Studies on the Purine and Pyrimidine Derivatives in Animal Tissues and in Cell Nuclei.

by

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CONTENTS.

ABBREVIATIONS.

The following abbreviations will be used in this thesis:-

DNA	Deoxyribonucleic Acid.
RNA	Ribonucleic Acid.
AMP	Adenosine-5'-monophosphate.
ADP	Adenosine-5'-diphosphate.
ATP	Adenosine-5'-triphosphate.
GMP	Guanosine-5'-monophosphate.
GDP	Guanosine-5'-diphosphate.
GTP	Guanosine-5'-triphosphate.
IMP	Inosine-5'-monophosphate.
XMP	Xanthosine-5'-monophosphate.
DPN	Diphosphopyridine nucleotide.
TPN	Triphosphopyridine nucleotide.
FAD	Flavin adenine dinucleotide.
FMN	Flavin mononucleotide.
CoA	Co-enzyme A.
GDPM	Guanosine diphosphate mannose.
CMP	Cytidine-5'-monophosphate.
CDP	Cytidine-5'-diphosphate.
CTP	Cytidine-5'-triphosphate.

UMP	Uridine-5'-monophosphate.
UDP	Uridine-5'-diphosphate.
UTP	Uridine-5'-triphosphate.
UDPG	Uridine diphosphate glucose.
UDPAG	Uridine diphosphate N-acetylglucosamine
UDPGA	Uridine diphosphate glucuronic acid
PRPP	5-Phosphoribosyl pyrophosphate.
TRIS	Tris-(hydroxymethyl)-aminomethane.
CN	Nuclei isolated in citric acid.
SN	Nuclei isolated in sucrose media.
NAN	Nuclei isolated in non-aqueous solvents
N.U.V.	Ninhydrin-positive and ultraviolet-ligh absorbing material.

SECTION I.

INTRODUCTION.

INTRODUCTION.

1.1 The Nucleic Acids.

It is now some time since Friedrich Miescher (1844-1895) brought to the attention of the scientific world the existence of a new phosphorus compound which he discovered in pus cell nuclei. The new compound was named by him "nuclein" and it is now known that it was in fact nucleoprotein. Miescher continued his studies using salmon sperm as a source of nuclear material and showed that the isolated sperm heads contained an acidic compound now recognised as nucleic acid. Subsequently it was made clear by reports from several laboratories both in Europe and America that nucleic acids are normal constituents of living cells, plant and animal, and that they can be divided into two main classes - the ribonucleic acids (RNA) and the deoxyribonucleic acids (DNA). RNA is found mainly in the cell cytoplasm, but it is known that the nucleus contains a small amount also. DNA, on the other hand, is exclusively a nuclear component.

The nucleic acids are large molecules built up from small units called nucleotides, so that the complete nucleic acid molecule could be termed a polynucleotide. Studies on the products of hydrolysis of nucleic acids have shown that

each nucleotide of RNA is composed of one molecule of ribose, one of phosphoric acid and one of a purine or pyrimidine base (adenine, guanine, cytosine or uracil). Each of the component nucleotides of DNA contains one molecule of deoxyribose, one of phosphoric acid and one of adenine, guanine, cytosine or thymine. 5-Methyl cytosine and 5-hydroxymethyl cytosine have also been identified in the hydrolysis products of certain deoxyribonucleic acids.

Mono-nucleotides of adenine, guanine, cytosine and uracil can be obtained from RNA by mild chemical hydrolysis and also by enzymic hydrolysis. Hydrolysis of RNA by dilute alkali yields two isomers of each nucleotide, the 2'-phosphate and the 3'-phosphate (Cohn, 1950, 1951) while hydrolysis of RNA with ribonuclease under conditions which do not permit the action of 5'-phosphatases yields the ribonucleoside-5'-phosphates (Cohn and Volkin, 1951).

1.2 The Free Nucleotides of Living Tissues.

Apart from the nucleic acids, other purine and pyrimidine derivatives are known to exist in living tissues. These compounds are in general ribonucleoside-5'-phosphates which may be obtained by brief extraction of the tissues with cold, dilute acid. The first nucleotide to be isolated from tissue extracts was inosine-5'-phosphate (IMP) which

was obtained from meat by Liebig in 1847. However, it is now believed to have arisen, in part at least, from the deamination of adenosine-5'-phosphate (AMP) which was found to exist in the free state in skeletal muscle tissue (Embden and Zimmermann, 1927; Embden and Schmidt, 1929) along with adenosine-5'-triphosphate (ATP)(Lohmann, 1929; Fiske and Subbarow, 1929). The occurrence of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) in various tissues has been known for about twenty years. H. von Euler and his collaborators between 1930 and 1937 were chiefly responsible for working out the structure of the two dinucleotides, each of which contains an AMP residue, but it was Kornberg who finally settled the question of the positions of the phosphate groups in the TPN molecule (Kornberg, 1950; Kornberg and Pricer, 1950). Another compound, flavin adenine dinucleotide (FAD), was isolated from sheep kidney in 1938 by Warburg and Christian. Like DPN and TPN, it is a coenzyme concerned with hydrogen transport, and contains an adenosine-5'-phosphate residue in its structure. The recently discovered coenzyme A (Lipmann, 1945) was also shown to contain adenosine-5'-phosphate (Baddiley and Thain, 1951).

In the earlier years, then, the only nucleotides known to occur in the free state in living organisms were the various adenosine-5'-phosphate derivatives mentioned in the previous paragraph. Corresponding compounds containing

the other naturally-occurring purine and pyrimidine bases had not been found until 1950 when uridine diphosphate glucose (UDPG) was discovered in yeast by Caputto, Leloir, Cardini and Paladini, and reported to be the coenzyme of the enzyme "galactowaldenase" which is involved in the interconversion of glucose-1-phosphate and galactose-1-phosphate. UDPG probably consists of a mixture of uridine diphosphate glucose and uridine diphosphate galactose (Smith and Mills, 1954b; Hurlbert and Potter, 1954). Since the discovery of UDPG in 1950 there has been a deluge of information concerning the isolation of many other new nucleotides from extracts of living cells.

The rapid advances made in this field owed their success largely to the advent of a new biochemical tool - ion exchange chromatography - which, combined with the technique of paper partition chromatography, made possible the isolation and identification of many new nucleotides. The technique of ion exchange chromatography as applied to the separation of nucleotides was developed by W.E. Cohn during the course of studies on the structure of RNA. Cohn (1951, 1955) has given extremely lucid accounts of the theoretical and practical aspects of fractionation of nucleotides by chromatography on ion exchange columns.

The isolation of many hitherto unrecognised nucleotides which rapidly followed the discovery of UDPG in yeast

(Caputto et al., 1950) is particularly vividly illustrated by many reports concerning the identification of uridine diphosphate linked through the pyrophosphate group to another phosphate group or to non-nucleotide groups other then glucose. Uridine-5'-diphosphate acetylglucosamine (UDPAG) was found in ethanol extracts of yeast (Cabib, Leloir and Cardini, 1953) together with DPN, AMP, UMP, IMP, GMP, ADP and UDPG, and also in rat liver extracts (Hurlbert and Potter, 1954). UDPG, UDPAG, UMP, UDP and UTP are known to exist in guinea pig liver (Smith and Mills, 1954a) and UDPG has been found in mammary gland (Smith and Mills, 1954b; Manson, 1956). Smith and Mills (1954a) and Storey and Dutton (1955) isolated UDP-glucuronic acid (UDPGA) from liver, and Pontis (1955) found UDP-galactosamine in the same tissue. Park, in 1952, drew attention to the occurrence of three UDP-derivatives in extracts of Staphylococcus aureus which had been grown in the presence of penicillin. All of these compounds contained UDP linked to an N-acetyl-amino-sugar while one was combined with L-alanine and another with a peptide composed of one molecule of L-lysine, one of D-glutamic acid and three of alanine. Very recently the occurrence of uridine diphosphate derivatives has been extended further by the isolation of UDP-galacturonic acid from a Type I pneumococcus (Smith, Mills and Harper, 1957c)

and of UDPG, UDPGA and UDPAG from certain other pneumococcal organisms (Smith, Mills and Harper, 1957b).

Most of the uridine nucleotides mentioned above are linked through the pyrophosphate groups to non-nucleotide substituents in the same way as in the adenosine coenzymes. Examples of this type of nucleotide were extended to cytidine when it was discovered that Lactobacillus arabinosus contained cytidine-5'-monophosphate together with related compounds (Baddiley and Mathias, 1954) which were shown to be CDP-ribitol and CDP-glycerol (Baddiley, Buchanan, Carss, Mathias and Sanderson, 1956). Similar CDP compounds (CDP-choline and CDP-ethanolamine) have been found by Kennedy and Weiss (1955; 1956) in liver and yeast. Guanosine diphosphate mannose (GDPM) has been demonstrated to occur in yeast (Gabib and Leloir, 1954) and in Penicillium chrysogenum (Ballio, Casinovi and Serlupi-Crescenzi, 1956). All four of the RNA bases are therefore known to occur in this type of combination in extracts of living organisms.

Further work involving the fractionation of preparations of ATP from rabbit muscle and from yeast revealed the presence of the triphosphates of guanosine and uridine (Bergkvist and Deutsch, 1953; Lipton, Morell, Friedon and Bock, 1953). The isolations of an adenosine tetraphosphate (Marrian, 1954) and an adenosine pentaphosphate (Sacks, 1955) from commercial preparations of ATP have been

reported. Many other reports of the isolation of soluble nucleotides from living cells have appeared, some of the most important of which have come from Potter and his colleagues in Wisconsin. They developed a system of ion exchange chromatography in which the elution of compounds from the column is effected by a gradually increasing concentration of eluting agent. The technique is termed "Gradient elution chromatography" and was first applied to the separation of the acids of the citric acid cycle (Busch, Hurlbert and Potter, 1952) with considerable success, and later to the fractionation of the acid-soluble nucleotides from several rat tissues (Schmitz, Hurlbert, Potter and White, 1954). Detailed accounts of the analyses of the free nucleotides of normal rat liver (Hurlbert, Schmitz, Brumm, and Potter, 1954; Schmitz, Hurlbert and Potter, 1954) of yeast (Schmitz, 1954a) of the Flexner-Jobling carcinoma of the rat (Schmitz, Potter and Hurlbert, 1955) and of rat Walker carcinoma (Schmitz, 1954b) were presented. From these and from subsequent reports from other laboratories it seemed that the mono-, di-, and tri-phosphates of cytidine, adenosine, guanosine and uridine existed generally in living tissues together with DPN, TPN, IMP and several UDP-derivative

Much less information is available concerning the existence of acid-soluble deoxyribonucleotides corresponding

to the ribonucleotides described above. Kanazir (1954) presented evidence for the presence of thymidylic acid (TMP) in the acid-soluble fraction of Escherichia coli which was exposed to ultraviolet irradiation, and Daoust and Cantero (1954) found the same compound in acid extracts of liver. More recently, however, Potter and Schlesinger (1955) reported the occurrence of the mono-, di- and tri-phosphates of thymidine and deoxycytidine in cold perchloric acid extracts of calf thymus tissue. There was no evidence for the existence of purine deoxynucleotides.

The vast amount of information concerning the acid-soluble nucleotides, which has accumulated since 1950, has been due in no small part to the introduction and remarkable success of the technique of ion exchange chromatography on columns.

1.3 The Coenzyme Function of Nucleotides.

The expansion of knowledge concerning the nature of the acid-soluble nucleotides of plant and animal tissues was accompanied by the realisation that many of them operated as coenzymes in certain biological transformations. In the earlier work (1930-1950) only the adenosine-5'-phosphate derivatives were known and had been found to function as coenzymes in hydrogen-transport systems. For example,

Warburg showed that TPN and flavin mononucleotide (FMN) participate in the oxidation of glucose-6-phosphate.

After 1950 and with the introduction of ion exchange chromatography some newly discovered nucleotides were shown to act as coenzymes. The UDPG, which was isolated from yeast (Caputto et al., 1950), was demonstrated by Garner and Crannis (1951) to function as the coenzyme in the enzymic conversion of galactose-1-phosphate to glucose-1-phosphate. The function of the other uridine diphosphate derivatives in enzymic reactions is less clear, but they appear to act as glycosyl donors. It was shown by Munch-Petersen, Kalckar, Cutolo and Smith (1953) that yeast contains a uridyl transfera which catalyses the reaction.

UDPG + pyrophosphate ⟷ UTP + α-glucose-1-phosph

The existence of this transferase was also demonstrated in isolated liver cell nuclei (Smith and Mills, 1954a; Mills, Ondarza and Smith, 1954) and in the lactating mammary gland (Smith and Mills, 1955). It was suggested by Smith and Mills (1955) the uridyl transferase system may be functional in the synthesis of lactose from glucose-1-phosphate. Smith and Mills (1954a) and Storey and Dutton (1955), who isolated UDPGA from liver, presented evidence for the participation of this nucleotide in glucuronide synthesis through a glycosyl transfer reaction.

UDPGA + R-OH ⟶ UDP + R-O-glucuronic acid

Smith, Mills and Harper (1957a; 1957b) have implicated UDP-glycosyl compounds in the biosynthesis of pneumococcal polysaccharides.

Cytidyl transferases have been found in liver and yeast and are responsible for the synthesis of CDP-choline and CDP-ethanolamine from CTP (Kennedy and Weiss, 1956). The CDP-choline and CDP-ethanolamine function in the biosynthesis of lecithin and phosphatidylethanolamine as "activated" forms of phosphorylcholine and phosphoryl-ethanolamine. It is possible that the CDP-glycerol and CDP-ribitol found by Baddiley et al. (1956) in L. arabinosus may in time be shown to possess similar functions to those of the cytidine coenzymes of Kennedy and Weiss (1956).

Sanadi and co-workers, in studies on the enzymic oxidation of α-ketoglutaric acid in a soluble enzyme preparation, found that GDP was a necessary co-factor, operating as the primary phosphate acceptor in the conversion of succinyl-CoA to succinate and CoA (Sanadi, Gibson, Ayengar and Ouellet, 1954; Sanadi, Gibson and Ayengar, 1954; Sanadi, Gibson, Ayengar and Jacob, 1956). It was demonstrated by Keller and Zamecnik (1955) that the incorporation of isotopically labelled amino-acid into liver microsomal protein exhibits a requirement for GTP. A guanosyl transfer reaction similar to those described above for the uridine and

cytidine derivatives has been described by Munch-Petersen (1955).

It is apparent, then, that the function of nucleoside-5'-phosphate compounds as co-factors in enzyme catalysed reactions has been extended from the well-known adenosine nucleotide coenzymes to include nucleotides of cytosine, guanine and uracil and it is becoming increasingly clear that these compounds play an important part in many biosynthetic reactions.

1.4 Biosynthesis of Acid-soluble Purine and Pyrimidine Derivatives.

I. Biosynthesis of Purines and Their Derivatives.

For a long time, it has been known that higher organisms are capable of synthesising purines *de novo* from smaller molecules, but the most important work on the biosynthesis of the purine ring system has been carried out during the last ten years by J.M. Buchanan, and G.R. Greenberg and their colleagues. The work was initiated by studies on the distribution of ^{13}C within the uric acid molecule as excreted by pigeons after the administration of small molecules labelled with ^{13}C.

Buchanan, Sonne and Delluva (1948) worked out a series of reactions for the degradation of the uric acid molecule in such a way that the individual carbon atoms of uric acid

could be isolated and assayed for ^{13}C content. It was thus found that CO_2 was incorporated chiefly into carbon 6 of the purine, the carboxyl group of glycine into carbon 4, and that formate was utilised almost exclusively for carbons 2 and 8 (Sonne, Buchanan and Delluva, 1948; Buchanan et al., 1948). These observations have been amply confirmed by work from other laboratories and it has also been demonstrated that some compounds which can give rise to formate or the "active one-carbon unit" derived from formate (serine, histidine and threonine) are capable of replacing formate as a precursor of positions 2 and 8 of the purine ring (Elwyn and Sprinson, 1950; Sakami, 1948; Sprinson and Rittenberg, 1952; Krasna, Peyser and Sprinson, 1952). Most of the subsequent work on purine biosynthesis was conducted on pigeon liver preparations in which the mechanism of biosynthesis of hypoxanthine was studied. Such preparations contain no xanthine oxidase so that when purine precursors are incubated with the liver systems, hypoxanthine but not uric acid accumulates. G.R. Greenberg, using a pigeon liver system, observed that ^{14}C-formate, ^{14}C-bicarbonate and ^{14}C-glycine could be incorporated into the hypoxanthine molecule (Greenberg, 1948; 1950). Subsequent work with extracts of pigeon liver confirmed that glycine, CO_2 and formate provide the precursor units from which hypoxanthine is synthesised (Greenberg, 1951a; Schulman, Sonne and

Buchanan, 1952), combining in the molar proportions 1:1:2 respectively.

The precursors of the nitrogen atoms of the purine ring have only recently been conclusively established. Shemin and Rittenberg (1947) found that nitrogen 7 of the uric acid molecule was derived from glycine and this was confirmed by Buchanan et al. (1948). Further investigations revealed that the nitrogenous precursor of positions 3 and 9 was the amide group of glutamine and that either glutamic acid or aspartic acid donated the nitrogen of position 1 in hypoxanthine synthesis in extracts of pigeon liver (Somne, Lin and Buchanan, 1953; Somne, Lin and Buchanan, 1956). Recently Buchanan, Flaks, Hartman, Levenberg, Lukens and Warren (1957) conclusively established aspartic acid as the specific donor of nitrogen atom 1.

While the question of the precursors of the purine ring system was being investigated, other relevant and interesting observations were made at the same time. In 1945, Stetten and Fox, working with Escherichia coli grown in the presence of sulphonamides, isolated from the culture medium a diazotisable amine which was later shown by Shive, Ackerman, Gordon, Getzendaner and Eakin (1947) to be 4-amino-5-imidazole carboxamide. On account of the resemblance of this compound to the purines, it attracted much attention in view of the possibility that it might function as a precursor

of the complete purine ring system. For example, it was found to serve as a precursor of rat nucleic acid purines (Miller, Gurin and Wilson, 1950) and of hypoxanthine in pigeon liver preparations (Schulman, Buchanan and Miller, 1950 Furthermore, Schulman and Buchanan (1952) demonstrated that it combined with formate in equimolar proportions to form hypoxanthine. However, Greenberg (1951b) and Schulman and Buchanan (1952) conducted a series of experiments which illustrated that 4-amino-5-imidazole carboxamide itself was not on the direct pathway of purine biosynthesis de novo. In the light of these observations and of evidence from Greenberg (1950, 1951a) that IMP was formed before hypoxanthine in pigeon liver extracts, it became clear that 4-amino-5-imidazole carboxamide ribotide was an intermediate in the synthesis of IMP from small molecule precursors and that IMP was the first purine compound to be formed being converted to inosine and subsequently to hypoxanthine in the liver extracts.

With the discovery of a new intermediate in nucleotide biosynthesis, 5-phosphoribosyl pyrophosphate (PRPP) by Kornberg, Lieberman and Simms (1954, 1955a, 1955b) and Lieberman, Kornberg and Simms (1954), the pathway of formation of IMP began to take shape. PRPP was found to combine with purines in an enzyme-catalysed reaction to form nucleotides according to the general equation

Purine + PRPP $\longleftrightarrow$ Purine nucleoside-5'-phosphate +

PRPP was also shown to be utilised by a purified pigeon liver enzyme system in the presence of glutamine to form 5-phosphoribosylamine (PRA), which could then condense with glycine to give glycinamide ribotide (GAR) (Hartman, Levenberg and Buchanan, 1955, 1956; Goldthwait, Peabody and Greenberg, 1955, 1956; Hartman, 1956). The GAR was then formylated to give formyl-glycinamide ribotide (FGAR) (Goldthwait, Peabody and Greenberg, 1954, 1956; Hartman et al., 1956; Warren and Flaks, 1956). The formate in this reaction was "activated" by a folic acid derivative. The following stage of the reaction sequence was shown by Levenberg and Buchanan (1956) to be the conversion of FGAR to 5-amino-imidazole ribotide (AIR) in the presence of glutamine with the intermediate formation of formyl-glycinamidine ribotide. The AIR was converted to 4-amino-5-imidazole carboxamide ribotide (AICAR) in the presence of CO_2 and aspartate (Lukens and Buchanan, 1956). 4-Amino-5-imidazole (N-succinyl) carboxamide ribotide was tentatively identified as an intermediate in this transformation. AICAR, in the presence of formate and a folic acid derivative, was then converted to formylaminoimidazole carboxamide ribotide, which could then cyclise to form IMP (Buchanan et al., 1956; Warren and Flaks, 1956). Buchanan and Schulman (1953) were the first to demonstrate the involvement of a folic acid derivative in

pigeon liver extracts in studies on the exchange of radio-active formate with carbon 2 of IMP.

The mode of de novo synthesis of IMP has thus been clarified by the use of extracts of pigeon liver. By fractionation of the extracts it has been shown that there are enzymes capable of catalysing many of the above reactions.

Further in vitro work on the formation of AMP and GMP from IMP has revealed the importance of these reactions in vivo in mammalian tissues and will be dealt with later in the "Discussion" section.

II. Biosynthesis of Pyrimidines and Their Derivatives.

The mechanism of biosynthesis of pyrimidines is not as well-defined as for purines. Heinrich and Wilson (1950) found that carbon 2 of rat nucleic acid pyrimidines is derived from CO_2 and that the intact glycine molecule and formate do not serve as precursors of nucleic acid uracil. Formate does, however, serve as a precursor for the 5-methyl group of thymine (Totter, Volkin and Carter, 1951). Wright, Miller, Skeggs, Huff, Wood and Wilson (1951) demonstrated that ureidosuccinic acid could serve as a precursor for the nucleic acid pyrimidines of a strain of Lactobacillus bulgaricus, and subsequently Wood and Wilson (1954) were able to show incorporation of ureidosuccinic acid into the nucleic acid pyrimidines in the rat. A report by Lagerkvist,

Reichard and Ehrensvärd (1951) suggested that the carbon chain of aspartic acid was incorporated into rat liver pyrimidines after deamination.

In 1947, orotic acid became a centre of interest when Mitchell and Houlahan reported that mutants of Neurospora, which require cytidine, uridine or uracil for growth, accumulated orotic acid in the culture medium during growth. After Reichard (1949) had observed that orotic acid could effectively serve as a precursor of nucleic acid purines in the rat, subsequent experiments established orotic acid as an important compound in pyrimidine biosynthesis and suggested that it was synthesised from aspartate by way of ureidosuccinic acid and dihydro-orotic acid (Reichard, 1952; Reichard and Lagerkvist, 1953; Lieberman and Kornberg, 1954; Cooper and Wilson, 1954).

