likely that the BCI are actually aggregates of microsomes produced by the fixation process, although this does not explain the disappearance of the BCI on starvation unless some change occurs in the microsomes which hinders aggregation.

1.4 The Chemical Composition of the Cytoplasmic Fractions.

Mitochondria:-

In the earlier papers of Claude (1940, 1941, 1943a), the elementary composition of the granules has been studied, and Claude (1946) states that they contain 10-12% N, 0.9-1.3% P, 0.82-1.16% S, 0.02-0.04% Fe and 0.02-0.035% Cu. The conclusion was reached that the mitochondria were complexes of phospholipid, protein and ribonucleic acid. Furthermore, Claude (1943b) reported on some preliminary findings concerning the localisation of enzymes in the cytoplasmic fractions. The nitrogen, phosphorus, RNA and lipid contents of the fractions obtained by Barnum and Huseby (1948) from mouse liver shown in Table 1 are in general agreement with those of other authors for liver tissue.

Phospholipid:-

Generally speaking, about 25 to 30% of the dry weight of the mitochondria is composed of lipid material, of which about 1/2 to 2/3 is phospholipid. Comparatively

Table 1.

The Chemical Composition of Cytoplasmic Fractions of Normal Mouse Liver

	Mg./g. wet weight of tissue			Rib. P as per cent tot. P	N/P	N/rib.	Composition of fractions based on their dry weight				
	N	P	Ribose (×2)				N	P	Nucleic acid (based on ribose)	Lipide	Phospholipide as per cent tot. lipide
							per cent	*per cent*	*per cent*	*per cent*	
E	*27.1* (9) [a]	*2.64* (6)	*5.4* (6)	*42.3* (6)	*10.3* (6)	*5.0* (6)	*9.1* (4)	*0.88* (4)	*3.9* (4)	*47.3* (2)	*15.7* (2)
	23.2–30.1	2.43–2.92	4.5–6.3	38.0–45.9	9.6–10.5	4.7–5.45	8.4–9.5	0.81–0.93	3.8–4.2	46.9–47.6	14.2–17.1
L	*4.83* (13)	*0.43* (13)	*0.64* (13)	*30.6* (13)	*11.2* (13)	*7.8* (13)	*12.1* (6)	*1.11* (6)	*3.7* (6)	*27.4* (7)	*56.6* (7)
	3.5–7.2	0.32–0.63	0.40–0.93	24.2–35.8	9.6–12.4	6.4–11.1	11.8–12.4	0.99–1.19	3.2–4.0	26.1–28.8	51.0–59.5
M	*5.02* (15)	*0.89* (15)	*1.93* (15)	*44.9* (15)	*5.6* (15)	*2.6* (15)	*10.3* (7)	*1.87* (7)	*9.1* (7)	*35.1* (7)	*62.7* (7)
	3.9–6.2	0.72–1.12	1.7–2.2	38.3–51.8	5.3–6.1	2.2–3.1	9.2–10.9	1.6–2.0	7.4–10.4	33.9–36.5	61.0–64.2
U	*1.54* (13)	*0.25* (13)	*0.92* (13)	*58.4* (13)	*6.4* (13)	*1.7* (13)	*13.2* (3)	*1.9* (3)	*16.0* (3)	*16.8* (5)	
	1.0–3.1	0.15–0.51	0.48–1.66	61–115	5.1–8.9	1.2–2.3	12.5–14.0	1.4–2.2	11.5–19.5	10.9–26.6	
S	*16.3* (11)	*0.93* (11)	*0.80* (9)	*17.7* (9)	*17.5* (11)	*21.8* (9)	*13.4* (3)	*0.73* (3)	*1.8* (3)		
	14.0–18.5	0.80–1.08	0.51–1.15	10.6–26.8	16.3–19.6	14.7–33.8	12.8–13.8	0.71–0.76	1.3–2.0		
X	0.86	0.033	0.14	87.7	25.8	6.8					

[a] Figures are averages of the number of individual experiments shown in parentheses. Ranges are shown below each average.

E. Whole Cytoplasm. L. Mitochondrial Fraction. M. Microsome Fraction

U&X Additional Submicroscopic Particles. S. Supernatant Fraction

little research has been carried out on this component, but Ada (1949) has studied its distribution, and with the aid of ^{32}P its metabolism. Other workers have studied its distribution throughout the cytoplasmic fractions (Barnum & Huseby, 1948, 1950; Schneider, 1946b).

Enzymes:-

A great deal of interest has centered on the mitochondria in view of the enzyme systems which appear to be associated with them. Initial experiments in this field however were subject to difficulties caused by incomplete separation of the various cytoplasmic fractions, and a large amount of the published data is of questionable value since the authors have not taken the necessary steps to purify and characterise the material which they were studying. Thus some enzymes have been reported in the nuclear fraction and in the mitochondria, this being due to contamination of the nuclei with mitochondria. Misleading conclusions have similarly been arrived at because of contamination of the mitochondria with microsomes. It is also necessary to bear in mind the fact that, in the course of separating one fraction from another, a co-factor necessary for the proper functioning of an enzyme may be lost, thus reducing the apparent activity of the enzyme. Furthermore, a fraction might appear to be very rich in

enzymic activity, but being itself small in bulk might only account for a small proportion of the activity of the whole cell. In view of these observations, Hogeboom and Schneider (1950a) have set down the following criteria which must be satisfied before any conclusions regarding the localisation of biochemical property may be reached.

1. The cell fraction must be cytologically defined in terms of the intracellular component which it represents.
2. The validity of the method of assay of the biochemical property must be established.
3. The biochemical property must be concentrated to a greater (preferably much greater) extent in the cell fraction than in the original tissue.

Besides these three points Hogeboom & Schneider strongly advocate the establishment of a balance sheet demonstrating the content of each fraction, and of the various combinations of fractions compared with the values obtained for whole tissue.

Considerable criticism has been levelled at the procedure of cytoplasmic fractionation, on the grounds that the artifacts produced as a result of the rupture of the cell are sufficient to invalidate any subsequent procedure which might be applied (Bradfield, 1950; Danielli, 1946). Such artifacts are of course obvious problems, and must at all times be considered when results are being

interpreted, but it would be unjustifiable to discard the procedure for this reason when the only other means of carrying out comparable studies are liable to produce equally misleading changes. Furthermore no other available technique lends itself to the study of such a diversity of biochemical properties within the cell with any degree of accuracy.

Certain enzymes have been clearly shown to be associated with the mitochondria. Thus there appears to be no doubt that most of the cytochrome oxidase and succinoxidase activity of rat liver and kidney (Hogeboom, Claude & Hotchkiss, 1946; Hogeboom et al., 1948; Schneider, 1946b,c, 1947; Schneider, Claude & Hogeboom, 1948) and of mouse liver (Schneider & Hogeboom, 1950b) is localised in the mitochondrial fraction, the small amounts found in other fractions being accounted for as contamination. So much reliance is placed on this specific localisation of the succinoxidase that it has been used as a means of estimating the contamination of the nuclear fraction with mitochondria. Among the other enzymes concentrated in this fraction are octanoic acid oxidase (Kennedy & Lehninger, 1948, 1949; Schneider, 1948) and oxalacetic acid oxidase (Schneider & Potter, 1949), but in both cases the addition of other fractions, themselves inactive, leads to increased

activity. The fact that these enzymes are found largely in the mitochondrial fraction demonstrates that these structures must play a part in the Krebs cycle reactions, although recent observations by Hogeboom and Schneider (1950a) on isocitric dehydrogenase suggest that the mitochondria are not solely responsible.

Of the enzymes not exclusively associated with the mitochondria are DPN-cytochrome c reductase (Hogeboom, 1949, Hogeboom & Schneider, 1950b) and TPN-cytochrome c reductase (Hogeboom & Schneider, 1950a) both of which are also found in the microsome fraction. Acid phosphatase, AMP-ase (Novikoff, Podber and Ryan, 1950) and ATP-ase (Novikoff et al., 1950; Schneider, 1946b, Schneider Hogeboom & Ross, 1950) as well as uricase (Schein, Podber & Novikoff, 1950) also appear to be largely recoverable from the mitochondrial fraction.

Recently a system capable of synthesizing ρ-amino hippuric acid has been shown to be localised in the mitochondria (Kielley & Schneider, 1950). Numerous other enzymes and substances of biological importance have been reported in the mitochondrial fraction, but it has not been established that they are localised within the mitochondria. Thus the presence of d-amino acid oxidase, α-glycerophosphate dehydrogenase and ribonuclease is reported by Claude (1944b).

Several authors have commented on the differences in enzyme distribution between normal liver mitochondria and mitochondria from hepatoma (Schneider, 1946b; Schneider & Hogeboom, 1950b; Schneider, Hogeboom & Ross, 1950).

Green, Loomis & Auerbach (1948) describe an enzyme system prepared by centrifuging at 2000 g homogenates of various tissues, and subsequently washing the sediment several times. This system has been shown to be capable of catalysing the complete oxidation of pyruvic acid, the fatty acids and amino acids to carbon dioxide and water, by way of the citric acid cycle, and has been named "cyclophorase" to indicate that it is a complex enzyme system. Harman (1950b) has examined this complex enzymatically and cytologically, and concludes that the mitochondria are the morphological units of the "cyclophorase system". This view is strongly contested by Schneider and Hogeboom (1951) on the grounds that most of the cyclophorase of whole tissue is not recoverable from and concentrated in the mitochondria; this is shown by the fact that d-isocitric dehydrogenase is found in higher concentrations in the supernatant fraction than elsewhere, although they are careful to remark on the possibility of the enzyme having been removed from the mitochondria in the course of fractionation.

Microsomes:

The elementary composition of the microsomes like that of the mitochondria has been studied by Claude (1946). The values quoted for rat liver are as follows:- 8.95% N, 1.74% P, 0.75% S, 0.01-0.02% Cu and 0.02-0.04% Fe. The low nitrogen figures are accounted for on the basis of an exceptionally high content of lipid, about 40 to 45% of the fraction. Of the remainder a considerable proportion is represented by ribonucleoprotein, and Claude (1947) considers the microsomes to be complex structures of phospholipid and nucleoprotein of the ribose type. The content of nitrogen, phosphorus, RNA and phospholipid in mouse liver microsomes is shown in Table 1 (Barnum & Huseby, 1948).

Phospholipids:-

About 40 to 45% of the dry weight of the microsomes is made up of phospholipids and these have been studied by Ada (1949), Barnum and Huseby (1948, 1950) and Claude (1946).

Enzymes:-

The enzymes of the microsomes have excited less attention than those of the mitochondria, and correspondingly less is known concerning them. Omachi, Barnum and Glick (1948) have demonstrated the concentration of an esterase, while Hogeboom (1949) has shown that DPN-cytochrome c reductase is found largely in this fraction, TPN-cytochrome c reductase is also found in the microsomes although to a

lesser extent than in the mitochondria (Hogeboom & Schneider, 1950a). Likewise ATP-ase is found to a very much lesser degree in the microsomes than in the mitochondria (Schneider, Hogeboom & Ross, 1950). Schneider and Hogeboom (1951) in their review on the subject mention other enzymes which have been less conclusively demonstrated in the microsomes.

Supernatant Fraction:

The supernatant remaining after the removal of mitochondria and microsomes constitutes the supernatant fraction, and for this reason its nature is less well defined. Variations in this fraction can usually be linked with the suspending medium and centrifugal fields employed in the separation. Generally speaking however, all particulate matter down to a size of 50 mμ. in diameter is sedimented in the microsome fraction, so that the supernatant for most practical purposes can be considered free from larger particles. In rat liver subjected to fractionation in sucrose about 32 to 44% of the total liver nitrogen is recovered in the supernatant fraction (Schneider & Hogeboom, 1951), while the values for other tissues are of the same order. The main component of the fraction appears to be protein in nature (Price, Miller & Miller, 1948), although a considerable proportion of the RNA of the cell is also

found there together with some lipid material which migrates centripetally because of its low specific gravity.

Enzymes:-

As might be expected, a great variety of enzymes has been found in the supernatant fraction. Thus LePage and Schneider (1948) reported that in rabbit liver and Flexner-Jobling carcinoma, all the enzymes connected with the glycolysis of glucose to lactic acid were present in this fraction, and that its total activity accounted for about 50% of that of the whole tissue. Cytochrome c of mouse and rat liver (Schneider, Claude & Hogeboom, 1948; Schneider & Hogeboom, 1950a), isocitric dehydrogenase (Hogeboom & Schneider, 1950a) and acid and alkaline phosphatase (Novikoff, Podber & Ryan, 1950) are also found to a large extent in the supernatant fraction.

1.5 The Pentose Nucleic Acid of the Cytoplasm.

The sugar part of the molecule of deoxypentose and pentose nucleic acids has been studied in relatively few cases, but has then proved to be deoxyribose and ribose respectively. It has therefore become common practice to refer to the deoxypentose nucleic acid as deoxyribonucleic acid (DNA), and to the pentose nucleic acid as ribonucleic acid (RNA). For the sake of convenience therefore it is proposed to make use of this terminology, it being under-

stood that it refers to deoxyribose and ribose only in the cases, e.g., liver, where these sugars have been identified, and in all other cases to deoxypentose and pentose.

Until the early part of this century it was generally believed that DNA was to be found exclusively in animal tissues, while RNA was characteristic of plant tissues. Feulgen and Rossenbeck (1924) demonstrated the presence of DNA in the nuclei of wheat germ, and assumed that the RNA was present in the cytoplasm. Jorpes (1934) was able to show the presence of RNA in pancreas, and when Behrens (1938) demontrated RNA in the cytoplasm of rye embryo, the early ideas of plant and animal nucleic acids were finally disproved. Claude (1940, 1941, 1943a,b) found that RNA was associated with the particulate matter of the cytoplasm of various animal tissues, and has since found it to be present in all the cytoplasmic fractions, while Schneider (1946b) has proved that all the DNA of the cell is recoverable from the nuclear fraction. However, although DNA is exclusively found in the nucleus, RNA is not a purely cytoplasmic constituent since Caspersson and Schultz (1940) and Davidson and Waymouth (1946) have found small amounts of RNA within the nucleus.

As a result of the demonstration of RNA in the cytoplasm, numerous workers have examined its distribution

throughout the cytoplasmic fractions of several tissues. The results obtained by some of these workers for liver are summarised in Table 2. Analysis of rat kidney has been carried out by Schneider (1946b) and Schneider and Potter (1949) while Schneider, Hogeboom and Ross (1950) have repeated this work on rat and mouse tumours. Muntwyler et al. (1950) have observed variations in the distribution of RNA in the liver fractions of rats maintained on a protein deficient diet, and Price, Miller and Miller (1948), Price, Miller, Miller and Weber (1949a,b, 1950) have studied the effects of feeding various carcinogenic and related dyes on the RNA distribution in rat liver.

1.6 The Biological Activity of the Nucleic Acids.

Several authors have sought to study the biosynthesis of the nucleic acids making use of isotopically labelled precursors. Thus Davidson and Raymond (1948) feeding ammonium citrate labelled with ^{15}N to pigeons and rats found incorporation of the isotope into the RNA of the livers, while there was negligible incorporation into the DNA. Plentl and Schoenheimer (1944) showed that labelled guanine was not incorporated into the tissue nucleic acids, while Brown, Roll, Plentl and Cavalieri (1948) demonstrated the incorporation of labelled adenine into the adenine, and to a lesser extent the guanine, of

Table 2.

The Distribution of RNA and DNA in Liver Cell Fractions.

Fraction	mg./100 g. fresh tissue DNAP	RNAP	Tissue	Method of Isolation	Reference
Homogenate	22.6	65.2	Rat Liver	Saline	Schneider (1946b)
Nuclear Fraction	23.4	4.9			
Mitochondrial Fraction	--	11.4			
Residue	--	47.8			
Homogenate	26.0	76.3	Rat Liver	0.88M-Sucrose	Hogeboom et al. (1948)
Cytoplasmic Extract	--	59.3			
Nuclear Fraction	25.8	14.4			
Mitochondrial Fraction	--	13.6			
Microsome Fraction+	--	26.5			
Supernatant Fraction+	--	20.5			
Nuclear Fraction		29.0	Rat Liver	0.14M-KCl	Price et al. (1948)
Mitochondrial Fraction		17.0			
Microsome Fraction		31.0			
Supernatant Fraction		9.0			
Nuclear Fraction		9.0	Rat Liver	0.88M-Sucrose	Price et al. (1948)
Mitochondrial Fraction		33.0			
Microsome Fraction		26.0			
Supernatant Fraction		20.0			
Homogenate	27.1	82.8	Rat Liver	0.25M-Sucrose	Schneider (1948)
Nuclear Fraction	26.8	11.4			
Mitochondrial Fraction	--	6.0			
Microsome Fraction	--	43.5			
Supernatant Fraction	--	19.2			

Table 2 (Contd.)

Homogenate	22.4	47.0	Rabbit Liver	0.25M-Sucrose	LePage & Schneider (1948)
Nuclear Fraction	22.3	13.1			
Mitochondrial Fraction	--	4.6			
Microsome Fraction	--	15.2			
Supernatant Fraction	--	14.8			
Cytoplasmic Extract		38.2	Rat Liver	0.88M-Sucrose	Hogeboom (1949)
Mitochondrial Fraction		10.4			
Microsome Fraction		21.8			
Supernatant Fraction		5.3			
Homogenate	28.5	78.9	Rat Liver	0.25M-Sucrose	Schneider & Potter (1949)
Nuclear Fraction	28.7	9.3			
Cytoplasmic Extract	--	70.1			
Mitochondrial Fraction	--	8.2			
Microsome Fraction	--	36.3			
Supernatant Fraction	--	26.2			
Homogenate	26.8	83.9	Rat Liver	0.25M-Sucrose	Muntwyler et al. (1950)
Nuclear Fraction	25.2	11.3			
Mitochondrial Fraction	--	4.3			
Microsome Fraction	--	41.6			
Supernatant Fraction	--	30.4			
Cytoplasmic Extract		69.5	Mouse Liver	Saline	Huseby & Barnum (1950)
Mitochondrial Fraction		8.6			
Microsome Fraction		30.3			
Supernatant Fraction*		18.0			

Table 2 (Contd.)

Homogenate	27.9	92.9	Mouse Liver	0.25M-Sucrose	Schneider, Hogeboom & Ross (1950)
Nuclear Fraction	22.4	10.2			
Mitochondrial Fraction	--	15.6			
Microsome Fraction	--	48.7			
Supernatant Fraction	--	15.3			

+ The authors carry out separate estimations on the washings from the mitochondrial fraction and microsome fraction. For the sake of uniformity, the content of RNAP in these has been added on to the subsequent fraction in each case.

* The authors obtain a further particulate fraction from the microsomal supernatant by exceedingly high speed centrifugation. For the sake of uniformity this has been included with the supernatant fraction.

tissue nucleic acids. Later Brown, Peterman and Furst (1948) and Furst, Roll and Brown (1950) found that feeding adenine labelled with ^{15}N to rats resulted in the labelling of the purines of whole viscera and liver RNA, while the corresponding incorporation into the DNA in resting liver was very small. LePage and Heidelberger (1951) however have shown that the purines of both RNA and DNA of rat liver and tumour take up ^{14}C labelled glycine quite rapidly. The apparent anomaly in these findings has been investigated by Furst and Brown (1951) who conclude that two pathways for the synthesis of DNA purines must exist.

Other workers have made use of radiophosphorus to study the incorporation of phosphorus into the molecule of RNA and DNA. By this means Brues, Tracey and Cohn (1944), Hammarsten and Hevesy (1946) and Davidson (1947) have shown that in resting liver the specific activity of the ribonucleic acid phosphorus (RNAP) is much higher than that of deoxyribonucleic acid phosphorus (DNAP).

With the demonstration that the RNA of the cell is distributed throughout the various cytoplasmic fractions and in the nucleus, it would obviously be of interest to study the uptake of ^{32}P by the RNAP of these fractions individually. In view of the association so frequently postulated between RNA and protein synthesis (Spiegelman

& Kamen, 1947; Caspersson, 1947; Thorell, 1947; Davidson, 1949) and also the opinions expressed concerning the synthesis of RNA and DNA (Marshak, 1948; Marshak & Calvet, 1949; Brachet, 1947 and Jeener & Szafarz, 1950a), such a study becomes not merely a matter of interest but one of necessity.

In such work it would be interesting to examine not only the specific activities of the phosphorus from the RNA in the different fractions, but also the quantitative distribution of the RNA. Previous work has involved the isolation of the RNA and DNA in order to measure the uptake of ^{32}P, but this procedure is neither quantitative nor convenient where small portions of tissues or tissue fractions are concerned. As a result, Davidson, Gardner, Hutchison, McIndoe, Raymond and Shaw (1949) and Davidson, Gardner, Hutchison, McIndoe and Shaw (1949) have examined the possibility of making use of the tissue fractionation procedure of Schmidt and Thannhauser (1945) as a means of obtaining a measure of the incorporation of ^{32}P into the RNAP and DNAP of various tissues. Several workers (Campbell & Kosterlitz, 1949; Holmes, 1949; Hull & Kirk, 1950a,b,c) have adopted this procedure without reservation as a means of studying the uptake of radiophosphorus by RNAP and DNAP. This involves the assumption that the

specific activities of the Schmidt and Thannhauser fractions corresponding to RNA and DNA are the same as those of the isolated materials. Jeener (1949a,b), Jeener and Szafarz (1950a,b) and Davidson, Frazer and Hutchison (1951) have shown that this assumption is not justifiable, and in company with Euler, Hevesy and Solodowska (1948), Friedkin and Lehninger (1949) and Marshak and Calvet (1949) have drawn attention to the presence of a small amount of highly active inorganic phosphate in the RNA fraction obtained by the Schmidt and Thannhauser procedure.

The possibility of this arising at least in part from incomplete removal of the acid soluble phosphorus compounds in the initial stages of the extraction has been recognised by Friedkin and Lehninger (1949) and Davidson, Frazer and Hutchison (1951). Both groups have applied various extractions such as washing the tissue initially with trichbracetic acid (TCA) containing sodium phosphate prior to the Schmidt and Thannhauser procedure. This treatment however did not eliminate the contaminating inorganic phosphate from the RNA fraction. Jeener (1949a, b) carried out repeated additions of inorganic phosphate to the RNA fraction followed by reprecipitation with magnesia mixture, in an attempt to remove all traces of the

highly active phosphorus, but found the method not completely successful. Jeener and Szafarz (1950 a,b) resorted to paper chromatography of the fraction using phenol as a solvent, a method which although not separating the nucleic acid components in any way apparently removed the last traces of inorganic phosphate.

Besides the presence of contaminating inorganic phosphate, Davidson, Frazer and Hutchison (1951) have shown that the RNA content of the fraction as estimated by pentose determination is invariably about 85% of that expected on the basis of the phosphorus content, and they have expressed the opinion that the RNA fraction contains phosphorus compounds other than acid soluble ribonucleotides and the inorganic phosphate generally considered to be derived from phosphoprotein.

Clearly then, before any successful attempt can be made to examine the incorporation of ^{32}P into the RNA of animal tissues, it is essential that a method should be devised yielding substances which can be readily identified, and which are free from contamination with other acid soluble phosphates. Such a method should at the same time be applicable to the small amounts of material which are available for example on the fractionation of the cellular components.

The primary object of this investigation has there-

fore been the development of a method which meets the above requirements, and the subsequent application of such a technique to the study of the uptake of ^{32}P into the RNAP of the cytoplasmic fractions. It is hoped by such an examination to obtain a greater understanding of the synthesis and function of the RNA of the cell.

PART II - METHODS.

2.1. Animals.

The animals used in the experiments to be described were as follows:-

a. Male albino rats varying in weight from 150 to 300 g. from the colony maintained in the department, and fed on a standard diet.

b. Male hooded rats varying in weight from 150 to 300 g. from the departmental colony, and fed on a standard diet.

c. Female rabbits weighing from 2.0 to 3.0 kg. from the departmental colony and fed on a standard diet.

d. Brown leghorn fowls from the departmental stock, fed on a standard diet. Cocks, laying and non-laying birds were used, and also cocks bearing the GRCH 15 tumour (Peacock, 1933).

2.2. The Administration of Radioactive Phosphorus.

Radioactive phosphorus (^{32}P as inorganic phosphate obtained from A.E.R.E. Harwell), except in the experiments involving the isolation of cytoplasmic fractions from sucrose homogenates, was made N with respect to HCl and subjected to 10 min. hydrolysis at 100°, in order to destroy any polyphosphates present. The solution was then neutralised by 5N-NaOH before use. The ^{32}P solution was

administered by subcutaneous injection between the shoulder blades, except in the experiments referred to above, when intramuscular injection in the thigh was used. In the initial experiments the dosage was 10 to 20 μc. ^{32}P/100 g. body wt., but latterly this was increased to 50 μc. ^{32}P/100 g. body wt.

2.3. The Excision of the Organs.

The animals were killed either by cervical dislocation or by exsanguination under ether anaesthesia. The abdomen was rapidly opened and the liver perfused with cold isotonic saline through the portal vein. The organs and tissues required were quickly removed, blotted to remove excess blood, weighed, and chilled on ice.

2.4. Preparation of Homogenates.

a. Where only whole tissue was being studied, and no attempt was being made to separate the cytoplasmic fractions, an "Atomix", Waring or Nelco blender fitted with an ice jacket was used. In this case the tissue was homogenised with 4 or 9 vol. of ice cold 0.9% saline at full speed for 6 min.

b. In the tissue fractionation experiments, it was necessary to adopt a procedure which would not bring about appreciable destruction of the nuclei, but which would effect maximum breakdown of the intact cells.

0.9% saline was used in the preliminary experiments as the suspending medium, and it was found that either the glass homogeniser of Potter and Elvehjem (1936), or the "Atomix" run at half-speed for 6 min. gave good results.

Experiments with 0.25M-sucrose however showed that the tissues were much more fragile in this medium, and that a very much milder homogenising technique was necessary. The apparatus of Potter and Elvehjem (1936) suffers from certain disadvantages in that it is very tedious when dealing with more than about 10 g. of tissue, that the degree of homogenisation is difficult to control from experiment to experiment, and that a fair amount of powdered glass was inevitably produced which contaminated the homogenate. As a result, a paddle-shaped blade was made to fit a Nelco blender, and it was found that 4 min. at full speed with a total volume of 75 ml. produced homogenates containing a minimum number of unbroken cells and a maximum number of intact, free nuclei. A photograph of this apparatus is shown in Fig.4.