Kornberg and his colleagues (Kornberg et al., 1954, 1955b; Lieberman et al., 1954, 1955) showed that UMP could be formed from orotic acid by yeast enzymes. The reaction took place in two stages:

(a) Orotic acid + PRPP ⟶ orotidine-5'-phosphate

(b) Orotidine-5'-phosphate ⟶ UMP + CO_2

Hurlbert and Reichard (1954) also found that orotic acid could give rise to UMP which was then either degraded to uridine and uracil or phosphorylated to uridine-5'-pyro-

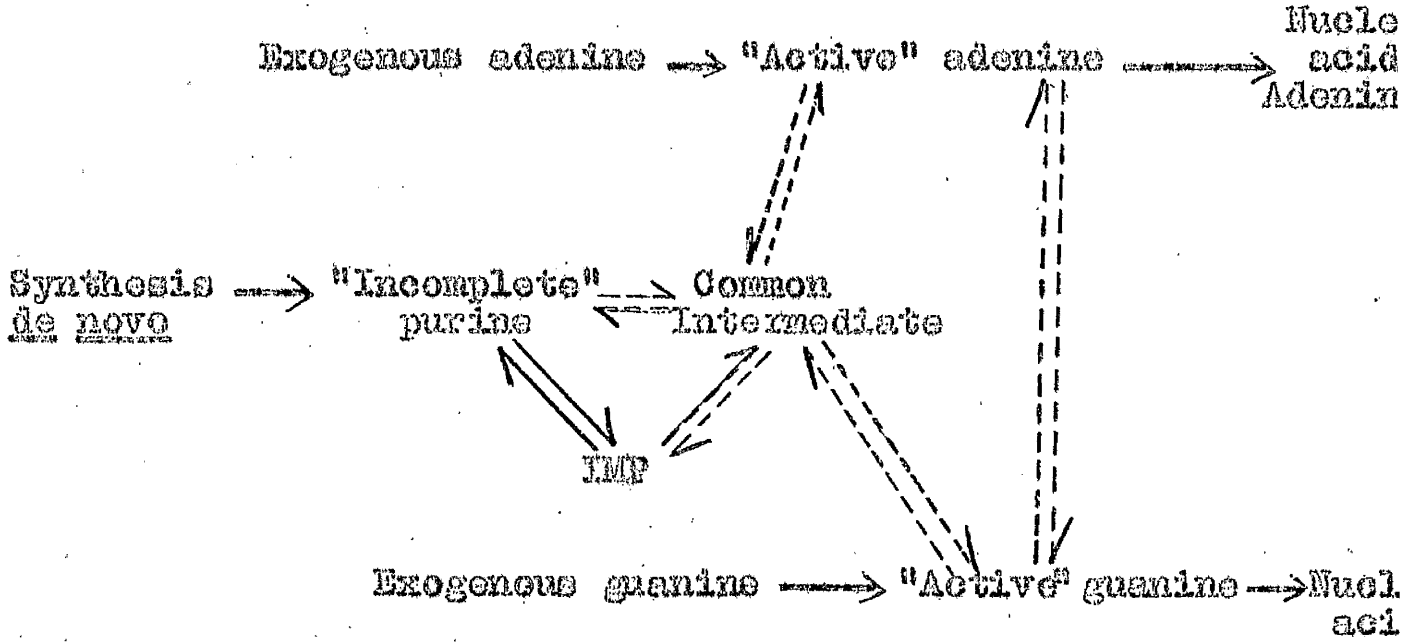
phosphate compounds. Recently Wu and Wilson (1956) provided evidence which strongly favours the hypothesis that ureidosuccinic acid is converted by enzymes in rat liver homogenates to dihydro-orotic acid and orotic acid before nucleotide (or nucleoside) formation and that orotic acid is on the direct pathway of pyrimidine nucleotide synthesis. They claim that the nuclear, mitochondrial and microsomal fractions of the rat liver are responsible for the conversion of ureidosuccinic acid to orotic acid while the cell sap fraction can convert the orotic acid to acid-soluble uracil derivatives.

1.5 Biosynthesis of the Nucleic Acids.

Although a vast amount of information has become available regarding the biosynthesis of the complete polynucleotide unit, much of it is of a somewhat indefinite nature. It is quite clear that animals are capable of synthesising nucleic acids without the aid of exogenous sources of purine and pyrimidine derivative (McCollum, 1909; Ackroyd and Hopkins, 1916). It is now known, of course, that purine and pyrimidine derivatives can be synthesised from small molecule precursors (Section 1.4).

Much work in the field of polynucleotide biosynthesis has been conducted by G.B. Brown and his collaborators in New

York. Many of their experiments have involved utilisation of preformed purines and pyrimidines into nucleic acids. Brown and Roll (1955) have offered a working scheme for purine interrelationships:-



Evidence is available which strongly supports the hypothesis that phosphorylated purine and pyrimidine derivatives occupy a key role in nucleic acid biosynthesis by operating as the building blocks for the polynucleotide. It is known that nucleoside-5'-phosphates exist in the free state in living cells (Section 1.2) and evidence suggesting that they are intimately concerned in nucleic acid biosynthesis has rapidly accumulated in the last few years.

As the adenosine phosphates were the first of the nucleotide series to be recognised in the acid-soluble fraction of living cells, much of the earlier evidence regard-

ing the role of the components of the acid-soluble fraction in nucleic acid biosynthesis was related to the adenine compounds, but with the recognition of phosphates of guanosine, cytidine and uridine in tissue extracts such studies were soon extended.

In 1953, LePage carried out *in vitro* experiments on the cells of the Ehrlich carcinoma, Gardner lymphosarcoma and on mouse liver. He observed that the *in vitro* systems readily incorporated 2-^{14}C-glycine into nucleic acid purines and that the acid-soluble adenine and guanine had a higher order of radioactivity than those of the nucleic acids. A significant feature of his work was that addition of certain purine compounds to the *in vitro* preparations led to dilution of the radioactivity reaching the nucleic acid purines. Adenine, adenosine, AMP, hypoxanthine and IMP were particularly effective as diluting agents. The work of Tyner, Heidelberger and LePage (1953) on rat liver and Flexner-Jobling carcinoma after administration of 2-^{14}C-glycine strongly implicated AMP as a precursor of the adenine of RNA and DNA. Edmonds and LePage (1956) continued the dilution studies in Ehrlich carcinoma cells in some detail, and showed that the effectiveness of a diluting agent depended on its conversion to inosine and IMP which were among the most effective diluents studied. AMP, another powerful diluent, did not however exert its action by prior conversion to inosine or IMP or to adenosine.

The Ehrlich carcinoma cells were also able to convert labelled inosine and IMP to adenine and guanine compounds.

In 1954, Marrian reported that 8-^{14}C-adenine injected into rats is rapidly and extensively incorporated into the AMP, ADP and ATP of the internal organs, thereby confirming the observations made by Goldwasser (1953) on pigeon liver homogenates and by Bennet (1953) with mice. Further work of a similar nature (Bennet and Krueckel, 1955) in which 4,6-^{14}C-adenine was administered to mice, proved that the labelled adenine was extensively utilised for the formation of acid-soluble AMP, ADP, ATP and IMP and for the formation of RNA and DNA in such a manner as to suggest that the acid-soluble nucleotides serve as precursors of the nucleic acids. The results of Bennet and Krueckel (1955) suggest that the "active adenine" of G.B. Brown, mentioned above, may well be in equilibrium with the adenine nucleotide pool of the tissues. It is unlikely that the observations of Weinfeld and Roll (1953) who found that adenosine-2' and 3'-phosphates are twice as effective as adenosine-5'-phosphate in serving as nucleic acid precursors, are of much significance in the search for the proximal precursors of polynucleotides, since the adenosine-5'-phosphate would be greatly diluted by the adenosine-5'-phosphate of the tissues.

Recently, Roll, Weinfeld, Carroll and Brown (1956) administered adenosine-2' and 3'-phosphates and guanosine-2'

and 3'-phosphates, labelled in the purine, ribose and phosphorus moieties, to rats. Determination of the extent of incorporation of the labelled compounds into the visceral nucleic acids revealed that all the nucleotides were extensively dephosphorylated, and that there was no evidence of incorporation of the intact nucleotides. The adenosine of the adenylic acids was the principal unit incorporated into RNA but adenine also appeared to be independently incorporated to a minor extent. Conversion of adenine to guanine took place without cleavage of the riboside. The guanine of the guanylic acid was mainly incorporated after having been split from the rest of the original nucleotide. Leibman and Heidelberger (1955) incubated ^{32}P-labelled ribonucleoside-2', -3', and -5'-phosphates with rat liver and Flexner-Jobling carcinoma slices and with suspensions of Ehrlich ascites cells. A study of the distribution of the isotope in the acid-soluble and nucleic acid fractions proved that the nucleotides were dephosphorylated and that ^{32}P incorporation into the nucleic acids was due to uptake of labelled inorganic phosphate resulting from nucleotide breakdown.

In the light of the finding that the mono-, di- and tri-phosphates of adenosine, guanosine, cytidine and uridine are present in tissue extracts, it became of interest to

ascertain at which level of phosphorylation a nucleotide would become a direct nucleic acid precursor.

The work of Grunberg-Manago, Ortiz and Ochoa (1956) who isolated from Azotobacter vinelandii an enzyme capable of catalysing the synthesis of an RNA-like polynucleotide suggests that the nucleoside-5'-diphosphates are the direct RNA precursors. Hurlbert and Potter (1954), Schmitz et al. (1954) and Brumm, Potter and Siekevitz (1956) carried out in vivo studies on the livers of rats which had received 6-^{14}C-orotic acid, 1-^{14}C-glucose and ^{32}P as inorganic phosphate prior to killing. The results of specific activity determinations on the isolated acid-soluble nucleotides indicated that the labelling of base, ribose and ester phosphate is very rapid and nearly the same for all members of each ribonucleoside series, so that it was not possible to establish the nucleoside diphosphates as the proximal precursors of the RNA.

Heidelberger, Harbers, Leibman, Takagi and Potter (1956) have shown that in a cell-free system prepared from rat liver, adenosine-5'-phosphate can be specifically incorporated into RNA without randomisation of its phosphate group. This is in contrast to the earlier work of Leibman and Heidelberger (1955) and of Roll et al. (1956) mentioned above. It would seem reasonable to suppose that in the cell-free system AMP may be incorporated into RNA by a process

analogous to that found in Azotobacter vinelandii (Grunberg-Manago, et al., 1956). Such a process would not involve any randomisation of the phosphate group of AMP. In the intact animal and in tissue slices on the other hand, AMP is presumably hydrolysed to adenosine and inorganic phosphate before it reaches the site of RNA synthesis. Consequently its phosphate would be expected to be incorporat randomly in the RNA molecule.

In 1952, Hurlbert and Potter showed that orotic acid was a specific precursor of the pyrimidine moieties of the nucleic acids. Two hours after orotic acid administration to rats, the liver was observed to contain about 35 per cent of the injected radioactivity in the form of metabolic derivatives of orotic acid, but no free orotic acid could be detected. 80 per cent of the radioactivity in the liver was originally in the acid-soluble fraction and gradually moved into the acid-insoluble fraction, which included the nucleic acids, where the activity could be accounted for in terms of the pyrimidine bases of RNA. Fractionation of the nucleo-tides in the acid-soluble fraction led to the isolation of radio-active uridine-5'-phosphate (Hurlbert, 1952). Time/ activity studies were then carried out in order to follow the shift of radioactivity from the acid-soluble fraction to the acid-insoluble fraction (Hurlbert, 1953) and evidence was presented that at early times orotic acid was incorporate into acid-soluble UMP, UDP, UTP and their several derivatives

and that the uridine compounds were rapidly interconverted *in vivo* (Hurlbert and Potter, 1954). The uridine phosphates appear to provide a direct precursor for uracil of the nuclear RNA and also to be a major, although possibly indirect, source of the uracil of cytoplasmic RNA, thereby strongly favouring the idea that the acid-soluble nucleotide pool can provide the proximal precursors for the nucleic acids. A cell-free system from rat liver was found to be capable of labelling the RNA uridine moiety when incubated with labelled uridine-5'-phosphate (Potter, Hecht and Herbert, 1956). Hecht and Potter (1956) carried out studies on the pathway of utilisation of orotic acid and cytidine for DNA synthesis *in vivo* in regenerating rat liver. The availability of radioactive precursors for synthesis of DNA was demonstrated many hours after injection of 6-^{14}C-orotic acid into normal and partially hepatectomised rats but labelled deoxyribonucleotides could not be detected in the acid-soluble fraction. That deoxyribonucleotides are, however, possible intermediates in DNA synthesis is supported by the work of Kornberg, Lehman and Simms (1956) with enzymes from *E. coli*. They observed that 2-^{14}C-thymidine was converted by their enzyme system into a polydeoxyribonucleotide and several acid-soluble nucleotides in such a way as to suggest that the thymidine was converted to thymidine-5'-phosphate and to

thymidine-5'-triphosphate from which the immediate polydeoxynucleotide precursor was formed.

1.6 The Present Study.

It was clear that the nucleoside-5'-phosphates in acid extracts of tissues were metabolically important compounds occupying a central role in the biosynthesis of the nucleic acids.

The present study was undertaken in order to investigate the nature of the acid-soluble nucleotides present in tissues which differed greatly with respect to DNA turnover rate, such as liver, intestinal mucosa and appendix, (Smellie, Humphrey, Kay and Davidson, 1955). It was considered possible that these tissues might reveal vastly different acid-soluble nucleotide patterns; for example, such a tissue as appendix with a very brisk DNA turnover might be expected to contain a soluble nucleotide pattern qualitatively and quantitatively different from that found in a tissue like intestinal mucosa in which the DNA renewal is not so active. Moreover, it seemed reasonable to suppose that the soluble nucleotides from appendix might be more active metabolically than those from intestinal mucosa.

At the outset of the present research, very little information was available concerning the soluble nucleotides of the cell nucleus. Since one of the main metabolic

differences between appendix and intestinal mucosa was the difference in DNA turnover rate, and since DNA is exclusively a nuclear constituent, it was considered advisable to compare also the soluble nucleotide complement of the nuclei derived from each of these tissues. Although one might expect nuclei to contain much acid-soluble deoxynucleotide material, work in this department had indicated that acid extracts of cell nuclei contain only negligible amounts of material reacting as deoxypentose (see Kay, Smellie, Humphrey and Davidson, 1956). Nevertheless, it seemed of some importance that information should be obtained concerning the nature and amounts of the soluble nucleotides of cell nuclei and that the results should be compared with those from the corresponding extracts of whole tissue. Furthermore it seemed important to examine the possibility that the nucleotides from nuclei would be metabolically more active than those from the cytoplasm since the RNA of the nucleus is known to be more active than the cytoplasmic RNA in most tissues including appendix and in intestinal mucosa (Smellie, et al., 1955).

It was also considered desirable to ascertain whether the acid-soluble nucleotides are uniformly distributed throughout the cytoplasmic fractions of the cell, as many reports had indicated that the energy generating system of the cell is located in the mitochondria and that the

microsomes do not possess the biochemical apparatus associated with oxidative phosphorylation. For example, the work of Siekevitz (1952) on the incorporation of labelled amino-acid into microsomal protein had suggested that the microsomes contain the enzymes responsible for the incorporation reaction, but that the energy required for the incorporation is provided by the respiratory enzyme systems of the mitochondria.

The most effective method of tackling these problems was undoubtedly to use the technique of ion exchange chromatography on columns used in conjunction with an extended gradient elution system, in order that nucleotides might be well separated from one another and isolated in quantities sufficiently large to permit identification and specific activity determination.

Since the work mentioned in Section 1.4 had implicated IMP as a key compound in nucleotide biosynthesis in vitro, it was hoped that it might be possible to demonstrate purine nucleotide interrelationships, which would confirm (in vivo in a mammalian system) the previous observations on IMP in vitro.

SECTION II.

EXPERIMENTAL.

EXPERIMENTAL.

2.1 Animals.

Rabbits and rats were used as experimental animals. In all experiments involving the use of ^{14}C-formate as a precursor of the purine ring system, young albino rabbits were used. They were killed by cervical dislocation; rats were killed by exsanguination under ether anaesthesia.

2.2 Tissues.

The tissues examined were liver, intestinal mucosa and appendix. Immediately after killing, the tissues were quickly excised and prepared for treatment with acid.

2.3a. Preparation of Acid Extract from Liver.

The liver was cleaned, weighed and minced finely with scissors. The mince, after chilling on ice, was thoroughly homogenised in 1 volume of ice-cold 1.2N $HClO_4$ in an M.S.E. Nelco blendor. The homogenate was centrifuged at 0° for 10 minutes at about 1,000 x g, and the supernatant fluid decanted. The precipitated material was re-extracted with 2 volumes of ice-cold 0.2N $HClO_4$ and centrifuged again at 0°. The combined supernatant fluids, contained in a conical flask surrounded by ice, were neutralised to pH 6 to 7 with 5N KOH using Universal Indicator Paper. The precipitate of $KClO_4$, a sparingly soluble salt, was

centrifuged down at 0° and discarded while the supernatant fluid which was usually pale greenish yellow in colour and opalescent, was filtered through Whatman No. 1 Filter paper, frozen in an ethanol/solid CO_2 mixture, and allowed to stand overnight in a deep-freeze cabinet. The extract was then thawed out in a refrigerator at about 4° and the small amount of $KClO_4$ which came out of solution during the course of this treatment was centrifuged down at 0° and discarded. This reduced the amount of $KClO_4$ in the extract and minimised the risk of applying to columns a perchlorate ion concentration which might interfere with the subsequent anion exchange chromatography.

The extract at this stage was ready for application to an anion exchange resin but was sometimes stored in a deep-freeze cabinet until required. When the volume of the extract was large, it was reduced to a convenient volume by lyophilisation. This further reduced the amount of $KClO_4$ applied to the column.

Liver which could not be processed immediately in the above fashion was packed in solid CO_2 and stored in a deep-freeze cabinet.

2.3b. Preparation of Acid Extract from Appendix.

After excision, the appendix was washed with 0.9% NaCl, freed from fat, weighed and cut up into small portions. The small pieces of appendix were quickly frozen round the inside of a round-bottomed flask, bearing a ground-glass neck,

by revolving the flask in an ethanol/solid CO_2 mixture. The tissue was then dried from the frozen state.

The thoroughly dry material was weighed and powdered finely in a glass mortar. The powder was homogenised in a Potter-Elvehjem type homogeniser in two volumes of ice-cold 0.6N $HClO_4$. After the first centrifugation at 0^0, the precipitated material and the supernatant fluid were further treated in the manner described for liver tissue.

2.3c. Preparation of Acid Extract from Intestinal Mucosa.

After excision, the small intestine was washed through with 0.9% NaCl and the mucosa scraped off the muscle layers with a spatula as quickly as possible. The mucosa was dried from the frozen state and treated with $HClO_4$ as described for appendix.

Each tissue was extracted with $HClO_4$ only two times so that, although the extraction of soluble substances was probably incomplete, the volume of extract and the perchlorate ion concentration were not increased to such an extent that the ensuing ion-exchange chromatography might prove to be troublesome.

2.4 Administration of Radioactive Isotopes.

In some experiments, rabbits were given an intramuscular injection (inside of thigh) of ^{14}C-sodium formate or of ^{32}P as inorganic phosphate, and were killed two hours later. In one experiment a rabbit was injected with

^{14}C-sodium formate intravenously (ear vein) and killed 15 minutes after administration of the isotope.

2.5 Isolation of Nuclei from Non-aqueous Solvents (NAN).

In several experiments, cell nuclei were isolated from rabbit intestinal mucosa and appendix using the method devised in this Department by Kay, Smellie, Humphrey and Davidson (1956). Portions of dried intestinal mucosa and appendix powders were used in these experiments, while the remaining portions of the powders were used for corresponding analyses on the whole tissue acid extracts.

The purified preparations of NAN were weighed and stored in a desiccator until required. Acid extracts of these preparations were made in the manner described in Sections 2.3b and 2.3c.

2.6 Preparation of Subcellular Fractions.

In two experiments, rabbit liver tissue was fractionated into the four subcellular fractions, nuclei, mitochondria, microsomes and cell sap, in order to conduct analyses on the individual cell fractions. In these experiments, the liver was quickly excised, cleaned, and, after mincing finely with scissors, was chilled on ice.

2.6a. Isolation of Nuclei from Sucrose/$CaCl_2$ medium. (sucrose nuclei - SN).

To a portion of the minced liver (about one-third of thTo a portion of the minced liver (about one-thirdded

followed by 8 volumes of 0.25M sucrose/0.0020M $CaCl_2$ and the mixture homogenised in a Potter-Elvehjem type homogeniser for about 2 minutes. After this period a small drop of the homogenate was examined microscopically after staining with 0.1% crystal violet in 0.1M citric acid, to determine the extent of liberation of nuclei. If necessary, homogenisation was continued for a further brief period and the homogenate again inspected microscopically. The homogenate was then centrifuged for 10 minutes at 600 x g. in an International refrigerated centrifuge. The supernatant fluid was decanted and the residue washed twice with 10 ml. of the sucrose/$CaCl_2$ solution at the same speed. The final sediment consisted mainly of liver cell nuclei.

2.6b. Preparation of Mitochondria, Microsomes and Cell Sap.

To a portion of the minced liver (about two-thirds of the whole liver) was added four volumes of 0.25M sucrose. The material was homogenised for two minutes in an M.S.E. Nelco Vendor equipped with a paddle-shaped rotor so as to give maximal disruption of cells with minimal damage to nuclei. When this stage had been reached, as determined by microscopic examination using the crystal violet stain (Section 2.6a), the homogenate was centrifuged at 600 x g. for 10 minutes in the International refrigerated centrifuge. The resulting residue consisted of nuclei, whole cells and debris. The supernatant fluid was decanted into cooled Lusteroid tubes and centrifuged for 10 minutes at 6,000 x g.

in the Spinco ultracentrifuge. The sediment consisted mainly of mitochondria. The supernatant fluid was decanted into cooled Lusteroid tubes and after a further period of centrifugation (60 minutes) at 40,000 x g. was separated into the microsomal (sediment) and cell sap (supernatant) fractions

2.6c. Preparation of Acid Extracts from the Subcellular Fractions.

The four subcellular fractions were treated with $HClO_4$ as described in Section 2.3a. The colours of the nuclear, mitochondrial and cell sap acid extracts were the usual pale greenish yellow, but the nuclear extract was much paler than the other two. The microsomal extract was intensely opalescent, almost white, probably due to the fact that much glycogen was sedimented along with the microsomes.

2.7a. Anion Exchange Chromatography - Preparation of the Resin.

The resin used was Dowex-1, X-10, 200-400 mesh, and was obtained from the Dow Chemical Company in the chloride form.

The extremes of fine and coarse particles were removed by sedimentation in distilled water in 5-litre beakers, and also by straining through a 100-mesh sieve. New resin was washed twice with 2N NaOH and with water until the supernatant fluid was neutral. Much coloured material was removed during this washing. The resin was further washed with acetone (three times), petroleum ether b.pt. 40°-60° (twice), acetone (twice) and with water until acetone vapour

was no longer detectable in the resin. This removed traces of small organic molecules present in the new resin.

The resin was used in the formate form and in order to prepare this, large columns of the resin (20 cm. x 3.3 cm.) were washed with 3M sodium formate until no more chloride ion appeared in the effluent liquid, with 1M ammonium formate in 6N formic acid, and finally with 90% formic acid. The latter washing removed more coloured material (yellow) from the resin, which swelled during the washing but shrank on further washing with distilled water. The resin, after the above treatment, was stored under water in large flasks until required.

Three types of glass tube were used for supporting columns of resin for chromatography, and these are shown in diagrammatic form in Figure 1.

The resin was poured as a thin aqueous suspension into a tube and packed with low air pressure. The column was further washed with about 10 bed-volumes of 1M ammonium formate in 6N formic acid, with about 5 bed-volumes of 90% formic acid and with 5 bed-volumes of water. It was observed that the swelling and shrinking which took place during the latter two washings disturbed the packing of the column and it was found necessary before application of acid extracts to the column to pour the resin from the tube and wash several times in distilled water by decantation from a beaker. Before repacking, the resin was allowed to stand under water for 2-3 hours, after which it was stirred very gently with a

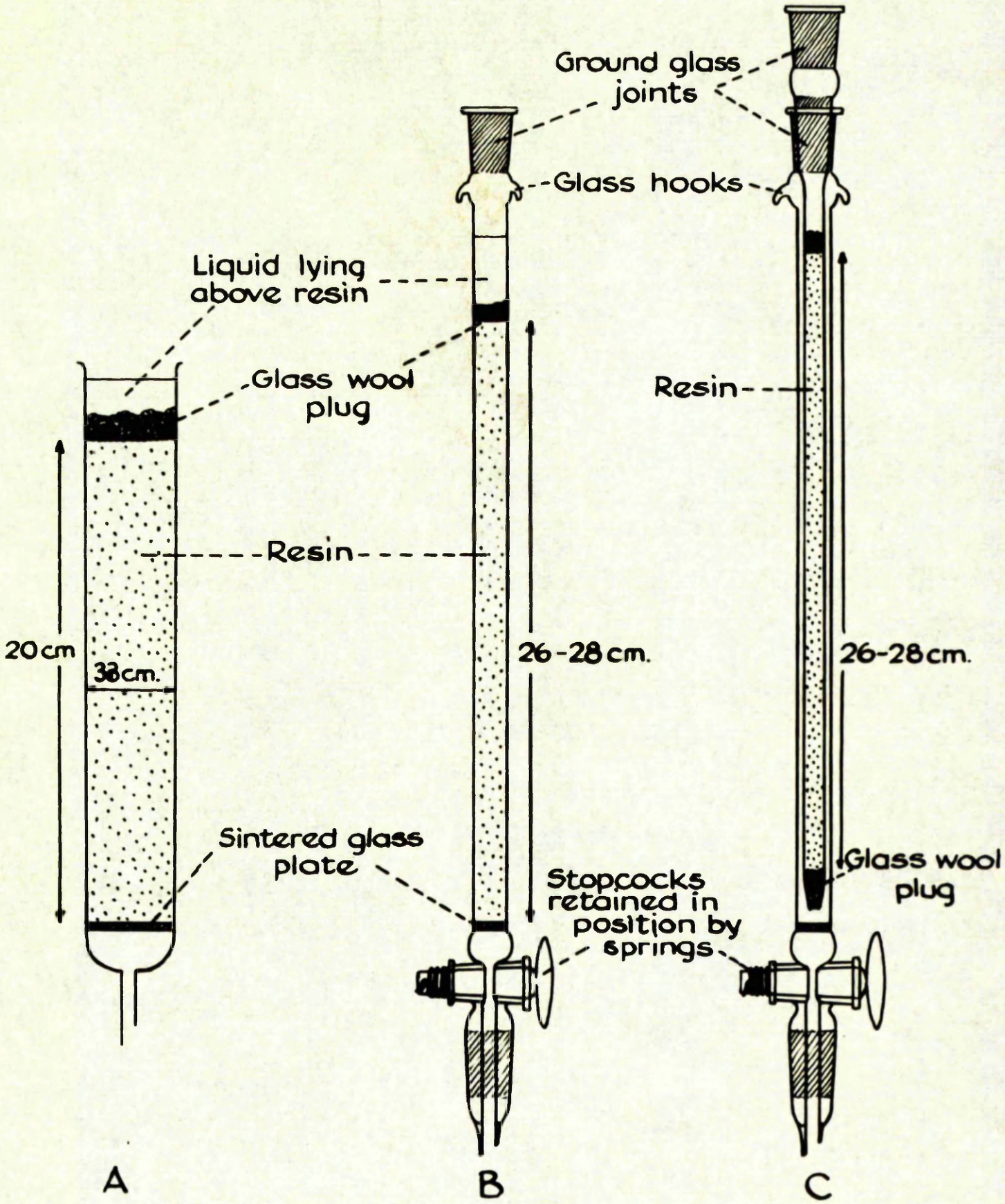

Ground glass joints
Glass hooks
Liquid lying above resin
Glass wool plug
Resin
Resin
20 cm
38 cm.
26-28 cm.
26-28 cm.
Sintered glass plate
Stopcocks retained in position by springs
Glass wool plug
A
B
C

Figure 1.

glass rod to remove air bubbles which had gathered on the surface of the resin layer. This routine ensured satisfactory packing of the column throughout the ensuing ion exchange chromatography. After packing, the surface of the resin was stirred gently and allowed to settle until it was level. Distilled water was passed through the column until the effluent was at the pH of the distilled water (pH 5 to 6), and finally, the surface of the resin, which was always kept under a short column of liquid, was covered with a small plug of glass wool in order to prevent any disturbance of the resin surface during elution.

At this stage, the resin was ready to take an acid extract. This was applied to the column under the pressure of a few cm. of mercury. The reservoir which was used for application of the extract was washed out with distilled water and the washings applied to the column. Distilled water was then passed through the column to remove compounds which were not strongly retained by the resin and when the effluent optical density at 260 mμ approached zero, the water wash was stopped. This effluent fluid was collected as one fraction and was retained for analysis. In some experiments it was collected in fractions and recorded on the flow charts.

2.7b. Anion Exchange Chromatography - Elution.

The apparatus for gradient elution is shown in Figure 2. Glass tubing, with small lengths of rubber tubing to provide flexibility, is used to connect the vessels

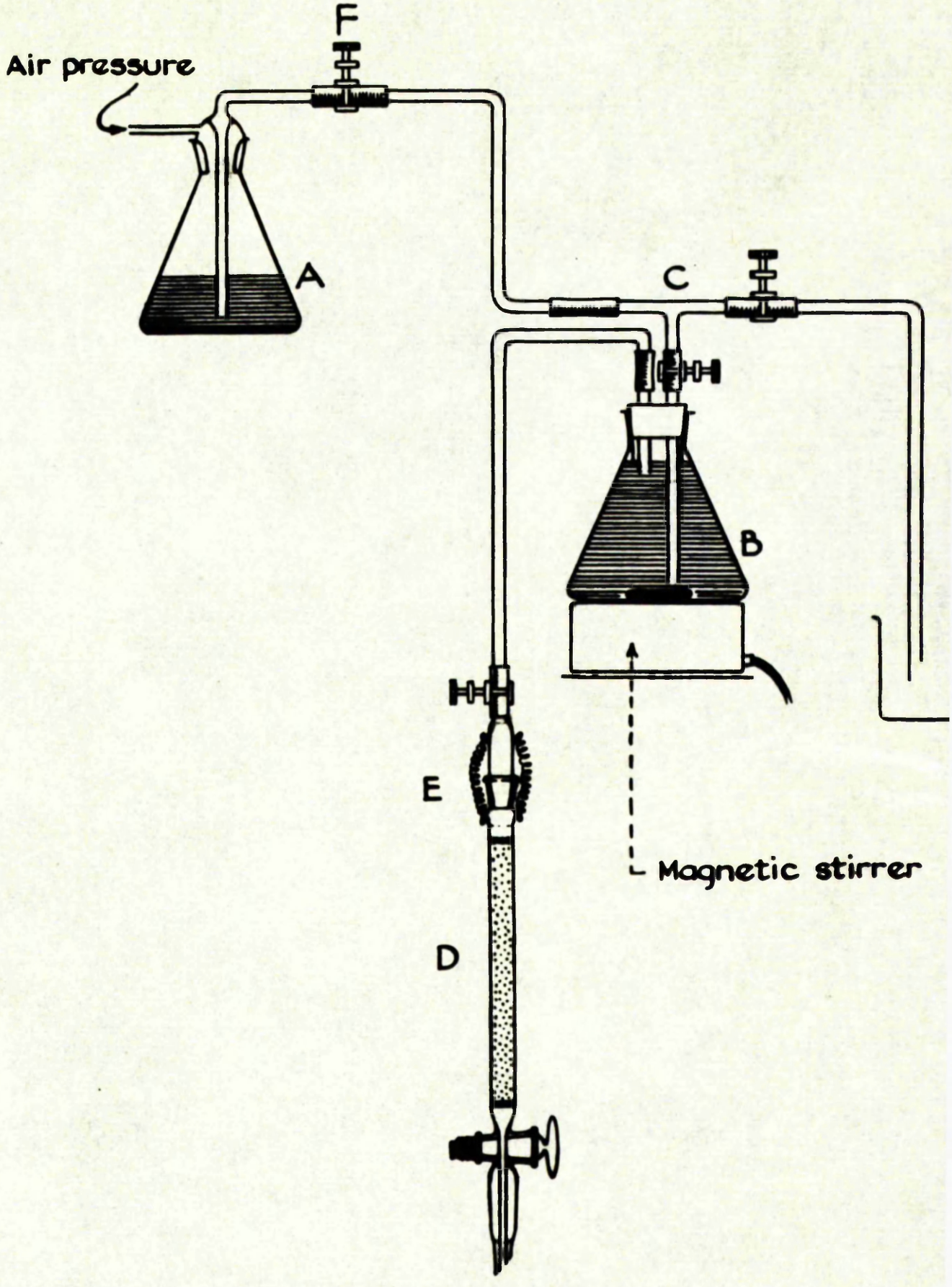


Figure 2.

Ground glass joints were greased lightly with Vaseline. It was found that silicone grease tended to spread throughout any glass system to which it was applied, and as this tendency may also include envelopment and consequent inactivation of resin particles, the Vaseline greasing was used in preference. An additional advantage of the use of Vaseline was the relative ease with which it could be removed from glassware during cleaning.

In Figure 2, the height of reservoir vessel (A) above the column (D) determines the rate of flow of eluting fluid through the resin. The stirrer in the mixing-flask (B) consists either of a piece of iron wire sealed off in a short length of polythene tubing, or of a piece of soft iron enclosed in a glass tube. The latter type of stirrer is further enclosed in a polythene bag to avoid risk of the glass tube or mixing-flask breaking while the stirrer is in operation. The T-piece arrangement at C is included in order to allow all the tubing between the reservoir and mixing-flask to be filled with eluting fluid prior to commencement of elution. A small column of liquid is always kept on top of the resin to avoid any possibility of the resin being forced dry. The ground-glass joint at E is secured with springs attached to glass hooks on the tubing. The screw-clip at F is shut off whenever the reservoir is replaced by another reservoir containing an eluent of greater eluting power. This

prevents introduction of air to the tube system and avoids sudden increases in concentration of fluid in the mixing-flask.

The elution scheme used in these experiments was similar to that employed by Hurlbert, Schmitz, Brumm and Potter (1954) but a number of modifications and improvements were introduced. These are shown in the Results section. The successive concentration ranges of elution power were obtained by substituting a more concentrated eluent for the eluent in the reservoir.

At the commencement of elution, the mixing-flask contained distilled water. The effluent was collected in a Matburn-Locarte or Shandon-Locarte automatic fraction collector equipped with a drop-counting mechanism. The Shandon-type collector was used in conjunction with the small resin-column (C in Figure 1) and takes small test-tubes suitable for the collection of small fractions in a scaled-down system. This apparatus is shown in Figure 3.

Gherkin, Martinez and Dunn (1953) have published an expression describing the variation of eluent concentration in gradient elution chromatography in this closed system. Lakshmanan and Lieberman (1953) have described another apparatus for gradient elution consisting of a dropping funnel which is connected to a mixing chamber provided with a magnetic stirrer. The rates of flow from the dropping funnel and from the mixing chamber are regulated independently

Figure 3.

This open system, however, was used in only one of the present series of experiments.

The elution of compounds absorbing ultra-violet light was followed by reading the optical density of each fraction in a Beckman Model DU spectrophotometer at 260 mμ & 275 mμ, using water in the blank cell. By plotting the optical density at 260 mμ against the tube number, an elution chart was constructed for each separation. These elution charts are shown in the Results section.

The ratio of optical density at 275 mμ (E_{275}) to that at 260 mμ (E_{260}) is characteristic of the various nucleotides (Hurlbert et al., 1954). These authors devised a system of rechromatography on Dowex-1, of peaks obtained in the above system in order to further resolve and characterise compounds eluted in the above system, but it was found in this investigation that the rechromatography was so laborious and time-consuming that it was abandoned in favour of chromatography on paper.

The technique of charcoal adsorption and elution (Buchanan, 1956) was tested for the isolation of peaks from column eluates. This technique, however, gave poor yields of nucleotides eluted with formic acid/ammonium formate, and for concentration of peaks eluted with formic acid solution lyophilisation was found to be the more efficient method.

2.8 Cation Exchange Chromatography.

Separation of amino-acids was carried out on Dowex-50 resin (X-4) according to the method of Moore and Stein (1954).

In order to estimate the amounts of amino-acids present in the column eluates, the quantitative ninhydrin estimation of Cocking and Yemm (1954) was used.

Elution charts with details of elution are shown in the Results section. Under the standard conditions employed it was possible to identify amino-acids from their positions on the elution chart.

2.9a. Chromatography of Nucleotides on Paper.

Compounds which were eluted from Dowex-1 columns with formic acid as the eluting solvent were located on flow charts as described in Section 2.7b, and the appropriate fractions were pooled.

The pooled compounds were dried from the frozen state, dissolved in a small volume of either 50% ethanol or water, and submitted to descending chromatography on paper (Whatman No. 1, washed with 2N acetic acid, and with water until neutral (- Hanes and Isherwood, 1949) using the neutral ethanol/ammonium acetate solvent of Paladini and Leloir (1952) - 7.5 vol. 95% ethanol, 3 vol. M ammonium acetate, pH about 7.5. The lower ends of the papers were serrated to permit even flow of the solvent from the ends of the

papers. Ethylenediamine tetra-acetic acid (versene) was added to the solvent for complexing metal impurities in the paper (Walker and Warren, 1951). This increases the yield of nucleotides and reduces tailing of spots. Ethylene-diamine tetra-acetic acid added to the solvent at a concentration of 10^{-2}M was found to produce satisfactory results (Smith, 1956). R_p values of nucleotides in relation to adenosine ($R_{adenosine}$) are given in Table 1. The separation of compounds in this solvent system has been shown by Paladini and Leloir (1952) to be temperature sensitive, and to allow for variation in Rp values, standard compounds were run with each set of chromatograms.

Spots were located on the chromatograms by inspection in ultra-violet light and the appearance of each chromatogram was recorded in diagrammatic form together with the time of running in the solvent. The behaviour of authentic samples of nucleotides and of certain bases and nucleosides in this chromatographic system were also recorded. Spots were cut out and eluted in small volumes of water or of 0.1N HCl and the eluates stored at 4° for further analyses.

A second solvent system was used for the separation of nucleotides on paper in two dimensions. Solutions of compounds in water were applied to sheets of Whatman No. 1 chromatography paper and run as descending chromatograms in 65% aqueous isopropanol made 2N with respect to HCl (Wyatt, 1951), to about 10 cm. from the ends of the papers. The

TABLE 1.

R-adenosine values of some nucleotides, nucleosides, bases and amino-acids in ethanol/ammonium acetate, pH 7.5.

Substance	R-adenosine
AMP	0.34
ADP	0.14
GMP	0.24
IMP	0.29
CMP	0.39
UMP	0.48
DPN	0.30
Adenosine	1.00
Guanosine	0.88
Inosine	0.92
Hypoxanthine	0.97
Xanthine	0.64
Uric Acid	0.44
Aspartic Acid	0.40
Glutamic Acid	0.45
Glycine	0.57
Glutathione	0.16

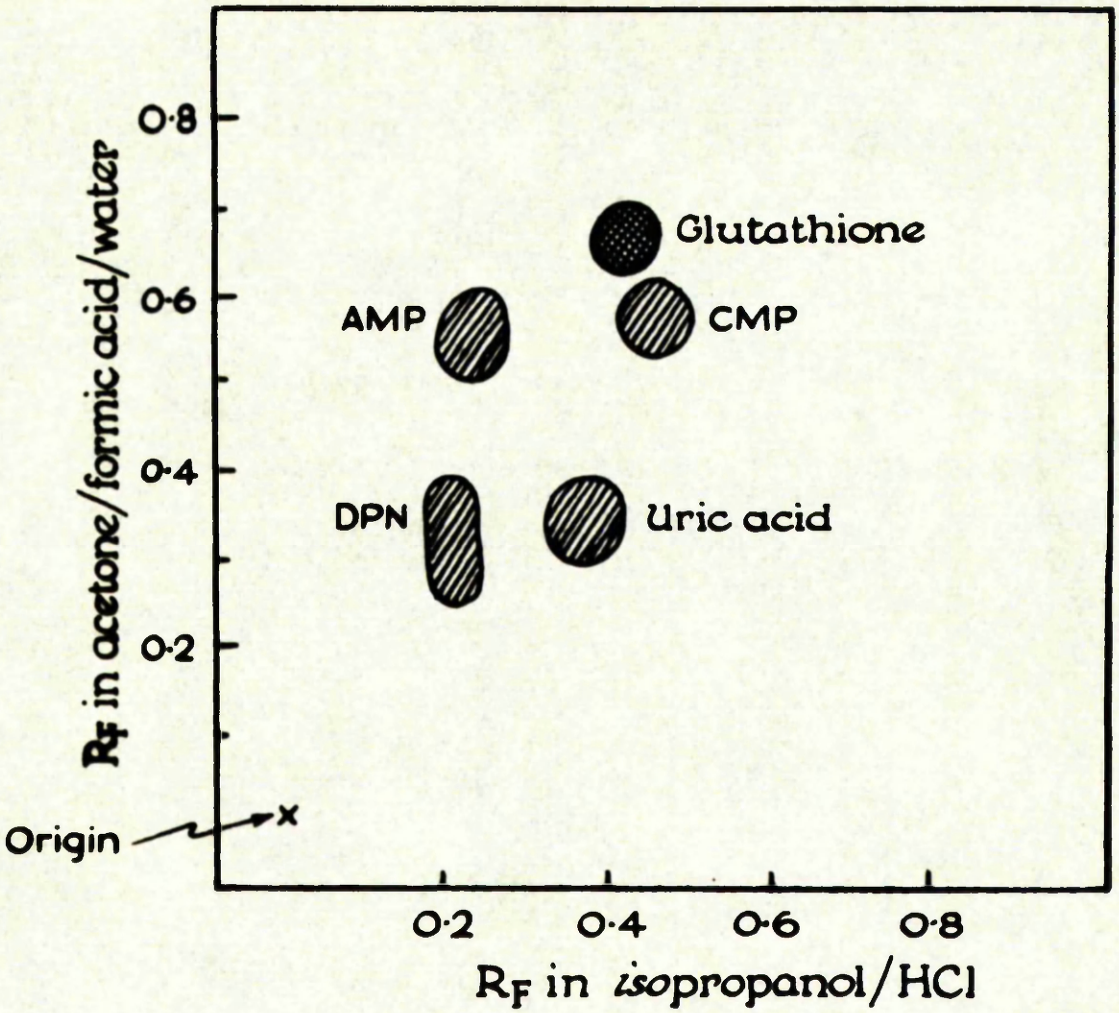

0·8
0·6
0·4
0·2
Origin
x
Glutathione
AMP
CMP
DPN
Uric acid
0·2
0·4
0·6
0·8
RF in acetone/formic acid/water
RF in isopropanol/HCl

Figure 4.

chromatograms were dried in a stream of cold air until all traces of the solvent had been removed. The papers were then run as ascending chromatograms in the solvent described by Burrows, Grylls and Harrison (1952) - acetone : formic acid : water (60 : 14 : 26) - for the separation of phosphoric esters. The location of U.V. absorbing compounds on such two-dimensional chromatograms, are shown in Figure 4.

Blank papers were run with all batches of test chromatograms and the areas corresponding to spots on test papers were cut out and eluted, the eluates being used in the blank cell in the Beckman spectrophotometer.

2.9b. Chromatography of Bases on Paper.

Nucleotides eluted from columns with formic acid as the eluting solvent were submitted to chromatography as described in Section 2.9a. In order to confirm the identification of the nucleotides and in some cases to isolate purine bases for specific activity determinations, the spots were further treated as follows. Portions of the paper eluates were taken to dryness in a vacuum desiccator using H_2SO_4 as the desiccant and the dry material hydrolysed to bases. Nucleotides, which were eluted from columns with formic acid/ammonium formate as the eluting solvent, were pooled and taken to dryness in evaporating dishes on a water-bath at 100°. Ammonium formate sublimed off during this process. The dry material from each pool was dissolved in 1.6 N HCl, transferred to a small tube dried again in a vacuum,

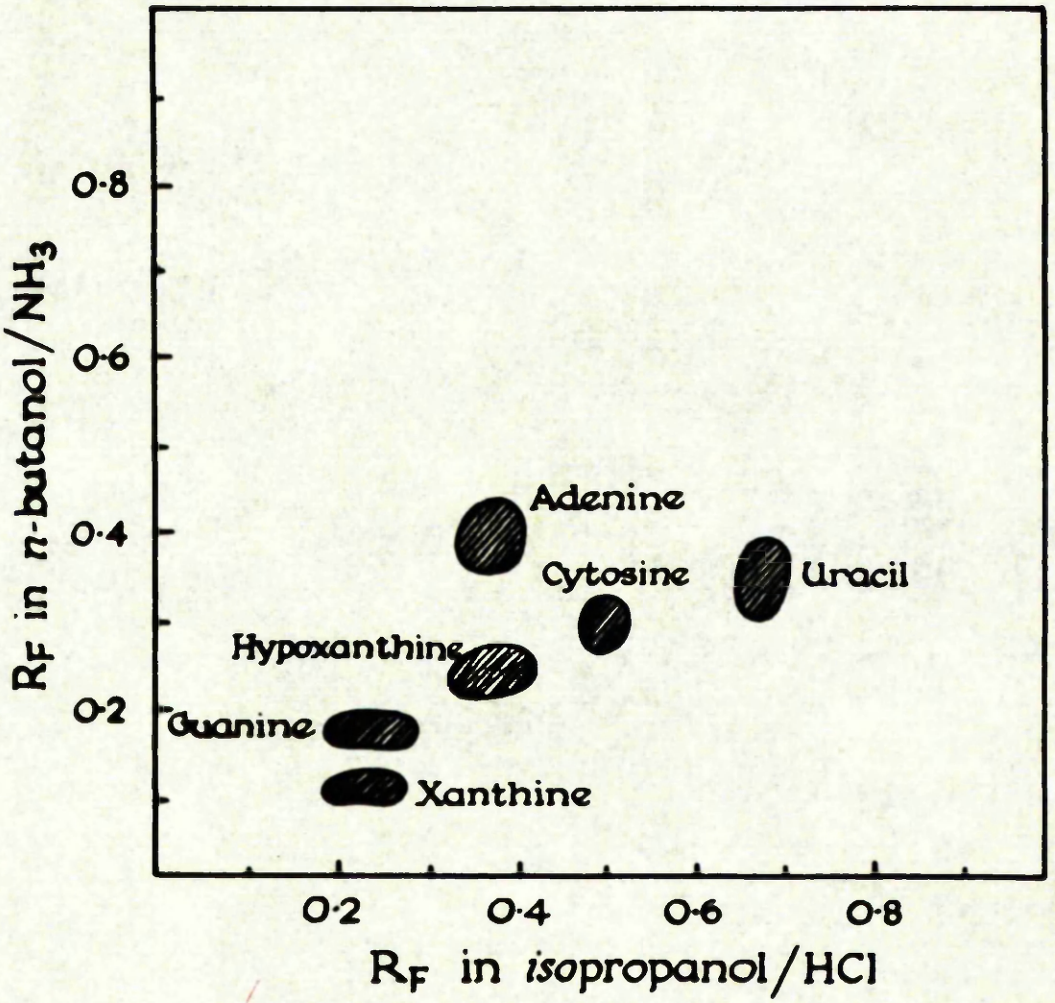

0·8
0·6
0·4
0·2
R_F in *n*-butanol/NH_3
Adenine
Cytosine
Uracil
Hypoxanthine
Guanine
Xanthine
0·2
0·4
0·6
0·8
R_F in *iso*propanol/HCl

Figure 5.