2.5. The Fractionation of Liver Tissue.

Preliminary experiments were carried out on saline homogenates. Homogenisation was obtained by means of the Potter and Elvehjem (1936) homogeniser, and the homogenate before being fractionated was strained through 2 layers of

Figure 4.

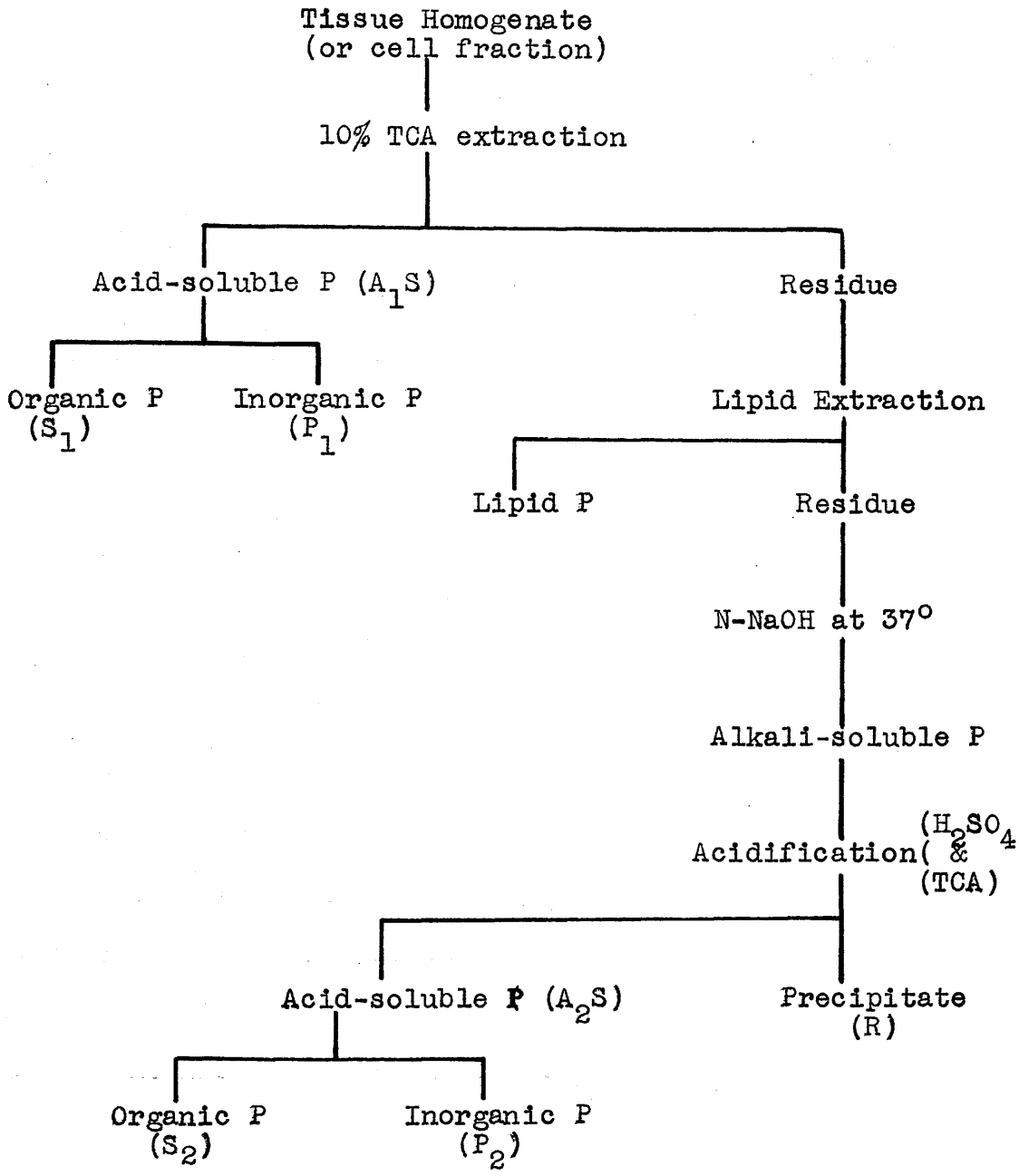
nylon to remove most of the connective tissue and any large pieces of unbroken tissue. Fractionation was then carried out by the method of Claude (1946) using an International Equipment Co. Model P.R.1 refrigerated centrifuge, with the high speed attachment when required. Later work was carried out on homogenates in 0.25M-sucrose, which after straining through nylon were fractionated by the method of Schneider (1948) described in the previous section, except that the microsome fraction was not washed.

2.6. Fractionation of Phosphorus Compounds in the Tissues.

Procedure 1. In early work when all the phosphorus containing fractions of the cellular constituents were studied, the method used was the modification of the Schmidt and Thannhauser (1945) procedure proposed by Davidson, Frazer and Hutchison (1951) (Fig.5).

In this procedure, the homogenate was treated with 0.5 vol. 30% (w/v) A.R. trichloracetic acid (TCA), and allowed to stand on ice for about 30 min. The precipitated material was centrifuged out and washed twice with a volume of ice-cold 10% (w/v) TCA equivalent to the original vol. of homogenate. The supernatant fluid and washings were set aside on ice for further fractionation as the acid-soluble fraction (A_1S). The precipitate was then extracted successively with acetone, ethanol, ethanol-

Figure 5.


Tissue Homogenate
(or cell fraction)
10% TCA extraction
Acid-soluble P (A_1S)
Residue
Organic P
(S_1)
Inorganic P
(P_1)
Lipid Extraction
Lipid P
Residue
N-NaOH at 37°
Alkali-soluble P
Acidification
(H_2SO_4 & TCA)
Acid-soluble P (A_2S)
Precipitate
(R)
Organic P
(S_2)
Inorganic P
(P_2)

$CHCl_3$ (3:1), ethanol-ether (3:1) (twice), and finally ether. The extracts were combined, taken to dryness on a water bath, and the residue extracted with $CHCl_3$ to provide the lipid fraction (lipid P).

The dry residue was treated with a volume of N-NaOH equal to that of the original homogenate, and incubated overnight at 37°. The alkaline digest was generally slightly turbid in accord with the experience of Davidson, Frazer and Hutchison (1951), but for the purposes of these experiments this insoluble matter was ignored. The digest was neutralised with 10N-H_2SO_4, and sufficient 30% (w/v) TCA added to bring the final concentration to 10%. The precipitate containing protein and DNA was centrifuged out and washed twice with 10% (w/v) TCA, the supernatant and washings being set aside for further fractionation as the acid-soluble fraction (A_2S). The solid residue was dissolved in N-NaOH to form the residual (R) fraction.

Both acid-soluble fractions A_1S and A_2S contained inorganic phosphate, and a portion of each was therefore treated with Mathison's (1909) reagent, in the ratio of 1 ml. reagent to 10 ml. of extract, in order to separate the inorganic and organic components. The mixture was made alkaline with ammonia and allowed to stand overnight in the cold. The precipitate of $Mg(NH_4)PO_4$ was filtered

off through a Whatman no.42 filter paper, washed with dilute ammonia and finally dissolved in N-HCl. This provided the inorganic fractions P_1 and P_2 while the mother liquors were taken as the organic phosphate fractions S_1 and S_2.

Procedure 2. The above procedure while suitable for the analysis of tissues and tissue fractions, did not lend itself to further studies of the ribonucleotide containing (A_2S or S_2) fractions. In order to make this possible further modifications of the separation scheme were adopted, the aim being threefold:-

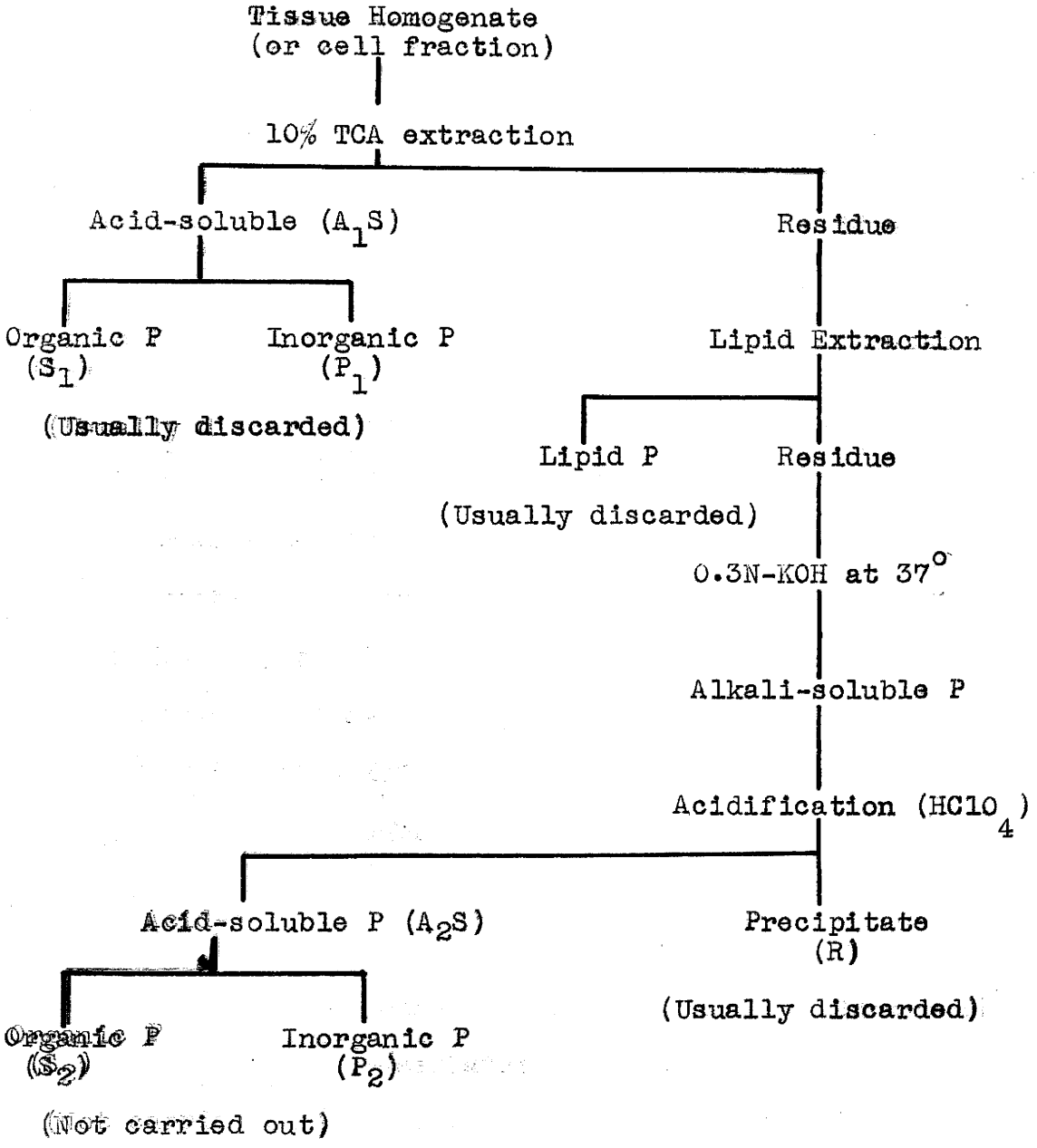
a. the concentration of the fraction,

b. the elimination of as much of the salt content as possible,

c. the use of a precipitant to replace the TCA, which would not absorb ultraviolet light.

The final technique developed is shown in Fig.6, and is similar to that of Davidson, Frazer and Hutchison (1951) initially, diverging at the stage of alkaline incubation. Here 0.3N-KOH was used to replace the N-NaOH in order to provide a metallic ion which could be readily removed; at the same time the volume of alkali was reduced to 1 ml./100 mg. dry powder. Incubation was carried out at 37° for 18 hr. The resulting digest, which was frequently

Figure 6.

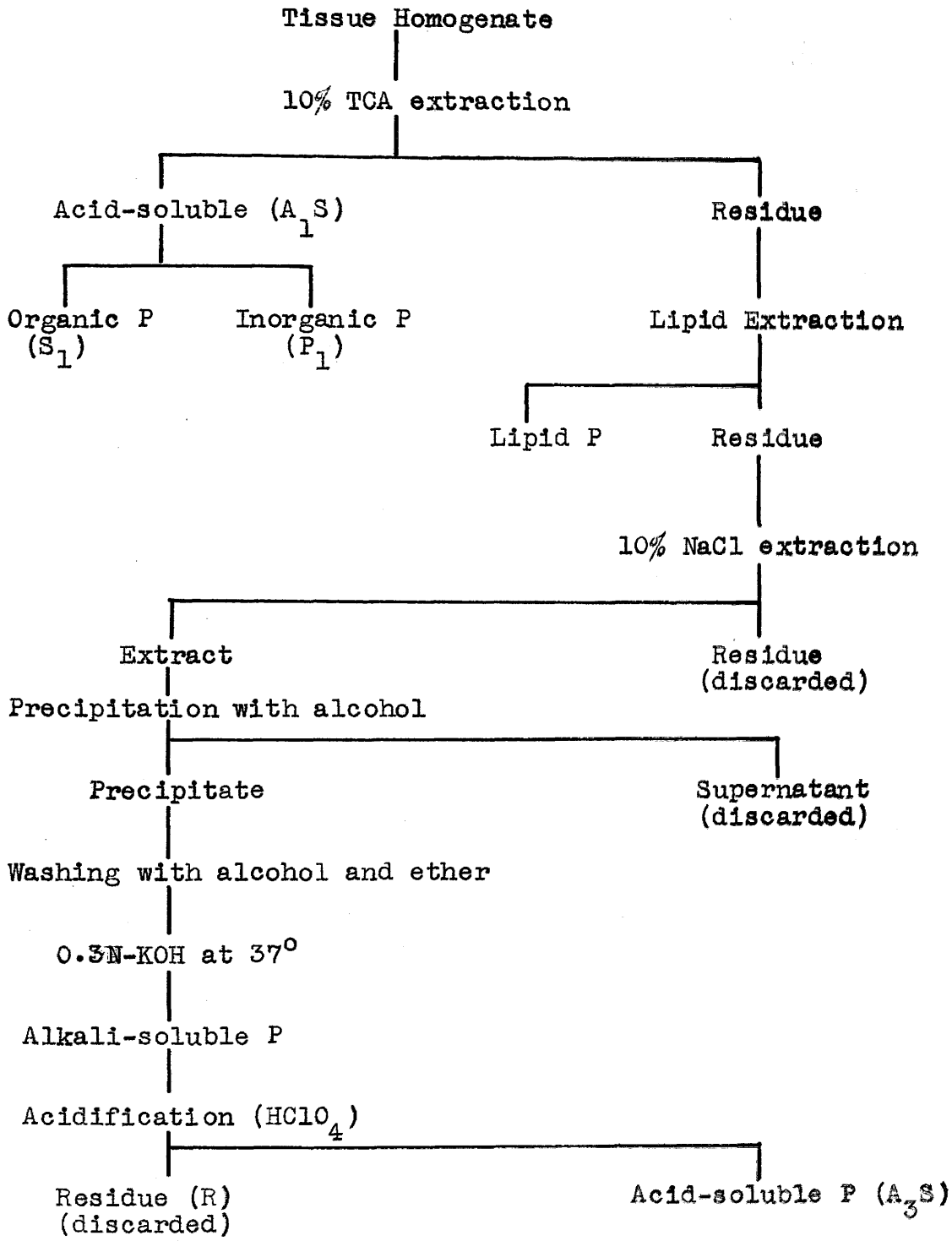

Tissue Homogenate
(or cell fraction)
10% TCA extraction
Acid-soluble (A_1S)
Residue
Organic P
(S_1)
Inorganic P
(P_1)
(Usually discarded)
Lipid Extraction
Lipid P
(Usually discarded)
Residue
0.3N-KOH at 37°
Alkali-soluble P
Acidification ($HClO_4$)
Acid-soluble P (A_2S)
Precipitate
(R)
(Usually discarded)
Organic P
(S_2)
Inorganic P
(P_2)
(Not carried out)

quite thick, was cooled on ice and adjusted to pH 1 by the addition of 60% $HClO_4$. By this means protein, DNA and the bulk of the K (in the form of the insoluble perchlorate) were precipitated and removed by centrifugation. The precipitate was washed twice with a small amount of N-$HClO_4$, the supernatant and washings combined and the pH adjusted to 4 with 5N-KOH. Any $KClO_4$ produced was centrifuged out and the supernatant then formed the acid-soluble (A_2S) fraction which contained the ribose mononucleotides derived from the tissue RNA. In most experiments, the R fraction was discarded since the interest was centered mainly on the A_2S fraction. When this was done, no attempt was made to wash the precipitate, in order to obtain maximum concentration of the A_2S.

The fraction A_2S obtained as described above was available for use as such for the subsequent analysis procedures.

Procedure 3. In later experiments when it had been found necessary to prepare a nucleotide fraction free from the phosphate esters (see later) which accompany the nucleotides in fraction A_2S, a further modification of the Schmidt and Thannhauser (1945) procedure was adopted. The scheme used is shown diagrammatically in Fig.7, the initial stages being unchanged from those of Davidson, Frazer and

Figure 7.


Tissue Homogenate
10% TCA extraction
Acid-soluble (A_1S)
Residue
Organic P (S_1)
Inorganic P (P_1)
Lipid Extraction
Lipid P
Residue
10% NaCl extraction
Extract
Residue (discarded)
Precipitation with alcohol
Precipitate
Supernatant (discarded)
Washing with alcohol and ether
0.3N-KOH at 37°
Alkali-soluble P
Acidification ($HClO_4$)
Residue (R) (discarded)
Acid-soluble P (A_3S)

Hutchison (1951). It proved advantageous however to separate the RNA from as much of the extraneous protein as possible before incubation with alkali. The procedure adopted was therefore to extract the dry powder, obtained after extraction of the lipids, three times at 100° for 1 hr. with a vol. of 10% (w/v) NaCl equivalent to that of the original homogenate. This extraction removed from the main bulk of the tissue, most of the RNA and DNA together with a small amount of proteinaceous material. The dissolved RNA and DNA were then precipitated by the addition of 2 vol. of absolute ethanol, the precipitate was washed with ethanol and ether and dried. The dry white powder obtained by this method was then incubated with 0.3N-KOH at 37° for 18 hr., in the proportion of 20 mg./ml., subsequent procedures being as described before.

2.7. The Technique of Ionophoresis.

Initial experiments were carried out using silica gel as the medium for ionophoresis as described by Consden, Gordon and Martin (1946), but these proved unsuccessful. The use of agar gel as described by Gordon, Keil, Sebesta, Knessl and Sorm (1950) however was more satisfactory. By this means separation of all four ribose mononucleotides was achieved, their positions being established by inspecting the surface of the gel in the radiations from an ultrabiolet lamp fitted with a filter as described by Holiday

and Johnson (1949). Elution was not attempted, but the areas of gel found to contain the nucleotides were cut out and the excised portions dissolved in water for the determination of the ultraviolet absorption spectra of the nucleotides. The agar solution was then digested for the determination of phosphorus.

This method, while moderately satisfactory, was rather clumsy, and not readily adapted to the analysis of the small amounts of the four nucleotides which would be available. It was therefore superseded by the method of ionophoresis on filter paper described by Durrum (1950) for amino acids.

The procedure was as follows:-

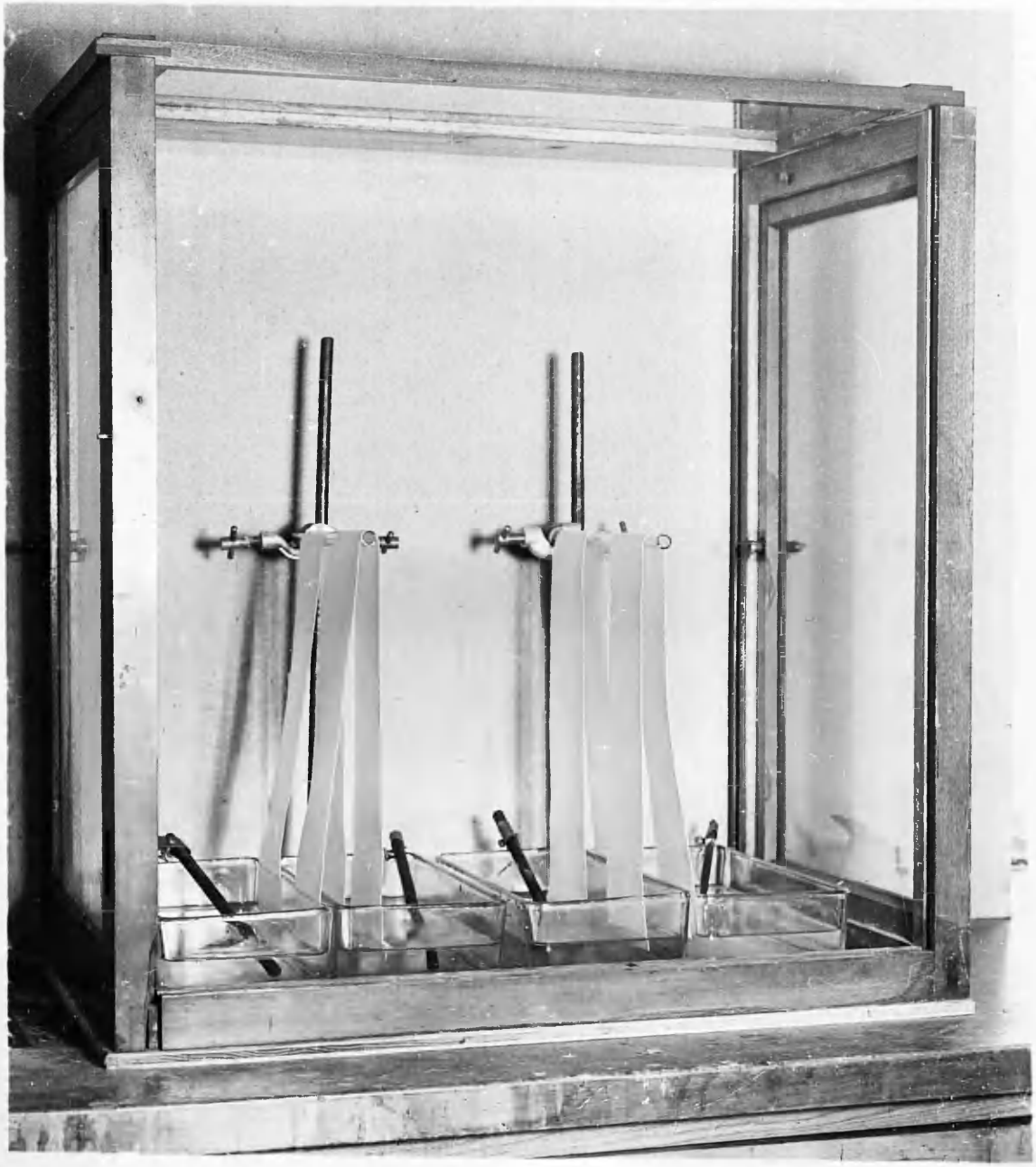
An accurately measured volume of the material to be analysed containing about 80 µg. P was applied in a narrow band about 3.5 cm. wide 5 cm. from one end of a strip of Whatman 3MM filter paper 72 cm. by 7 cm. The application was made from an Agla micrometer syringe, and by holding the paper in a current of warm air it was not difficult to spot up to 500 µl. (Care was necessary that too much heat was not used since this resulted in the spontaneous formation of the isomeric nucleotides.) The strip was then moistened with 0.02M-citric acid/trisodium citrate buffer at pH 3.5 obtained by a fiftyfold dilution of a mixture of 3 parts

of M-citric acid and 1 part of M-trisodium citrate solutions. The strip was then suspended over a glass rod so that the two ends were immersed to a depth of 1 cm. in two separate glass dishes containing the same buffer. The dishes used were refrigerator dishes and the volume of buffer in each was 500 ml., three strips of paper being accommodated in each pair of vessels (Fig.8). Carbon electrodes placed in each dish were connected to a source of direct current so that the anode was in the vessel further away from the spot on the paper. The direct current supply consisted of a full wave valve rectifier capable of a smoothed output of 600V. at 300 ma., which on small load actually delivered about 800 V. The output was controlled by means of a Variac transformer in the input side, thus making available a continuous range of 100 to 800 V.

Complete separation of adenylic acid, cytidylic acid, guanylic acid and uridylic acid was obtained in amounts of the order of 200 to 300 µg. of each by running at a potential gradient of 11 V./cm. for 18 hr., the current flowing being about 0.5 to 1.0 ma./cm. The apparatus was covered during the run with a glass case to minimise evaporation, and although it proved possible to obtain the same separation in a shorter time by increasing the voltage applied, this procedure resulted in heating of the paper

Figure 8. Ionophoresis Apparatus showing four papers set up during a run.

Figure 8.



and increased evaporation which gave rise to artifacts. Moreover, water condensed on the case and was liable to drip on to the papers.

Samples of the four purified ribose mononucleotides were supplied by Nutritional Biochemicals Inc., guanylic and cytidylic acids supplied by B.D.H., and adenylic acid made by Ernst. Bischoff Co., Inc., were also used in the preliminary experiments.

2.8. Location of the Ribonucleotides.

On completion of the run, the papers were dried by suspending them in the radiations from a pair of 250 W. 125 V. industrial infra-red lamps connected in series. The bands were located by inspecting the papers in ultra-violet light by the procedure of Holiday and Johnson (1949), and were then marked lightly in pencil. When a permanent record was desired, a photograph was made by the method of Markham and Smith (1949b).