Hydrolysis was carried out as described by Wyatt (1952) in 12N $HClO_4$ for 1 hour at 100°. Each acid hydrolysate was neutralised to pH 7 with 5N KOH and the solution made acid again with two drops of 6N HCl. The precipitated $KClO_4$ was centrifuged down and washed once with about 0.5 ml. 1.6N HCl. The combined supernatant fluids from each hydrolysate were then reduced in volume to about 0.3 ml. in a vacuum desiccator. The $KClO_4$ which came out of solution during this process was centrifuged down and the supernatant fluid was applied to a sheet of Whatman No.1 chromatography paper 12 cm. from the edge on the long axis and 5 cm. from the edge on the short axis.

This routine ensured that the solution was always acid and that guanine, which is less soluble at neutral pH, was kept in solution. The 1.6N HCl wash produced higher yields of base. Also, the precipitation of as much perchlorate ion as possible improved the subsequent chromatography on paper. In preliminary chromatography it was found that the presence of $HClO_4$ in the hydrolysate applied to the paper tended to saturate the paper, and, when warm air was used to hasten evaporation, the paper was frequently charred and destroyed at the point of application.

The chromatograms were run in the descending solvent of Wyatt (1951), 65% aqueous isopropanol made 2N with respect to HCl, to about 10 cm. from the end of the papers. They were then dried in a stream of cold air until the last traces of solvent had disappeared. The dry papers were turned through 90° and run in the ascending solvent described by MacNutt (1952),

n-butanol saturated with water, 100 ml., concentrated ammonia solution, 1 ml. - until the solvent front was 3 cm. from the top of the paper. After chromatography, the papers were dried in warm air and bases located by inspection in ultra-violet light. The positions of bases on such chromatograms are shown in Figure 5. It can be seen from this diagram that the bases xanthine, guanine and hypoxanthine lie near the origin and may not be completely separated from each other and from extraneous material at the origin. A method for complete isolation of these bases is described in Section 2.9c.

Blank papers were run with all batches of test chromatograms.

2.9c. Further Chromatography of Bases on Paper.

After chromatography on paper of bases, as described in Section 2.9b, spots were located in ultra-violet light, cut out and stapled to strips of Whatman No. 1 paper as shown in Figure 6. The strips were run as adsorption chromatograms in distilled water. In this system, any NH_4Cl present in the spots after the preceding two-dimensional chromatography travelled to the solvent front.

The strips were dried and the bases again located in ultra-violet light. The spots were cut out together with the corresponding areas from blank papers and eluted by

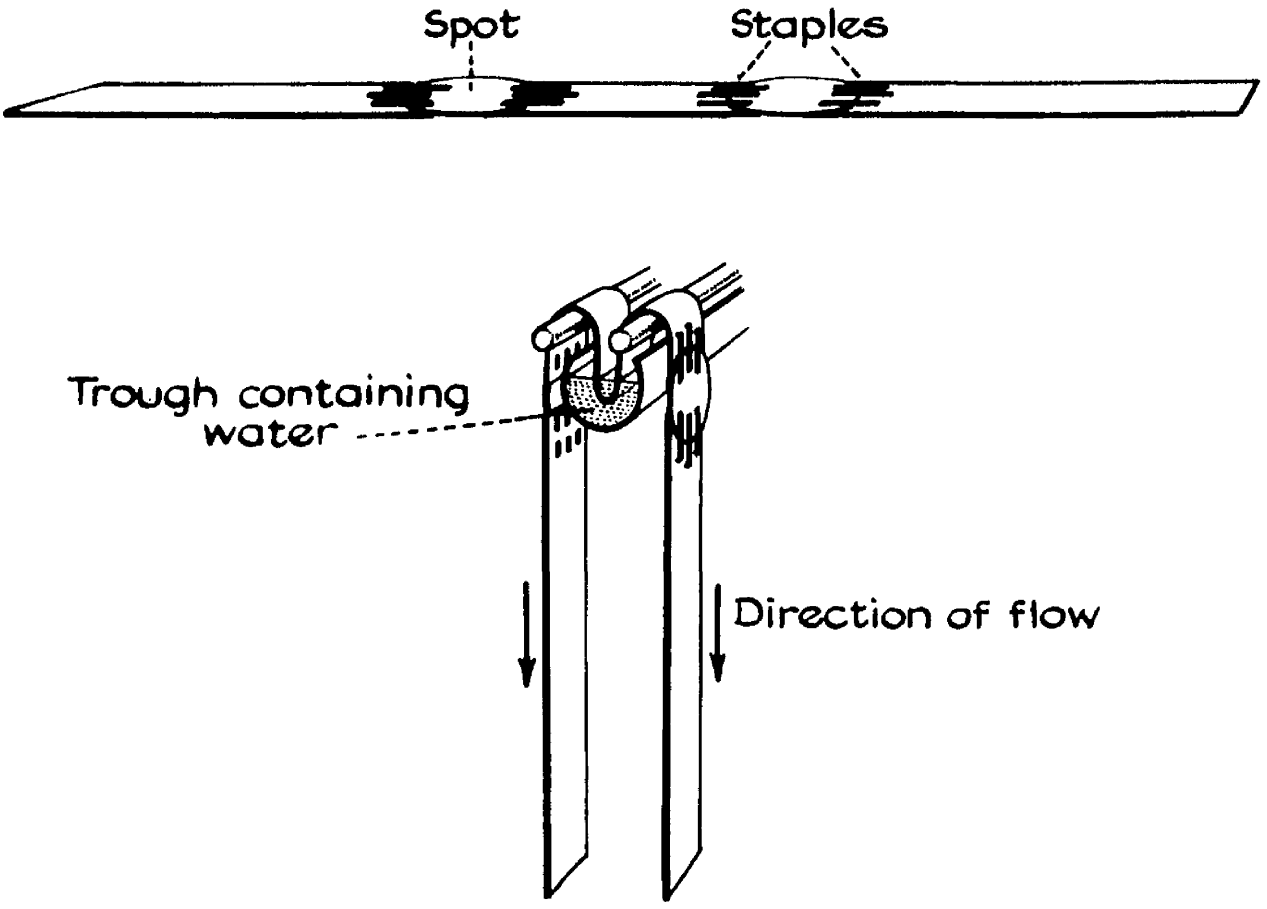


Figure 6.

capillary flow using 0.1N HCl for adenine, cytosine and uracil, and 1.6N HCl for guanine, xanthine and hypoxanthine, 0.3 to 0.4 ml. of eluate being collected.

It was found, in the routine of plating out radioactive purines on flat nickel planchettes for specific activity determinations, that the adsorption chromatography procedure was necessary in order to obtain the material in sufficiently pure form to provide infinitely thin layers for counting.

In addition, the procedure gave confirmatory evidence of the identity of the base present in the two-dimensional chromatograms. Rp values of bases on the strips were:-

Adenine	0.2
Guanine	0.2
Xanthine	0.4
Hypoxanthine	0.5
Cytosine	0.6
Uracil	0.6

2.9d. Chromatography of Amino-Acids on Paper.

Peaks which were located on flow charts by the quantitative ninhydrin method of Cocking and Yemm (1954), were pooled, dried from the frozen state and dissolved in small volumes of water.

Portions of these solutions were taken to dryness in a vacuum desiccator, dissolved in about 0.3 ml. of 6N HCl and sealed off in thick-walled glass tubes for hydrolysis at 110° for 18 hours. After cooling, the hydrolysates were transferred to small test-tubes and the HCl was removed *in vacuo* over Na OH pellets. The dry material was dissolved in about 0.4 ml. distilled water. Portions of these solutions were applied to sheets of Whatman No. 1 chromatography paper. Portions of the solutions before hydrolysis were also applied to chromatography paper.

The papers were run in two dimensions using the following solvent systems:-

descending: pyridine : amyl alcohol : water (35:35 :30) (de Verdier and Agren, 1948).

OR

butanol : acetic acid : water (4:1:5) (Partridge, 1948).

ascending: phenol : water : ammonia. The phenol was saturated with water in a separating funnel and run off into a petri dish in which the chromatogram (cylinder) was placed. The aqueous layer was poured into a small beaker and after the addition of a few ml. concentrated ammonia solution, was stood in the bottom of the chromatography tank. A second beaker containing a few crystals of KCN was also placed in the tank to prevent oxidation of the phenol

After drying in a warm-air oven, the papers were washed in benzene or ether contained in a flat, open tray, and dried again.

Spots were located by spraying the papers with a ninhydrin spray reagent containing 0.2% ninhydrin in 60% ethanol with 1% acetic acid.

Comparisons of these chromatograms were made with corresponding chromatograms of standard mixtures of amino-acids.

2.10 Ionophoresis on Paper.

After lyophilisation of peaks which were eluted from Dowex-1-formate with 1N formic acid, the material, dissolved in water, was applied to strips of Whatman No. 3MM paper (72 cm. x 7 cm.) for ionophoresis in a citrate buffer, pH 3.5, by the method of Davidson and Smellie (1952).

The papers were then dried and the ultra-violet light absorbing areas marked off lightly in pencil. In order to determine the location of ninhydrin-positive compounds, 1 cm. strips were cut from one edge of each ionophoretogram and were sprayed with the ninhydrin reagent (Section 2.9d). Ultra-violet light absorbing bands and ninhydrin-positive bands on the remaining portions of the ionophoretograms were cut out and eluted with water by capillary flow. Analyses were carried out on the pooled eluates from a number of corresponding ionophoretograms. The 1 cm. strips were preserved as permanent records of the separations.

2.11 Autoradiography.

When radioactive materials were submitted to ionophoresis on paper, one set of ionophoretograms was retained for photography in ultra-violet light (Markham and Smith, 1949). The ionophoretograms were then left in contact with strips of Kodak Industrex type D X-ray film for periods of 2 to 3 weeks for the preparation of autoradiographs.

2.12 Ultra-violet Absorption Measurements.

These measurements were made in the Beckman Model DU spectrophotometer on five types of solution:-

(i) lyophilised column eluates dissolved in water.

(ii) water eluates from papers chromatographed in the ethanol ammonium acetate solvent. These eluates were further examined in the Beckman spectrophotometer after making the solutions 0.1N with respect to HCl and subsequently after raising the pH to at least 11 by addition of 1N NaOH.

(iii) HCl eluates from papers run in the two-dimensional solvent for separation of bases. The solutions were also examined after making alkaline (at least pH 11) by addition of 1N NaOH, or 2N NaOH - see Section 2.9c.

(iv) HCl eluates from adsorption chromatograms as obtained after the procedure described in Section 2.9c.

(v) water and HCl eluates from ionophoretograms.

Measurements were made in the Beckman spectrophotometer at 5 mμ intervals from 215 mμ to 290 mμ when absorption spectra were being plotted, and at 290 mμ and at the λ_{max} when bases were to be quantitatively estimated (Crosbie, Smellie and Davidson, 1953).

2.13 Phosphorus Estimations.

Phosphorus was estimated in paper eluates by the method of Allen (1940) and by a micromodification of the same technique.

2.14 Ribose Estimations.

The ribose content of certain compounds after paper chromatography in ethanol/ammonium acetate or in acetone/formic acid/water, was estimated by the orcinol reaction as described by Kerr and Seraidarian (1945).

A reagent blank and a series of ribose standard solutions were estimated along with each batch of test samples.

2.15 Enzymic Assay for Diphosphopyridine Nucleotide.

The presence of DPN in column eluates and in eluates from paper chromatograms run in ethanol/ammonium acetate, was confirmed enzymically using alcohol dehydrogenase. The method of preparation of alcohol dehydrogenase and the enzymic assay procedure were those described by Racker (1950).

2.16 Determination of Radioactivity - ^{14}C-labelled Purines.

Purine bases finally isolated as in Section 2.9c were treated as follows. 0.1 ml. of the eluate was applied to the raised central plateau of a nickel planchette which had been thoroughly cleaned, and the solution was dried under infra-red lamps.

Plated samples were counted at infinite thinness in end-window counters connected to Panax scaling units, for 10-minute periods. Background counts were carried out with blank planchettes in position in the counters.

It was found desirable to count samples containing 1.6N HCl without delay. HCl at this concentration attacks the metal planchette and the sample area tends to become encrusted and no longer reliable for counting.

SECTION III.

RESULTS.

RESULTS.

3.1 The Distribution of Acid-Soluble Nucleotides in Whole Tissue and in Cell Nuclei.

Several experiments were carried out to determine whether the acid-soluble nucleotide content of cell nuclei differed from that of the corresponding whole tissue. In these experiments, nuclei were isolated from non-aqueous media (Section 2.5), the tissues examined being rabbit appendix and rabbit intestinal mucosa.

3.1a. The Acid-Soluble Nucleotides of Whole Rabbit Appendix.

The elution pattern from ion exchange chromatography of the acid-soluble nucleotides of whole rabbit appendix is shown in Chart 1. This was one of the first separations carried out and had four elution ranges as shown on the chart. (In some experiments to be described later, a fifth elution range was included at the beginning of the chromatogram).

For such separations on whole tissue extracts a standard column of Dowex-1-formate of dimensions 26.0 cm. x 1.0 cm. was always used in order to facilitate interpretation of the ion exchange chromatograms from the same and from other tissues.

For every ion exchange chromatogram, a complete set of data was drawn up, but since the presentation of the complete data in all cases would require an unreasonable

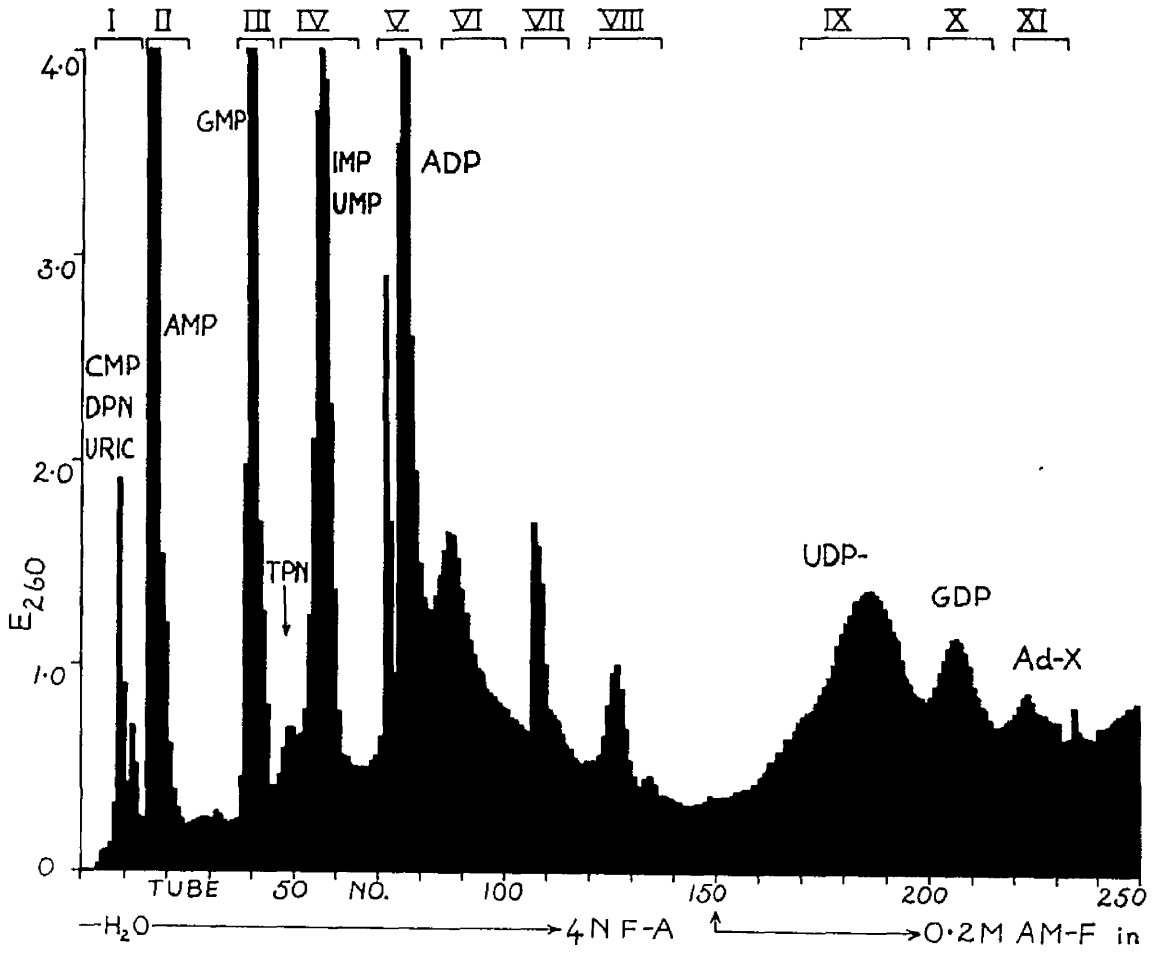
CHART 1.

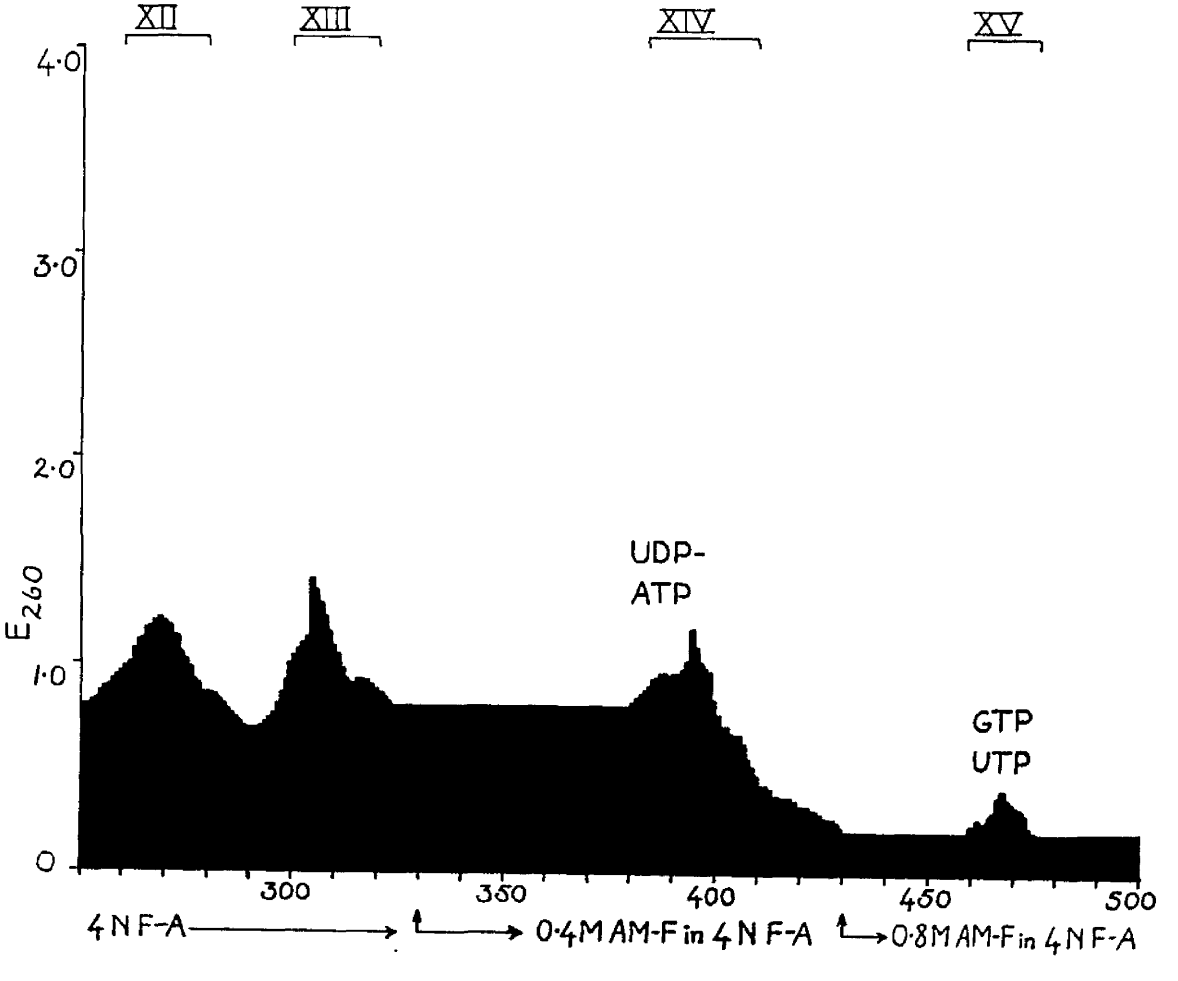
Elution chart from the ion exchange chromatography of acid-soluble compounds absorbing at 260 mμ, obtained from 24 g. (wet weight) of rabbit appendix. The appendix tissue was a portion of the pooled material from four albino rabbits, each of which had received 1 mc. ^{14}C-sodium formate two hours before killing.

Column:- Dowex-1-formate; 26.0 cm. x 1.0 cm.
Mixing Volume:- 500 ml. Fractions:- 100 drops per tube, about 6 ml. per tube.

Readings at 260 mμ which were too great to be plotted on the elution chart were, peak II 9.25, 43.0, 12.0; peak III, 4.14, 4.04; peak IV 4.3; peak V 5.0.

At tube 230, a leak developed in the glass covering on the magnetic stirrer with the production of some ultra-violet-absorbing material which temporarily interfered with the elution pattern.


I
II
III
IV
V
VI
VII
VIII
IX
X
XI
4·0
3·0
2·0
1·0
0
E_{260}
CMP
DPN
URIC
AMP
GMP
TPN
IMP
UMP
ADP
UDP-
GDP
Ad-X
TUBE NO.
50
100
150
200
250
$-H_2O$
4 N F-A
0·2 M AM-F in


XII
XIII
XIV
XV
4·0
3·0
2·0
1·0
0
E_{260}
UDP-
ATP
GTP
UTP
300
350
400
450
500
4 N F-A
0·4 M AM-F in 4 N F-A
0·8 M AM-F in 4 N F-A

amount of space, a composite table containing all the necessary information is shown only for Chart 3 (Table 4).

Hydrolysis of the material in the peaks in Chart 1 followed by paper chromatography of the bases revealed that several peaks contained more than one nucleotide. This was suggested in the first instance by the variation in $E_{275}:E_{260}$ ratios of fractions emerging from the column. Table 2 gives these ratios found for nucleotides in column eluates in the present series of experiments. The ratios are similar to those given by Hurlbert et al. (1954). Uric acid is included in Table 2 as it was found to be present in many of the tissue extracts examined.

In Chart 1, the elution pattern was obscured temporarily beyond tube 230 by the presence of some extraneous ultraviolet-absorbing material (see legend to Chart 1), but bases were satisfactorily isolated after hydrolysis of compounds eluted by 0.2M ammonium formate in 4N formic acid and by 0.4M ammonium formate in 4N formic acid.

The general features of the elution chart are evident by inspection and the positions of compounds are similar to those of the corresponding compounds found in rat liver extracts by Schmitz, Potter, Hurlbert and White (1954), who used the same elution system under similar conditions.

Certain peaks, however, require further comment. By examination of $E_{275}:E_{260}$ ratios of column eluates, and by determination of bases present in individual fractions

TABLE 2.

$E_{275} : E_{260}$ ratios of the mono-, di- and tri- phosphates of cytidine, adenosine, guanosine, and uridine, and of inosine-5'-phosphate and uric acid, as found in colum eluates.

Nucleotides, etc.	$E_{275} : E_{260}$ ratio.
Cytidine-5'-phosphates	1.0 - 2.0
Adenosine-5'-phosphates	0.38 - 0.44
Guanosine-5'-phosphates	0.70 - 0.75
Uridine-5'-phosphates	0.55 - 0.65
Inosine-5'-phosphate	0.30 - 0.35
Uric Acid	1.90 - 2.50

after hydrolysis with perchloric acid and two-dimensional paper chromatography, it was found that peak I contained GMP, DPN and uric acid eluted in that order. In peak III, the amount of TPN was small but appeared to be eluted immediately after GMP while in peak IV IMP preceded UMP. Grégoire, Grégoire and Limozin (1957) fractionating the acid-soluble nucleotides from Micrococcus lysodeikticus found that DPN was eluted before GMP, GMP before TPN and IMP after UMP in their system which was similar to the one employed here. Bellio, Casinovi and Serlupi-Crescenzi (1956) in the course of studies on the soluble nucleotide distribution in Penicillium chrysogenum also found that DPN preceded GMP but that TPN preceded GMP and IMP preceded UMP.

Immediately following AMP and before peak III a compound containing adenine was eluted. The $E_{275}:E_{260}$ ratios of the fractions in this peak differed from those of AMP and are given in Table 3. Following ADP are three peaks, VI, VII and VIII, all of which contain adenine and phosphorus. They were not identified further, but their $E_{275}:E_{260}$ ratios are included in Table 3. The $E_{275}:E_{260}$ ratios of these peaks and of the small peak between AMP and GMP are higher than the ratios obtained for AMP and ADP.

In Chart 1, peak IX contains a derivative or derivatives of UDP and peak XIV contains a derivative of UDP and/or UDP itself. In these experiments, compounds containing UDP were not characterised fully and were denoted on Charts thus, UDP-.

TABLE 3.

E_{275} : E_{260} ratios of some fractions from certain peaks shown in Chart 1.

Peak	Tube No.	E_{275} : E_{260} ratio.
Between II and III	26	0.52
	28	0.56
	30	0.62
	32	0.46
	34	0.51
V	78	0.42
	80	0.45
	82	0.48
VI	84	0.53
	86	0.57
	88	0.60
	90	0.59
VII	106	0.64
	108	0.65
	110	0.61
	112	0.56
	114	0.53
	116	0.55
VIII	126	0.48
	128	0.48
	130	0.50
	132	0.49
	134	0.58
	136	0.59
	138	0.55

Peak XI was not fully characterised. It contained adenine, had an $E_{275}:E_{260}$ ratio of 0.4, characteristic of the adenosine nucleotides, and emerged from the column at the same point as the ADP-X peak obtained by Schmitz et al. (1954) from rat liver extracts, and by Manson (1956) from extracts of goat mammary gland.

CDP and CTP, which appeared in some of the ion exchange chromatograms to be described, were not detected in this experiment but were, however, found to be present in another extract of whole appendix (see Chart 2) and in extracts of appendix NAN (see Chart 3).

In another experiment the appendices from three rabbits were pooled and extracted with 1.0N perchloric acid. Chart 2 is the elution chart of ion exchange chromatography of the resulting extract. These appendices were excised with all possible speed, immediately chilled on ice and were homogenised in the ice-cold perchloric acid within six minutes of the death of the animal. In this respect they differed from the appendices used in the experiment illustrated in Chart 1. In this case there was an appreciably longer time lag between the death of the animals and the freezing of the minced tissue in a flask prior to lyophilisation.

From Chart 2 it is clear that speed of manipulation and strict observance of cold experimental conditions throughout the excision and cleaning of the tissue, altered

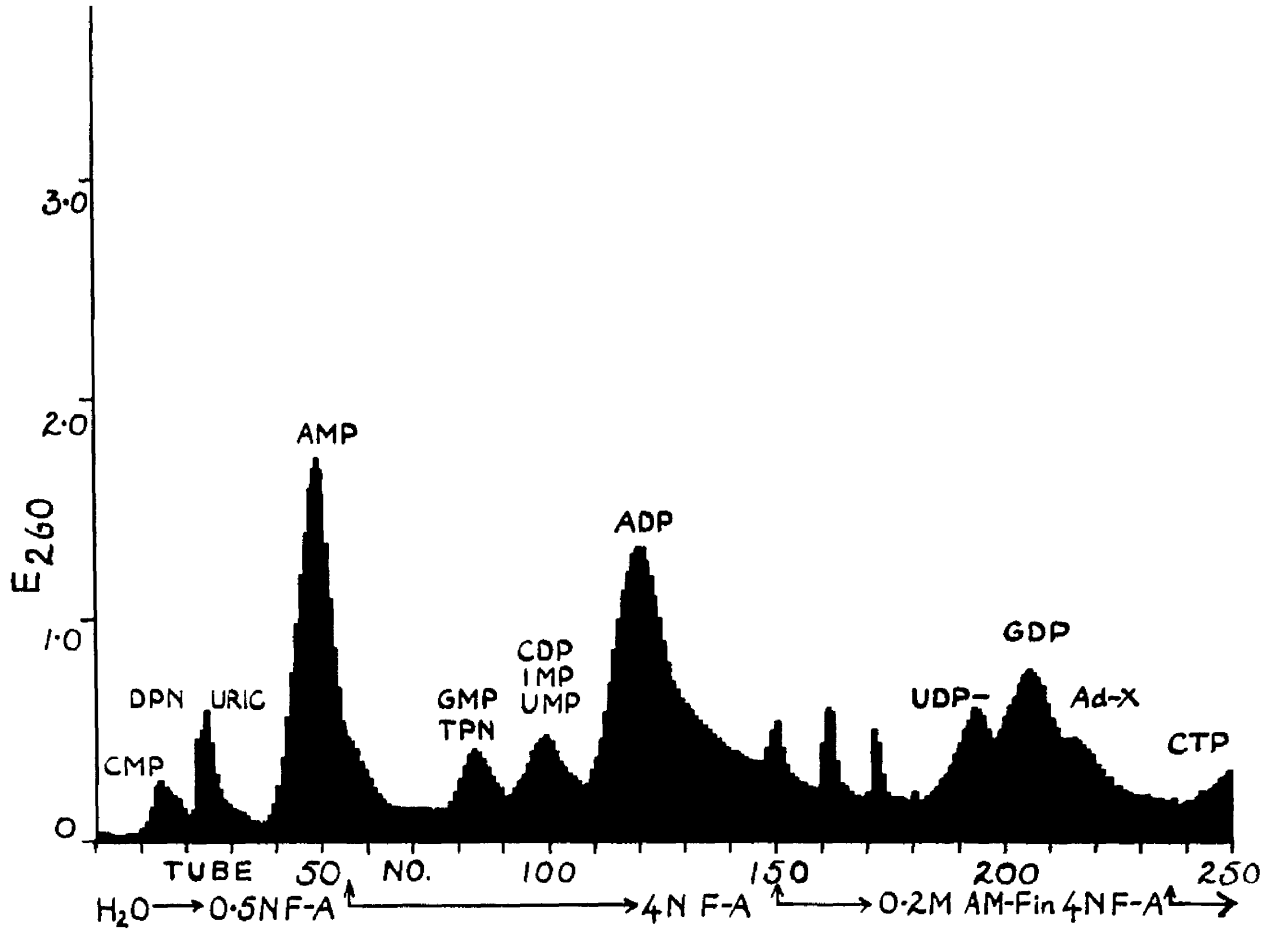
CHART 2.

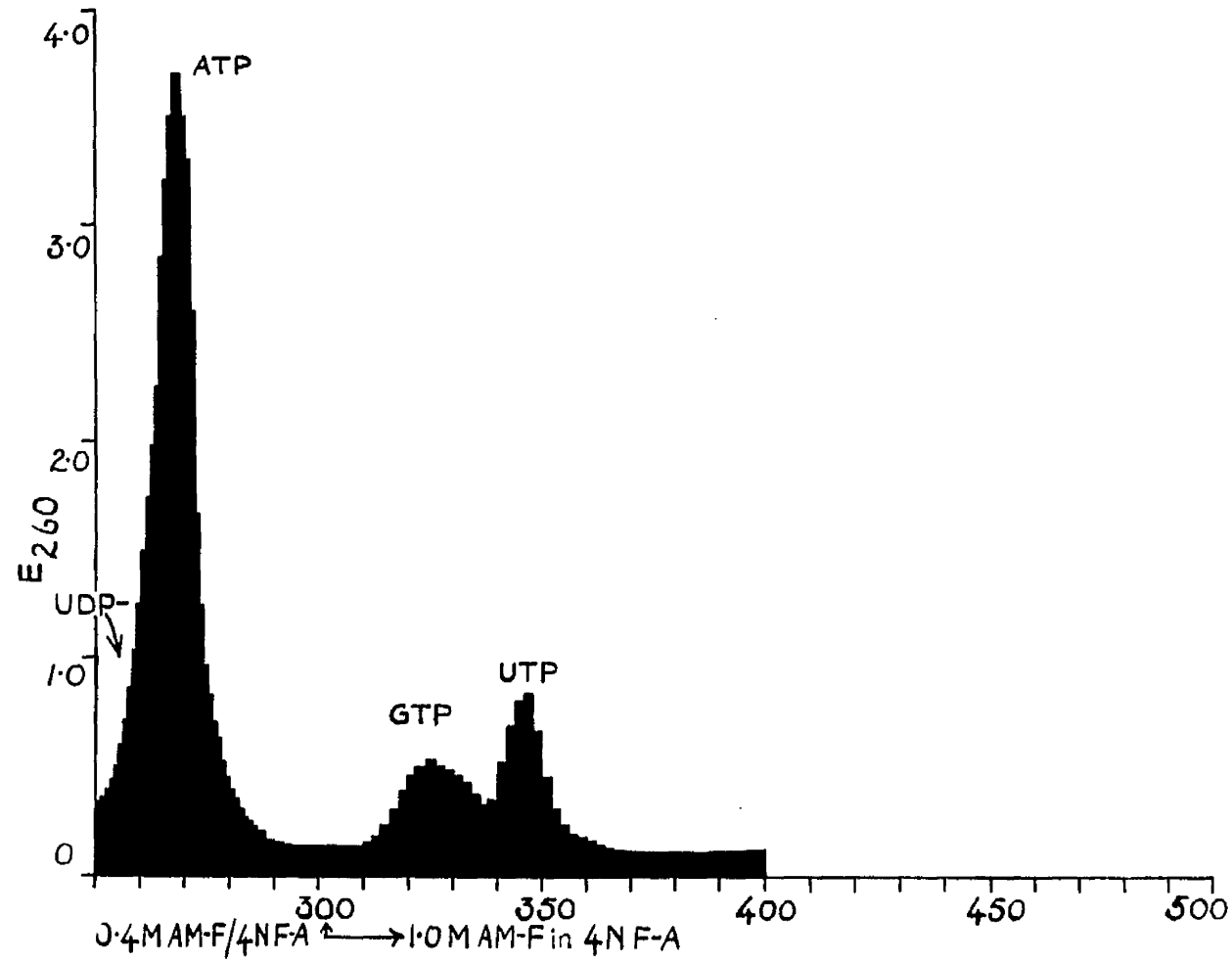
Elution chart from the ion exchange chromatography of acid-soluble compounds absorbing at 260 mμ, obtained from 24 g. (wet weight) of rabbit appendix. The appendix tissue was obtained from three rabbits.

Column:- Dowex-1-formate; 26.0 cm. x 1.0 cm.

Mixing Volume:- 500 ml.

Fractions:- 50 drops per tube up to tube 47, 90 drops per tube from tube 48 to tube 153, 100 drops per tube thereafter. 100 drops per tube corresponded approximately to 6.0 ml.


3·0
2·0
1·0
0
E260
AMP
ADP
GDP
CDP
IMP
UMP
GMP
TPN
DPN
URIC
CMP
UDP–
Ad-X
CTP
TUBE 50 NO. 100 150 200 250
H2O → 0·5N F-A
4N F-A
0·2M AM-F in 4N F-A


4·0
3·0
2·0
1·0
0
E260
ATP
UDP-
GTP
UTP
300 350 400 450 500
0·4M AM-F/4N F-A
1·0 M AM-F in 4N F-A

the appearance of the subsequent elution pattern in that triphosphates were present in greater amounts. This is particularly obvious in the case of ATP.

In two other experiments with rabbit appendix tissue which had been stored in the deep-freeze cabinet at -10° for periods of two to six months, it was found that the triphosphates and diphosphates had greatly decreased in quantity while the monophosphates had increased. The NFB fraction in these experiments was greatly increased.

The experiment illustrated in Chart 1 was carried out with radioactive material. The specific activities of the purine bases derived from the purine nucleotides shown on Chart 1 are given in the next section (Table 5).

3.1b. The Acid-Soluble Nucleotides of Rabbit Appendix Cell Nuclei.

Chart 3 is the elution chart from ion exchange chromatography of acid-soluble nucleotides from nuclei isolated from rabbit appendix in non-aqueous media (NAM). Table 4 gives the complete set of chromatographic and spectral data relating to the compounds shown on the elution chart.

An extra elution range was used in this experiment, the elution being started with 1N formic acid in the reservoir vessel. The NFB fraction is not included on the chart.

CHART 3.

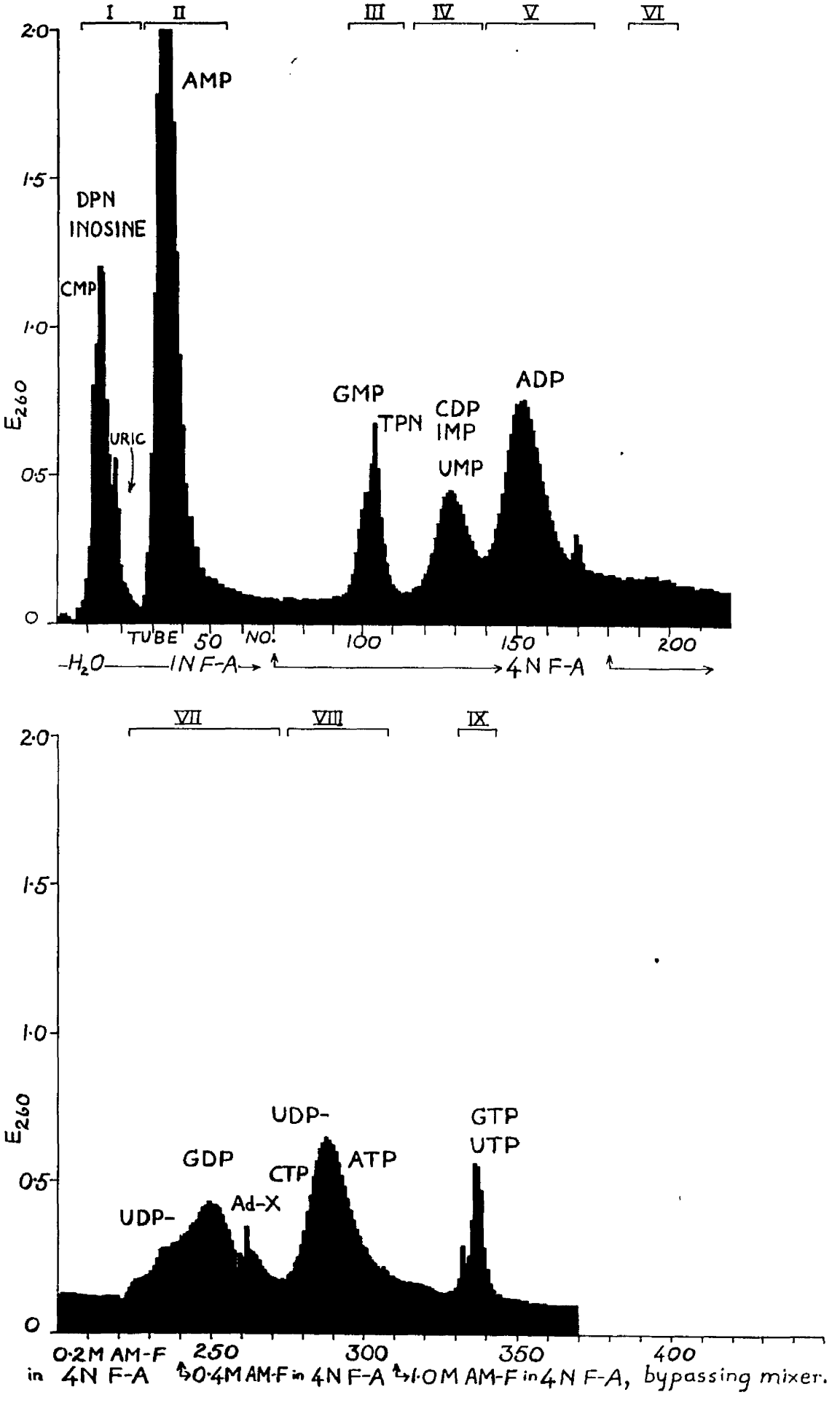
Elution chart from the ion exchange chromatography of acid-soluble compounds absorbing at 260 mμ, obtained from 350 mg. (dry weight) of rabbit appendix NAN. The NAN were prepared from 7.4 g. (wet weight) of appendix tissue from an albino rabbit which had received 1 mc. ^{14}C-sodium formate two hours before killing.

Column:- Dowex-1-formate; 26.0 cm. x 0.7 cm.

Mixing Volume:- 250 ml. Fractions:- 40 drops per tube, about 2.5 ml. per tube.

Prior to application to the column, the extract was made slightly alkaline by the addition of a drop of ammonia solution.

Readings at 260 mμ which were too great to be plotted on the elution chart were, peak II 2.25, 2.55, 2.45, 2.2.


I
II
III
IV
V
VI
2·0
1·5
1·0
0·5
0
E_{260}
AMP
DPN
INOSINE
CMP
URIC
GMP
TPN
CDP
IMP
UMP
ADP
TUBE NO.
50
100
150
200
$-H_2O$
1 N F-A
4 N F-A
VII
VIII
IX
UDP-
GDP
Ad-X
CTP
ATP
GTP
UTP
250
300
350
400
0·2 M AM-F in 4 N F-A
0·4 M AM-F in 4 N F-A
1·0 M AM-F in 4 N F-A, bypassing mixer.

Peak I, like the corresponding peak from the whole appendix extract contained CMP and DPN, and probably also uric acid as a very high $E_{275}:E_{260}$ ratio was found in fractions 18-23. Uric acid was not detected on paper chromatograms but this substance is difficult to locate on paper particularly when it is present in small quantity, probably because its ultraviolet absorption maxima are at 280-284 mμ and at 230-233 mμ, while the maximum transmission of light from the ultraviolet lamps used was in the region of 260 mμ. (In later experiments, uric acid was more easily located and identified by ionophoresis on paper of the appropriate fractions after a modification of the elution technique - see Section 3.3).

Inosine was identified in peak I, Chart 3, by comparison with the chromatographic and spectral properties of an authentic sample of inosine. Hypoxanthine was obtained after acid hydrolysis of this compound.

The extract of appendix NAN was applied to the column at alkaline pH (about pH 8.0) and under such conditions, inosine, which has a weakly dissociable H^+ was retained on the resin as an anion. This probably accounts for the absence of inosine from peak I on Chart 1, as in that experiment the sample was placed on the column at a pH of 6.0 - 7.0.

In the present investigation, inosine was detected in all samples which were applied to the resin column at alkaline pH (with the exception of liver SN extract).

Table 4 indicates that another substance was present in peak I. This substance corresponded to guanine both before and after hydrolysis with perchloric acid.

The relative amounts of the components of peak I are shown below:-

Component	Relative Amount
Inosine	+++++
CMP	+
DPN	+
Guanine	+ +

Peak III contained GMP together with a small amount of TPN. CDP is probably also present in this part of the chromatogram as a trace of cytosine was found in an acid hydrolysate of the dry material obtained from the pooled fractions of this part of the chromatogram.

The major components of peak IV were IMP and UMP. The adenosine nucleotides were probably obtained from the overlapping portion of peak V but it is noteworthy that Grégoire et al. (1957) found two adenosine nucleotides associated with their ADP peak from extracts of Micrococcus Lysodeikticus and that Siekevitz and Potter (1955) found an adenosine nucleotide immediately preceding ADP in their fractionation of the acid-soluble nucleotides in rat liver mitochondria.

TABLE 4.

This table contains the results which led to the identification of compounds eluted from the Dowex-1-formate column as shown in Chart 3.

The table is divided into three parts:

Part One deals with column chromatographic position, paper chromatographic position and also gives the $E_{275}:E_{260}$ ratios of certain fractions from the column.

Part Two shows the spectral data in 0.1N HCl of spots found on paper chromatograms as laid out in Part One.

Part Three gives the spectral data of bases in HCl solution, the bases having been obtained from the spots in Part One by acid hydrolysis.

TABLE 4. Part One.

PEAK	TUBE No.	$\frac{E_{275}}{E_{260}}$ ratio
I	9	1.07
	10	1.26
	15	0.41
	16	0.44
	19	1.12
	20	1.14
II	36	0.39
III	98	0.61
	102	0.66
	111	0.53
IV	120	0.62
	127	0.60
	133	0.56
	137	0.58
	140	0.52
V	145	0.44
	153	0.42
	163	0.48

TABLE 4. Part One (Continued).

PEAK	TUBE No.	$\frac{E_{275}}{E_{260}}$ ratio
VI	195	0.41
VII	285	0.46
	250	0.61
	259	0.47
VIII	272	0.52
	290	0.48
	310	0.43
IX	333	0.65
	337	0.65
	339	0.55

TABLE 4. Part One (Continued).

PEAK	Spots obtained from the material contained in the peaks of Chart 3 after chromatography in the ethanol/ammonium acetate solvent	Radenosine value of spot.
I	a b c d e	0.37 0.51 0.74 0.94 1.10
II		0.48
III	a b c	0.27 0.41 0.53
IV	a b c d e	0.20 0.49 0.61 0.75 0.96
V	a b c	0.37 0.52 0.76

TABLE 4. Part Two.