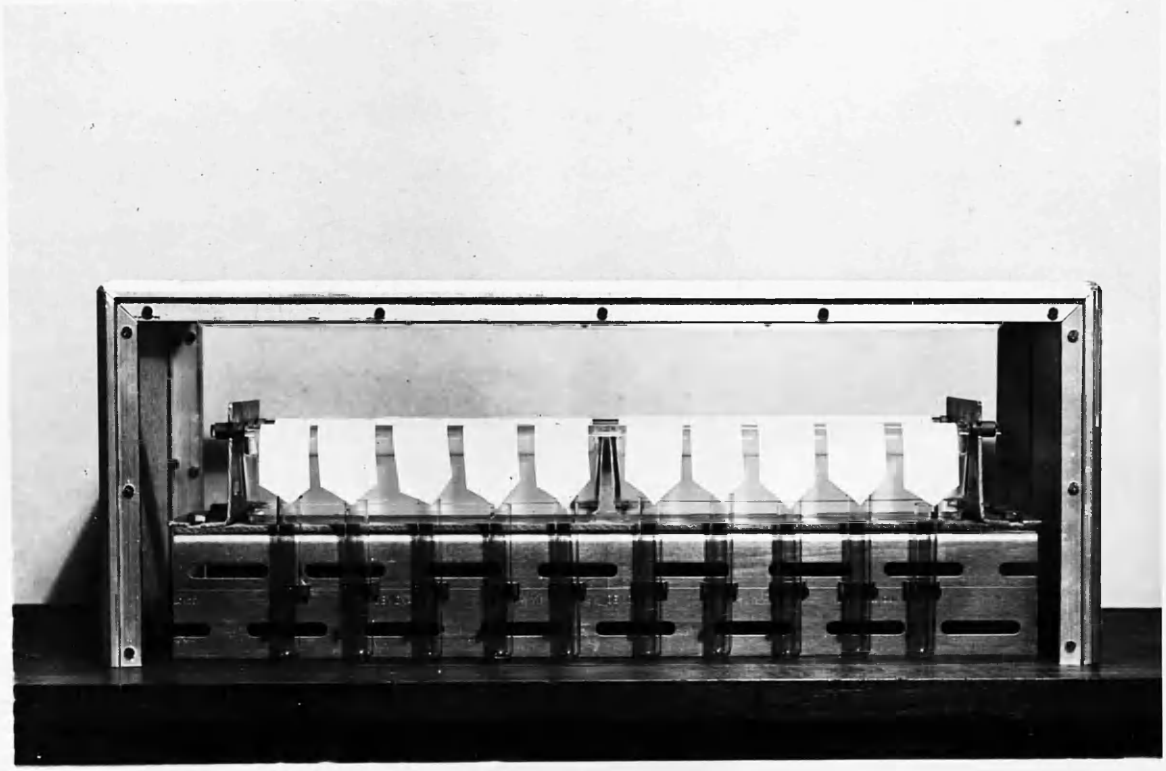
2.9. The Elution and Identification of the Spots.

The bands located in the ultraviolet light were cut out and eluted by the method of Consden, Gordon and Martin (1947) into graduated pyrex tubes in the apparatus shown in Fig.9. The volume of the eluate was adjusted to 5 ml. with distilled water, sufficient 5N-HCl having been added to give a final concentration of 0.1N. The ultra-

Figure 9. Elution Apparatus showing a series of paper strips being eluted into graduated pyrex tubes.

Figure 9.

Elution Apparatus.



violet absorption spectrum of each eluate was determined on the Beckman Model DU Spectrophotometer or a Unicam SP 500 quartz spectrophotometer, but owing to the high blank from the paper, only the maxima could be established. Identification was carried out firstly from the positions of the bands, and secondly by relating the ultraviolet absorption spectra of the eluates to those of the four nucleotides at the same pH (Fig.10).

As a further check on the identity of the nucleotides in the early experiments, samples of the eluates were hydrolysed with $HClO_4$ according to the method of Marshak and Vogel (1950), the bases separated by the paper chromatographic procedure of Wyatt (1951), and subsequently characterised by their ultraviolet absorption spectra. In every case only the appropriate base was present. These separations and determinations have been kindly carried out by Dr. G. Crosbie of this department.

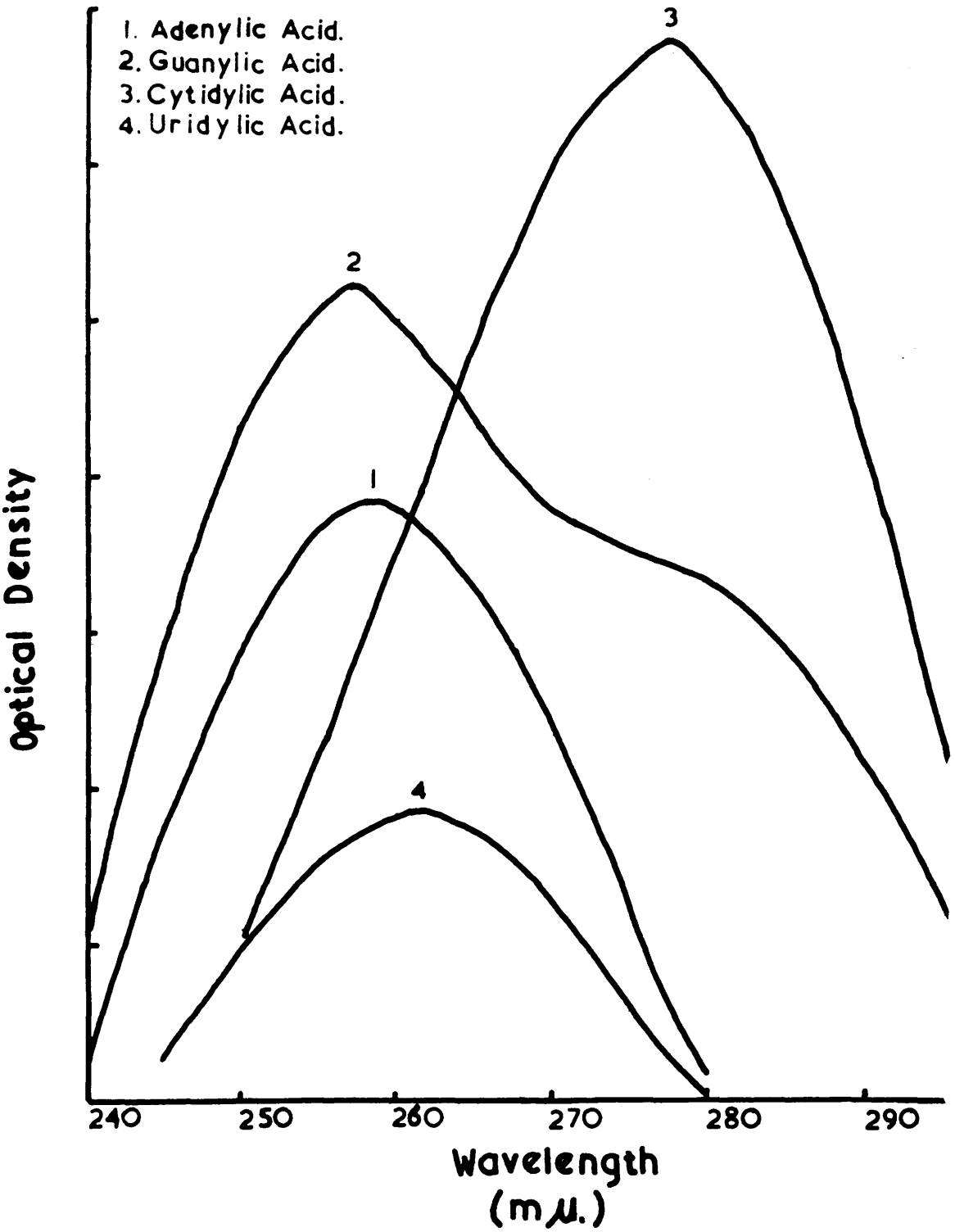
After identification, the quantity of nucleotide present in each eluate was determined by P estimation.

2.10. The Purification of Ribonucleic Acids.

a. A sample of commercial yeast RNA made by B.D.H., was purified by dissolving in a minimum of dilute alkali and repeated precipitation with 10 vol. of glacial acetic acid. The final precipitate was washed free of acetic acid with ethanol and dried with ether.

Figure 10.

The U.V. Absorption Spectra of the Nucleotides in 0·1N-HCl.



b. Samples of rabbit and rat liver RNA and RNA isolated from the cytoplasmic fractions were prepared by the method of Davidson, Frazer and Hutchison (1951), traces of glycogen being removed by incubation with salivary amylase immediately prior to deproteinising.

The hydrolysis of these purified materials before ionophoresis was carried out by incubating with 0.3N-KOH at 37° for 18 hr., in the proportion of 1 ml. alkali per 5 to 10 mg. of RNA. Acidification was carried out as with tissue digests.

2.11. The Estimation of Phosphorus.

Determination of phosphorus was carried out by a modification of the method of Allen (1940). When necessary the sensitivity of the method (normally 20 to 200 µg.) could be increased by working on a half or fifth scale, the latter being used for determination of phosphorus in the eluates from ionophoresis.

Reagents:-

a. 10N-sulphuric acid.

b. 100 vol. hydrogen peroxide (M.A.R.)

c. 8.3% (w/v) ammonium molybdate solution.

d. 20% (w/v) sodium metabisulphite.

e. Amidol, 1% (w/v) in d.

Procedure:-

The sample (containing between 20 and 200 µg. P)

in which P was to be estimated was pipetted into a 30 ml. microkjeldhal flask, followed by a glass ball and 1.2 ml. of 10N-H_2SO_4. The contents were then digested on a gas or electric digestion rack with the aid of a few drops of 100 vol. H_2O_2. When digestion was complete, the flask was allowed to cool and 21.35 ml. of distilled water were added followed by 1 ml. of 8.3% (w/v) ammonium molybdate and 2 ml. of a freshly prepared 1% (w/v) solution of amidol in 20% (w/v) sodium metabisulphite giving a final vol. of 25 ml. After shaking, the intensity of the blue colour was measured between 10 and 30 min. after the addition of the reagents, on a Hilger spekker absorptiometer fitted with Ilford red no. 608 filters. The reagent blank was then subtracted, and the phosphorus content obtained from standard tables. These tables were constructed from the readings obtained using varying amounts of a stock solution of inorganic P containing 20 µg.P/ml.

2.12. The Assay of Radioactivity.

For the determination of radioactivity, portions of the solutions were pipetted into a liquid counter (Type M 6, manufactured by 20th Century Electronics) attached to a conventional probe unit and scaling unit (Type 200, manufactured by Dynatron Radio Ltd.). All specific activities were calculated as counts/min./100 µg. P,

corrections being applied for slight variations in efficiency between the counters used, and also for decay of the ^{32}P back to the time of injection.

2.13. Autoradiographs.

Autoradiographs of the strips of filter paper after ionophoresis of radioactive materials were made using Kodak Industrex Type D X-ray film. The procedure was as follows:-

The ultraviolet absorbing areas on the papers were lightly outlined in pencil, and a strip of X-ray film placed on top of the paper so as to cover all these areas. The two were then stapled together, and heavy pencil marks made on the filter paper at both ends of the strip of film. The paper and film were then sandwiched tightly between two sheets of hardboard which were clamped together. The whole was then wrapped in lightproof paper, and placed in a drawer well clear of any radioactive material. After 14 days' exposure, the film was separated from the filter paper, developed for about 15 min. in Ilford ID 19 developer, washed in water, and fixed normally. At the same time ultraviolet photographs of the filter papers were made by the method of Markham and Smith (1949b).

2.14. The Separation of very small Quantities of Organic and Inorganic Phosphate.

The precipitation of inorganic P as $Mg(NH_4)PO_4$ is

not convenient when amounts of both are very small. The method used on such occasions has therefore been as follows:- The volume of solution was usually 3 to 4 ml.; 0.24 ml. of 10N-H_2SO_4 was added as in the micro-modification of the Allen procedure; this was immediately followed with 0.2 ml. of 8.3% (w/v) amidol in 20% (w/v) sodium metabisulphite solution without any digestion. Any blue colour which developed was then due to inorganic phosphate. The total volume was adjusted to 5 ml. with distilled water, and the intensity of the colour measured. The solution was then extracted with isobutanol which removed the blue phospho-molybdate complex. The isobutanol extract and aqueous layers then contained the inorganic and organic phosphate components respectively, and could be used for radioactivity determinations. Estimation of the organic phosphate was carried out by determination of the total phosphate in an aliquot of the original and calculating the organic part by difference.

PART III - RESULTS.

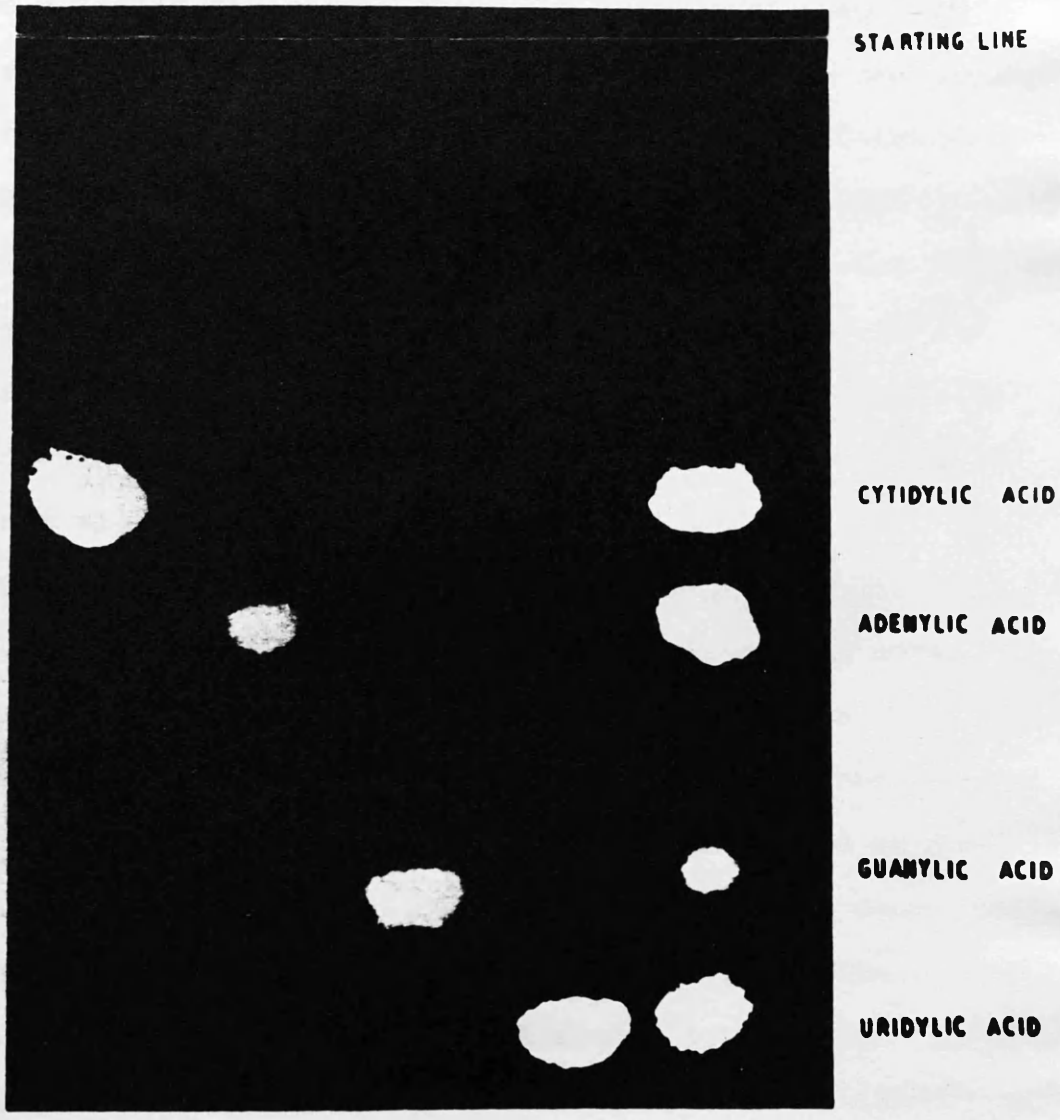
3.1 The Separation of the Pure Nucleotides.

The separation of the four pure ribose mononucleotides obtained by ionophoresis on filter paper is illustrated in Fig.11. In order to demonstrate the separation, a wide strip of paper was used, the four pure nucleotides being applied singly and in a mixture at intervals along the starting line. The paper was then subjected to ionophoresis in the normal manner, and after drying was photographed in ultraviolet light by the method of Markham and Smith (1949b). The spots in order from left to right are cytidylic, adenylic, guanylic and uridylic acids and finally a mixture of all four. From the photograph it is obvious that an adequate separation can be achieved by this method, and that the nucleotides separate in the order uridylic, guanylic, adenylic and cytidylic acids in decreasing order of mobility.

The separation obtained at pH 3.5 agrees well with the values for net charge per molecule of ribonucleotide as a function of pH calculated by Cohn (1950) from the data of Levene and Bass (1931), although it was found in practice that all four nucleotides moved more rapidly in the direction of the anode than might be expected from such considerations. This anomaly may be explained at least in part by the very strong endosmotic flow which takes place

Figure 11. The separation of the pure nucleotides by ionophoresis. The nucleotides were applied singly and as a mixture at intervals along the starting line and subjected to ionophoresis in the normal fashion.

Figure 11.


STARTING LINE
CYTIDYLIC ACID
ADENYLIC ACID
GUANYLIC ACID
URIDYLIC ACID

in the direction of the anode, and which may be measured by running under identical conditions, a separate strip of paper to which a spot of an un-ionised materal such as glucose had been applied at the starting point. The paper was dried in the normal manner after running, and the position of the glucose determined by spraying the paper with one of the conventional reagents for its detection , such as aniline hydrogen phthalate (Partridge, 1949). By this means, it was found that the glucose spot was located immediately behind cytidylic acid, i.e., nearer to the starting point. Some of the factors which must be considered in connection with ionophoresis on paper have been dealt with by Durrum (1951).

3.2 The Recovery of the Nucleotides after Ionophoresis.

The results shown in Table 3 illustrate experiments in which the recoveries of the pure nucleotides were determined after being run singly. The phosphorus content of the nucleotide solutions was determined, and an accurately measured volume of each was then applied to separate papers. On completion of the run, the papers were dried, the spots located, cut out and eluted by the usual methods. The phosphorus content of each eluate was then determined, and it will be seen from the table that the recoveries obtained were quite adequate.

Table 3.

The Recovery of Pure Nucleotides when applied to Paper singly, run and eluted.

Nucleotide	µg. P applied	µg. P recovered	Percentage Recovery
1. Cytidylic Acid	25.0	24.6	98.5
2. " "	19.2	19.0	99.0
3. " "	25.4	24.1	95.5
1. Adenylic Acid	20.7	20.0	97.0
2. " "	20.6	20.1	97.5
3. " "	20.7	19.8	96.0
1. Guanylic Acid*	19.6	19.0	97.0
2. " " *	19.6	19.2	98.0
3. " " *	10.0	9.4	94.0
1. Uridylic Acid	24.7	23.7	96.5
2. " "	23.2	22.8	98.5
3. " "	23.3	23.7	101.0

* Previously purified by ionophoresis.

In Table 4 the above procedure was repeated on papers to which accurately measured amounts of all four nucleotides had been applied. Once again the recoveries proved to be satisfactory.

The guanylic acid used in both sets of experiments was contaminated with small amounts of adenylic acid and free guanine, and was therefore subjected to purification by ionophoresis before use.

As a check that the phosphorus determination was a valid method of estimating the nucleotides, an experiment was carried out in which the pure nucleotides were determined by phosphorus estimation, and by the ultraviolet absorption of the bases obtained from them after digestion and chromatography. One portion of the solution was used for phosphorus estimation, from which the amount of nucleotide present was calculated: a second portion was evaporated to dryness, digested with $HClO_4$, the bases separated by chromatography, and determined by ultraviolet absorption measurements. It will be observed from Table 5 that the amounts of nucleotides determined by these two methods are in agreement.

3.3 The Comparison of Analysis of a Nucleotide Mixture by Ionophoresis and by Chromatography.

One further check on the validity of the ionophoretic procedure is illustrated by the results in Table 6

Table 4.

The Recovery of Pure Nucleotides when applied to Paper as a mixture, run and eluted.

Nucleotide	µg. P applied	µg. P. recovered	Percentage Recovery
1. Cytidylic Acid	19.2	18.3	95.0
2. " "	25.0	24.6	98.0
3. " "	25.4	25.0	98.0
1. Adenylic Acid	20.6	19.4	94.5
2. " "	20.7	19.6	94.5
3. " "	20.7	19.7	95.0
1. Guanylic Acid*	10.0	10.2	102.0
2. " " *	10.0	9.9	99.0
3. " " *	10.0	9.8	98.0
1. Uridylic Acid	23.2	22.0	95.0
2. " "	24.7	25.4	102.0
3. " "	23.3	21.8	93.5

* Previously purified by ionophoresis.

Table 5.

Comparison of the estimation of the pure nucleotides (a) by phosphorus estimation, and (b) by determination of the base liberated by perchloric acid digestion and separated by the paper chromatographic method of Wyatt (1951).

Nucleotide	Concn./ml. of nucleotide calculated from P	Concn./ml. of nucleotide calculated from the ultraviolet absorption of the base
	mg.	mg.
Cytidylic Acid	10.4	9.8
Adenylic Acid	9.6	9.3
Guanylic Acid	1.9	1.9
Uridylic Acid	9.9	9.4

Table 6.

Analysis of a mixture of pure nucleotides (a) by ionophoresis and (b) by chromatography of the bases derived from the nucleotides by $HClO_4$ hydrolysis and separated by the method of Wyatt (1951). The results are expressed as molar ratios relative to adenylic acid as 10.

Nucleotide	Ionophoresis	Chromatography.
Cytidylic Acid	10.8	10.8
Adenylic Acid	10.0	10.0
Guanylic Acid*	9.0	9.3
Uridylic Acid	13.4	12.3

* A correction has been applied for approximately 4% free guanine present in the sample of guanylic acid used.

in which comparison is made between the analysis of a mixture of nucleotides by ionophoresis of the nucleotides and chromatography of the bases derived from them. It will be noticed that there is in general good agreement except in the case of uridylic acid, where it was invariably found that the figure obtained by ionophoresis was slightly higher than that found by chromatography. Further experiments carried out with pure uridylic acid alone revealed that the uridylic acid estimated both by phosphorus and ultraviolet measurements from eluates of an ionophoretic run were in agreement, and tallied with the concentration by weight of uridylic acid in the original solution. It would appear therefore that the correct figure in Table 6 is that obtained by ionophoresis, and that the value obtained by the chromatographic procedure is low.

3.4 The Effect of Alkaline Incubation on the Nucleotides.

Marrian, Spicer, Balis and Brown (1951) have shown that when cytidylic acid is incubated in N-alkali at 37° for 18 hr., partial deamination occurs with the formation of uridylic acid. These authors suggest the use of either 0.3N-alkali at 37° or N-alkali at room temp., in which conditions they state that no deamination takes place. This observation has been confirmed by taking portions of cytidylic acid which had been incubated for 18 hr., in N-KOH at 37° and 25° and in 0.3N-KOH at 37°, and submitting

them to ionophoresis. Only after incubation in N-KOH at 37° was any detectable amount of uridylic acid produced, the quantity involved being about 10% of the original cytidylic acid.

A mixture of all four nucleotides was then divided into four parts, one of which was maintained as a control while the others were incubated in N-KOH at 37° and at 25° and in 0.3N-KOH at 37° respectively. The pH of each fraction was then adjusted to 4 with 60% $HClO_4$, the precipitate of $KClO_4$ centrifuged out, and portions of the supernatants applied to the papers in the usual way. The results of this experiment are illustrated in Table 7 which shows that only cytidylic acid is affected by the variations in conditions, and that it is not deaminated by incubation in 0.3N-KOH at 37° although there is appreciable deamination in N-KOH at 37°, with a resultant increase in uridylic acid.

3.5 The Hydrolysis of RNA by 0.3N-KOH.

The extent of liberation of acid-soluble phosphates from RNA by incubation in 0.3N-KOH at 37° for 18 hr. is illustrated in Table 8 from which it is clear that the phosphorus of the RNA is liberated quantitatively in the form of acid-soluble phosphates. The fact that only about 97% of the RNAP is recovered in the acid supernatant is

Table 7.

Comparison of the analysis of a mixture of pure nucleotides by ionophoresis before and after incubation for 18 hr. in various strengths of alkali at different temperatures. The results are expressed as molar ratios taking adenylic acid as 10.

Nucleotide	Unincubated	0.3N-KOH at 37°	N-KOH at 25°	N-KOH at 37°.
Cytidylic Acid	11.0	10.8	10.3	9.9
Adenylic Acid	10.0	10.0	10.0	10.0
Guanylic Acid	9.1	9.0	9.0	9.4
Uridylic Acid	13.1	13.4	13.0	15.0
Percentage P Recovered	98.0	98.5	100.0	92.0

Table 8.

Liberation of acid soluble phosphates from a specimen of yeast RNA by alkaline hydrolyses followed by acidification. In each case 37.5 mg. RNA was incubated with 5 ml. 0.3N KOH at 37° for 18 hr.

	P content of the alkaline digest in µg.	P content of the acid supernatant in µg.	Percentage recovery
1.	3150	3070	97.5
2.	3128	3025	96.7
3.	3143	3035	96.6

almost certainly due to small losses incurred in the process of acidifying, removing precipitated $KClO_4$, and washing the precipitate.

3.6 The Analysis of Specimens of Purified RNA.

The technique of ionophoresis was applied to the analysis of the ribonucleotides obtained by the digestion of several samples of purified RNA from yeast, rabbit and rat liver. The separation of nucleotides achieved from hydrolysates of a sample of yeast RNA is illustrated in Fig.12 from which it is clear that separations comparable with those achieved with mixtures of the pure materials are also obtained with the RNA hydrolysates. The results of the analyses of a number of different samples of RNA by ionophoresis of the nucleotides and chromatography of the bases are shown in Table 9 in which the results are expressed as molar ratios relative to adenylic acid as 10. Agreement between the two methods is close, with the exception of the uridylic acid. The discrepancy in this case is considerably larger than in the mixture of nucleotides (Table 6), but Dr. G. Crosbie of this department has confirmed the ionophoretic values by ultraviolet determinations on eluates from ionophoretic runs of the specimen of yeast RNA.

One other aspect of Table 9 which deserves consideration is the recovery of nucleotide P which is about 93%.

Figure 12. Ultraviolet photograph of the separation by ionophoresis of the four nucleotides from a hydrolysate of a specimen of yeast RNA.

Figure 12.