Peak and Spot	Spectral data at pH 1 of the spots obtained from ethanol/ ammonium acetate paper chromatograms.						Probable identity of compound
	λ_{max_1} mμ	λ_{max_2} mμ	λ_{min_1} mμ	λ_{min_2} mμ	$\frac{230}{260}$	$\frac{250}{260}$	
I a	257.5	-	236	-	0.57	0.93	DPN
b	279	-	241	-	1.76	0.67	GMP
c	270	247.5	265	228	0.87	1.32	Guanine
d	250	-	223	-	0.27	1.23	Inosine
e	262.5	-	-	-	0.52	0.75	Adenosine
II	258	-	232	-	0.29	0.91	AMP
III a	275	256	232	-	0.75	0.96	GMP
b	259	-	236	-	0.66	0.89	TPN
c	272	-	243	-	1.0	0.90	Cytidine

TABLE 4. Part Two (continued).

Peak and Spot	Spectral data at pH 1 of the spots obtained from ethanol/ ammonium acetate paper chromatograms.						Probable identity of compound
	λ_{max1} mμ	λ_{max2} mμ	λ_{min1} mμ	λ_{min2} mμ	$\frac{280}{260}$	$\frac{250}{260}$	
IV a	257	-	228	-	-	0.92	ADP
b	258	-	230	-	0.39	0.87	AMP+IMP
c	262	-	232	-	0.42	0.73	UMP
d	272	252	263	228	0.93	1.20	Guanosine
e	252	-	223	-	0.42	1.09	Inosine
V a	257.5	-	231	-	0.25	0.83	ADP
b	260	-	232	-	0.29	0.79	AMP
c	267	-	239	-	0.90	0.88	*

* Jaklik (1956) and Grégoire et al. (1957) have found compounds with spectra having maxima at 267 mμ associated with the ADP fraction.

TABLE 4. Part Three.

Peak and Spot	Spectral data, in HCl solution, of bases obtained from spots after hydrolysis in 12 N perchloric acid. λ_{max_1} mμ	λ_{max_2} mμ	λ_{min_1} mμ	λ_{min_2} mμ	280/260	250/260	Base present
I a	Amount too small to measure						
b i	274	-	245	-	1.65	0.26	
b ii	273	-	245	-	1.12	0.80	Cytosine
c	273	249	264	226	0.89	1.37	Guanine
d i	248	-	216	-	-	1.54	Hypoxanthin
d ii	265	-	231	-	0.12	0.68	Adenine (?)
e	262.5	-	229	-	0.41	0.80	Adenine
II	262.5	-	229	-	0.37	0.76	Adenine
III a	249	-	225	-	0.81	1.46	Guanine
b	262.5	-	233	-	0.49	0.86	Adenine
c	277	-	-	-	1.17	0.30	Cytosine

TABLE 4. Part Three (continued).

Peak and Spot	Spectral data, in HCl solution, of bases obtained from spots after hydrolysis in 12 N perchloric acid. λmax_1 mμ	λmax_2 mμ	λmin_1 mμ	λmin_2 mμ	$\frac{280}{260}$	$\frac{250}{260}$	Base
IV a	Amount too small to measure						
b i	251	-	221	-	0.70	1.04	Hypoxanthine
b ii	262.5	-	230	-	0.50	0.77	Adenine
b iii	259	-	228	-	0.28	0.82	Uracil
c i	271.5	252	236	-	0.98	0.98	Guanine
c ii	259	-	230	-	0.30	0.88	Uracil
d	Amount too small to measure						
e	244	-	222.5	-	0.66	1.44	Hypoxanthine
V a	262.5	-	-	-	0.35	-	Adenine
b	262.5	-	-	-	0.35	-	Adenine
c	Amount too small to measure						

TABLE 4. Part Three (continued).

Peak and Spot	Spectral data, in HCl solution, of bases obtained from spots after hydrolysis in 12 N perchloric acid. λ_{max_1} mμ	λ_{max_2} mμ	λ_{min_1} mμ	λ_{min_2} mμ	$\frac{230}{260}$	$\frac{250}{260}$	Base present
VI	262.5	"	"	"	0.36	"	Adenine
VII a	270	249	265	224	0.84	1.34	Guanine
b	262.5	"	"	"	0.36	0.76	Adenine
c	273	"	242	"	1.02	0.57	Cytosine
d	259	"	224	"	0.19	0.84	Uracil
VIII a	262.5	"	"	"	0.36	0.76	Adenine
b	"	"	"	"	"	"	Cytosine (trace
c	259	"	"	"	"	0.62	Uracil
IX a	275	248	223	"	0.74	1.40	Guanine
b	262.5	"	"	"	"	"	Adenine
c	260	"	228	"	0.21	0.80	Uracil

The small peak eluted after ADP had $E_{275}:E_{260}$ ratios of 0.48 - 0.50 (see Table 4, Part Two). This compound emerges from the column in a position similar to peaks VI, VII and VIII in Chart 1.

Peak VIII contained only a trace of GTP, while peak IX in addition to GTP and UTP contained a compound of adenine. At this stage of the ion exchange chromatography the full eluting power of the last elution range was used by by-passing the mixing vessel.

Table 5 gives the specific activities of purine bases isolated from hydrolysates of the corresponding purine nucleotides shown in Charts 1 and 3. Peaks shown in Chart 1 were concentrated by adsorption on charcoal (B.D.H. activated charcoal) with subsequent elution by 50% aqueous ethanol containing a trace of ammonia (Mills, 1954; Buchanan, 1956). Yields of nucleotides eluted from Dowex-1-formate by eluents containing ammonium formate were low when the charcoal adsorption procedure was used. Formate ion interferes with the elution process from charcoal (Mills, 1954).

It can be seen from Table 5 that in the whole appendix extract the mono-, di- and triphosphates of adenosine have specific activities (for adenine) of the same order. The corresponding nucleotides in the appendix NAN extract have specific activities of the same order but the values are slightly higher than those obtained for the whole tissue extract. The hypoxanthine of the IMP from whole

TABLE 5.

Specific activities of purine bases derived from the acid-soluble purine nucleotides of whole rabbit appendix and of rabbit appendix NAN. The animals received an intramuscular injection of 1 mc. 14C-sodium formate 2 hours before sacrifice.

Base	Nucleotide from which base was obtained.	Specific activity; counts per min. per μ mole of bases.	
		Whole appendix Chart 1	Appendix NAN Chart 3
Adenine	AMP	7,460	8,080
Adenine	ADP	6,970	10,350
Adenine	ATP	6,600	7,930
Guanine	GMP	8,380	7,780
Guanine	GDP	2,410	3,640
Guanine	GTP	-	2,410
Hypoxanthine	IMP	6,950	-
Adenine	DPN	-	5,075
Adenine	TPN	7,300	7,570
Adenine	Ad-x	5,600	8,230
Adenine	peak VI	5,580	
Adenine	peak VII	6,100	
Adenine	peak VIII	5,950	
Adenine	peak VI		9,200
Guanine	peak I		1,470

appendix has a specific activity of the same order of magnitude as those obtained for the adenine of AMP, ADP and ATP in the same extract. The guanine in GMP from both whole tissue and NAN has a specific activity similar to that of the adenosine nucleotides, but in the GDP in both extracts it shows a value less than half that in the corresponding GMP. The GTP-guanine from NAN also has a low specific activity.

The comparisons between whole tissue and NAN in the previous paragraph were made bearing in mind that the NAN were obtained from a single rabbit appendix while the whole tissue extract was prepared from the pooled appendix tissue from four rabbits. However, all five rabbits were young albinos of approximately equal weight, all of which had been treated in exactly the same way, receiving 1 mc. ^{14}C-sodium formate two hours before killing.

3.1c. The Acid-Soluble Nucleotides of Whole Rabbit Intestinal Mucosa.

Charts 4, 5 and 6 show the elution patterns from ion exchange chromatography of the acid-soluble nucleotides of whole rabbit intestinal mucosa.

The general features of Charts 4 and 5 are similar, but in Chart 6 a much improved separation of compounds eluted with low concentrations of formic acid was effected by inclusion of an initial elution range approaching 0.5 N formic acid and by collecting smaller fractions in the first part of the chromatogram.

In Chart 6 inosine was present in peak I along with GMP and DPN. Uric acid was so well separated from the other compounds eluted in this range that it formed a separate peak (peak II).

The principal features of each chart are evident by inspection, but certain factors require further comment.

The apparent absence of uric acid on Chart 4 cannot be regarded as significant since uric acid was detected in Chart 5 and particularly well in Chart 6. All three tissue extracts were prepared in the same way.

As cytidine in all three stages of phosphorylation was found in a later experiment in which the acid-soluble nucleotides of intestinal mucosa NAN were fractionated on Dowex-1-formate, the mono-, di- and triphosphates of

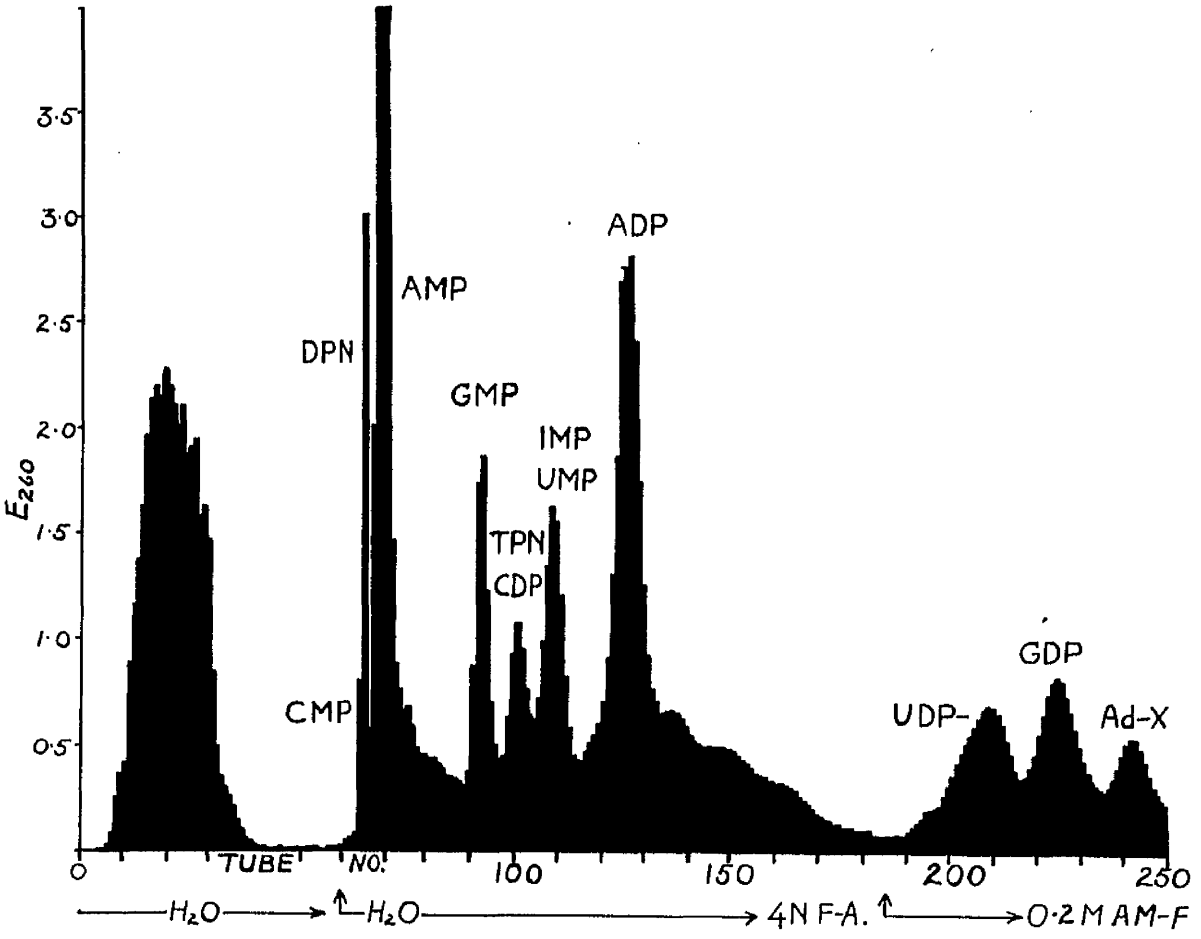
CHART 4.

Elution chart from the ion exchange chromatography of acid-soluble compounds absorbing at 260 mμ, obtained from 20 g. (wet weight) of rabbit intestinal mucosa.

Column:- Dowex-1-formate; 25.0 cm. x 1.0 cm.

Mixing Volume:- 500 ml. Fractions:- 100 drops per tube, about 6.0 ml. per tube.

Readings at 260 mμ which were too great to be plotted on the elution chart were, AMP 11.3, 17.9, 4.3.


3·5
3·0
2·5
2·0
1·5
1·0
0·5
E_{260}
DPN
AMP
ADP
GMP
IMP
UMP
TPN
CDP
CMP
GDP
UDP-
Ad-X
0
TUBE NO.
100
150
200
250
H_2O
H_2O
4N F-A.
0·2 M AM-F

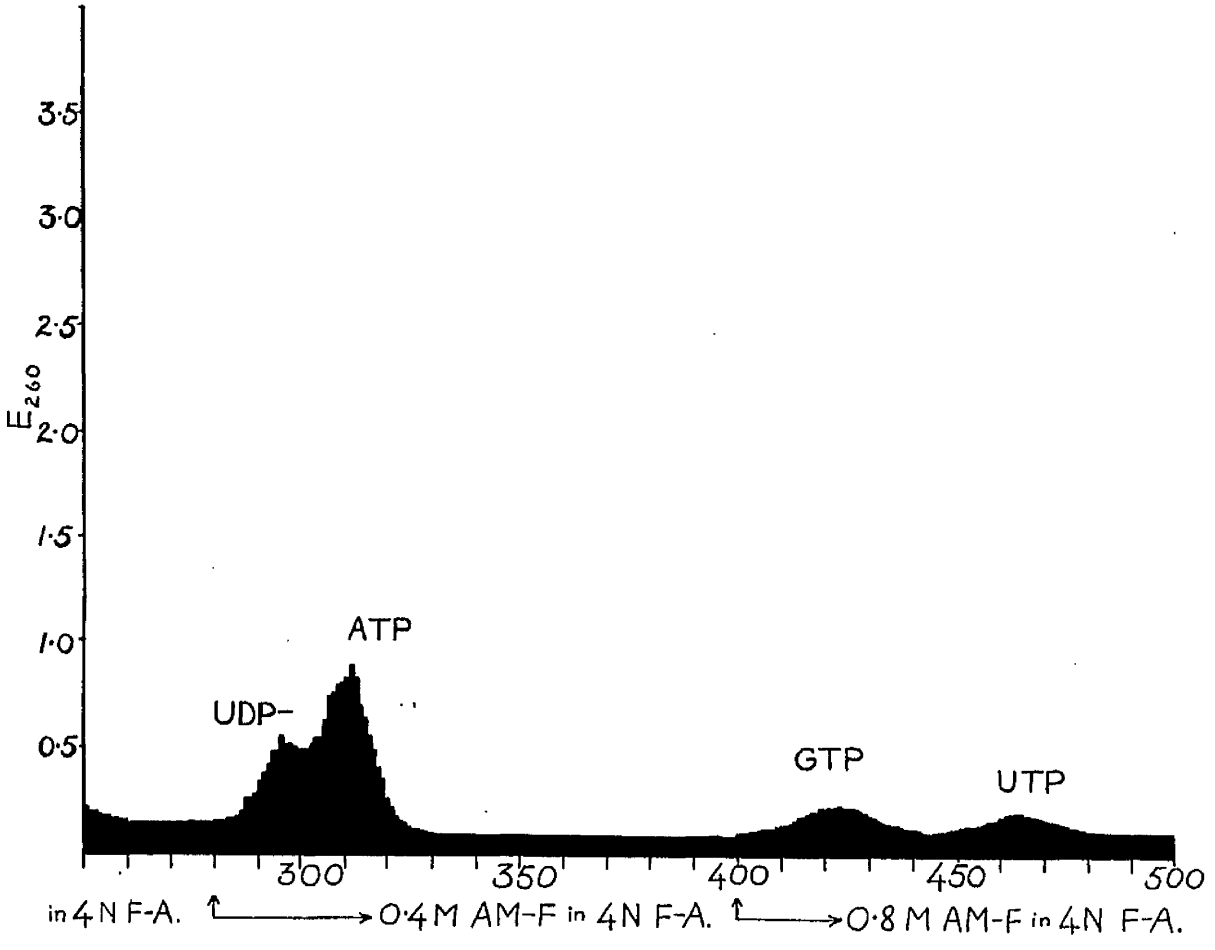

3·5
3·0
2·5
E_{260}
2·0
1·5
1·0
0·5
ATP
UDP-
GTP
UTP
300
350
400
450
500
in 4N F-A.
0·4 M AM-F in 4N F-A.
0·8 M AM-F in 4N F-A.

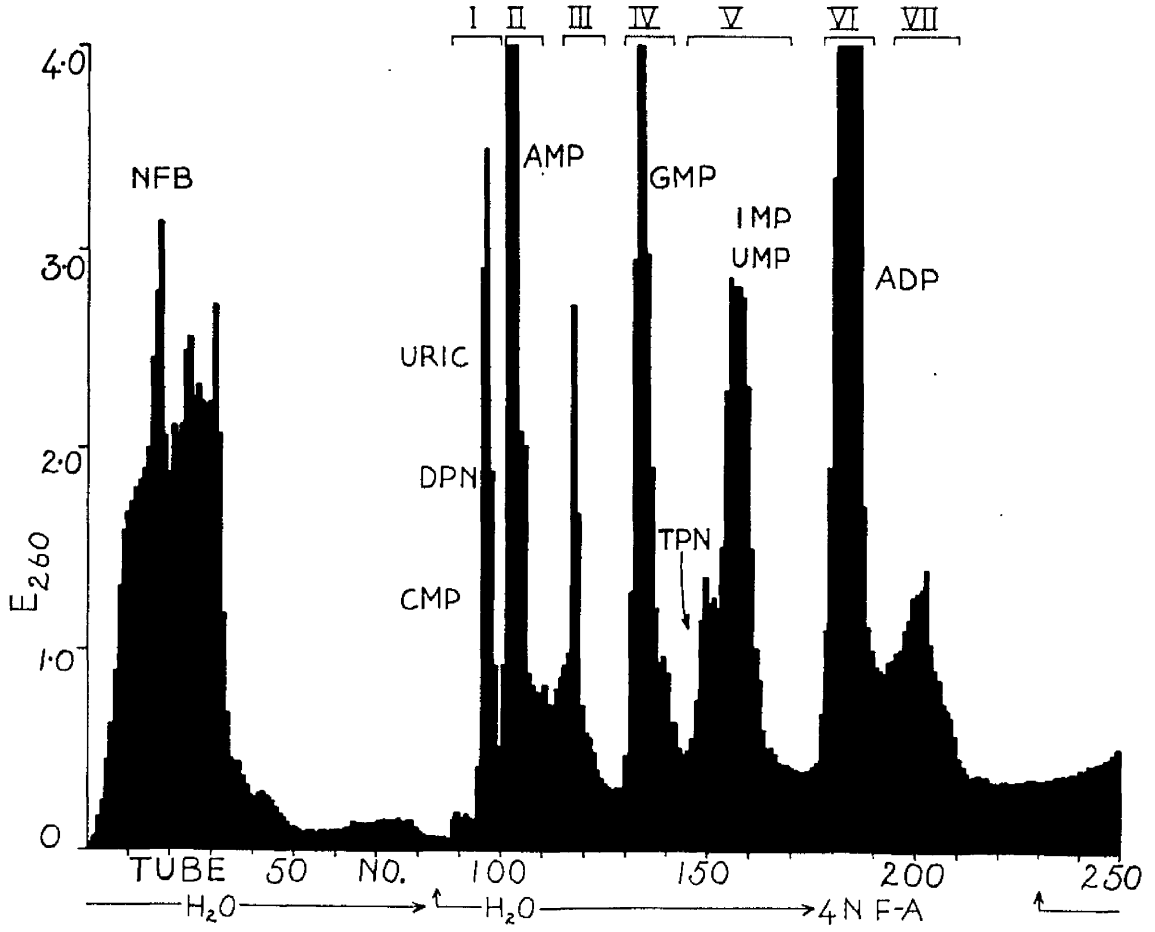
CHART 5.

Elution chart from the ion exchange chromatography of acid-soluble compounds absorbing at 260 mμ, obtained from 20 g. (wet weight) of rabbit intestinal mucosa. The mucosa tissue was a portion of the pooled material obtained from four albino rabbits, each of which had received 1 mc. ^{14}C-sodium formate two hours before killing.

Column:- Dowex-1-formate; 26.0 cm. x 1.0 cm.

Mixing Volume:- 500 ml. Fractions:- 50 drops per tube, about 3.0 ml. per tube.

Readings at 260 mμ which were too great to be plotted on the elution chart were, peak II 11.7, 4.6; peak IV 4.5, 4.25; peak VI 5.15, 6.93, 7.58, 7.95, 6.76, 4.50.


I
II
III
IV
V
VI
VII
4·0
3·0
2·0
1·0
0
E_{260}
NFB
AMP
GMP
IMP
UMP
ADP
URIC
DPN
TPN
CMP
TUBE NO.
50
100
150
200
250
H_2O
H_2O
4 N F-A

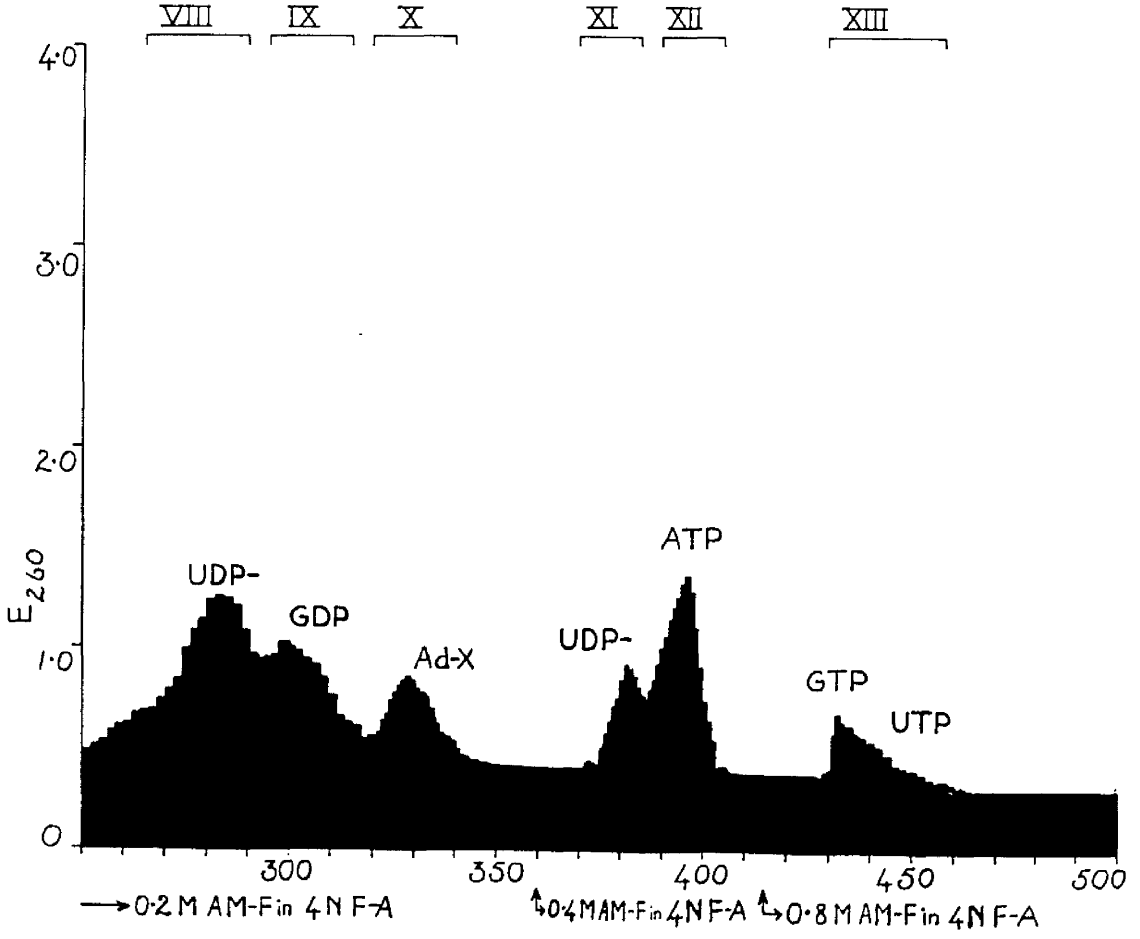

VIII
IX
X
XI
XII
XIII
4·0
3·0
2·0
1·0
0
E_{260}
UDP-
GDP
Ad-X
UDP-
ATP
GTP
UTP
300
350
400
450
500
0·2 M AM-Fin 4 N F-A
0·4 M AM-Fin 4 N F-A
0·8 M AM-Fin 4 N F-A

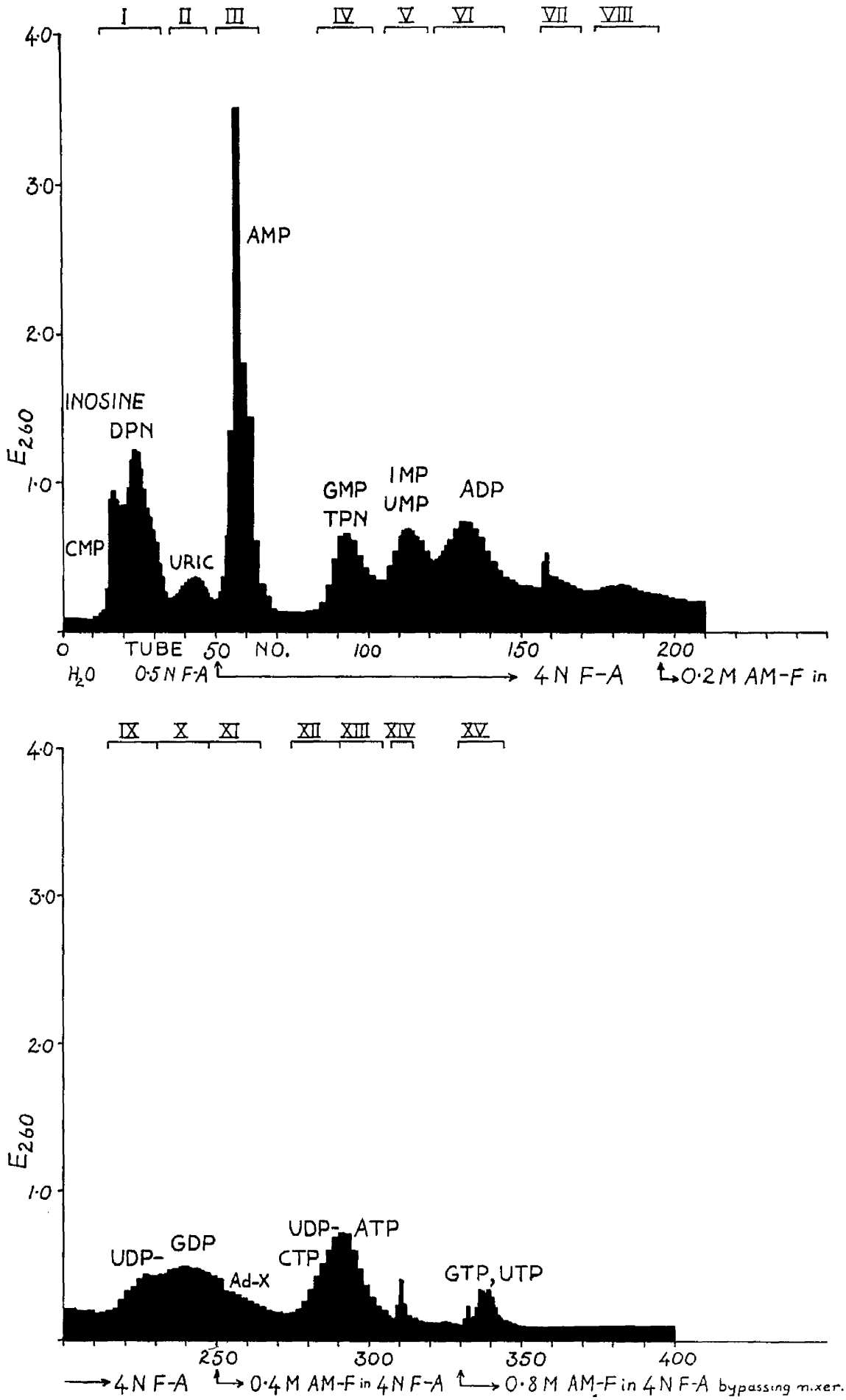
CHART 6.

Elution chart from the ion exchange chromatography of acid-soluble compounds absorbing at 260 mμ, obtained from 15 g. (wet weight) of rabbit intestinal mucosa. The mucosa was obtained from one rabbit which had received 1 mc. ^{14}C-sodium formate (marginal ear vein) 15 minutes before killing.

Column:- Dowex-1-formate; 20.0 cm. x 1.0 cm.

Mixing Volume:- 500 ml. Fractions:- 45 drops per tube from tubes 1 to 46, 75 drops per tube from tubes 47 to 190, and 100 drops per tube from tube 191. 100 drops per tube represents about 6.0 ml.

Prior to application to the column the sample was made slightly alkaline by the addition of a drop of ammonia solution.


I
II
III
IV
V
VI
VII
VIII
4·0
3·0
2·0
1·0
E_{260}
AMP
INOSINE
DPN
CMP
URIC
GMP
TPN
IMP
UMP
ADP
0
TUBE
50
NO.
100
150
200
H_2O
0·5 N F-A
4 N F-A
0·2 M AM-F in
IX
X
XI
XII
XIII
XIV
XV
4·0
3·0
2·0
1·0
E_{260}
UDP-
GDP
Ad-X
UDP-
CTP
ATP
GTP, UTP
250
300
350
400
4 N F-A
0·4 M AM-F in 4 N F-A
0·8 M AM-F in 4 N F-A bypassing mixer.

cytidine must be present in acid extracts of whole intestinal mucosa. In most experiments with intestinal mucosa, intestinal mucosa NAN, appendix and appendix NAN, CDP and CTP, if detected, were present in amounts rather smaller than the amount of CMP present. However, in one experiment with whole appendix (Chart 2), CMP and CDP were present in approximately equal proportions while CTP was slightly more abundant.

The regions of Charts 4, 5 and 6 following AMP and preceding GMP must be compared with each other and with the corresponding region of Chart 1. These fractions have in common $E_{275}:E_{260}$ ratios higher than that for AMP and lower than that for GMP. All show the presence of a compound or compounds containing adenine. This unidentified material appears in Chart 5 as a well-defined peak, peak III. The $E_{275}:E_{260}$ ratios of these fractions are shown in Table 6.

Following ADP and preceding UDP-, some compounds which do not correspond to ADP or to UDP- were eluted. All contained adenine and phosphorus. In Chart 6, one of the peaks, peak VIII, may contain a flavine nucleotide. The pooled fractions from this region of the chromatogram were pale yellow in colour and after acid hydrolysis gave adenine and other products of hydrolysis similar to those obtained by hydrolysis of an authentic sample of FAD (see Figure 7). Table 7 gives the $E_{275}:E_{260}$ ratios of these fractions from Charts 4, 5 and 6.

TABLE 6.

E_{275} : E_{260} ratios of fractions following AMP and preceding GMP in Charts 4, 5 and 6.

Chart No.	Tube No.	E_{275} : E_{260} ratio.
4	75	0.48
	77	0.46
	79	0.46
	81	0.52
	83	0.52
5	111	0.55
	113	0.52
	115	0.50
	117	0.59
	119	0.50
	121	0.59
6	67	0.45
	69	0.48
	71	0.53
	73	0.56
	75	0.56

TABLE 7.

E_{275} : E_{260} ratios of fractions eluted immediately after ADP in Charts 4, 5 and 6.

Chart No.	Tube No.	E_{275} : E_{260} ratio.
4	139	0.55
	143	0.50
	147	0.47
	151	0.48
	155	0.55
	159	0.46
	163	0.47
5	193	0.48
	197	0.56
	199	0.61
	201	0.67
	205	0.61
6	149	0.56
	151	0.69
	155	0.61
	159	0.53
	177	0.48
	185	0.47

The pattern of elution after introduction of 0.2M ammonium formate in 4N formic acid is similar in Charts 4, 5 and 6, but in Chart 6, compounds containing adenine were found after ATP in peak XIV and XV. In this experiment (see also Chart 3) the full eluting power of the last elution range was put into effect by bypassing the mixing vessel.

In Chart 6, the final modification of the techniques for detection, purification and identification of bases was used (see Section 2.9c).

3.1d. The Acid-Soluble Nucleotides of Rabbit Intestinal Mucosa Cell Nuclei.

The pattern of acid-soluble nucleotides obtained from rabbit intestinal mucosa NAN is shown in Chart 7. The extract of NAN in this experiment was applied to the column at pH 6.0 to 7.0.

The $E_{275}:E_{260}$ ratios following AMP and preceding GMP are entirely characteristic of AMP unlike the corresponding fractions from Charts 1, 2, 4, 5 and 6. It would appear then, that the unidentified component occurring in this part of the ion-exchange chromatograms from whole tissue is a component of the cytoplasm.

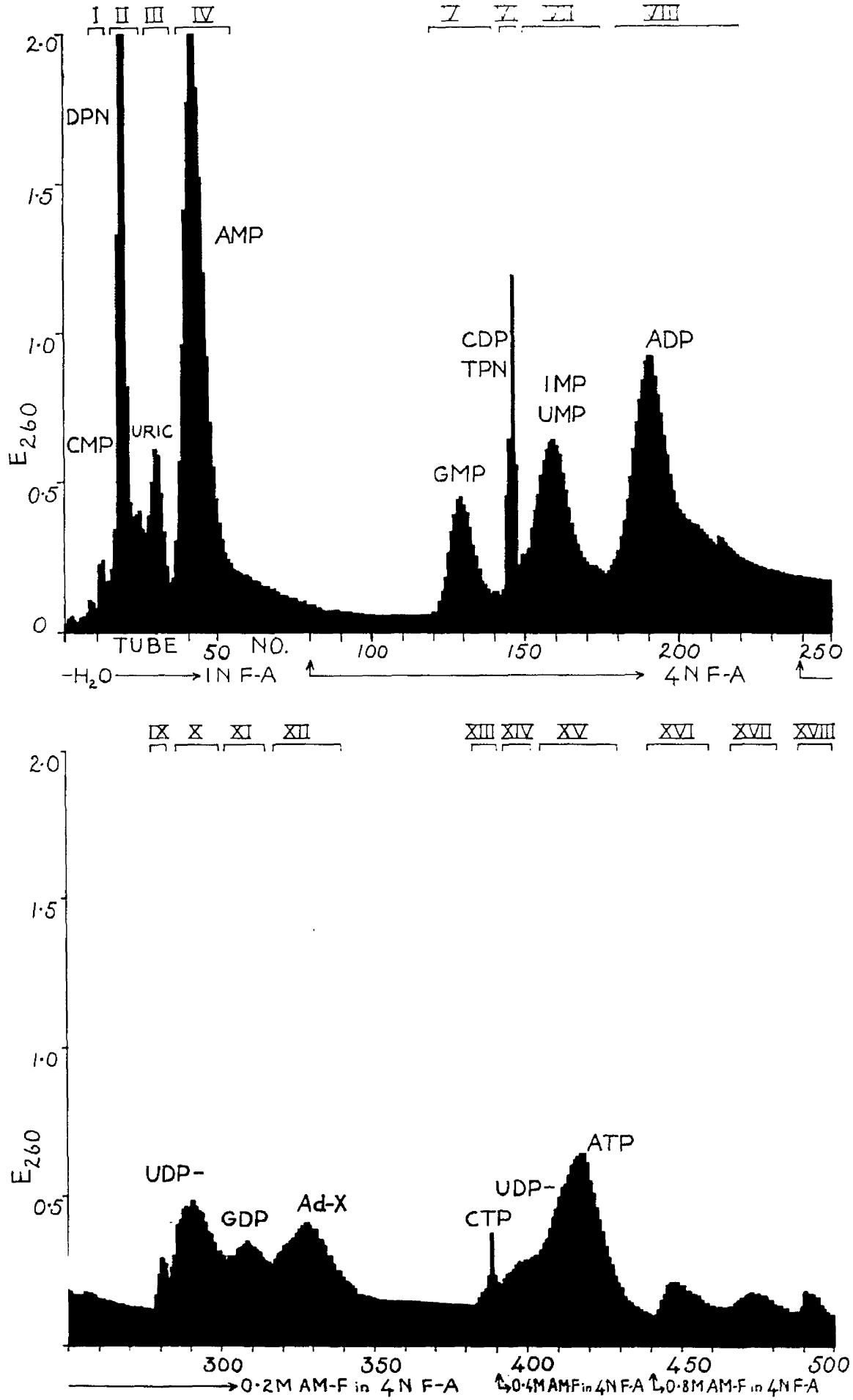
The fractions in the small peak eluted immediately after ADP have $E_{275}:E_{260}$ ratios higher than those for the fractions contained in the ADP peak.

CHART 7.

Elution chart from the ion exchange chromatography of acid-soluble compounds absorbing at 260 mμ, obtained from 1.1 g. (dry weight) of rabbit intestinal mucosa NAN. The NAN were prepared from 9.2 g. (dry weight) of mucosa tissue obtained from two rabbits.

Column:- Dowex-1-formate; 26.0 cm. x 0.7 cm.
Mixing Volume:- 250 ml. Fractions:- 40 drops per tube, about 2.5 ml. per tube.

Readings at 260 mμ which were too great to be plotted on the elution chart were, peak II 2.45, 2.05; peak III, 2.1, 2.1.


I II III IV
V VI VII VIII
2·0
1·5
1·0
0·5
0
E_{260}
DPN
AMP
CMP
URIC
CDP
TPN
IMP
UMP
ADP
GMP
TUBE NO.
50
100
150
200
250
$-H_2O$
1 N F-A
4 N F-A
IX X XI XII
XIII XIV XV
XVI XVII XVIII
UDP-
GDP
Ad-X
UDP-
CTP
ATP
300
350
400
450
500
0·2 M AM-F in 4 N F-A
0·4 M AM-F in 4 N F-A
0·8 M AM-F in 4 N F-A

Peaks XVI, XVII and XVIII did not contain sufficient material to permit isolation and identification of bases but the $E_{275}:E_{260}$ ratios found in this part of the chromatogram suggest that peaks XVII and XVIII contain GTP and UTP respectively.

In another experiment with NAN from the intestinal mucosa tissue obtained from rabbits which receive 1 mc. of ^{14}C-sodium formate two hours before killing, guanine and uracil were isolated after acid hydrolysis, from the regions of the chromatogram which correspond to peaks XVII and XVIII in Chart 7. In the same experiment, adenine was obtained by acid hydrolysis of the peak corresponding to peak XVI in Chart 7.

The radioactive NAN mentioned in the last paragraph were isolated from a portion of the pooled intestinal mucosa tissue from four rabbits each of which had received 1 mc. ^{14}C-sodium formate two hours before killing. Another portion of the same pool of mucosa tissue was used for the preparation of a whole tissue acid extract (see Chart 5).

Table 8 gives the specific activities of purine bases isolated from the nucleotides found in the extracts of nuclei and of whole tissue from this pool of mucosa tissue.

Also included in Table 8 are the specific activities of purine bases isolated from the purine nucleotides found in the acid extract of whole intestinal mucosa obtained from a rabbit which received 1 mc. ^{14}C-sodium formate 15 minutes before killing.

TABLE 8.

Specific activities of purine bases derived from the acid-soluble purine nucleotides of whole rabbit intestinal mucosa and of rabbit intestinal mucosa NAN.

Columns I and II give the results from experiments in which a group of four albino rabbits received 1 mc. of ^{14}C-sodium formate each two hours before killing. The pooled mucosa tissue from the four rabbits was lyophilised, ground to a powder and thoroughly mixed. The powder was divided into two portions, one for whole tissue extraction and the other for isolation of NAN.

Column III gives the results from an experiment in which an albino rabbit received 1 mc. of ^{14}C-sodium formate 15 minutes before killing.

TABLE 8.

Base	Nucleotide from which base was obtained.	Specific activity in counts per minute per µm. of base.		
		I Whole mucosa Two hours Chart 5	II Mucosa NAN Two hours Chart 7	III Whole mucosa 15 minutes Chart 6
Ad	AMP	8,090	6,970	2,000
Ad	ADP	6,450	6,870	2,100
Ad	ATP	5,330	7,350	1,950
Gu	GMP	6,680	-	4,230
Gu	GDP	7,460	7,500	4,550
Gu	GTP	-	5,350	3,900
Hx	IMP	6,000	-	8,540
Ad	DPN	-	9,300	510
Ad	TPN	6,850	7,350	950
Hx	from inosine			4,040

The purpose of this last experiment was to ascertain whether any large differences in specific activities of purine nucleotide bases could be demonstrated at very short time intervals between administration of ^{14}C-formate and the killing of the animal. The work of Abrams and Bentley (1955a; 1955b; 1955c) on soluble enzyme extracts from rabbit bone marrow, and of Williams and Buchanan (1953) and Greenberg (1950; 1951a) on pigeon liver extracts, had implicated inosinic acid as a central compound in purine biosynthesis.

Table 8 shows that after two hours, the specific activities of the purine bases from the adenosine-5'-phosphates, from the guanosine-5'-phosphates and from inosinic acid of both whole intestinal mucosa and intestinal mucosa NAN were of the same order.

After 15 minutes, however, large differences in specific activity were observed in the nucleotides of whole intestinal mucosa. The bases from AMP, ADP and ATP had similar specific activities which were about half the values obtained for GMP, GDP and GTP. The adenine in DPN and TPN had low specific activities. The value obtained for hypoxanthine in IMP was much greater than the values found for any of the other nucleotides and was about two times as great as the specific activities of guanine from the guanosine-5'-phosphates. Hypoxanthine from inosine had a specific activity about half that from IMP.

3.2 The Distribution of Acid-Soluble Nucleotides in the Sub-Cellular Fractions of Rabbit Liver.

After the series of experiments involving comparison of the acid-soluble nucleotide content of whole tissue with that of the corresponding nuclei had been carried out, it was considered desirable to take the investigation one stage further by examining the distribution of acid-soluble nucleotides in the various cytoplasmic fractions of the cell. Accordingly, rabbit liver, which was the most convenient tissue for this purpose, was fractionated by the technique of differential centrifugation in sucrose media to give nuclei, mitochondria, microsomes and cell sap. The acid extracts of these fractions were submitted to ion exchange chromatography on Dowex-1 resin.

3.2a. The Acid-Soluble Nucleotides of Rabbit Liver Cell Nuclei.

Chart 8 shows the pattern of the ion exchange chromatography of acid-soluble nucleotides present in nuclei which were isolated in a sucrose/calcium chloride solution as described in Section 2.6a.

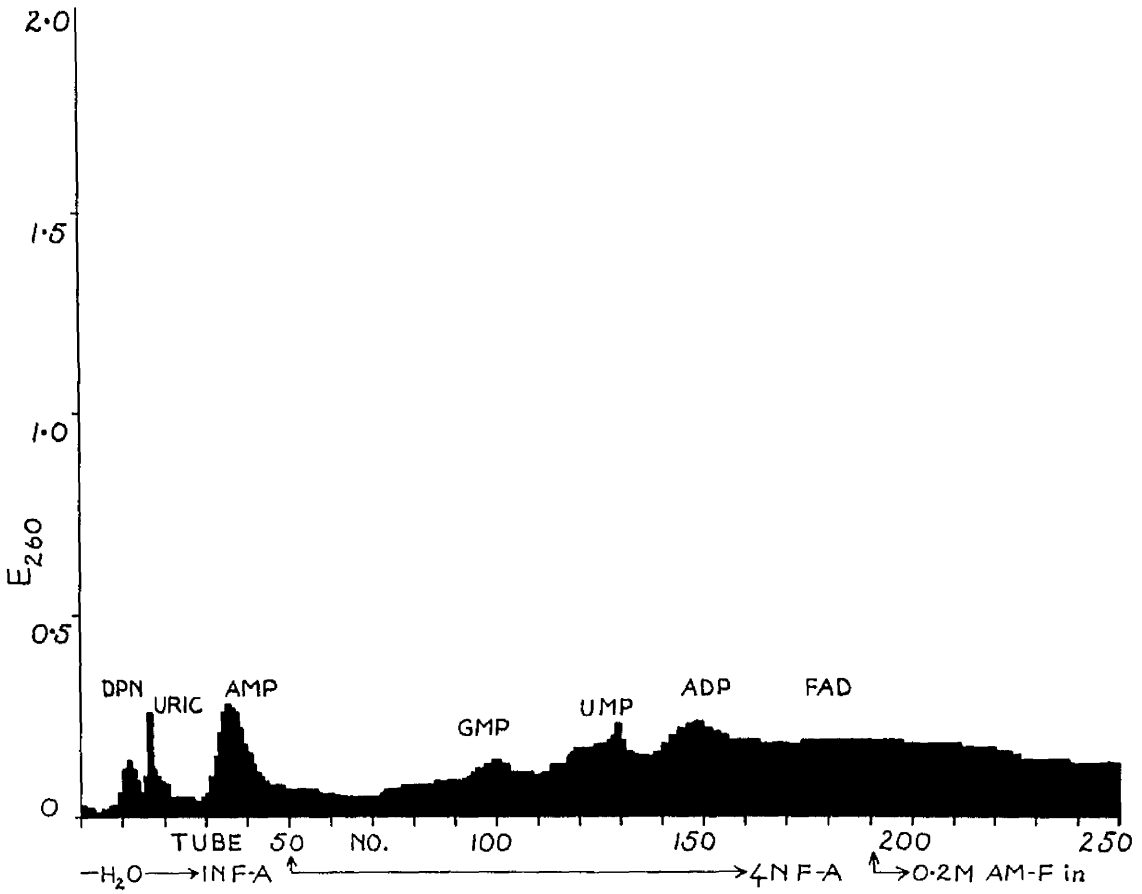
The identification of nucleotides in this system was made by observation of position on the ion exchange chromatogram, calculation of $E_{275}:E_{260}$ ratios of fractions emerging from the column, and isolation and identification of bases by chromatography on paper in two dimensions after acid hydrolysis of the material in the pooled fractions. The

CHART 8.