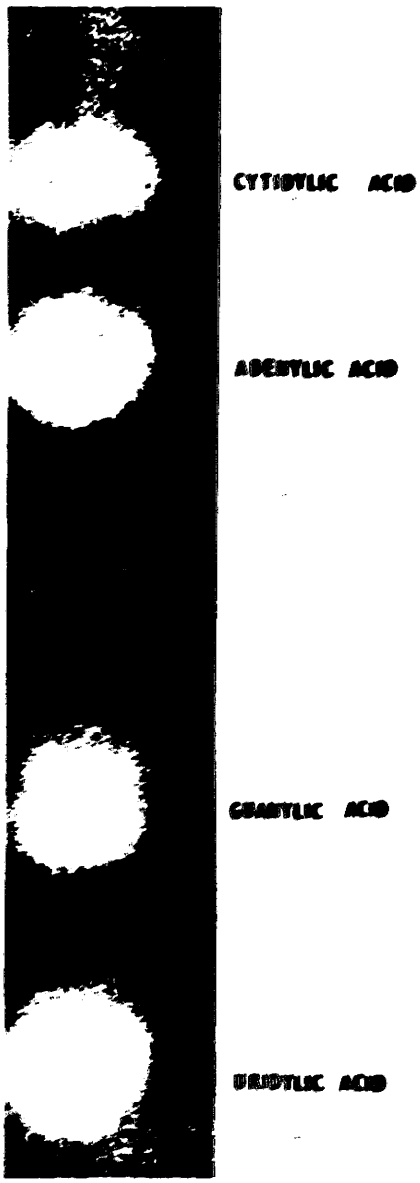


Table 9.

The analysis of several samples of RNA by ionophoresis of the nucleotides and chromatography of the bases. The results are expressed as molar ratios relative to adenylic acid as 10.

Nucleotide	Yeast RNA		Rabbit Liver RNA		Rat Liver RNA	
	P estm.	Base	P estm.	Base	P estm.	Base
Cytidylic Acid	8.8	8.1	13.9	14.3	14.5	13.9
Adenylic Acid	10.0	10.0	10.0	10.0	10.0	10.0
Guanylic Acid	12.4	11.5	18.8	16.9	17.6	17.5
Uridylic Acid	10.4	7.9	10.9	7.1	10.1	8.1
Percentage Recovery	93.7	-	92.0	-	93.8	-

This might be due either to the presence in the RNA originally of a small amount of non-nucleotide P, or to loss of P from the nucleotides during hydrolysis. From the results in Table 7 it would appear that the former explanation is the more likely as there is no evidence of loss of P from the nucleotides in this case.

3.7 The Ionophoresis of the A_2S Fraction of Rat Liver.

The method of ionophoresis having proved satisfactory when dealing with pure nucleotides and hydrolysates of samples of purified RNA from various sources, the next step was its application to the analysis of the rather less pure A_2S fractions obtained by the modified Schmidt & Thannhauser (1945) procedure 2. When this was done, it was found that separations comparable with those obtained with the purified materials were obtained when the same conditions were employed. An ultraviolet photograph of a strip of filter paper on which such a separation had been carried out is shown in Fig.13 where it will be seen that the nucleotides have been adequately separated. It was found however that if the length of the run was curtailed, an extra component, which showed only faintly in the ultraviolet photograph, was apparent, preceding uridylic acid. In order to demonstrate the presence of this additional component which we have called "A", a short run on a 57 cm. paper strip at 14 V./cm. for 6 hr. sufficed, although this

Figure 13. Ultraviolet photograph of the separation of the four nucleotides from a rat liver A_2S fraction (Long run).

Figure 13.

was inadequate for the complete separation of the nucleotides. Fig.14 is an ultraviolet photograph of such a strip of paper, and "A" is the faint slightly crescent-shaped area preceding uridylic acid. In the normal procedure for the separation of the nucleotides on a longer paper, this fast moving component was run off the paper into the buffer in the anode compartment. For each A_2S therefore two runs were usually employed, -- a short run for the separation of the fast moving component "A" (Fig.14), and a long run for the separation of the nucleotides (Fig.13). The component "A" was found to be present both in solutions obtained by acidifying the alkaline digest with $HClO_4$, and in solutions obtained by acidification of the digest with TCA and subsequent removal of the TCA by repeated extraction with ether. As a means of detecting the presence of TCA the reaction between TCA and α-naphthol in ultraviolet light described by Eggleton, Elsden and Gough (1943) was used.

The nature of "A" will be discussed at greater length later.

By eluting the four nucleotides from the long run alone and by estimating their phosphorus content, it was possible to obtain a measure of the relative compositions of the RNA of different animal tissues. In Table 10 the

Figure 14. Ultraviolet photograph of a short ionophoretic run of an A_2S fraction from rat liver showing the crescent shaped area "A" preceding uridylic acid.

Figure 14.

Ionophoresis of A_2S Fraction. (Short run)

(U.V. Photograph)

Table 10.

Comparison of the analysis of the A_2S and RNA prepared from the cytoplasm of several different batches of rat liver. Results are expressed as molar ratios relative to adenylic acid as 10.

Expt. No.	Adenylic Acid	Guanylic Acid	Cytidylic Acid	Uridylic Acid	Purine/ Pyrimidine ratio.
1. RNA	10.0	17.6	15.9	11.2	1.02
A_2S	10.0	17.0	16.8	11.2	0.97
2. RNA	10.0	17.7	16.6	12.3	0.96
A_2S	10.0	17.6	15.7	11.5	1.01
3. RNA	10.0	17.2	14.9	9.5	1.11
A_2S	10.0	18.7	15.2	11.6	1.07
4. RNA	10.0	17.7	14.5	10.4	1.11
A_2S	10.0	19.7	14.4	11.1	1.16
Mean RNA	10.0	17.6	15.5	10.9	1.05
A_2S	10.0	18.3	15.5	11.4	1.05

analysis of the A_2S from the whole cytoplasm of rat liver and of the RNA isolated from the same material is compared for several groups of animals. The results are expressed as molar ratios relative to adenylic acid as 10. It will be noticed that in all cases, guanylic acid is present in the largest proportion, while cytidylic acid follows next. There is also reasonable correlation between the results from the A_2S and isolated RNA, while the figures for the different experiments indicate a similar composition for the RNA in each case.

3.8 The Analysis of fraction A_3S prepared from the Saline Extract of Dry Tissue Powder.

The study of the A_3S prepared by procedure 3 illustrated in Fig.7 proved to have several advantages as will be seen later. Not the least of these was the fact that from rather more than a gram of dry tissue powder which proved quite difficult to dissolve in 0.3N-KOH, there was obtained about 150 mg. of material which was readily soluble in water. Using this technique it was therefore possible to obtain a solution containing the nucleotides in higher concentration than had hitherto proved feasible, and which at the same time contained fewer degradation products of the tissue proteins. The recovery of the tissue RNA was not quantitative, but proved to be of the

order of 70%, and since, as will be seen later, the composition of the RNA in the various cytoplasmic fractions appears to be reasonably homogeneous, it seems to be valid to consider the composition of the RNA extracted by procedure 3 as representative of the tissue concerned. The results illustrated in Table 11 show the molar ratios of the four nucleotides in the RNA from several different tissues. In rat and rabbit livers, it will be noted that the relative proportions of the nucleotides agree with those found for isolated rat and rabbit liver RNA and also for crude rat and rabbit liver A_2S (Tables 9,10). The relative proportions of the nucleotides in all the fowl tissues studied proved to be similar, and to differ little from those found in rats and rabbits.

3.9 Preliminary Experiments with ^{32}P.

Several authors (Marshak & Vogel, 1950b; Jeener & Szafarz, 1950a,b) have stressed the difficulty of removing traces of contaminating ^{32}P from the nucleotide-containing fraction of animal tissues, and Davidson, Frazer and Hutchison (1951) have demonstrated that if a small amount of radioactive phosphate is added to a homogenate of non-radioactive rat liver along with TCA, there is still considerable activity in the A_2S fraction obtained by the method of Schmidt and Thannhauser (1945) even after exceedingly exhaustive washing of the tissue with TCA and TCA

Table 11.

Composition of the RNA of several tissues. The analyses were carried out on the ribonucleotide fraction obtained by procedure 3, and the results are expressed as molar ratios relative to adenylic acid as 10.

Tissue	Adenylic Acid	Guanylic Acid	Cytidylic Acid	Uridylic Acid
Rat Liver 1.	10.0	17.5	13.9	10.9
Rat Liver 2.	10.0	17.6	14.3	10.8
Rabbit Liver	10.0	16.9	14.6	10.3
Liver from Cock 1.	10.0	16.5	13.4	10.3
Liver from Cock 2.	10.0	17.7	13.9	10.8
Liver from Laying Hen	10.0	16.7	13.6	10.2
Liver from Cock bearing GRCH 15 breast tumour	10.0	18.1	14.5	11.1
GRCH 15 Tumour from Cock	10.0	18.4	14.5	11.1

containing sodium phosphate.

Experiments have therefore been carried out to investigate the efficiency of ionophoresis in removing such contaminating ^{32}P. Small amounts of inorganic radioactive phosphate were added to a spot of the mixed nucleotides on paper, and the mixture submitted to ionophoresis. The progress of the inorganic phosphate band was followed by means of a "Panax" monitor set, and ionophoresis was generally continued until this band was approaching the end of the paper. The position of the band was obtained after drying the paper by moving the monitoring counter slowly over its surface. It was found that all the activity was located in one well defined area, and this was marked in pencil. On subsequent examination of the paper in ultraviolet light, the radioactive region proved to be well clear of the four nucleotides. When the separate nucleotides were eluted, the amount of contaminating ^{32}P as determined in a 20th Century Electronics Type M 6 liquid counter was found to be negligible.

In another experiment, a small amount of radioactive phosphorus was added to the paper at the starting point along with the normal quantity of a non-radioactive rat liver A_2S, and the whole submitted to a normal short run. The paper was then dried, the ultraviolet absorbing areas

including "A" marked in pencil and the "Panax" monitor moved slowly over the paper. On this occasion the single area which exhibited radioactivity was found to coincide with component "A". Determination of the activity of the nucleotides once again indicated negligible contamination from added ^{32}P.

One further check on the removal of contaminating radioactive phosphate from the A_2S fractions was then applied. The experiment of Davidson, Frazer and Hutchison (1951) in which 0.88 µc. ^{32}P was added to a homogenate of 4 g. of non-radioactive rat liver tissue along with TCA was duplicated. The precipitated material was washed four times with ice-cold 10% TCA and subjected to the usual modified Schmidt and Thannhauser (1945) procedure, the alkaline digest being precipitated with $HClO_4$. The ribonucleotides were separated by ionophoresis, eluted and the eluates assayed for radioactivity which proved to be negligible.

3.10 The Ionophoresis of the A_2S Fraction from the Livers of Rats which had received ^{32}P.

Inorganic radioactive phosphate was administered to rats in doses from 20 to 50 µc./100 g. body wt., and the animals killed at specified times thereafter. The livers were perfused with saline, excised, homogenised

and then submitted to the modified Schmidt and Thannhauser (1945) procedure (Fig.6). The ribonucleotides were separated by ionophoresis both by long and short runs. The papers were photographed in ultraviolet light and were then left in contact with Kodak Industrex Type D X-ray film for 14 days for the preparation of autoradiographs. Comparison of the ultraviolet photographs and autoradiographs for both long and short runs is made in Figs. 15 and 16. From these photographs it is clear that there are present particularly in the short runs phosphorus-containing spots which are strongly radioactive, but which are not apparent in the ultraviolet photograph. The first of the non-nucleotide spots and that showing most strongly on the autoradiograph corresponds to the ultraviolet absorbing area "A". About half way between "A" and uridylic acid there is found on the autoradiograph a second intense spot which has been designated "C". It will be noticed that the front edge of the nucleotide block in the ultraviolet photograph does not correspond with the front edge on the autoradiograph. Closer inspection reveals that there are in fact two bands in this region on the autoradiograph, one of which corresponds with the uridylic acid spot, the other being slightly in front of it. This component we have called "D". At the other end of the block of nucleotides, it was again

Figure 15. Autoradiograph (A) and ultraviolet photograph (B) of the separation of the nucleotides from an A_2S fraction of rat liver by ionophoresis. Components "D" and "E" are visible on the autoradiograph but not on the ultraviolet photograph. The A_2S was prepared from the liver tissues of rats which had received 50μc.^{32}P/100g. body wt. 2 hr. before sacrifice.

Figure 15.

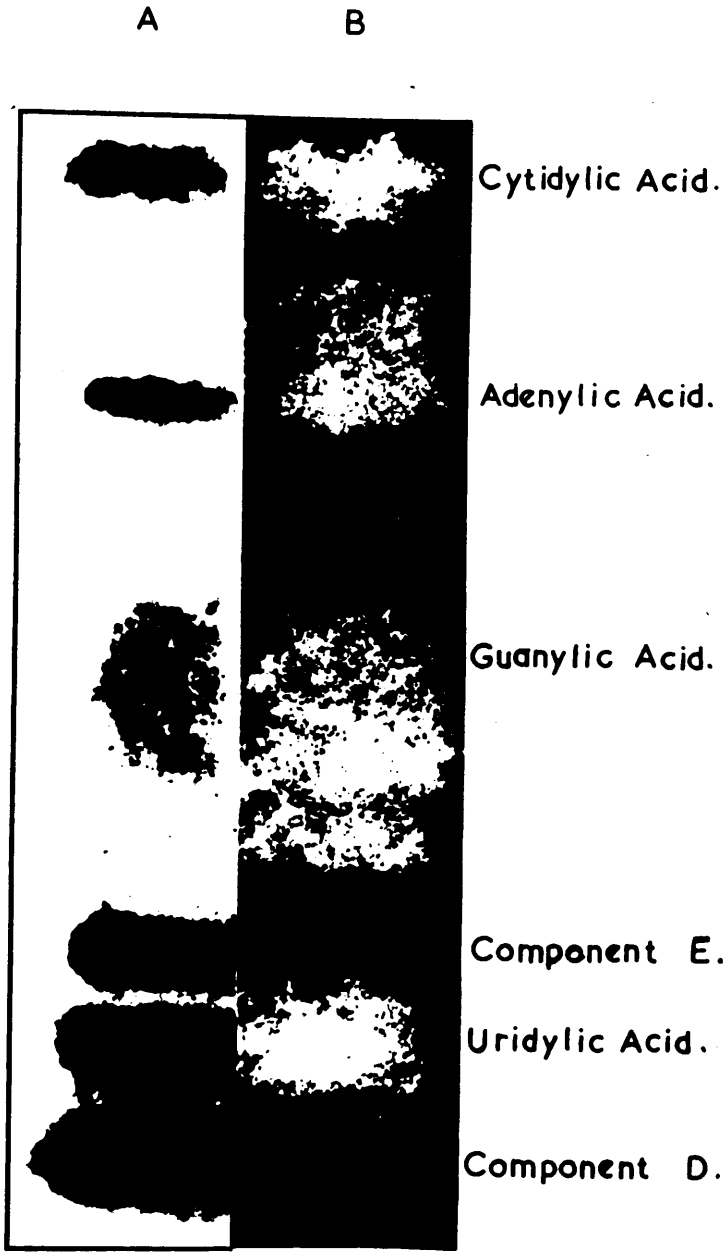
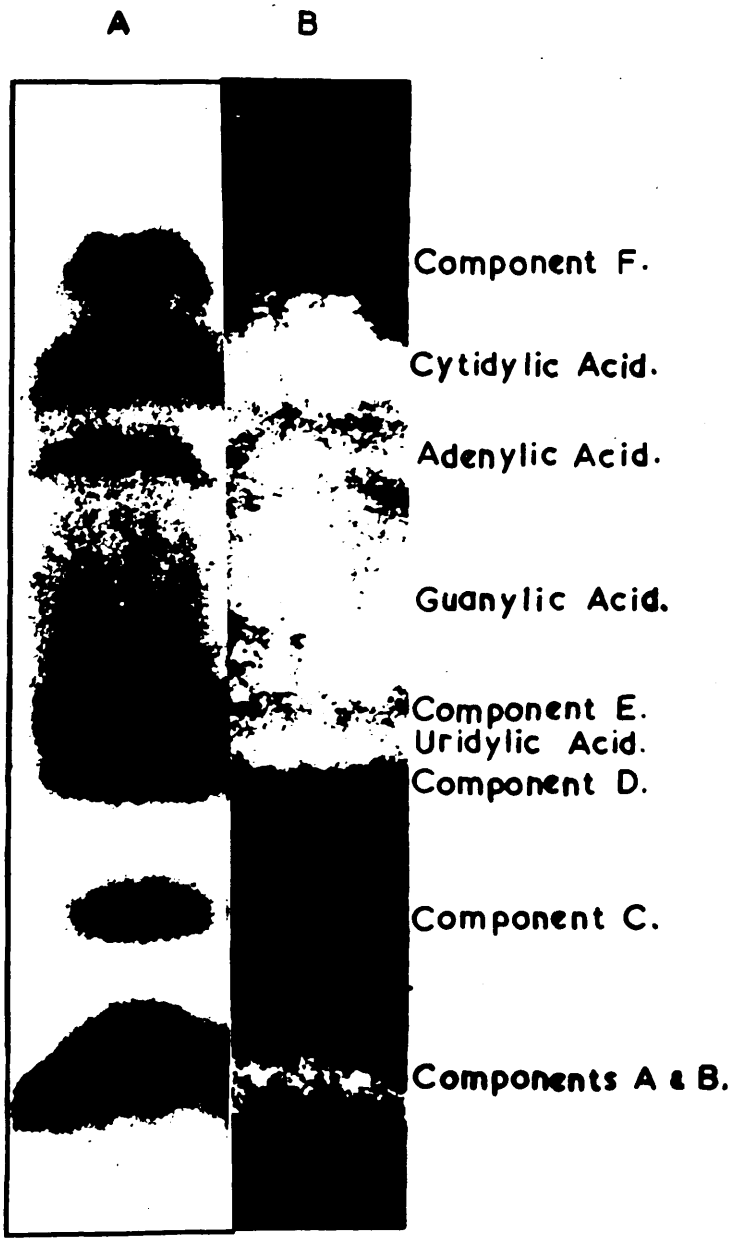

A
B
Cytidylic Acid.
Adenylic Acid.
Guanylic Acid.
Component E.
Uridylic Acid.
Component D.

Figure 16. Autoradiograph (A) and ultraviolet photograph (B) of a short ionophoretic run of an A_2S fraction from rat liver, showing components "A", "B", "C", "D", "E" and "F". The animals received 50μc.^{32}P/100g. body wt. 2 hr. before killing.

Figure 16.


A
B
Component F.
Cytidylic Acid.
Adenylic Acid.
Guanylic Acid.
Component E.
Uridylic Acid.
Component D.
Component C.
Components A & B.

found that there was lack of correlation between the ultraviolet photograph and the autoradiograph, there being an additional spot on the latter located between the cytidylic acid region and the origin: this area has been called "F".

The autoradiographs have therefore provided very useful information by locating the positions on the papers of other phosphorus containing substances which were not detectable by the other methods used. The presence of these additional components had been suspected because the total phosphorus recovered from the strip when the nucleotides and "A" were added together amounted only to about 80% of the phosphorus applied to the paper initially.

When the ultraviolet absorbing area "A" was eluted, it was found possible to separate the eluate into an inorganic and an organic constituent, both of which contained phosphorus. The inorganic portion which was separated by developing the blue phosphomolybdate complex on an undigested eluate and extracting with isobutanol was designated "B", while the organic portion which must be that responsible for the ultraviolet absorption retained the name "A". The other components "C", "D" and "F" contained no inorganic phosphate, nor were they visible on examination of the paper in ultraviolet light. The relationship of all these components to the nucleotides is

apparent from the photographs in Figs. 15 and 16.

When the phosphorus content of all these areas was taken into account in conjunction with the nucleotides, the recovery of the phosphorus applied to the paper approached 100%. The results of some of these experiments are shown in Table 12, in the earlier of which all the additional components are not listed since at the time of analysis they had not been located. One striking feature of the figures is the considerable proportion of the A_2S phosphorus which is non-nucleotide despite the fact that it has previously been generally assumed by users of the Schmidt and Thannhauser method that this fraction contains only nucleotide phosphorus together with a minute amount of "phosphoprotein" phosphorus. It will be noted that there is considerable variation in the amount of phosphorus in the components "A", "B", "C", "D", and "F"; this is in part due to the fact that the exact position of these spots was not located before the paper was cut up for elution, the procedure being to cut the whole area between uridylic acid and "A" into strips approximately 2 cm. wide, elute each separately and determine the phosphorus in each. The subsequently developed autoradiograph served as a key from which the phosphorus content of the various strips was allocated to the various components. The phosphorus content of these components was in most cases so low as to

Table 12.

Separation by ionophoresis of the components of the ribonucleotide fraction of rat liver tissue submitted to the modified Schmidt and Thannhauser procedure. For an explanation of "A", "B", "C", "D" and "F" see Fig.16 and text.

Expt.	Components in µg.P/100 µg. total P in fraction A_2S									
Whole Liver	Adenylic Acid	Guanylic Acid	Cytidylic Acid	Uridylic Acid	A	B	C	D	F	Total
1.	14.2	24.2	20.6	16.7	4.3	3.8	6.1	6.5	-	89.1
2.	14.6	25.0	24.0	16.4	1.9	2.2	8.1	8.0	-	96.2
3.	13.3	23.5	20.8	15.3	1.4	1.5	9.7	11.0	-	96.5
4.	13.2	25.4	21.5	15.9	3.5	2.0	6.9	3.0	-	91.2
5.	12.8	25.8	19.9	18.2	4.6	5.6	8.7	4.6	-	100.2
6.	14.0	26.4	20.6	15.9	3.8	2.5	2.4	6.2	-	92.8
Whole Cytoplasm										
1.	14.1	23.8	21.8	17.1	3.6	1.4	-	-	-	81.8
2	12.3	28.4	19.7	15.4	6.0	1.5	4.5	4.2	1.1	93.1

preclude the cutting of narrower strips for elution since the spekker readings on the existing eluates were frequently only about twice those of the reagent blank, thus making the accuracy of the estimation poor. The nucleotides themselves appear to be remarkably consistent in amount accounting for about 75% of the total phosphorus in the fraction. Moreover, the relative amounts of the nucleotides is of the same order in each case; in rat liver guanylic acid was always most abundant, while the cytidylic acid was also high in comparison with adenylic and uridylic acids.

The specific activities of the nucleotides and other components of several whole liver A_2S fractions is shown in Table 13. In this table some of the components were not analysed and no values are therefore quoted. In three cases, "A" and "B" were not separated, and in these instances the combined value is shown. It will be observed that in every case the activities of the nucleotides are very much lower than those of the corresponding whole fractions, and particularly of the additional components. This is most noticeable 2 hr. after injection, the increased activity of the nucleotides 24 hr. after injection tending to diminish the effect of the concomitants on the activity of the whole fractions. Of the nucleotides, uridylic acid invariably exhibits the highest activity, a fact which must be regarded with some suspicion in view of its proximity to

Table 13.

Specific activities in counts/min./100 µg. P of the phosphorus fractions separated in Table 8. The results were obtained from the liver tissues of rats, the first three of which had received 20 µc. ^{32}P/100 g. body wt., and the remainder, 50 µc. ^{32}P/100 g. body wt. In three cases, "A" and "B" were not separated, and the results for the combined materials are printed between the two columns.

Time after Injection	Adenylic Acid	Guanylic Acid	Cytidylic Acid	Uridylic Acid	"A"	"B"	"C"	"D"	"F"	Whole Fraction
Whole Tissue										
2 hr.	134	133	174	390	660	5700	-	-	-	524
2 hr.	116	35	77	240	750	2950	-	-	-	314
2 hr.	285	95	300	390	660	3050	-	-	-	825
2 hr.	485	184	344	660	5075		1350	1590	-	-
2 hr.	723	431	490	990	1500	7800	2000	2600	-	2050
2 hr.	293	278	275	702	1820	3120	1180	775	-	1280
2 hr.	287	249	234	525	2000	3320	1000	1720	-	1050
24 hr.	2150	2090	2040	2290	950	2550	3600	2650	-	2850

Table 13 (Contd.)

Time after Injection	Adenylic Acid	Guanylic Acid	Cytidylic Acid	Uridylic Acid	"A"	"B"	"C"	"D"	"F"	Whole Fraction
Whole Cytoplasm										
2 hr.	702	370	544	965	500	19800	2350	2800	-	1730
2 hr.	975	570	810	870	4160	21300	6700	2300	-	2560
2 hr.	300	255	215	588	2470	4270	1120	3020	-	1190
2 hr.	1240	785	1165	2325	14180		3960	3400	6750	3500
24 hr.	2075	1970	2010	2320	5700		4180	3050	-	2640

the more active components "A", "B", "C" and "D" (see later).

3.11 Comparison of the Specific Activities of the Nucleotides from the Isolated RNA and the A_2S of whole Cytoplasm.

In view of the possibility mentioned above that uridylic acid obtained by ionophoresis of an A_2S fraction might be contaminated with a more active component, it was decided to compare the activities of all the nucleotides obtained by ionophoresis of a hydrolysate of RNA isolated from rat liver cytoplasm with the activities of the nucleotides obtained from an A_2S fraction prepared from an aliquot of the same material. An autoradiograph of a strip of paper on which the nucleotides from a sample of radioactive RNA had been separated is shown in Fig.17 alongside the corresponding ultraviolet photograph. It will be observed that in this case there is no evidence of additional phosphorus containing substances besides the nucleotides. The results of this experiment are shown in Table 14. It is clear from such figures that the activity of the uridylic acid obtained from the isolated RNA is in all cases but one substantially lower than that from the A_2S fraction, and that the values for uridylic acid in the isolated RNA are comparable with those obtaining for the other nucbotides. The values found for the other nucleo-

Figure 17. Autoradiograph (A) and ultraviolet photograph (B) of the separation of nucleotides from an isolated RNA. The RNA was prepared from the liver cytoplasm of rats which had received 50μc. ^{32}P 24 hr. before sacrifice. The tendency for the isomeric guanylic acids to separate is visible on the autoradiograph.

Figure 17.

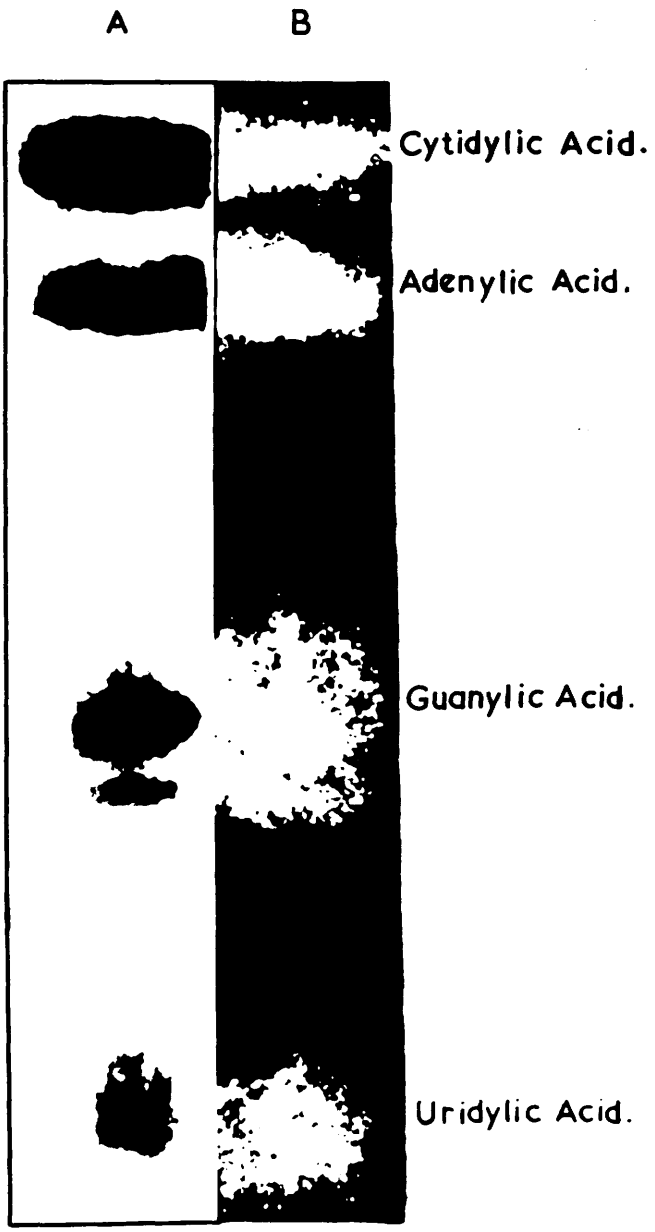

A
B
Cytidylic Acid.
Adenylic Acid.
Guanylic Acid.
Uridylic Acid.

Table 14.

Comparison of the specific activities (ct./min./100µg.P) of the nucleotides obtained from the RNA and A_2S obtained from rat liver cytoplasm. 50µc.^{32}P/100 g. body wt. was administered to the animals.

Expt. No.	Time after injection	Adenylic Acid	Guanylic Acid	Cytidylic Acid	Uridylic Acid	Whole Fraction
1. RNA	2 hr.	541	310	453	385	605
A_2S	2 hr.	702	370	544	965	1730
2. RNA	2 hr.	1190	640	690	880	864
A_2S	2 hr.	975	570	810	870	2560
3. RNA	2 hr.	355	170	350	294	454
A_2S	2 hr.	350	310	255	610	1210
4. RNA	2 hr.	235	133	164	187	236
A_2S	2 hr.	300	255	215	588	1190
5. RNA	24 hr.	2020	1750	1920	1940	2210
A_2S	24 hr.	2075	1970	2010	2320	2640

tides are not in good agreement with one another although the discrepancy is much smaller than in the case of uridylic acid. One possible explanation of this anomaly is that only a small proportion of the total RNA present in the cytoplasm is recovered on isolation, while all the RNA is represented in the A_2S fraction. It seems likely therefore that the isolated material is not a representative sample of the cytoplasmic RNA since several authors (Jeener, 1949a; Jeener & Szafarz, 1950a; Barnum & Huseby, 1950, and Davidson, McIndoe & Smellie, 1951) have demonstrated differences in uptake of ^{32}P into the RNA of the different cytoplasmic fractions. Moreover, since a very high proportion of the cytoplasmic RNA is to be found in the microsome fraction in which the specific activity of the RNAP has been found to be low, it is quite possible that the isolated RNA comes preferentially from this source. Apart from these possibilities it is clear in addition that the uridylic acid obtained from the A_2S was contaminated with some other highly active component which we have called "E" (Fig.15). The amount of phosphorus in this component would appear to be small since there is in general good agreement between the estimates of the molar proportions of uridylic acid in the isolated RNA and in the A_2S fraction (Table 10), although the tendency is for the A_2S

figure to be higher than that found for the RNA. One other feature of the results obtained in this experiment is noteworthy:- even in the isolated RNA the figures for the activities of the nucleotides are in most cases considerably lower than the activity of the whole RNA. The significance of this finding is of itself difficult to assess, but taken in conjunction with the fact that the recoveries of nucleotide phosphorus from the isolated RNA although approaching close to 100% has never actually been 100%, it seems not impossible that the isolated RNA may contain some highly active contaminant. This was confirmed by autoradiographs of short ionophoretic runs of isolated RNA hydrolysates which indicated the presence of an additional component corresponding to "C" in Fig.16.

3.12 The Elimination of the Concomitants.

In view of the number and amount of the concomitants "A", "B", "C", "D", "E" and "F" (Figs.15 and 16) in the A_2S fraction obtained by the initial modification of the Schmidt and Thannhauser (1945) procedure, it was obviously desirable to find some means of eliminating them or at least reducing them in number or extent.

At first it seemed most likely that the origin of these substances would be either acid-soluble material which had not been completely extracted in the initial washings

with TCA, or some phospholipid which had remained even after the exhaustive extraction applied. Experiments were therefore carried out in which the number of washings with 10% TCA was increased to 5, and in which the lipid extraction was supplemented by two extractions of 30 min. with boiling ethanol-chloroform (3:1). On ionophoresis of the A_2S obtained after such drastic prior treatment however it was found that there was no diminution in either number or amount of the concomitants as evidenced by phosphorus and radioactivity determinations.

In several cases, ionophoresis of a radioactive A_2S was carried out down one edge of a wide strip of paper, the paper dried in the usual fashion, and then subjected to chromatography in the form of ascending chromatograms at right angles to the direction of ionophoresis. The solvents utilised were the isobutyric acid/ammonia, isopropanol/HCl and methanol/formic acid systems of Magasanik et al. (1950), Wyatt (1951) and Bandurski and Axelrod (1951) respectively. After running, the chromatograms were dried and the uridylic acid located in ultraviolet light as before. The uridylic acid lane was then cut up, eluted in strips, and phosphorus and radioactivity determinations carried out on each eluate. All these solvents reduced the activity of the uridylic acid to about that of the other nucleotides, and to a level comparable with that found for the uridylic acid obtained from

an isolated RNA prepared from the same starting material.

Such procedures were clumsy and at the best qualitative, and it was therefore considered desirable to find some means of obtaining an A_2S fraction as far as possible free from these contaminants. The experiments on exhaustive extraction of the acid-soluble and lipid substances of the tissue indicated that the concomitants were probably protein bound and were liberated only after alkaline incubation. An attempt was made to eliminate some of these concomitants by separating the RNA from the bulk of the protein before it was incubated with alkali. This procedure involved the extraction of the dry lipid free residue of the tissue with 10% saline as previously described (procedure 3).

At this stage owing to the apparent heterogeneity of the RNA of the cell, it was decided to carry out further experiments on the validity of such methods on the cytoplasmic fractions themselves, and further results on this work are presented later.

3.13 Results of Analysis of Cytoplasmic Fractions.

A number of preliminary experiments were carried out in order to investigate the separation of cytoplasmic fractions, and to obtain some data on the distribution of phosphorus compounds in them using the Schmidt and Thann-

hauser (1945) procedure as modified by Davidson, Frazer and Hutchison (1951). The results obtained were rather scattered, largely due to the method of expression, viz., mg.P/100 g. fresh tissue. This did not take into account the fact that homogenisation was difficult to control from experiment to experiment with the result that a variable proportion of unbroken cells was found in the nuclear fraction. At the same time, straining of the homogenate through nylon, which proved to be desirable, removed variable amounts of intact tissue. These experiments furthermore were carried out on saline homogenates made very slightly alkaline with NaOH so that both mitochondria and microsomes were partially agglutinated and the nuclear and mitochondrial fractions contaminated. In view of these variations, the use of average values was rejected, but the results of one typical experiment are illustrated in Table 15. From these figures it will be noted that the distribution of RNA is comparable with that found by other workers. A very high proportion of the DNA content of the homogenate is found in the nuclear fraction indicating that there has been little destruction of nuclei. Since the ratio of RNA:DNA in the nucleus is of the order of 1:3, the contamination of the cytoplasmic fractions with nuclear RNA must be very small.

Table 15.

The application of the Schmidt and Thannhauser separation procedure as modified by Davidson et al. (1951) to an isotonic saline homogenate at pH9 of rat liver tissue and to the fractions obtained from it by differential centrifugation. All results are expressed in mg. P/100 g. fresh tissue.

Fraction	Whole Homogenate	Total of Cell Fractions	Nuclear Fraction	Mitochondria Fraction	Microsome Fraction	Supernatant Fraction
ASP	101.8	101.7	16.6	7.1	7.1	70.9
L	118.7	103.9	46.6	18.0	36.0	3.3
Alkaline Digest	138.0	119.5	56.9	14.1	29.5	19.0
A_2S	106.0	91.4	35.2	9.6	28.4	18.2
S_2	96.5	90.9	35.4	10.0	28.0	17.5
P_2	3.1	2.2	0.8	0.3	0.3	0.8
R	30.2	27.3	23.4	3.1	0.4	0.4

3.14 The Specific Activities of the phosphorus containing Fractions from the Cytoplasmic Constituents.

In the experiments described above, the uptake of ^{32}P by the various cytoplasmic fractions was studied simultaneously. The rats were treated with ^{32}P at the rate of 10 µc./100 g. body wt., 2 hr. prior to sacrifice, and the radioactivity of each of the phosphorus containing fractions was assayed for the determination of specific activities. The results of these studies corresponding to the analyses in Table 15 are illustrated in Table 16, and are of course subject to the same limitations. In general however the same pattern was found throughout all the preliminary experiments. Perhaps the most important feature was that the specific activities of the ribonucleotide-containing fractions from the mitochondria and supernatant were of the same order while that from the microsomes was considerably lower.

3.15 The Ionophoresis of the A_2S fraction from the Cytoplasmic Constituents.

In view of the results previously obtained with whole tissue and whole cytoplasm in which it was found that there was considerable contamination of the A_2S with highly active P compounds, it was obviously desirable to determine the true specific activities of the ribonucleotides from

Table 16.

The specific activities of the fractions analysed in Table 15. The animals received 10µc.^{32}P/100 g. body wt. 2 hr. before sacrifice. Results are expressed as counts/min./100µg. P.

Fraction	Whole Homogenate	Nuclear Fraction	Mitochondria Fraction	Microsome Fraction	Supernatant Fraction.
AS	6080	5050	5700	5150	6400
L	765	600	628	770	1070
Alkaline Digest	-	-	-	-	-
A_2S	446	436	535	160	435
S_2	354	366	376	153	306
P_2	1450	1540	2020	63	1680
R	44	16	-	-	-

the different cytoplasmic fractions and also to determine whether the additional components were limited to one fraction or were common to all. At the time of these experiments only the presence of "A" and "B" had been established, and no concrete evidence as to the contamination of the uridylic acid had been obtained. The results of four experiments in which the specific activities of the nucleotides from the three cytoplasmic fractions of isotonic saline homogenates of rat liver tissue were determined are shown in Table 17. The animals used in these experiments had received 20 μc.^{32}P/100 g. body wt., two hours before killing.

3.16 Results obtained using the 0.25M-Sucrose Procedure of Schneider (1948).

In view of the criticisms which have been levelled at the separation of the cytoplasmic constituents from saline homogenates, it was considered desirable that future experiments should be carried out in sucrose. Electron micrographs of the mitochondria and microsomes obtained in saline were kindly prepared by Dr. I.M. Dawson of the Chemistry Department of this University, and revealed that both types of particle were grossly agglutinated, and that the mitochondrial fraction was contaminated with considerable quantities of microsomes (Fig.18a,b). In view of these

Table 17.

The Specific Activities (counts/min./100µg. P) of the components of the A_2S fraction from whole tissue and the three cytoplasmic constituents obtained from rat liver tissue by differential centrifugation. The separation was carried out in isotonic saline, and the ^{32}P was administered to the animals in doses of 20µc./100 g. body wt.

Expt.No.	Component of A_2S	Whole Tissue	Mitochondrial Fraction	Microsome Fraction	Supernatant Fraction
1.	Cytidylic Acid	550	485	128	455
	Adenylic Acid	280	540	131	364
	Guanylic Acid	340	480	62	287
	Uridylic Acid	740	665	196	510
	"A" plus "B"	3375	3840	3520	5368
	Whole A_2S	1570	1320	746	1279
2.	Cytidylic Acid	300	204	65	375
	Adenylic Acid	285	216	128	260
	Guanylic Acid	121	188	33	135
	Uridylic Acid	482	325	120	314
	"A"	660	500	50	180
	"B"	3050	3600	4000	4660
	Whole A_2S	825	785	530	725

Table 17 (Contd.)

Expt.No.	Component of A_2S	Whole Tissue	Mitochondrial Fraction	Microsome Fraction	Supernatant Fraction
3.	Cytidylic Acid	77	160	81	50
	Adenylic Acid	116	130	8	112
	Guanylic Acid	28	130	17	66
	Uridylic Acid	240	215	37	138
	"A"	750	128	500	1600
	"B"	2950	2300	220	1900
	Whole A_2S	314	320	120	270
4.	Cytidylic Acid	174	122	11	181
	Adenylic Acid	134	135	- *	230
	Guanylic Acid	133	68	- *	127
	Uridylic Acid	390	510	178	340
	"A"	660	375	- *	750
	"B"	5700	1260	750	4800
	Whole A_2S	524	500	284	550

*No counts measurable in eluates.

Figure 18. Electron micrographs of (A) the mitochondrial fraction x 14,000 and (B) the microsome fraction x 27,000 isolated from a saline homogenate of rat liver tissue.

Figure 18.

A.

B.

findings, experiments were carried out on rat liver tissue homogenised in 0.25M-sucrose in order to confirm the results obtained in Table 17. The ^{32}P was administered intramuscularly in doses of 50 μc./100 g. body weight, the fractions isolated by the Schneider (1948) procedure, and submitted to the modified Schmidt and Thannhauser separation. The A_2S fraction obtained from an aliquot of the whole cytoplasm and from each of the cytoplasmic constituents was analysed by both long and short ionophoretic runs. The results of specific activity determinations are shown in Table 18.

The fractionation on this occasion was carried out on a large scale in an M.S.E. "High Speed Centrifuge", operated in the cold room, where it was observed that the temperature of the microsomal supernatant after running for 1 hr. at full speed was 15°. The centrifugal fields obtained with this centrifuge were comparable with those used with the International Refrigerated Centrifuge using the high speed attachment. In this experiment as in all subsequent fractionation experiments, NaF was added to the homogenate to yield a final concentration of 0.02M in order to minimise enzymic phosphorylation reactions during the time of isolation. The components obtained were divided into two portions from one of which the A_2S fraction was prepared in the normal

Table 18.

The specific activities (counts/min./100μg. P) of the components of the A_2S from whole cytoplasm and the three cytoplasmic fractions, compared with the specific activities of the nucleotides obtained from the RNA isolated from the cytoplasmic constituents. The separation of these constituents was carried out in 0.25M-sucrose, and ^{32}P was administered intramuscularly in doses of 50μc./100 g. body wt. 2 hr. before killing.

Fraction		Adenylic Acid	Guanylic Acid	Cytidylic Acid	Uridylic Acid	"A" + "B"	"C"	"D"	Whole Fraction
Whole Cytoplasm	A_2S	1240	785	1165	2325	14180	3960	3400	3500
	RNA	-	-	-	-	-	-	-	-
Mitochondrial Fraction	A_2S	810	537	608	1833	10600	3800	3580	2700
	RNA	660	425	565	602	-	-	-	895
Microsome Fraction	A_2S	609	358	453	943	13500	3890	4320	2000
	RNA	631	334	310	359	-	-	-	435
Supernatant Fraction	A_2S	2235	1315	1685	2240	16500	3260	1950	3050
	RNA	1702	990	1173	1220	-	-	-	1900

fashion, while the other was used for the isolation of the RNA by the procedure of Davidson, Frazer and Hutchison (1951).

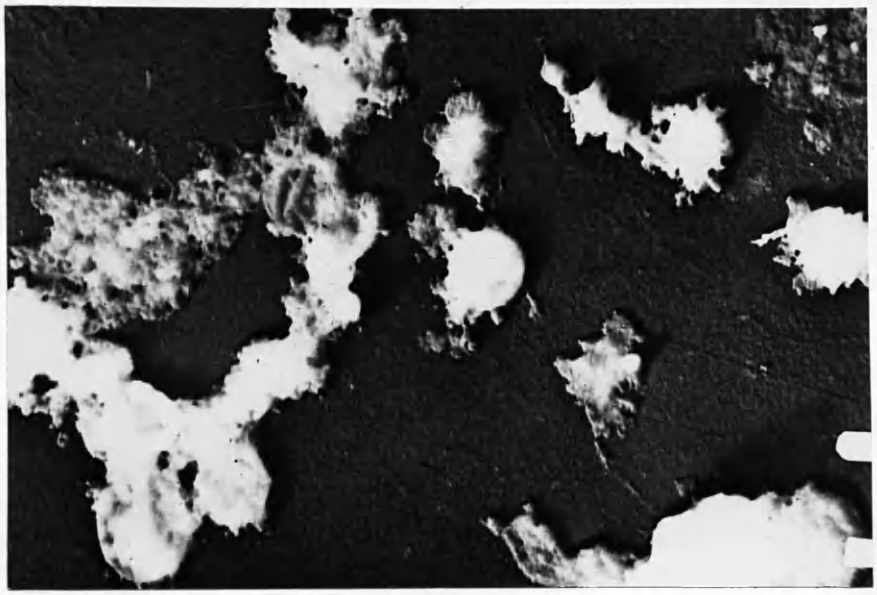
During the course of these fractionation experiments, samples of the mitochondrial and microsome fractions were examined by Dr. I.M. Dawson of the Chemistry Department of this University in the electron microscope. The fractions were fixed in sucrose with osmic acid, and then washed with distilled water to remove the sucrose. Electron micrographs of mitochondria and microsomes obtained in sucrose are illustrated in Fig.19a,b. Careful searching of the fields in both cases showed that there was no contamination of the mitochondria with nuclei, nuclear debris or microsomes. Similarly, there was no contamination of the microsomes with mitochondria.

From the results in Table 18, it is clear that the results obtained in the sucrose experiments are similar to those obtained in saline in that the microsome fraction again exhibits the lowest uptake of ^{32}P 2 hr. after injection. Comparison of the specific activities of the nucleotides from the A_2S and RNA shows them to be similar except in the case of uridylic acid which invariably exhibits a higher activity in the A_2S than in the RNA. This discrepancy makes it quite clear that there is contamination of the uridylic acid with an organic phosphate of considerable

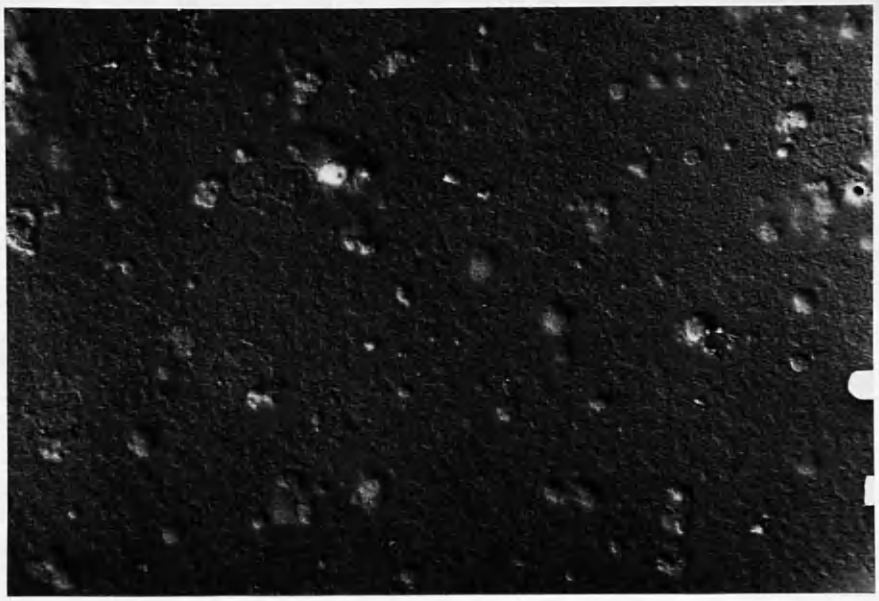
Figure 19. Electron micrographs of (A) the mitochondrial fraction x 14,000 and (B) the microsome fraction x 14,000 isolated from a homogenate of rat liver tissue in 0.25M-sucrose.

Figure 19.

A.



B.



specific activity, and this component has been designated "E". In this experiment, components "A" and "B" were not separated, and the figures quoted apply to the mixture of the two. All the components have proved to be present in each of the cytoplasmic fractions. The comparison of the activities of the nucleotides with those of the whole A_2S make it quite obvious that the activity of the whole fraction bears no relationship whatever to that of the nucleotides. Even the isolated RNA has a slightly higher activity than that of any of the nucleotides derived from it.

In view of the fact that the autoradiographs indicated that the chief contaminant of uridylic acid "E" Fig.15 lay at the back edge of the uridylic acid spot, and that "D" was located slightly in front of it, it was decided to attempt to obtain the true activity of the uridylic acid by cutting the spot into three areas. The first, "E", was cut 1 cm. inside the ultraviolet absorbing area extending for 3 cm. in the direction of guanylic acid. The second which was taken as uncontaminated uridylic acid extended from the first cut to the front edge of the uridylic acid spot, while the third, "D", comprised the 3 cm. immediately in front of uridylic acid. The results of such an experiment are shown in Table 19 where the activities of the

Table 19.

The specific activities (ct./min./100µg. P) of the nucleotides, "D" and "E" of the A_2S from the three cytoplasmic fractions, compared with the specific activities of the nucleotides obtained from the RNA isolated from the same fractions. The fractionation was carried out in 0.25M-sucrose, and ^{32}P was administered intramuscularly in doses of 50µc./100 g. body wt. 2 hr. before killing.

Fraction		Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	"D"	"E"	Whole fraction
Mitochondrial Fraction	A_2S	559	323	469	749	2660	1595	2360
	RNA	458	269	310	438	-	-	1295
Microsome Fraction	A_2S	405	191	325	314	2645	722	1710
	RNA	400	138	212	223	-	-	359
Supernatant Fraction	A_2S	978	434	838	1110	2805	2030	4280
	RNA	798	340	683	512	-	-	2000

nucleotides separated from the A_2S and isolated RNA of the three cytoplasmic fractions are shown side by side. It is clear from these figures that this procedure is not by any means completely satisfactory since even the area taken to represent uncontaminated uridylic acid exhibits an activity considerably in excess of the uridylic acid from the isolated RNA. Of interest also are the figures for the acitivities of the areas "D" and "E" which explain the high values for uridylic acid found when the entire area of ultraviolet absorption is taken.

3.17 The Composition of the RNA from the Cytoplasmic Fractions.

The heterogeneity of the RNA of the cytoplasmic fractions with respect to the uptake of ^{32}P has been illustrated, and itwas therefore desirable to consider the composition of the RNA from the three sources. The results illustrated in Table 20 are the figures obtained for the analysis of the RNA isolated from the three cytoplasmic fractions in the experiment carried out in the previous section. From these figures it would appear that there is no major difference between the fractions, although the guanylic acid content of the microsome RNA appears to be somewhat higher than that of the other two components of the cytoplasm.

Table 20.

The relative compositions of the RNA isolated from the three cytoplasmic fractions fractionated in 0.25M-sucrose. The results are expressed as molar ratios relative to adenylic acid as 10.

Nucleotide	Mitochondrial Fraction	Microsome Fraction	Supernatant Fraction
Adenylic Acid	10.0	10.0	10.0
Guanylic Acid	18.1	20.2	18.5
Cytidylic Acid	16.0	16.1	15.1
Uridylic Acid	12.1	11.6	11.9

3.18 The Relationship between Specific Activity and Time after Injection.

A series of experiments was carried out to examine the variation with time of specific activities of the nucleotides from the three cytoplasmic fractions. In previous experiments with rats some difficulty was found in relating the final activity of the nucleotides to the dose of ^{32}P administered. It was therefore decided to carry out this series of experiments on rabbits, which have the advantage that one animal provides all the necessary tissue, thus obviating the possibility of non-uniform uptake by different members of the group. At the same time by using rabbits it was possible to draw a sample of blood at any interval after the injection of isotope, from which the specific activity of the inorganic phosphate of the blood could be determined.

The procedure adopted was as follows:-

Radioactive phosphorus was administered to female rabbits of the same strain by intramuscular injection in the thigh. The dose of ^{32}P used was 50 μc./100 g. body wt. The animal's ears were then shaved, and after 2 hr. a sample of blood (about 5 ml.) was withdrawn from the ear vein. This was treated with 0.5 vol. 30% (w/v) TCA. The precipitated proteins were removed by centrifugation,

and the clear supernatant was then treated with Mathison's (1909) reagent for precipitation of inorganic phosphate. This precipitate was filtered off through a Whatman no.42 filter paper, washed with ammonia, dissolved in N-HCl, and was then used for the determination of phosphorus and radioactivity. The supernatant from this precipitate provided the blood acid-soluble phosphate fraction.

At the end of the appropriate time interval, the animal was killed by cervical dislocation, a sample of blood collected, the liver perfused with saline and removed. Fractionation of the cytoplasmic constituents was carried out by the Schneider (1948) procedure (Fig.3), washing of the fractions being omitted. These were then submitted to the modified Schmidt and Thannhauser (1945) procedure (Fig.6). The nucleotides were separated by ionophoresis of the ribonucleotide (A_2S) fraction, and after elution radioactivity and phosphorus determinations were carried out. In order to overcome variations in the amount of ^{32}P administered, the results of specific activity determinations were calculated relative to the specific activity of the blood inorganic phosphate at 2 hr. from each animal, giving the "Relative Specific Activity" (R.S.A.).

$$\text{Relative Specific Activity (R.S.A.)} = \frac{\text{Specific Activity of fraction}}{\text{Specific Activity of blood inorganic phosphate at 2 hr.}} \times 10^3$$

In this series of experiments the activity of the uridylic acid was obtained by taking only the area extending from 1 cm. in front of the rear edge of the ultra-violet absorbing spot to the front edge of the same, which although not giving an accurate value for the activity was at least a considerable improvement on taking the whole area.

The results of this series of experiments are illustrated in Figs.20, 21, 22, 23 and 24. In Fig.20, the variation with time after injection of the relative specific activities of the blood inorganic and acid-soluble phosphates (1,2), whole cytoplasm inorganic and acid-soluble phosphates (3,4) and of the whole cytoplasm lipid phosphorus (5) are illustrated. From this figure as might be expected it will be noted that the blood and tissue inorganic phosphate (1,3) R.S.A.s fall very rapidly over the first 18 hr., after which the slope of the curves becomes similar to those of the blood and tissue acid-soluble phosphates (2,4) and lipid phosphorus (5). The R.S.A. of the lipid phosphorus on the other hand rises steadily from 2 hr. to 24 hr. before beginning to fall.

In Figs. 21, 22 and 23 the variation with time of R.S.A.s of the four nucleotides derived from each of the three cytoplasmic fractions in turn are illustrated.

Figure 20.

The variation with time of the relative specific activities of some components of rabbit blood and liver.

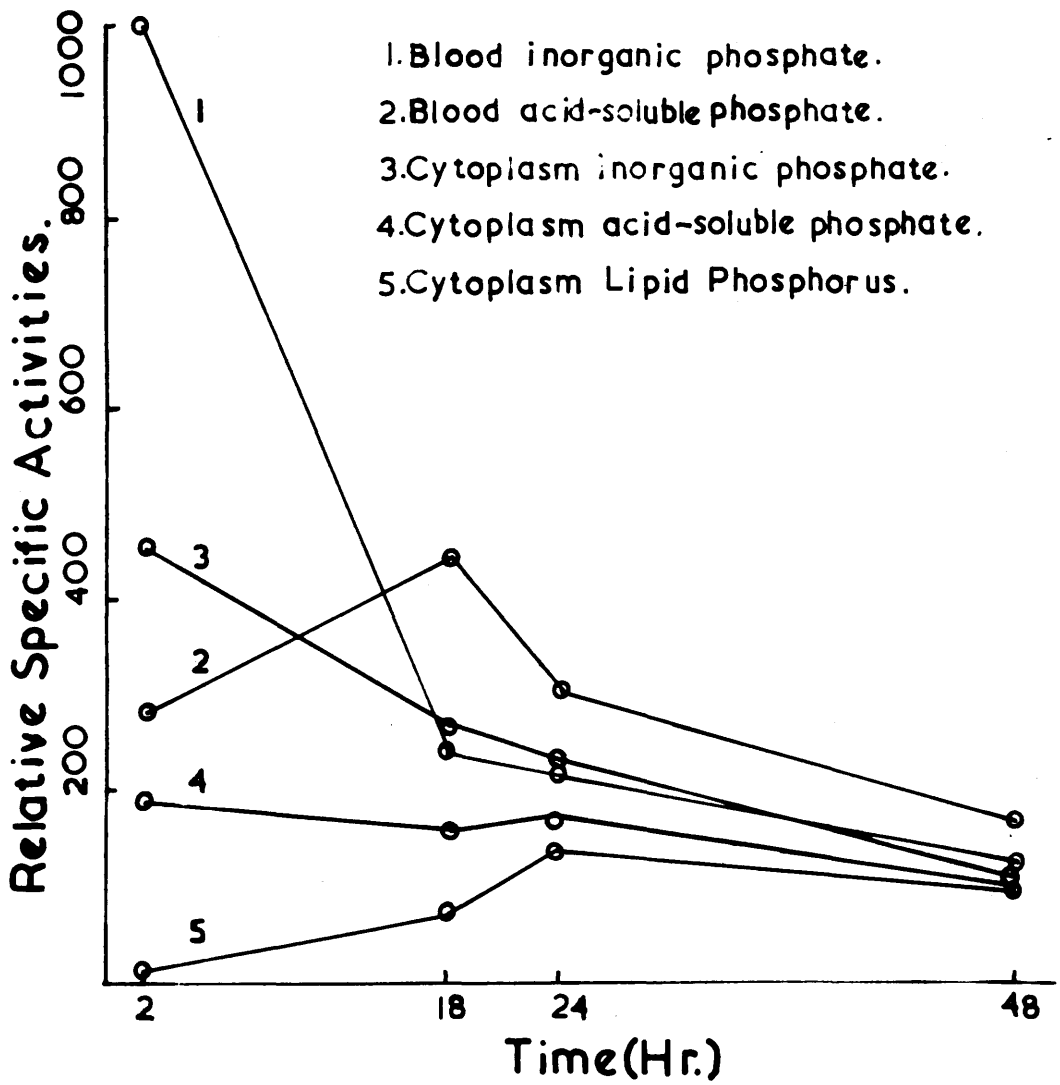


Figure 21.

The variation with time of the relative specific activities of the nucleotides derived from the RNA of the mitochondrial fraction.

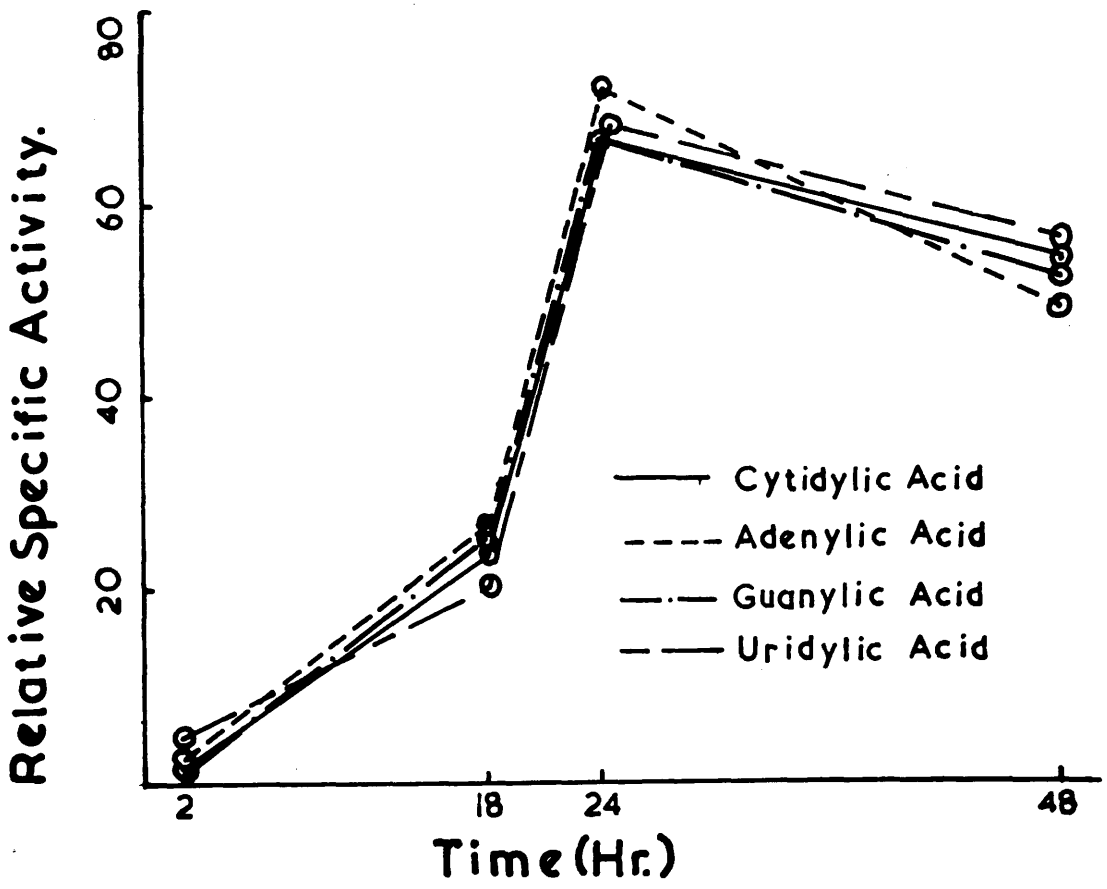


Figure 22.

The variation with time of the relative specific activities of the nucleotides derived from the RNA of the microsome fraction.

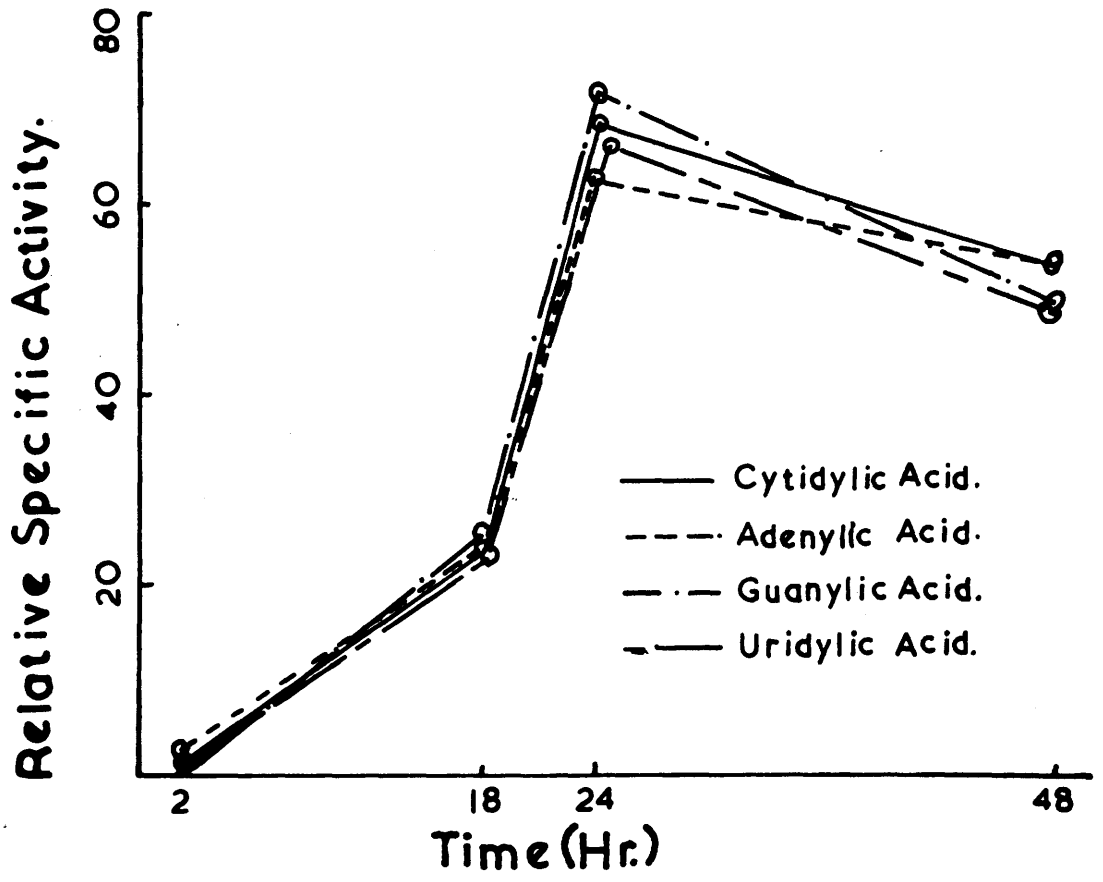
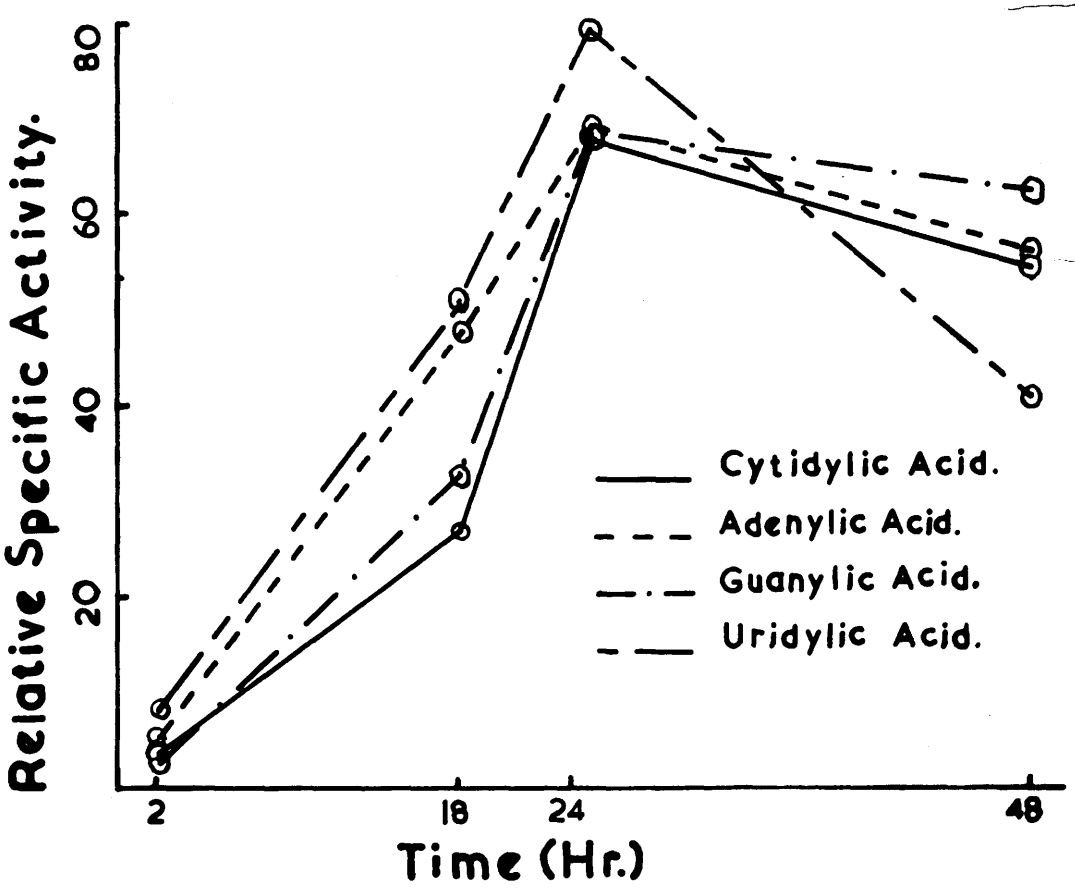


Figure 23.

The variation with time of the relative specific activities of the nucleotides derived from the RNA of the supernatant fraction.

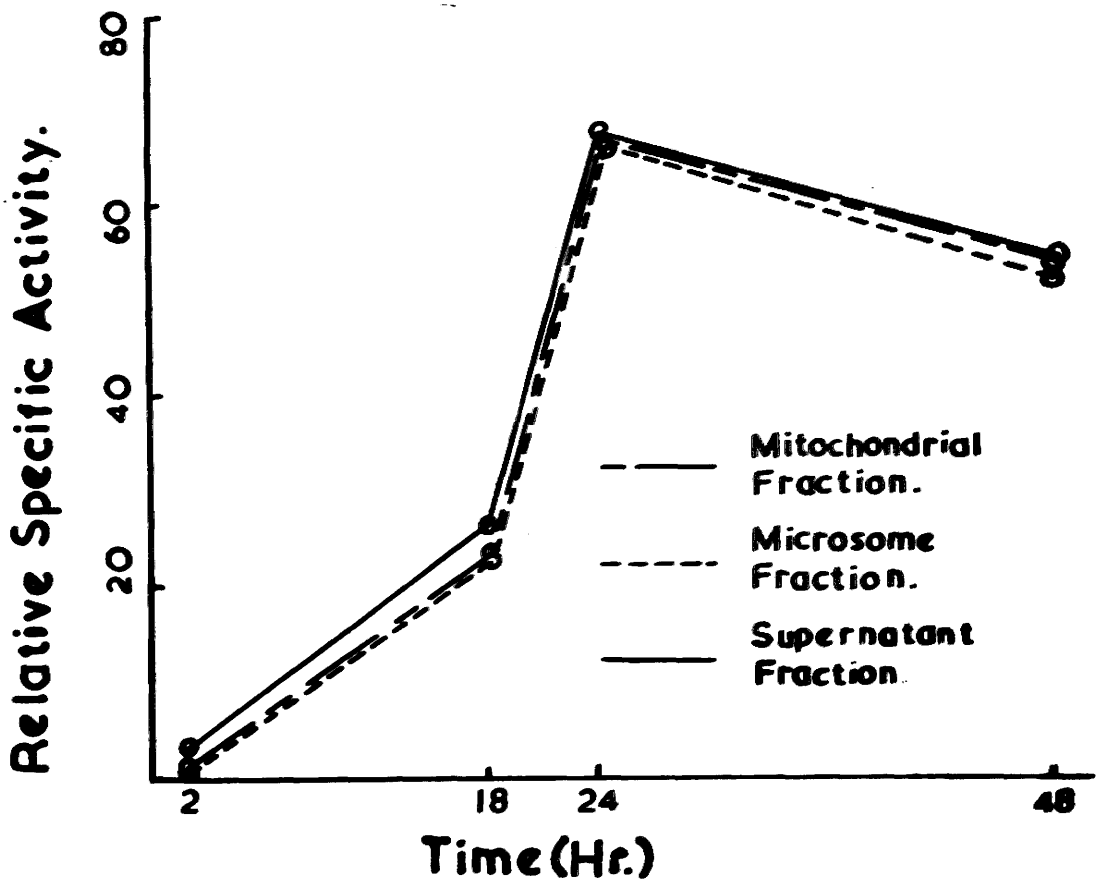


From these figures it is apparent that, certainly in the case of the mitochondrial and microsome fractions, the uptake of ^{32}P into the four nucleotides follows exactly the same pattern, although there might appear to be at any one time interval a considerable difference between any particular pair of nucleotides. These differences however are ironed out when seen in the perspective of the whole curve. In the supernatant fraction (Fig.23) uridylic acid and to a lesser extent adenylic acid seem to behave in a slightly different fashion from the other two nucleotides at 18 and 24 hr. In the case of uridylic acid this is almost certainly due to contamination with "E", while the one value for adenylic acid (18 hr.) which is anomalous might easily be due to errors. Generally however, the pattern which emerges is that the four nucleotides in each cytoplasmic fraction behave in a similar fashion, reaching peak uptake of ^{32}P about 24 hr. after injection.

Comparison of the three fractions is made in Fig.24 where the variations with time of the R.S.A.s of the cytidylic acid from them is plotted. From this figure it is clear that each of the cytoplasmic fractions behaves in the same way, although at the earlier time intervals there would appear to be some differences between them. These

Figure 24.

The variation with time of the relative specific activities of the cytidylic acids derived from the ribonucleic acids of the three cytoplasmic fractions.



differences, like the differences between the individual nucleotides within a fraction, however, fade into insignificance when considered in the perspective of the rest of the time curve.

The limited number of determinations which have so far been carried out make it impossible to predict the location of the maximum of the curves plotted in Fig.24, but it would seem certain that this must fall between 24 and 48 hr. after injection. It is obviously necessary to carry out many more experiments in this series in order to fill in the curve, and also to overcome difficulties due to variations in the metabolism of the particular animals used. In view of these considerations it is impossible to vouch for each of the points quoted on the figures as being representative of a group of animals, but the main facts emerging are that each of the nucleotides in any of the cytoplasmic fractions behaves in the same manner as does the RNA from each fraction.

3.19 The Specific Activities of the Nucleotides from the Mitochondrial Fraction obtained by different methods.

It has been shown that the Specific Activity of the uridylic acid obtained by ionophoresis of the A_2S fraction prepared as shown in Fig.6 indicated that there was contamination with some highly active material. The work of

Mitchell and Moyle (1951a,b) suggested that this contamination might be due to glycerophosphate which has a pK value intermediate between that of uridylic and guanylic acids. This was tested, by the ionophoresis of known quantities of uridylic acid plus α-glycerophosphate. Results indicated that glycerophosphate did in fact occupy a position overlapping uridylic acid after ionophoresis. Various attempts were made to free from contamination the A_2S fraction from a radioactive mitochondrial preparation. These included incubation with acid phosphatase from prostate, alkaline phosphatase from intestinal mucosa and also treatment with 2N-$HClO_4$ at 100°. It was found that both phosphatases destroyed the uridylic acid, and that the $HClO_4$ hydrolysis did not remove the contaminant.

A large sample of mitochondria from radioactive rat liver was prepared, and divided into 3 parts. From the first of these, the A_2S fraction was prepared in the normal fashion, while the second portion was utilised for the isolation of the RNA. The third aliquot was treated by procedure 3 outlined in Fig.7 yielding what has been called the A_3S fraction. Ionophoresis of all three hydrolysates was then carried out in the usual manner, the nucleotides being eluted, counted and the phosphorus content of each eluate measured. The results of this

experiment are illustrated in Table 21 from which it is clear that the primary object has been obtained by use of procedure 3, the uridylic acid activity of the A_3S fraction approximating to that of the other nucleotides. At the same time, except in the case of uridylic acid, there is better agreement between the specific activities of the nucleotides from the A_3S and A_2S than between the A_3S and RNA. The reason for this discrepancy is not clear; it may be due to contamination of all the nucleotides obtained by ionophoresis from the A_2S and A_3S fractions. This however does not seem likely as one might expect that at least one of the nucleotides obtained by these methods would be free from contamination. Furthermore autoradiographs of strips of paper on which ionophoresis of radioactive A_3S fractions had been carried out showed no evidence of contamination of the nucleotide region.

One possible explanation is that the full isolation of the RNA involves precipitation from glacial acetic acid, and deproteinising by the method of Sevag, Lackman and Smolens (1938), procedures which are quite drastic and could bring about partial degradation of the RNA. This difficulty is obviated by the use of procedure 3 which does not require the use of glacial acetic acid or deproteinisation. It is possible that the labelling of the RNA is

Table 21.

The specific activities (counts/min./100µg.P) of the nucleotides of the RNA of rat liver mitochondria obtained by ionophoresis of (a) hydrolysed RNA, (b) A_2S (fig.6), (c) A_3S (fig.7).

Nucleotide	Isolated RNA	A_2S	A_3S
Adenylic Acid	512	482	565
Guanylic Acid	297	412	370
Cytidylic Acid	315	430	455
Uridylic Acid	340	1335	562

not homogeneous, and that the more biologically labile phosphate groups are also the more chemically labile. If then during the isolation of the RNA some loss of the more active nucleotides occurred, the activity of the final product would be lower than might have been expected, but this requires further investigation.

3.20 The Determination of the True Specific Activities of all four Nucleotides, by Ionophoresis of the A_3S (procedure 3) from the three Cytoplasmic Fractions.

The partial isolation of the RNA (procedure 3) was then applied to the analysis of the nucleotides from the three cytoplasmic fractions isolated in 0.25M-sucrose. The animals were male albino rats, and they received 50 μc. ^{32}P/100 g. body weight intramuscularly 2 hr. before sacrifice. The results of specific activity determinations are illustrated in Table 22, from which it is clear that the uridylic acid activity is of the same order as that of the other nucleotides in each of the cytoplasmic fractions. Furthermore, the microsome fraction again exhibits the lowest activity, as was previously recorded. In each of the fractions the activities of the nucleotides is considerably less than that of the whole A_3S despite the partial isolation procedure. This is in accord with the findings for the RNA isolated from the three cyto-

Table 22.

The true specific activities (counts/min./100µg. P) of the four nucleotides derived from the RNA of the three cytoplasmic fractions obtained by ionophoresis of the A_3S (Procedure 3). The animals (male albino rats) received 50µc. ^{32}P 2 hr. before killing.

Fraction	Adenylic Acid	Guanylic Acid	Cytidylic Acid	Uridylic Acid	Whole Fraction
Whole Cytoplasm	383	201	360	286	720
Mitochondrial Fraction	289	182	215	232	450
Microsome Fraction	189	109	141	119	278
Supernatant Fraction	650	303	575	433	1260

plasmic fractions (Table 19). Of the nucleotides, adenylic acid exhibits the highest activity in each of the fractions, while guanylic acid is invariably found to have the lowest activity.

PART IV - DISCUSSION.

4.1 Methods of Analysis of RNA.

Since the development of the technique of chromatography on filter paper by Consden, Gordon and Martin (1944), numerous authors have sought to apply similar methods to the separation of the degradation products of RNA with a view to its analysis. Vischer and Chargaff in 1947 first investigated the possibilities of chromatography in this respect, and in the following year they (1948a) described the separations of purine and pyrimidine bases as well as several related compounds achieved by the use of a variety of solvents. They next turned their attention to the hydrolysis and analysis of samples of RNA (Vischer & Chargaff, 1948b), utilising two of the previously studied solvent systems. The procedure was twofold, a preliminary hydrolysis with methanolic HCl, followed by formic acid hydrolysis of the pyrimidine residues, and proved to be rather complicated and by no means quantitative. The analysis of a sample of yeast RNA by this method is included in Table 23. About the same time, Hotchkiss (1948) studied the separation of purine and pyrimidine bases and nucleosides on filter paper using n-butanol saturated with water as the solvent. Preliminary experiments with the purified materials proved reasonably satisfactory, but once again the hydrolysis of RNA proved to be a pitfall.

Table 23.

The composition of the RNA from several different sources as determined by various authors. All results have been calculated relative to adenylic acid as 10.

Source of RNA	Adenylic Acid	Guanylic Acid	Cytidylic Acid	Uridylic Acid	Reference
Yeast	10.0	9.7	9.4	3.1	Vischer & Chargaff (1948b)
Yeast	10.0	9.7	6.1	7.0	Chargaff et al. (1950)
Yeast	10.0	9.6	7.5	6.7	" "
Yeast	10.0	10.6	8.6	8.2	" "
Yeast	10.0	10.5	8.0	10.2	" "
Yeast	10.0	5.4	5.9	15.4	Carter (1950)
Yeast	10.0	12.2	7.8	9.0	Smith & Markham (1950)
Yeast	10.0	10.8	7.9	8.9	Wyatt (1951)
Yeast	10.0	9.7	9.4	3.1	Beale et al. (1950)
Yeast	10.0	12.3	7.8	11.2	Boulanger & Montreuil (1951b)
Yeast	10.0	12.4	8.8	10.4	Current Experiments
Pig Pancreas	10.0	24.5	12.5	2.8	Vischer & Chargaff (1948b)
Pig Pancreas	10.0	22.5	9.8	4.6	Chargaff et al. (1950)
Pig Pancreas	10.0	24.5	12.6	2.8	Beale et al. (1950)
Pig Liver	10.0	16.3	16.1	7.7	Chargaff et al. (1950)
Sheep Liver	10.0	16.8	13.4	5.6	" "
Beef Liver	10.0	14.6	10.9	6.6	" "
Calf Liver	10.0	16.2	11.1	5.3	" "

Table 23 (Contd.)

Source of RNA	Adenylic Acid	Guanylic Acid	Cytidylic Acid	Uridylic Acid	Reference
Calf Liver	10.0	18.0	14.9	8.4	Volkin & Carter (1951a
Calf Liver	10.0	18.8	16.1	8.7	" "
Calf Spleen	10.0	19.8	17.7	8.6	" "
Calf Thymus	10.0	23.2	14.2	6.7	" "
Calf Pancreas	10.0	35.1	17.1	9.7	" "
Beef Pancreas	10.0	23.0	10.0	3.9	Boulanger & Montreuil (1951b)
Rabbit Liver	10.0	20.1	16.8	9.9	Volkin & Carter(1951a)
Rabbit Liver	10.0	19.2	16.1	9.5	" "
Rabbit Liver	10.0	18.8	13.9	10.9	Current Experiments.
Rabbit Liver*	10.0	16.9	14.6	10.3	Current Experiments.
Rat Liver	10.0	17.2	18.9	8.6	Volkin & Carter(1951a)
Regenerating Rat Liver	10.0	19.0	18.3	9.3	" "
Rat Liver	10.0	17.6	15.9	11.2	Current Experiments.
Rat Liver	10.0	17.7	16.6	12.3	" "
Rat Liver	10.0	17.2	14.9	9.5	" "
Rat Liver	10.0	17.7	14.5	10.4	" "
Rat Liver*	10.0	17.5	13.9	10.9	" "
Rat Liver*	10.0	17.6	14.3	10.8	" "
Mouse Liver	10.0	16.2	12.9	8.6	Volkin & Carter(1951a)
Mouse Hepatoma	10.0	15.5	15.5	8.3	" "
Carcinomatous Human Liver					
Unaffected Tissue	10.0	32.9	28.8	8.3	Chargaff et al.(1950)

Table 23 (Contd.)

Source of RNA	Adenylic Acid	Guanylic Acid	Cytidylic Acid	Uridylic Acid	Reference
Metastases	10.0	41.4	43.2	7.2	Chargaff et al.(1950)
GRCH 15 Fowl Tumour	10.0	33.6	20.1	9.0	Beale et al. (1950)
Cock Liver*	10.0	16.5	13.4	10.3	Current Experiments.
Cock Liver*	10.0	17.7	13.9	10.8	" "
Liver from Laying Hen*	10.0	16.7	13.6	10.2	" "
Liver from Cock bearing GRCH 15 Tumour*	10.0	18.1	14.5	11.1	" "
GRCH 15 Tumour from Cock*	10.0	18.4	14.5	11.1	" "

*Analysis was carried out on the partially isolated RNA obtained by procedure 3.

Markham and Smith (1949a,b) described a method for the detection on filter paper of spots containing the purine or pyrimidine bases, based on their strong ultraviolet absorption and also reported on the separation of the purine and pyrimidine bases, nucleosides and several related compounds in different solvent systems.

The separation of mononucleotides by paper chromatography was first described by Vischer, Magasanik and Chargaff (1949), and was utilised for the analysis of some specimens of RNA (Chargaff, Magasanik, Doniger & Vischer, 1949). This procedure, which did not obtain resolution of guanylic and uridylic acids, made use of a system composed of isobutyric acid/water in an atmosphere of ammonia, was later modified by Magasanik, Vischer, Doniger, Elson and Chargaff (1950) by incorporating the ammonia in the solvent itself. Using this technique, determination of guanylic acid and uridylic acid was carried out by differential spectrophotometry. Chargaff, Magasanik, Vischer, Green, Doniger and Elson (1950) then applied the method to the analysis of RNA from yeast and several mammalian tissues, the results of which are illustrated in Table 23.

Smith and Markham (1950) carried out careful studies of the separation of the components of yeast RNA obtained by hydrolysis with HCl, and were able to obtain consistent

figures for its analysis by separation of the purine bases and pyrimidine nucleotides obtained by this hydrolysis. The results of such an analysis are shown in Table 23. This method was then applied by Markham and Smith (1950) to the study of virus RNA. The use of starch columns was continued by Beale, Harris and Roe (1950) who separated the four nucleosides by means of an n-butanol/water system at room temp. Two methods of hydrolysis were utilised; firstly the aqueous pyridine method of Bredereck, Martini and Richter (1941), and secondly the hydrolysis of the nucleotides obtained by alkaline hydrolysis of the RNA using human prostate acid phosphatase (Schmidt, Cubiles & Thannhauser, 1947) which proved to be more satisfactory. By this method Beale et al. (1950) were able to obtain figures for the composition of some different samples of RNA which are shown in Table 23.

Carter (1950) proposed the use of several buffered solvent systems for the separation of various degradation products of RNA, and has applied one of these to the analysis of a specimen of yeast RNA hydrolysed to the mononucleotides with barium hydroxide. In this case there was no resolution of cytidylic and uridylic acids; moreover it is clear from the figures quoted (Table 23) that there is an enormous preponderance of uridylic acid, while the value

for cytidylic acid is low. This would suggest that the hydrolysis with baryta has brought about deamination of the cytidylic acid as described by Marrian et al. (1951) and confirmed above.

Further improvements on their earlier methods of analysis of RNA were made by Markham and Smith (1951), who also described the separation of nucleotides on paper using a solvent system composed of ammonium sulphate, water and isopropanol. This solvent, like those of Carter (1950), did not resolve the two pyrimidine nucleotides although the isomeric purine nucleotides were separated from one another. The technique of chromatography of the purine bases and pyrimidine nucleotides was also improved although the authors found it necessary to apply a correction for hydrolysis of the pyrimidine nucleotides which suggests that the conditions were not so quantitative as might have been desired.

Wyatt (1951) using the method of hydrolysis with perchloric acid described by Marshak and Vogel (1950a) and a solvent system of isopropanol/HCl obtained excellent separations of all four bases produced by the hydrolysis of RNA. He found figures for the analysis of a specimen of yeast RNA comparable with those found by Smith and Markham (1950) for the same specimen of RNA by their method (Table 23). There appears however to be a slight discrepancy

between the two methods since Markham and Smith (1951) in a subsequent paper apply a 5% upwards correction for the hydrolysis of the pyrimidine nucleotides. When such a correction is applied to the analysis of yeast RNA quoted by Smith and Markham (1950) (Table 23), the values obtained for the pyrimidine nucleotides are appreciably higher than those found by Wyatt (1951) (Table 23). Very recently, Boulanger and Montreuil (1951a,b) have described the use of several phenolic solvents for the quantitative separation of the ribonucleotides, and have applied these to the analysis of samples of RNA hydrolysed with ammonium or sodium hydroxide (Table 23). In this case the nucleotides were detected by means of the phosphate detecting spray reagent of Hanes and Isherwood (1949), and estimation was carried out by phosphorus determinations on duplicate chromatograms.

A new departure was made by Cohn (1949) when he made use of anion and cation exchange resins to separate the bases and nucleotides from mixtures of the pure materials, and also of the nucleotides obtained by alkaline hydrolysis of yeast RNA. In all these experiments, good recoveries were obtained, and the results were very promising. The technique was further improved by Cohn (1950) who achieved good separations of all the nucleotides, and demonstrated

the presence of the isomeric adenylic and guanylic acids in samples of rat liver RNA. Volkin and Carter (1951a) made use of this technique to analyse several specimens of RNA from animal tissues, the results of which are shown in Table 23.

During this time, the purely chemical methods of analysis were not entirely neglected. Thus Kerr, Seraidarian and Wargon (1949a,b) described a method based on the precipitation of the purine bases from sulphuric acid hydrolysate of RNA with silver sulphate, leaving the pyrimidine nucleotides in solution. The two groups of substances were then determined by differential spectrophotometry. Fairley, Seagram and Loring (1950) subsequently made use of a similar technique to examine the RNA of the mitochondrial fraction of beef pancreas.

The criteria of a satisfactory method of analysis of RNA for our purposes were as follows:-

1. The method of hydrolysis must yield the mononucleotides.
2. The hydrolysis to mononucleotides must be quantitative, and must involve no intercoversion of the breakdown products.
3. The method of separation must be adequate for all four nucleotides.
4. The method of estimation of the separated components must be accurate and valid.

Examination of all the methods available made it clear that very few were therefore suitable for our purposes. Methods involving separations of the bases and nucleosides were ruled out since for isotope studies using ^{32}P it was necessary to use the nucleotides, but even the methods of separation of the nucleotides were in some instances unsuitable since they did not achieve resolution of all four components (Carter, 1950; Magasanik et al., 1950; and Markham and Smith, 1951). The technique of Cohn (1950) appears to be quite suitable, but was not published until much of our work was in progress, and in addition it could not be readily adapted to the small quantities of material at our disposal. The paper chromatographic method of Boulanger and Montreuil (1951), likewise, was not published until much of our work was completed, but it suffers from the disadvantage that the spots cannot be exactly located on the chromatogram which is to be used for analysis. The presence of phenol precludes the location of the nucleotides by the method of Holiday and Johnson (1949) or Markham and Smith (1949a). Boulanger and Montreuil locate the nucleotides on a duplicate chromatogram by using the Hanes and Isherwood (1949) reagent for the detection of phosphates, a method which in our hands has not proved satisfactory for the detection of the nucleotides.

4.2 The Technique of Ionophoresis.

It has been demonstrated that at the time of commencement of these studies, none of the existing methods for the analysis of RNA was suitable, and that it was necessary to adopt a new approach to the problem. It was therefore decided to attempt a separation of the nucleotides based on their differing mobilities in an electric field. The photographs in Figs.11 and 12 make it clear that an adequate separation of the nucleotides can be achieved by the method of ionophoresis on filter paper, while the results illustrated in Tables 3 and 4 show that the recoveries of the pure materials from the paper are satisfactory. The figures shown in Table 8 demonstrate that the RNAP is liberated quantitatively into the material to be analysed on incubation with alkali and acidification, while from Table 7 it will be seen that although alkaline hydrolysis can cause interconversion of cytidylic acid and uridylic acid this does not occur in the conditions utilised. From this Table also it will be noted that using 0.3N-alkali all the original material was recovered as nucleotide phosphorus. In the case of the samples of isolated RNA studied (Table 9) the nucleotide phosphorus recovered accounted for about 93% of the original RNAP, indicating that the hydrolyses, separations and estimations if not absolutely quantitative

were very close to being so especially since it seemed not impossible that the 7% phosphorus unaccounted for might not all have been nucleotide phosphorus initially.

The method of phosphorus determination was well established, and has been carefully checked in respect of its use as a means of estimating the quantity of mononucleotide (Table 5). The overall validity of the method of ionophoresis has been further checked by comparison of the analysis of mixtures of nucleotides and of samples of RNA with results obtained by the totally different method of chromatography of the bases (Tables 6 and 9). These tables indicate that in the cases of adenylic, guanylic and cytidylic acids, good agreement is obtained; in the case of uridylic acid, however, the ionophoretic value is invariably slightly higher than that obtained by chromatography of the bases. Experiments carried out on the recovery of uridylic acid based on phosphorus estimations and also on ultraviolet determinations on the eluates from ionophoretic runs showed quite clearly that the uridylic acid recovered was the same whether determined by phosphorus or ultraviolet measurements. Moreover this accounted almost exactly for that applied to the paper initially both in terms of phosphorus and weight. Finally Dr. Crosbie, who carried out the base chromatography, has concluded that there is a loss of uracil in the course of the perchloric

acid hydrolysis. It would appear therefore that the ionophoretic method for the analysis of RNA satisfies the criteria set out above, and can be adopted as a valid method.

The composition of RNA from different sources determined by ionophoresis of the nucleotides, and by the other methods discussed above is shown in Table 23, which also includes the results obtained from the analysis of the partially isolated RNA obtained by the saline extraction of the lipid free tissue powder (Procedure 3). These figures indicate that the ionophoretic method yields results which are comparable with the more recent and more carefully established techniques. In the case of the samples of yeast RNA there is considerable variation in the analysis figures. It is difficult to estimate to what extent these variations are due to the method of isolation of the RNA, and to what extent they are due to faults in the methods of analysis, but it would appear that the degree of degradation of the RNA is greatly dependent on the isolation procedure, and that this can alter considerably the composition of the final material.

In the case of the RNA from animal tissues, two facts are apparent, the high content of guanylic acid in every sample, and the high cytidylic acid value. These results

have been obtained by a variety of techniques of analysis applied to numerous tissues, and it would appear therefore that the high guanylic and cytidylic acid content of the tissues studied is in fact characteristic. In RNA from pancreas and from tumour tissues, the guanylic acid content is greatly increased. The high concentration of ribonuclease in pancreas renders the results for that tissue suspect, since degradation of RNA by this enzyme preferentially removes the pyrimidine nucleotides and adenylic acid, leaving a core very rich in guanylic acid (Magasanik & Chargaff, 1951).

Similar considerations may apply to the RNA from tumour tissue since here again high guanylic acid values are recorded (Beale et al., 1950; Chargaff et al., 1950). In our analyses of the GRCH 15 tumour of the fowl when procedure 3 was applied, i.e., when any ribonuclease would have minimum opportunity to degrade the RNA, the values quoted were obtained. On one occasion when the cytoplasmic material remaining after the isolation of nuclei in citric acid was used, after standing at room temperature for some time, values similar to those obtained by Beale et al. (1950) were obtained.

4.3 The Analysis of the Schmidt and Thannhauser (1945) Ribonucleotide Fraction.

The exact nature of the A_2S fraction obtained by the

application of the Schmidt and Thannhauser (1945) procedure has for some time been the subject of controversy. Thus numerous authors (Jeener, 1949a,b; Marshak & Calvet, 1949; Marshak & Vogel, 1950b; Jeener & Szafarz, 1950 a,b; Davidson, Frazer & Hutchison, 1951) have commented upon the presence in this fraction of inorganic phosphate which they have found difficult to remove by purely chemical methods. Recently Szafarz and Paternotte (1951) have described in detail a chromatographic method for its removal, without however achieving separation of the nucleotides from one another. The iomphoretic analysis which has been described, not only achieves the removal of all inorganic phosphate from this fraction, but also provides a means of separating the four nucleotides into four discrete spots (Fig.13).

Davidson, Frazer and Hutchison (1951) besides noting the presence in the A_2S fraction of inorganic phosphate suggested that there might be present other organic phosphates in addition to the ribonucleotides. This was based on the observation that only about 85% of the phosphorus of the fraction could be accounted for on the basis of pentose determinations, and while the inorganic phosphate admittedly accounted for some of the remainder, a large proportion of this extra phosphorus was neither nucleotide nor inorganic phosphate. Application of the iono-

phoretic procedure has confirmed that a large proportion of the A_2S P is not nucleotide in origin, and that nucleotide phosphorus in fact accounts for only about 75% of the total (Table 12). It is therefore not satisfactory to estimate the RNA content of a tissue by application of the Schmidt and Thannhauser (1945) procedure alone since such analyses will yield results that are high by some 33%.

Short ionophoretic runs (Fig.14) indicated where some of the additional phosphorus was to be found, but it was not until autoradiographs of papers on which the A_2S fraction from the livers of rats which had received ^{32}P before killing, were prepared that exact location of all the remaining phosphorus was possible. It is clear from Figs. 15 and 16 that at least five phosphorus containing components are present in the A_2S fraction over and above the inorganic phosphate which coincides with the most rapidly moving concomitant. It will be noticed on the autoradiographs (Fig.16) that fogging of the photographic emulsion is most intense in the region of these additional components, and since between them they comprise only about 25% of the total phosphorus of the fraction, it is to be expected that they will exhibit a high activity.

That this is the case will be seen from the results in Table 13 where the specific activities of all the separate components of the A_2S fraction are illustrated.

From the figures, it is apparent that the most active component is "B" which seems to correspond to the "phosphoprotein" or P_2 phosphorus of Davidson, Frazer and Hutchison (1951). Owing to the presence of these additional phosphorus containing substances in the fraction A_2S, the activities of the nucleotides themselves bear no relationship whatsoever to the activity of the whole fraction, and it is therefore quite unjustifiable to assume that the specific activity of the ribonucleotide fraction obtained by the procedure of Schmidt and Thannhauser represents the true activity of the RNAP itself.

Of the nucleotides, guanylic acid invariably exhibits the lowest activity, while uridylic acid is usually higher than any of the others. The proximity of the uridylic acid to the concomitants "A", "B", "C" and "D" aroused suspicion as to whether this high value was in fact correct, or was due to contamination with an undetected phosphorus containing compound of high specific activity. That the latter was the case is obvious from the comparison of the specific activities of the nucleotides from an isolated RNA and an A_2S prepared from the same material (Table 14), and from the results illustrated in Table 19 where the area around the uridylic acid was cut into three portions each being assayed separately for phosphorus and radioactivity.

An investigation of these additional components

proved that they do not originate either in the acid-soluble or in the lipid phosphate compounds of the tissue. Unfortunately the method of ionophoresis does not lend itself to preparation of these substances in quantity, and no attempt has therefore been made so far to identify them. The recent papers of Mitchell and Moyle (1951a,b), who detected the presence of a glycero-phospho-compound in the ribonucleotide fraction of certain micro-organisms, suggested that something similar might be found in the ribonucleotide fraction of animal tissues. In experiments in which a mixture of α-glycero-phosphate and uridylic acid was applied to filter paper and run in the normal fashion, the two components were not resolved. This observation does not prove the presence of glycero-phosphate in the A_2S fraction, but indicates nevertheless that it is a possible contaminant. It is more difficult to trace the origin of such substances in animal tissue than in the experiments of Mitchell and Moyle (1951b), but the presence of these components in each of the cytoplasmic fractions weighs against localisation in any particular cytological unit.

Of the concomitants, "E" proved the most obstinate to eliminate and since it affected both the activity and phosphorus determinations of uridylic acid (Tables 10, 14,

18, 19 and 21), it was necessary to adopt the further modification of the Schmidt and Thannhauser (1945) procedure shown in Fig.7, which amounted to a partial isolation of the RNA.

The results obtained using this procedure, and also from isolated specimens of RNA (Tables 21, 22), disprove the earlier conjecture that uridylic acid exhibits a markedly higher activity than the other nucleotides. The highest activity is invariably associated with adenylic acid, and the lowest with guanylic acid as was observed by Volkin and Carter (1951b) for several tissues. This latter finding is perhaps of some significance in view of the conclusions regarding the structure of RNA reached by Magasanik and Chargaff (1951) who found that the action of ribonuclease on RNA left an undigested residue exceedingly rich in guanylic acid. They suggested that the main skeleton of the RNA contained a high proportion of guanylic acid. If this were so, it seems not impossible that the "backbone" of the molecule may be comparatively inert biologically, the side chains providing the functional portion. The guanylic acid obtained on complete hydrolysis of the molecule would be diluted with guanylic acid from this inert core and would therefore exhibit a lower activity than might be expected. On the other hand, the proportion of adenylic acid in this

unhydrolysed core is also high (Magasanik and Chargaff, 1951) and on the same analogy the activity of the total adenylic acid should likewise be lower than that of the pyrimidine nucleotides, which are liberated almost in their entirety by the enzymic digestion. The possibility of heterogeneous labelling of the nucleotides from RNA has already been mentioned in connection with the discrepancies between the specific activities of the nucleotides obtained from isolated RNA, fraction A_2S and fraction A_3S (Table 21).

It has been observed that the specific activity of the whole isolated RNA is often considerably in excess of the specific activities of the nucleotides obtained from it by ionophoresis (Tables 14,19). This factor in conjunction with the observation that only about 93% of the RNAP was recovered in the form of nucleotide phosphorus (Table 9) suggested that even the isolated RNA might be contaminated with some highly active phosphate. Evidence for the presence of such a substance has been found on autoradiographs prepared from short ionophoretic runs of the isolated RNA from an animal which had received ^{32}P. The extra spot appeared in a position corresponding to component "C" of the A_2S fraction, and was present on short ionophoretic runs of hydrolysates of isolated RNA and also

of the A_3S fraction (procedure 3).

The presence of this hitherto unsuspected component in samples of isolated RNA makes it essential to separate the individual nucleotides in isotope studies on the uptake of radioactive phosphorus by animal tissues, rather than to determine the specific activity of the isolated RNA.

4.4 The Use of Procedure 3.

In view of the extent of the contamination of the fraction A_2S by phosphorus containing substances, and the tedium of carrying out the complete isolation procedure for RNA, the partial isolation (procedure 3) of the RNA became necessary. This technique suffers from one major disadvantage in that only about 70% of the RNA in any given material is obtained. While this is of no consequence, from the point of view of subsequent analysis, if all the starting material is homogeneous it means that no figure for the RNA content of a sample of tissue can be obtained. Furthermore if the starting material is not reasonably homogeneous there is a danger of preferentially extracting one of the components, a feature which could be misleading.

In the case of rat liver, for instance, the tissue RNA is composed of RNA from the three cytoplasmic fractions and the nucleus. If the composition of any of these constituents differed markedly from the others, it would not

be valid to determine the composition of the saline extracted material since this might not be representative of all components. In our experience, the composition of the RNA from the different cytoplasmic fractions is similar (Table 20) and this is in accord with the findings of Elson and Chargaff (1951) although the values obtained by these authors do not agree closely with our own. Consequently we have considered it valid to analyse the A_3S fraction of animal tissues and to consider these results representative of the whole RNA. This procedure is at least as satisfactory as the analysis of isolated samples of RNA since the initial stages are similar, and the subsequent procedures much less vigorous.

Specific activity determinations on the nucleotides obtained from an A_3S fraction are open to similar criticisms. In common with other workers we have found a non-uniform uptake of ^{32}P into the RNA of the three cytoplasmic fractions at short intervals after injection (Tables 17,18, 19 and 22). Consequently it is not valid to determine the specific activity of the nucleotides obtained by ionophoresis of the A_3S fraction of whole tissue and to consider them representative of the whole RNA. The same objection of course applies to samples of RNA isolated from heterogeneous material.

It would seem therefore that only so long as the

property being studied can be demonstrated to be homogeneously distributed throughout the starting material is it justifiable to apply procedure 3 to its study.

4.5 Cytoplasmic Fractionation Procedures.

It will be seen from Table 15 that the distribution of RNA throughout the three cytoplasmic fractions obtained by Claude's (1946) procedure is comparable with that found by other authors by several methods (Table 2). At the same time, the RNA content of the nuclear fraction (Table 15) is very high, indicating contamination of the nuclear sediment with whole cells, mitochondria or both. There is, moreover, in the mitochondrial fraction a measurable quantity of DNA which although small nevertheless provides definite evidence that some of the nuclei have been destroyed in the course of homogenisation. The extent of contamination of the mitochondrial RNA with RNA from the nucleus may be estimated on the assumption that the ratio of RNA:DNA in the nucleus is about 1:3, and on this basis contamination is very slight. Several varieties of homogeniser and of homogenisation techniques have been used in an attempt to obtain maximum rupture of whole cells with minimum destruction of nuclei, but it has proved quite impossible to produce a homogenate containing simultaneously no whole cells and no broken nuclei. The results obtained by several authors (Table 2) indicate that they have achieved

more satisfactory conditions of homogenisation than ours, since none of them find any DNA in the cytoplasmic fractions, and at the same time there is little evidence of contamination of the nuclear fraction with RNA from cytoplasmic sources. Homogenisation within such narrow limits must indeed be a difficult task.

Electron micrographs of the mitochondrial and microsome fractions obtained from saline homogenates (Figs.18a,b) of rat liver make it clear that the mitochondria separated from such homogenates are grossly agglutinated and contaminated with microsomes. Similarly, the microsome fraction is agglutinated and liable to sediment along with the mitochondria.

Corresponding electron micrographs of mitochondria and microsomes prepared from 0.25M-sucrose homogenates of rat liver (Figs.19a,b) show that there is much less evidence of agglutination, and no obvious contamination of one fraction with the other. Clearly, the sucrose procedure is desirable for any studies in which it is necessary to compare the properties of one of the cytoplasmic fractions with another.

4.6 The Incorporation of Isotopes into the RNA of the Cytoplasmic Constituents.

In view of our findings concerning the contamination of the Schmidt and Thannhauser (1945) ribonucleotide

fraction with non-nucleotide phosphates, the results expressed in Table 16 for the specific activities of the A_2S fractions from the three cytoplasmic constituents are of no absolute value. It is indeed questionable whether they have even a relative worth. We have therefore applied the technique of ionophoresis to the separation of the nucleotides from the ribonucleotide (A_2S) fraction obtained from the cytoplasmic constituents by application of the modified Schmidt and Thannhauser (1945) procedure.

The results obtained (Tables 17,18 and 19) make it certain that the nucleotides from the RNA of each of the cytoplasmic constituents are found in association with the previously mentioned concomitants, and that the specific activity of the whole A_2S fraction is no measure of the specific activity of the constituent nucleotides. Even in the case of the isolated RNA (Table 18), the specific activities of the whole materials are considerably in excess of that of any of the constituent nucleotides except in the case of the microsome fraction. It appears therefore that even the procedure of isolating the RNA from a sample of tissue provides no guarantee of its purity, and that it is necessary to separate the RNA of the tissue into its constituent ribonucleotides.

The difficulty of removing all these contaminating substances from the nucleotides is illustrated in Tables 18,

and 19 from which it will be seen that ionophoresis certainly provides adequate purification of adenylic, guanylic and cytidylic acids, but that the uridylic acid is not completely free from non-nucleotide phosphates. This discrepancy has been overcome by the use of procedure 3 (Table 21) which shows similar activities of all four nucleotides derived from the same material by procedure 3 and by isolation of the RNA followed by ionophoresis. The substance responsible for the contamination of the uridylic acid obtained by ionophoresis of the A_2S fraction has clearly been eliminated by the use of this partial isolation procedure.

Examination of the specific activities of the nucleotides obtained by ionophoresis of the fraction A_2S from each of the cytoplasmic constituents indicates that with the exception of uridylic acid (which we know to be contaminated) adenylic acid exhibits the highest activity while guanylic acid has the lowest. When the true value for uridylic acid (Table 22) is taken into account, this finding still holds, and is in agreement with our findings for whole tissue RNA and also with those of Volkin and Carter (1951b) for several tissues.

The cytoplasmic fractions themselves, whether prepared from saline or sucrose homogenates, exhibit the same

pattern. 2 hr. after injection of ^{32}P, the specific activities of the nucleotides from the microsome fraction are always lowest, while those from the supernatant fraction are invariably highest (Tables 17,18,19 and 22). Jeener (1949a) and Jeener and Szafarz (1950a) obtained comparable results for experiments on the uptake of ^{32}P into the RNA of the cytoplasmic fractions of mouse embryo, chick embryo and pigeon crop gland. Jeener and Szafarz (1950a) however found considerable difference between these growing tissues and resting rat liver in which they found the highest specific activity, 2 hr. after injection of ^{32}P, in the RNA from the non-sedimentable material, the lowest activity being not in the smallest particles but in the largest. The cytoplasmic fractionation technique used by these authors is very different from any of the other methods described. It seems likely that all the mitochondria and a large proportion of what we know as microsomes are sedimented in their first sediment, while the small particles obtained by them consist of the smaller microsomes and still smaller particulate matter not normally sedimented at all. Furthermore, although these authors take considerable trouble to rid the ribonucleotide fraction of any inorganic phosphate before determining radioactivity, they have not found, nor apparently suspected, the presence of contaminat-

ing organic phosphates. Consequently, the accuracy of their results is doubtful, since they have not characterised the cytoplasmic fractions which they were analysing, and since there is almost certainly considerable contamination of their ribonucleotide fraction with organic phosphates of high specific activity.

Barnum and Huseby (1950) using more conventional methods of cytoplasmic fractionation carried out similar investigations at different time intervals after injecting the radioactive phosphate. Recognising the difficulties of contamination of the ribonucleotide fractions obtained from the cytoplasmic constituents, they have isolated the RNA in each case. Once again the greatest uptake of isotope was found to occur in the supernatant RNA, but in this case there was no significant difference between the uptake of ^{32}P into the mitochondrial and microsome fractions. It must be emphasised however that the isolation of the RNA is not sufficient to guarantee freedom from contamination, particularly in the supernatant fraction (Table 18).

Marshak and Calvet (1949) have studied the uptake of ^{32}P into the RNA of the nucleus and of two cytoplasmic particulate fractions obtained by centrifugation of the citric acid supernatant, after the removal of nuclei. They found that the uptake of ^{32}P into the RNA of the most readily sedimentable particles was lower than that into the

RNA of the smaller particles. The nature of the cytoplasmic particles obtained by these workers is highly suspect since it is recognised that acid conditions such as they used bring about aggregation of the cytoplasmic constituents. As a result, no reliance can be placed on the differentiation between the two types of particle claimed by Marshak and Calvet. Moreover, apart from doubts as to the nature of the cytoplasmic particles, no attempt was made to purify the ribonucleotide fraction obtained by the application of the Schmidt and Thannhauser (1945) procedure, so that the results obtained have no absolute value and are of little relative use.

The incorporation of ^{15}N into the purines and pyrimidines of RNA from the cytoplasmic fractions of regenerating rat liver has been studied by Reichard (1950) using essentially the fractionation technique of Hogeboom et al. (1948). He found that the isotope content of any base from each of the cytoplasmic fractions was the same, and reached the very cautious conclusion that the evidence did not preclude the existence of ribonucleic acids of different biological activity within the cytoplasm, but that more sensitive methods of cell fractionation would be necessary to illustrate such differences.

Recently, Hultin, Slautterback and Wessel (1951) have published a brief note on the in vivo incorporation

of ^{15}N and ^{32}P into the ribonucleic acids of chick liver cytoplasmic fractions. In the ^{15}N experiments, it was observed that in three intervals up to 1 hr. after injection of the isotope, uptake was greatest in the RNA from the cell fluid, and lowest in the RNA from the mitochondria. Experiments with ^{32}P, in which an attempt was made to separate the nucleotides by ion exchange chromatography, provided but sketchy results, the authors being unable to measure the uptake of ^{32}P into the adenylic or uridylic acids. The general conclusion derived from these experiments however was that the uptake of isotope into guanylic and cytidylic acids was similar, and that the greatest incorporation was into the RNA from the supernatant fraction, the smallest into the RNA from the mitochondria.

There is clearly some difference of opinion on this matter, but in view of the repeated findings of different specific activities in the ribonucleic acids from the three cytoplasmic fractions at short intervals after injection of ^{32}P, it seems that the ribonucleic acids from these fractions are separate entities, and not manifestations of the same material in different states of aggregation. Before any more definite conclusions can be reached, it will be necessary to characterise the cytoplasmic fractions used, and to obtain from them RNA free from radioactive contaminants.

4.7 The Variation with Time of the Incorporation of Isotopes into the Ribonucleic Acids of the Cytoplasmic Fractions.

Jeener and Szafarz (1950a) have suggested that the RNA of the cell is synthesised within the nucleus, and subsequently passed through the nuclear membrane into the cytoplasmic supernatant whence it is gradually incorporated, firstly into the small particles and then into the large particles. In support of this hypothesis they quote the well established evidence that at short intervals after the injection of ^{32}P the specific activity of the nuclear RNA is much higher than that of any of the cytoplasmic fractions (Marshak, 1948; Marshak & Calvet, 1949; Barnum & Huseby, 1950; Jeener & Szafarz, 1950a; Davidson, McIndoe & Smellie, 1951). They also use as evidence their finding that the specific activities of the ribonucleic acids within the cytoplasm are highest in the non-sedimentable material and lowest in the largest cytoplasmic particles.

In order to test this hypothesis, it was decided to construct a curve showing the variations with time of the specific activities of the nucleotides from the RNA of the three cytoplasmic fractions. Several authors have carried out such studies, but in all cases some essential aspect

has been neglected. Thus Marshak and Calvet (1949) used cytoplasmic particles of dubious nature, and carried out radioactive assays on unpurified ribonucleotide fractions. The work of Barnum and Huseby (1950) is much more reliable, but still leaves some doubt since there is no guarantee that their isolated RNA is free from contamination. Moreover although the curve which they have constructed contains many determined points, the studies have not been carried far enough to estimate the peaks of the curves for the cytoplasmic ribonucleic acids.

In our studies, we have attempted to overcome the shortcomings of the previous workers, in that our fractions have been characterised by electron microscopy, and precautions have been taken to determine the true specific activities of the nucleotides. The time intervals have been chosen to give a fairly rapid indication of the positions of the peaks of the curves, and the relation of the specific activities to that of the blood inorganic phosphate at 2 hr. should eliminate errors due to variations in dosage of isotope.

The results illustrated in Figs. 21, 22 and 23 show that within each of the cytoplasmic fractions, the nucleotides behave in an almost identical fashion. At any particular time interval, there are certainly differences

between the nucleotides, and these are consistent at the earlier time intervals. When these are viewed in relation to the variations in Relative Specific Activities with time, they fall into a lesser perspective. The important feature is that the slopes of the curves for each nucleotide in each fraction follow almost exactly the same pattern, and since the turnover is related to this slope, it may be said that each of the nucleotides within a fraction is turning over at the same rate.

In Fig.24 the variation with time of the Relative Specific Activities of the cytidylic acids from each of the cytoplasmic fractions is plotted on the same graph. Since the four nucleotides in each fraction behave in the same fashion, it is permissible to take one of these and to use it for comparison between the fractions. From Fig.24, it is clear that the RNA of each of the cytoplasmic fractions behaves in exactly the same way in respect of the uptake of ^{32}P, the individual curves being so close as to be virtually superimposed on one another.

This evidence is in direct contradiction to the hypothesis of Jeener and Szafarz (1950a), which would involve a more rapid uptake of ^{32}P into the RNA of the supernatant than into the RNA of the microsomes, and a more rapid incorporation of ^{32}P into the RNA of the microsomes than into that of the mitochondria. Jeener and Szafarz

(1950a), at one time interval, obtained evidence of such an occurrence, but in view of our own findings, their hypothesis is untenable. Their conclusions therefore serve to emphasise the danger of making interpretations from experimental evidence which is not complete.

While our results rule out the possibility of the RNA of the supernatant fraction giving rise to that of the microsome fraction, which in turn gives rise to that of the mitochondria, they do not detract from the hypothesis that nuclear RNA is a precursor of the cytoplasmic RNA. Indeed, the results of Potter, Recknagel and Hurlbert (1951) who studied the uptake of ^{14}C administered in the form of orotic acid lends further support to this suggestion. These authors examined the incorporation of the ^{14}C into the nuclear RNA, the RNA of the mitochondria and that of the remaining supernatant of rat liver. They found that as with ^{32}P the ^{14}C was incorporated most rapidly into the nuclear RNA, the peak of the time curve occurring about 4 hr. after injection of the orotic acid. Uptake of the isotope by the ribonucleic acids of the mitochondrial and supernatant fractions was much slower, and occurred at about the same rate in both cases. Moreover, the peaks of these two curves fall about 20 hr. after injection although the exact positions of the peaks are difficult to

estimate since the next point is at 91 hr. These findings however resemble our own with ^{32}P, and this is of particular interest since it suggests that the nucleotide molecule is turned over as a whole, and that the phosphorus is not metabolised independently of the rest of the molecule.

The homogeneity of the ribonucleic acids from the three cytoplasmic fractions with respect to uptake of isotopes, and composition, poses the question of their uneven distribution throughout the cytoplasmic fractions. It is possible that this provides a key to the problem of function or synthesis, since these may be related to the distribution, but it is certain that further and more intensive research will be necessary before definite conclusions as to the synthesis and function of the ribonucleic acids of the cytoplasm can be achieved.

Summary.

1. A method for the separation of ribonucleotides by ionophoresis on filter paper is described.

2. A procedure for the partial isolation of RNA from animal tissues is described.

3. The technique of ionophoresis has been applied to the analysis of samples of isolated and partially isolated ribonucleic acid from various sources.

4. It has also been applied to the analysis of the ribonucleotide fraction obtained from animal tissues by a modification of the Schmidt and Thannhauser separation procedure.

5. The technique of ionophoresis together with that of partial isolation of the RNA is particularly useful in experiments with radioactive phosphorus, since it enables the ribonucleotides to be isolated free from contamination by non-nucleotide radioactive substances.

6. By a combination of ionophoresis and autoradiography, it has been demonstrated that the ribonucleotide fraction obtained by the Schmidt and Thannhauser procedure contains, in addition to the ribonucleotides, small amounts of inorganic phosphate of high specific activity probably derived from phosphoprotein, and at least 5 other protein bound phosphorus containing substances.

7. The presence of these additional components which amount to about 25% of the ribonucleotide fraction, renders estimates of the RNA content of animal tissues by the Schmidt and Thannhauser procedure high by about 30%.

8. In the cases of animals which have received ^{32}P 2 hr. before killing, the presence of these additional components which have high specific activities makes it impossible to obtain any estimate of the specific activity of the RNA by measurements on the ribonucleotide fraction itself.

9. Specific activity determinations on the nucleotides separated by ionophoresis from the partially isolated RNA and from isolated RNA indicate that adenylic acid exhibits the highest value, while guanylic acid exhibits the lowest.

10. The technique of ionophoresis has been applied to the analysis of the ribonucleic acids from the cytoplasmic fractions. It was found that the composition of each was similar.

11. Ionophoresis of the ribonucleotides from the three cytoplasmic fractions 2 hr. after injection of ^{32}P showed that adenylic acid in each case had the highest specific activity and guanylic acid the lowest.

12. Of the three fractions, the microsome fraction invariably showed the lowest uptake of ^{32}P into the ribonucleotides 2 hr. after injection.

13. It was found that the uptake of radioactive phosphate into each of the ribonucleotides of any of the fractions varied with time in the same fashion.

14. Using cytidylic acid as representative, it was found that the variation with time of the specific activities of the nucleotides from each of the fractions was the same.

Bibliography.

Ada, G.L. 1949
Biochem. J. 45, 422.

Altmann, R. 1894
Die Elementsorganismen und ihre Bezeihungen zu den zellen, Leipzig.

Allen, R.J.L. 1940
Biochem. J. 34, 858.

Bandurski, R.S. & Axelrod, B. 1951
J. biol. Chem. 193, 405.

Barnum, C.P. & Huseby, R.A. 1948
Arch. Biochem. 19, 17.

Barnum, C.P. & Huseby, R.A. 1950
Arch. Biochem. 29, 7.

Beale, R.N., Harris, R.J.C. & Roe, E.M.F. 1950
J. chem. Soc. 1397.

Behrens, M. 1938
Hoppe-Seyl. Z. 253, 185.

Bensely, R.R. 1947
Anat. Rec. 98, 609.

Bensely, R.R. & Hoerr, N.L. 1934
Anat. Rec. 60, 449.

Berg, W. 1934
Z. mikr-anat. Forsch. 36, 146.

Boulanger, P. & Montreuil, J. 1951a
Bull. Soc. Chim. biol. 33, 784.

Boulanger, P. & Montreuil, J. 1951b
Bull. Soc. Chim. biol. 33, 791.

Brachet, J. 1947
Cold. Spr. Harb. Sym. quant. Biol. 12, 18.

Bradfield, J.R.G. 1950
Biol. Rev. 25, 113.

Bredereck, H., Martini, A. & Richter, F. 1941
Ber. dtsch. chem. Ges. 74B, 694.

Brown, G.B., Peterman, M.L. & Furst, S.S. 1948
J. biol. Chem. 174, 1043.

Brown, G.B., Roll, P.M., Plentl, A.A. & Cavalieri, L.F. 1948
J. biol. Chem. 172, 469.

Brues, A.M., Tracey, M.M. & Cohn, W.E. 1944
J. biol. Chem. 155, 619.

Campbell, R.M. & Kosterlitz, H.W. 1949
J. Endocrinol. 6, 171.

Carter, C.E. 1950
J. Amer. chem. Soc. 72, 1466.

Caspersson, T. 1947
Symp. Soc. exp. Biol. 1, 127.

Caspersson, T. & Schultz, J. 1940
Proc. nat. Acad. Sci., Wash. 26, 507.

Chantrenne, H. 1947
Biochim. Biophys. Acta 1, 437.

Chargaff, E., Magasanik, B., Doniger, R. & Vischer, E. 1949
J. Amer. chem. Soc. 71, 1513.

Chargaff, E., Magasanik, B., Vischer, E., Green, C.,
Doniger, R. & Elson, D. 1950
J. biol. Chem. 186, 51.

Claude, A. 1940
Science, 91, 77.

Claude, A. 1941
Cold. Spr. Harb. Sym. quant. Biol. 9, 263.

Claude, A. 1943a
Science 97, 451.

Claude, A. 1943b
Biol. Symp. 10, 111.

Claude, A. 1944a
J. exp. Med. 80, 19.

Claude, A. 1944b
A.A.A.S. Research Conference on Cancer pp. 223.

Claude, A. 1946
J. exp. Med. 84, 51.

Claude, A. 1947
Harvey Lect. 43, 121.

Claude, A. & Fullam, E.F. 1945
J. exp. Med. 81, 51.

Claude, A. & Fullam, E.F. 1946
J. exp. Med. 83, 499.

Cohn, W.E. 1949
Science, 109, 377.

Cohn, W.E. 1950
J.Amer. chem. Soc. 72, 1471.

Consden, R., Gordon, A.H. & Martin, A.J.P. 1944
Biochem. J. 38, 224.

Consden, R., Gordon, A.H. & Martin, A.J.P. 1946
Biochem. J. 40, 33.

Consden, R., Gordon, A.H. & Martin, A.J.P. 1947
Biochem. J. 41, 590.

Cunningham, L., Griffin, A.C. & Luck, J.M. 1950
Cancer Res. 10, 194.

Dalton, A.J., Kahler, H., Kelly, M.G., Lloyd, B.J. & Striebich, M.J. 1949
J. Nat. Cancer Inst. 9, 439.

Danielli, J.F. 1946
Nature, Lond. 157, 755.

Davidson, J.N. 1947
Cold Spr. Harb. Sym. quant. Biol. 12, 50.

Davidson, J.N. 1949
Ann. Rev. Biochem. 18, 155.

Davidson, J.N., Frazer, S.C. & Hutchison, W.C. 1951
Biochem. J. 49, 311.

Davidson, J.N., Gardner, M., Hutchison, W.C., McIndoe, W.M., Raymond, W.H.A. & Shaw, J.F. 1949
Biochem. J. 44, xx.

Davidson, J.N., Gardner, M., Hutchison, W.C., McIndoe, W.M. & Shaw, J.F. 1949
1st. Int. Congr. Biochem. Abstr. p.252.

Davidson, J.N., McIndoe, W.M. & Smellie, R.M.S. 1951
Biochem. J. 49, xxxvi.

Davidson, J.N. & Raymond, W.H.A. 1948
Biochem. J. 42, xiv.

Davidson, J.N. & Waymouth, C.J. 1946
J. Physiol. 105, 191.

Dounce, A.L. 1950
Ann. N.Y. Acad. Sci. 50, 982.

Durrum, E.L. 1950
J. Amer. chem. Soc. 72, 2943.

Durrum, E.L. 1951
Science 113, 66.

Eggleton, P., Elsden, S.R. & Gough, N. 1943
Biochem. J. 37, 526.

Elson, D. & Chargaff, E. 1951
Fed. Proc. 10, 180.

Euler, H. von., Hevesy, G. & Solodowska, S. 1948
Ark. Kemi Min. Geol. 26 A, 1.

Fairley, J.L., Seagram, H.L. & Loring, H.S. 1950
Fed. Proc. 9, 169.

Feulgen, R. & Rossenbeck, H. 1924
Hoppe-Seyl. Z. 135, 203.

Friedkin, M. & Lehninger, A.L. 1949
J. biol. Chem. 177, 775.

Furst, S.S. & Brown, G.B. 1951
J. biol. Chem. 191, 239.

Furst, S.S., Roll, P.M. & Brown, G.B. 1950
J. biol. Chem. 183, 251.

Green, D.E., Loomis, W.F. & Auerbach, V.H. 1948
J. biol. Chem. 172, 389.

Gordon, A.H., Keil, B., Sebesta, K., Knessl, O. & Sorm, F. 1950
Czech. Chem. Com. 15, nos.1, 2.

Hammarsten, E. & Hevesy, G. 1946
Acta physiol. scand. 11, 335.

Hanes, C.S. & Isherwood, F.A. 1949
Nature, Lond. 164, 1107.

Harman, J.W. 1950a
Exp. Cell Res. 1, 394.

Harman, J.W. 1950b
Exp. Cell Res. 1, 381.

Hogeboom, G.H. 1949
J. biol. Chem. 177, 847.

Hogeboom, G.H., Claude, A. & Hotchkiss, R.D. 1946
J. biol. Chem. 165, 615.

Hogeboom, G.H., Schneider, W.C. & Pallade, G.E. 1947
Proc. Soc. exp. Biol. N.Y. 65, 320.

Hogeboom, G.H., Schneider, W.C. & Pallade, G.E. 1948
J. biol. Chem. 172, 619.

Hogeboom, G.H. & Schneider, W.C. 1950a
J. biol. Chem. 186, 417.

Hogeboom, G.H. & Schneider, W.C. 1950b
J. Nat. Cancer Inst. 10, 983.

Holiday, E.R. & Johnson, E.A. 1949
Nature, Lond., 163, 216.

Holmes, B.E. 1949
Brit. J. Radiol. 22, 487.

Hotchkiss, R.D. 1948
J. biol. Chem. 175, 315.

Hull, W. & Kirk, P.L. 1950a
J. gen. Physiol. 33, 325.

Hull, W. & Kirk, P.L. 1950b
J. gen. Physiol. 33, 335.

Hull, W. & Kirk, P.L. 1950c
J. gen. Physiol. 33, 343.

Huseby, R.A. & Barnum, C.P. 1950
Arch. Biochem. 26, 187.

Jeener, R. 1949a
Nature, Lond. 163, 837.

Jeener, R. 1949b
Bull. Soc. Chim. biol., Paris, 31, 731.

Jeener, R. & Szafarz, D. 1950a
Arch. Biochem. 26, 54.

Jeener, R. & Szafarz, D. 1950b
Experientia, 6, 59.

Jorpes, E. 1934
Biochem. J. 28, 2102.

Kennedy, E.P. & Lehninger, A.L. 1948
J. biol. Chem. 172, 847.

Kennedy, E.P. & Lehninger, A.L. 1949
J. biol. Chem. 179, 957.

Kerr, S.E., Seraidarian, K. & Wargon, M. 1949a
J. biol. Chem. 181, 761.

Kerr, S.E., Seraidarian, K. & Wargon, M. 1949b
J. biol. Chem. 181, 773.

Kielley, R.K. & Schneider, W.C. 1950
J. biol. Chem. 185, 869.

Lagerstedt, S. 1949
Acta. Anat., Suppl. 9.

Lehninger, A.L. & Kennedy, E.P. 1948
J. biol. Chem. 173, 753.

Lepage, G.A. & Heidelberger, C. 1951
J. biol. Chem. 188, 593.

Lepage, G.A. & Schneider, W.C. 1948
J. biol. Chem. 176, 1021.

Levene, P.A. & Bass, L.W. 1931
Nucleic Acids. Chemical Catalog Co., New York.

Magasanik, B. & Chargaff, E. 1951
Biochim. Biophys. Acta. 7, 396.

Magasanik, B., Vischer, E., Doniger, R., Elson, D. & Chargaff, E. 1950
J. biol. Chem. 186, 37.

Markham, R. & Smith, J.D. 1949a
Nature, Lond. 163, 250.

Markham, R. & Smith, J.D. 1949b
Biochem. J. 45, 294.

Markham, R. & Smith, J.D. 1950
Biochem. J. 46, 513.

Markham, R. & Smith, J.D. 1951
Biochem. J. 49, 401.

Marrian, D.H., Spicer, V.L., Balis, M.E. & Brown, G.B. 1951
J. biol. Chem. 189, 533.

Marshak, A. 1948
J. cell. comp. Physiol. 32, 381.

Marshak, A. & Calvet, F. 1949
J. cell. comp. Physiol. 34, 451.

Marshak, A. & Vogel, H.J. 1950a
Fed. Proc. 9, 85.

Marshak, A. & Vogel, H.J. 1950b
J. cell. comp. Physiol. 36, 97.

Mathison, G.C. 1909
Biochem J. 4, 233

Miescher, F. 1897
Die histochemischen und physiologischen Arbeiten.
Leipzig.

Mitchell, P. & Moyle, J. 1951a
J. gen. Microbiol. 5, 966.

Mitchell, P. & Moyle, J. 1951b
J. gen. Microbiol. 5, 981.

Muntwyler, E., Seifter, S. & Harkness, D.M. 1950
J. biol. Chem., 184, 181.

Novikoff, A.B., Podber, E. & Ryan, J. 1950
Fed. Proc., 9, 210.

Omachi, A., Barnum, C.P. & Glick, D. 1948
Proc. Soc. exp. Biol., N.Y., 67, 133.

Pallade, G.E. & Claude, A. 1949
J. Morph., 85, 35.

Partridge, S.M. 1949
Nature, Lond. 164, 443.

Peacock, P.R. 1933
J. Path. Bact. 36, 141.

Plentl, A.A. & Schoenheimer, R. 1944
J. biol. Chem. 153, 203.

Potter, V.R. & Elvehjem, C.A. 1936
J. biol. Chem. 114, 495.

Potter, V.R., Recknagel, R.A. & Hurlbert, R.B. 1951
Fed. Proc. 10, 646.

Price, J.M., Miller, E.C. & Miller, J.A. 1948
J. biol. Chem. 173, 345.

Price, J.M., Miller, E.C., Miller, J.A. & Weber, G.M. 1949a
Cancer Res. 9, 96.

Price, J.M., Miller, E.C., Miller, J.A. & Weber, G.M. 1949b
Cancer Res. 9, 398.

Price, J.M., Miller, E.C., Miller, J.A. & Weber, G.M. 1949c
Cancer Res. 10, 18.

Reichard, P. 1950
Acta chem. scand. 4, 861.

Schein, A.H., Podber, E. & Novikoff, A.B. 1950
Fed. Proc. 9, 224.

Schmidt, G. & Thannhauser, S.J. 1945
J. biol. Chem. 161, 83.

Schmidt, G., Cubiles, R. & Thannhauser, S.J. 1947
Cold Spr. Harb. Sym. quant. Biol. 12, 161.

Schneider, W.C., 1946a
J. biol. Chem. 164, 241.

Schneider, W.C., 1946b
J. biol. Chem. 165, 585.

Schneider, W.C. 1946c
Cancer Res. 6, 685.

Schneider, W.C. 1947
Cold Spr. Harb. Sym. quant. Biol. 12, 211.

Schneider, W.C. 1948
J. biol. Chem. 176, 259.

Schneider, W.C., Claude, A. & Hogeboom, G.H. 1948
J. biol. Chem. 172, 451.

Schneider, W.C. & Hogeboom, G.H. 1950a
J. biol. Chem. 183, 123.

Schneider, W.C. & Hogeboom, G.H. 1950b
J. Nat. Cancer Inst. 10, 969.

Schneider, W.C., Hogeboom, G.H. & Ross, H.E. 1950
J. Nat. Cancer Inst. 10, 977.

Schneider, W.C. & Potter, V.R. 1949
J. biol. Chem. 177, 893.

Sevag, M.G., Lackman, D.B. & Smolens, J. 1938
J. biol. Chem. 124, 425.

Smith, J.D. & Markham, R. 1950
Biochem. J. 46, 504.

Thorell, B. 1947
Cold Spr. Harb. Sym. quant. Biol. 12, 247.

Vischer, E. & Chargaff, E. 1947
J. biol. Chem. 168, 781.

Vischer, E. & Chargaff, E. 1948a
J. biol. Chem. 176, 703.

Vischer, E. & Chargaff, E. 1948b
J. biol. Chem. 176, 715.

Vischer, E., Magasanik, B. & Chargaff, E. 1949
Fed. Proc. 8, 263.

Volkin, E. & Carter, C.E. 1951a
J. Amer. chem. Soc. 73, 1516.

Volkin, E. & Carter, C.E. 1951b
J. Amer. chem. Soc. 73, 1519.

Warburg, O. 1913
Pflug. Arch. 154, 599.

Wyatt, G.R. 1951
Biochem. J. 48, 584.