Elution chart from the ion exchange chromatography of acid-soluble compounds absorbing at 260 mμ, obtained from rabbit liver SN. The SN were prepared from 20 g. of liver tissue.

Column:- Dowex-1-formate; 26.0 cm. x 0.7 cm.
Mixing Volume:- 250 ml. Fractions:- 40 drops per tube, about 2.5 ml. per tube.

Prior to application to the column, the sample was made slightly alkaline by the addition of a drop of ammonia solution.


2·0
1·5
1·0
0·5
0
E_{260}
DPN
URIC
AMP
GMP
UMP
ADP
FAD
TUBE 50 NO. 100 150 200 250
$-H_2O \rightarrow$ IN F-A
4N F-A
0·2M AM-F in

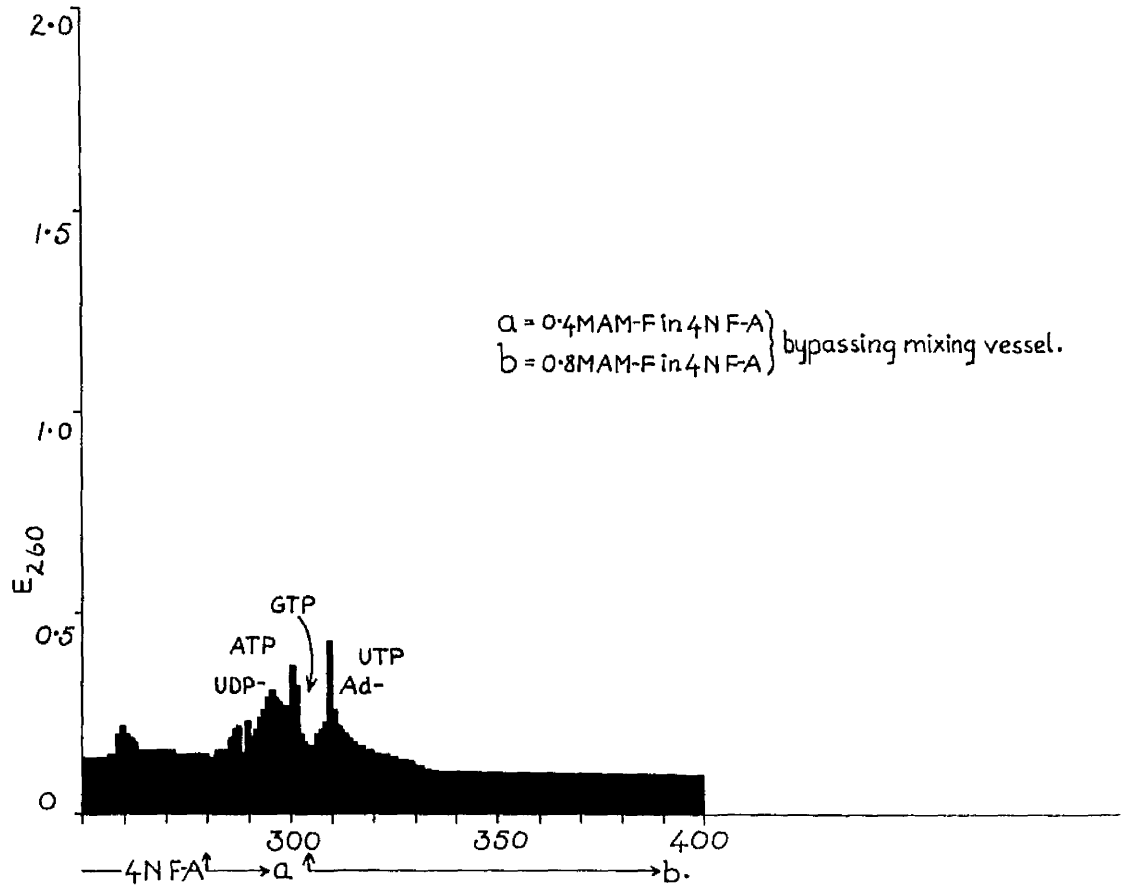

2·0
1·5
1·0
0·5
0
E_{260}
GTP
ATP
UDP-
UTP
Ad-
300 350 400
4N F-A
a
b.
a = 0·4MAM-F in 4N F-A
b = 0·8MAM-F in 4N F-A
bypassing mixing vessel.

FIGURE 7.

Diagram of a two-dimensional paper chromatogram of a 12 N $HClO_4$ hydrolysate of an authentic sample of FAD.

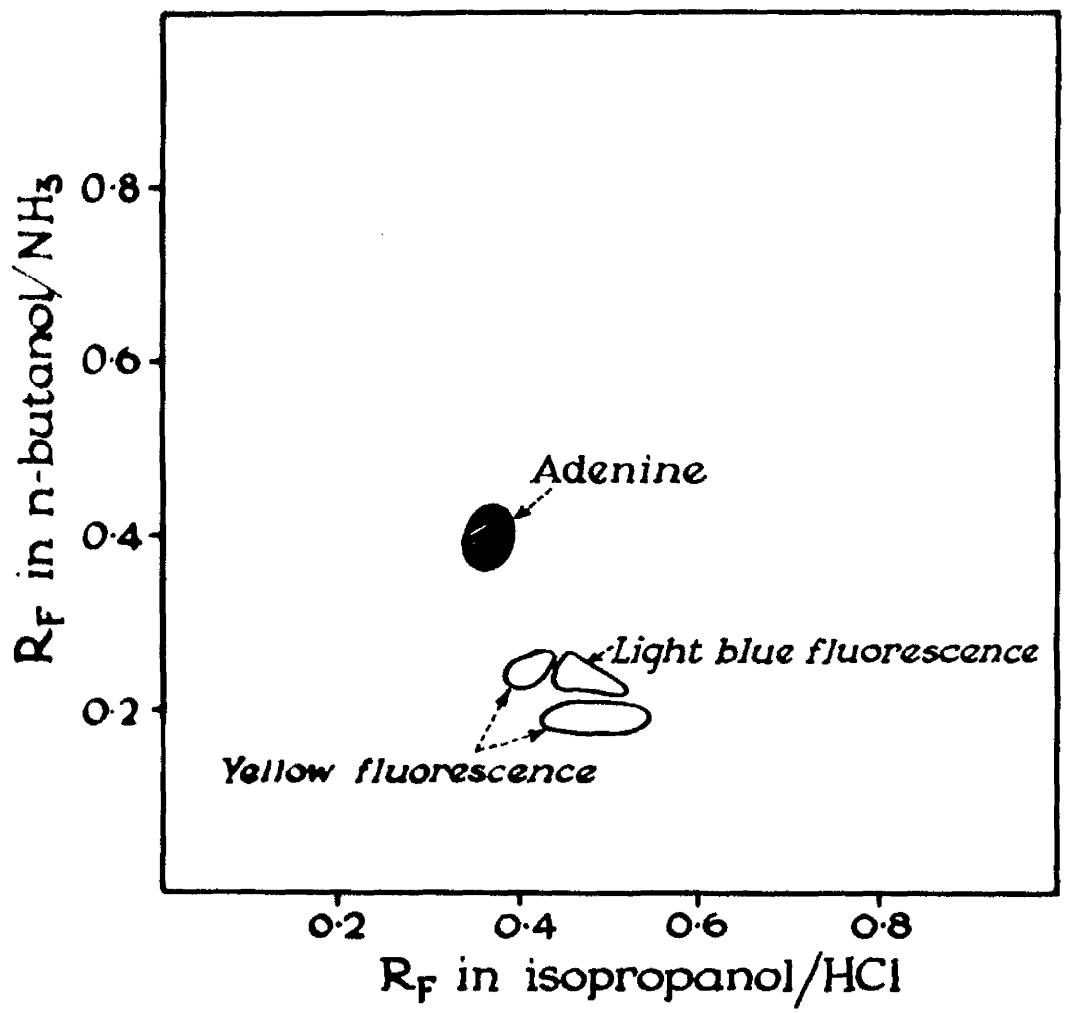

0·8
0·6
0·4
0·2
R_F in n-butanol/NH_3
Adenine
Light blue fluorescence
Yellow fluorescence
0·2
0·4
0·6
0·8
R_F in isopropanol/HCl

Figure 7.

obvious scarcity of material did not permit the use of paper chromatography in the ethanol/ammonium acetate solvent and of further chromatography of bases as described in Section 2.9c.

Fractions 170-220 when pooled and concentrated by partial lyophilisation gave a yellow-coloured solution. Acid hydrolysis of the dry material yielded adenine and other products of hydrolysis similar to those obtained by acid hydrolysis of pure FAD. Figure 7 shows a diagram of the two-dimensional paper chromatography of the hydrolysate of the authentic FAD sample. In addition to adenine two yellow fluorescent spots and one bright blue fluorescent spot were observed on the chromatogram by inspection in ultraviolet light.

The last peak to be eluted from the column contained UTP and a compound which gave adenine after hydrolysis with acid. The occurrence of compounds of adenine in this region of the ion exchange chromatogram was observed in other experiments (see Sections 3.1b, 3.1c and 3.1d).

3.2b. <u>The Acid-Soluble Nucleotides of Rabbit Liver Mitochondria.</u>

The ion exchange chromatogram of the compounds absorbing at 260 mμ obtained from a rabbit liver mitochondrial extract is depicted in Chart 9.

In peak I, uric acid was taken to be present largely

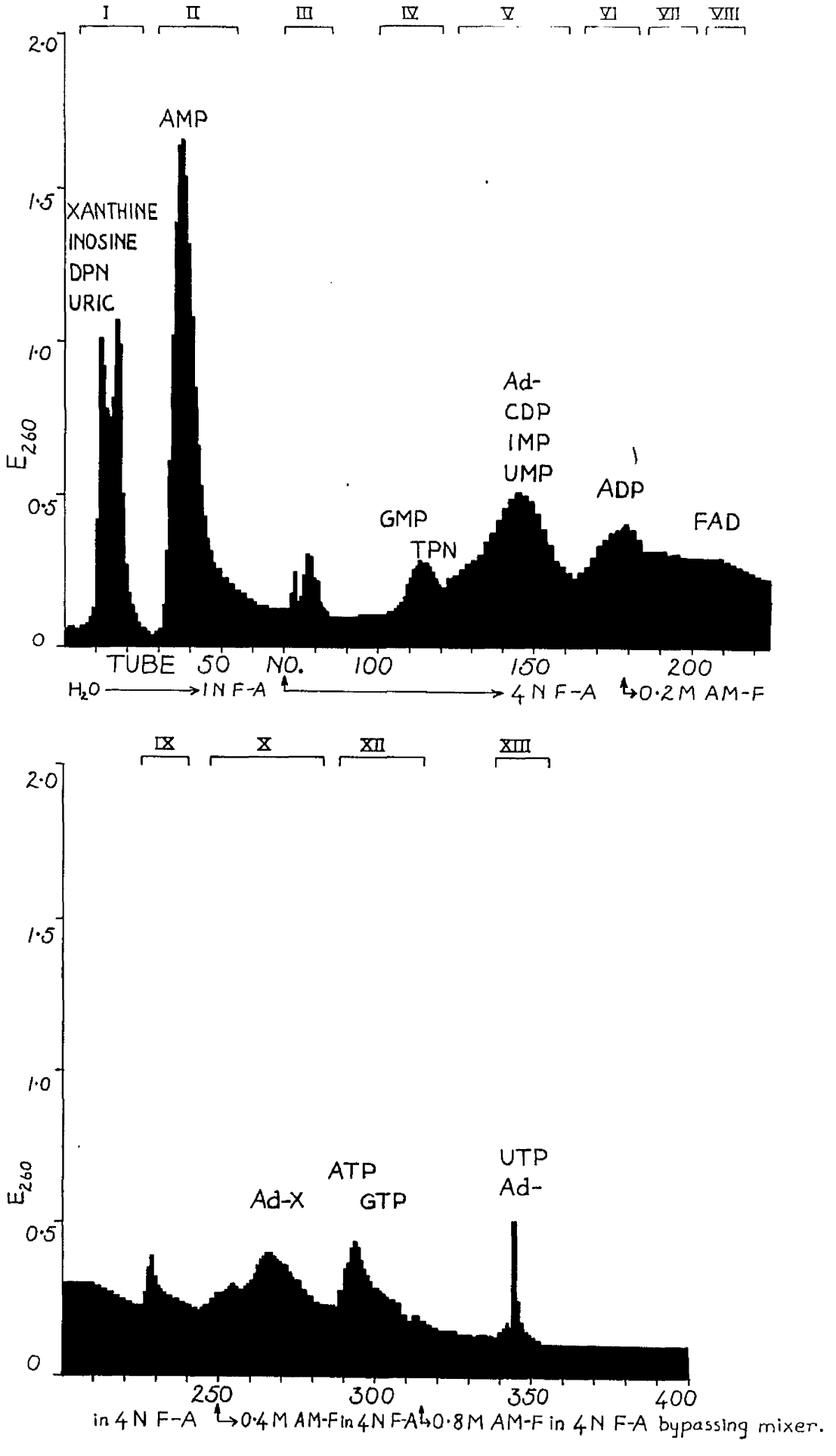
CHART 9.

Elution chart from the ion exchange chromatography of acid-soluble compounds absorbing at 260 mμ, obtained from rabbit liver mitochondria. The mitochondria were prepared from 50 g. of liver tissue.

Column:- Dowex-1-formate; 26.0 cm. x 0.7 cm.
Mixing Volume:- 250 ml. Fractions:- 40 drops per tube, about 2.5 ml. per tube.

Prior to application to the column, the sample was made slightly alkaline by the addition of a drop of ammonia solution.

The mitochondria in this experiment were obtained from the same liver as the microsomes of Chart 12. Both the mitochondria and the microsomes were treated with perchloric acid immediately after isolation.


I
II
III
IV
V
VI
VII
VIII
2·0
1·5
1·0
0·5
0
E_{260}
AMP
XANTHINE
INOSINE
DPN
URIC
Ad-
CDP
IMP
UMP
ADP
GMP
TPN
FAD
TUBE 50 NO. 100 150 200
H_2O → IN F-A → 4 N F-A ↳0·2 M AM-F
IX
X
XII
XIII
2·0
1·5
1·0
0·5
0
E_{260}
Ad-X
ATP
GTP
UTP
Ad-
250 300 350 400
in 4 N F-A ↳0·4 M AM-F in 4 N F-A ↳0·8 M AM-F in 4 N F-A bypassing mixer.

on the basis of a high $E_{275}:E_{260}$ ratio in tubes 19-23. The relative amounts of the four components of peak I are shown below.

Component	Relative Amount
Xanthine	+ + +
Inosine	+ + + + + +
DPN	+
Uric Acid	Trace

Peak III on hydrolysis with acid yielded one base only, adenine. The $E_{275}:E_{260}$ ratios of the fractions in this peak were about 0.46, a ratio lower than that observed for the corresponding peaks in previous ion exchange chromatograms of whole appendix and whole intestinal mucosa extracts. It is possible that the change of reservoir eluent from 1N formic acid to 4N formic acid hastened the elution of the "tail" of AMP and that peak III contains AMP in addition to another unknown compound.

The amount of TPN in peak IV was about one-half that of GMP.

Peak V, in addition to CDP, IMP and UMP contained another compound which yielded adenine after acid hydrolysis. This compound was present in fairly large quantity and was not ADP. The relative amounts of the components of this peak are given on the following page.

Component	Relative Amount
GDP	Trace
IMP	+
UMP	+ +
Adenine-compound	+ + +

The region of the chromatogram shown on Chart 9 as peak VIII was tentatively identified as FAD. The pooled fractions of this peak were yellow in colour. After hydrolysis with perchloric acid, products similar to the hydrolysis products of an authentic sample of FAD were obtained (see Figure 7).

Acid hydrolysis of peak IX produced the bases adenine and uracil, the latter probably resulting from the degradation of a UDP derivative. The adenine of this peak was probably derived from the overlapping portions of peaks VIII and X.

Peak X contained a large amount of a nucleotide of adenine preceded by a smaller amount of a nucleotide of uracil. The adenine nucleotide emerged from the column at the point at which the ADP-X of Schmitz et al. (1954) would be expected to emerge. GDP could not be detected in this part of the chromatogram.

Peak XII contained ATP and GTP in the approximate ratio of 8 to 1. UTP together with an equal amount of a

compound which yielded adenine after acid hydrolysis were found in peak XIII (see Section 3.1b).

In another experiment with rabbit liver mitochondria, a good separation of compounds eluted in the 1N formic acid range was obtained. Part of the elution chart from this experiment is shown in Chart 10. It is of interest that, in this experiment, the isolated mitochondria were stored in a deep-freeze cabinet at -10° for a few days prior to perchloric acid extraction (see Chart 2). This may account for the occurrence of large amounts of xanthine and hypoxanthine on Chart 10. It is of considerable interest that the microsomal fraction (Chart 11) corresponding to the mitochondria of Chart 10 contained hypoxanthine and xanthine in peak I. These microsomes were stored at -10° for a few days before homogenising in perchloric acid. It is also worth noting at this stage that the microsomal fraction (Chart 12), corresponding to the mitochondrial fraction of Chart 9, contained no hypoxanthine, nor did the mitochondrial fraction. These two extracts (Charts 9 and 12) were prepared immediately after isolation of the cytoplasmic particles. However, the storage time is not the only factor involved, for hypoxanthine was found in the MFB fraction in several experiments, so that the pH of the extract on application to the column must be a critical factor in determining whether hypoxanthine will be retained on the resin.

CHART 10.

Part of the elution chart from the ion exchange chromatography of acid-soluble compounds absorbing at 260 mμ, obtained from rabbit liver mitochondria.

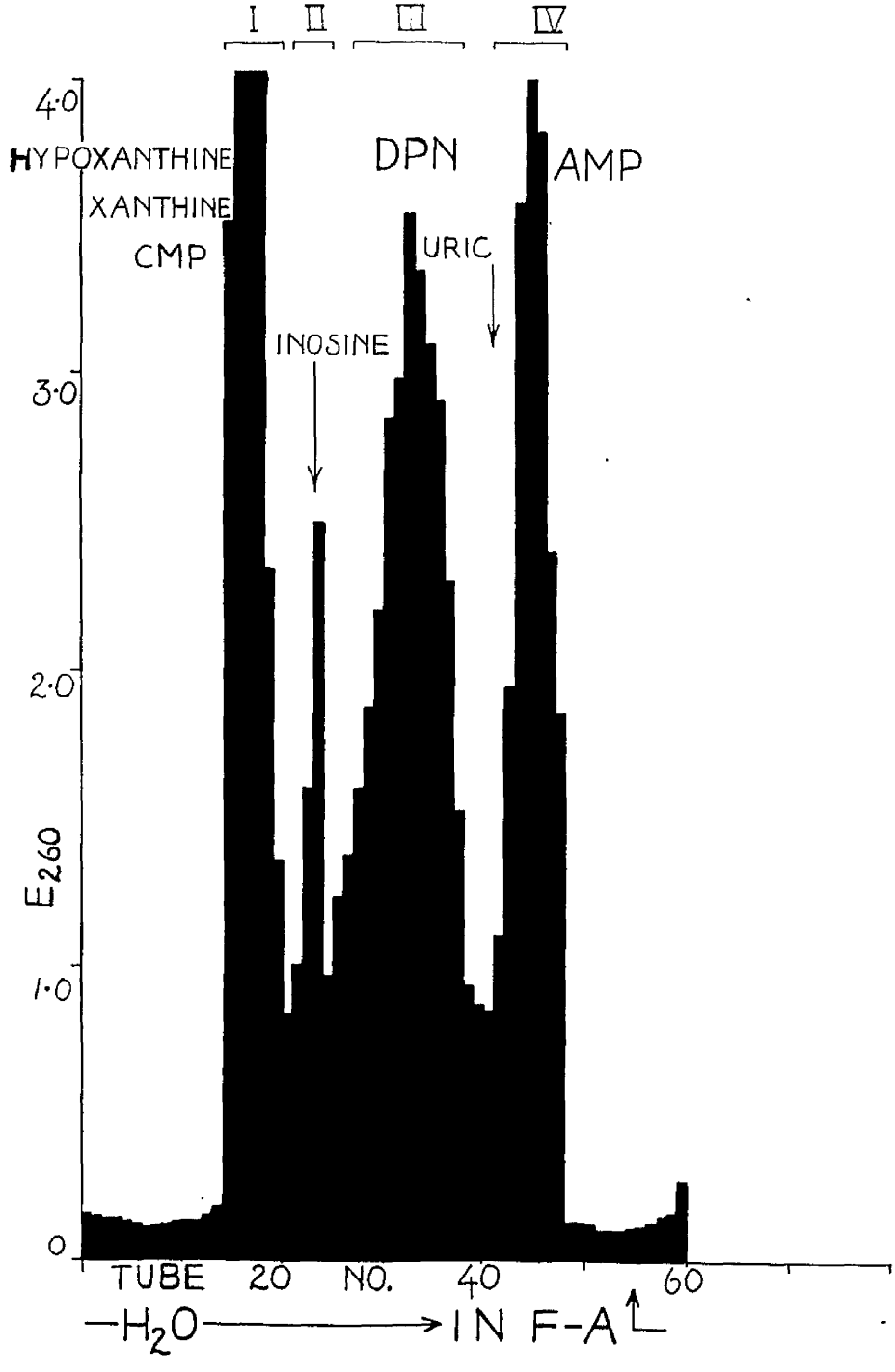
Column:- Dowex-1-formate; 26.0 cm. x 0.7 cm.

Mixing Volume:- 250 ml. Fractions:- 40 drops per tube, about 2.5 ml. per tube.

Prior to application to the column, the sample was made slightly alkaline by the addition of a drop of ammonia solution.

Readings at 260 mμ which were too great to be plotted on the elution chart were, peak I 10.7, 8.95, 5.07; peak IV, 4.04.

The mitochondria in this experiment were obtained from the same liver as the microsomes of Chart 11. Both the mitochondria and the microsomes were stored in a deep-freeze cabinet for a few days prior to acid extraction.


I
II
III
IV
4·0
3·0
2·0
1·0
0
E260
HYPOXANTHINE
XANTHINE
CMP
INOSINE
DPN
URIC
AMP
TUBE 20 NO. 40 60
—H2O——→IN F-A

The relative amounts of the components of peak I, Chart 10, are given below.

Component	Relative Amount.
Hypoxanthine	+ + +
Xanthine	+ + + + +
CMP	+

3.2c. The Acid-Soluble Nucleotides of Rabbit Liver Microsomes.

Charts 11 and 12 are the elution charts from the ion-exchange chromatography of the acid-soluble nucleotides extracted from two preparations of rabbit liver microsomes.

The microsomes of Chart 11 were stored at -10° for about 3 days prior to treatment with perchloric acid, while the microsomes of Chart 12 were treated with the cold perchloric acid immediately after isolation from the cold sucrose medium (Section 2.6b). In both experiments, the isolation of microsomes was carried out with all possible speed and great care was always taken to ensure that all operations were carried out at 0°.

As the acid-soluble nucleotide contents of the nuclear mitochondrial, microsomal and cell sap fractions were being investigated in these experiments, it was inevitable that some of the acid extracts of the sub-cellular fractions from any one rabbit liver preparation were stored at -10° for periods of twelve to twenty-nine

CHART 11.

Elution chart from the ion exchange chromatography of acid-soluble compounds absorbing at 260 mμ, obtained from rabbit liver microsomes.

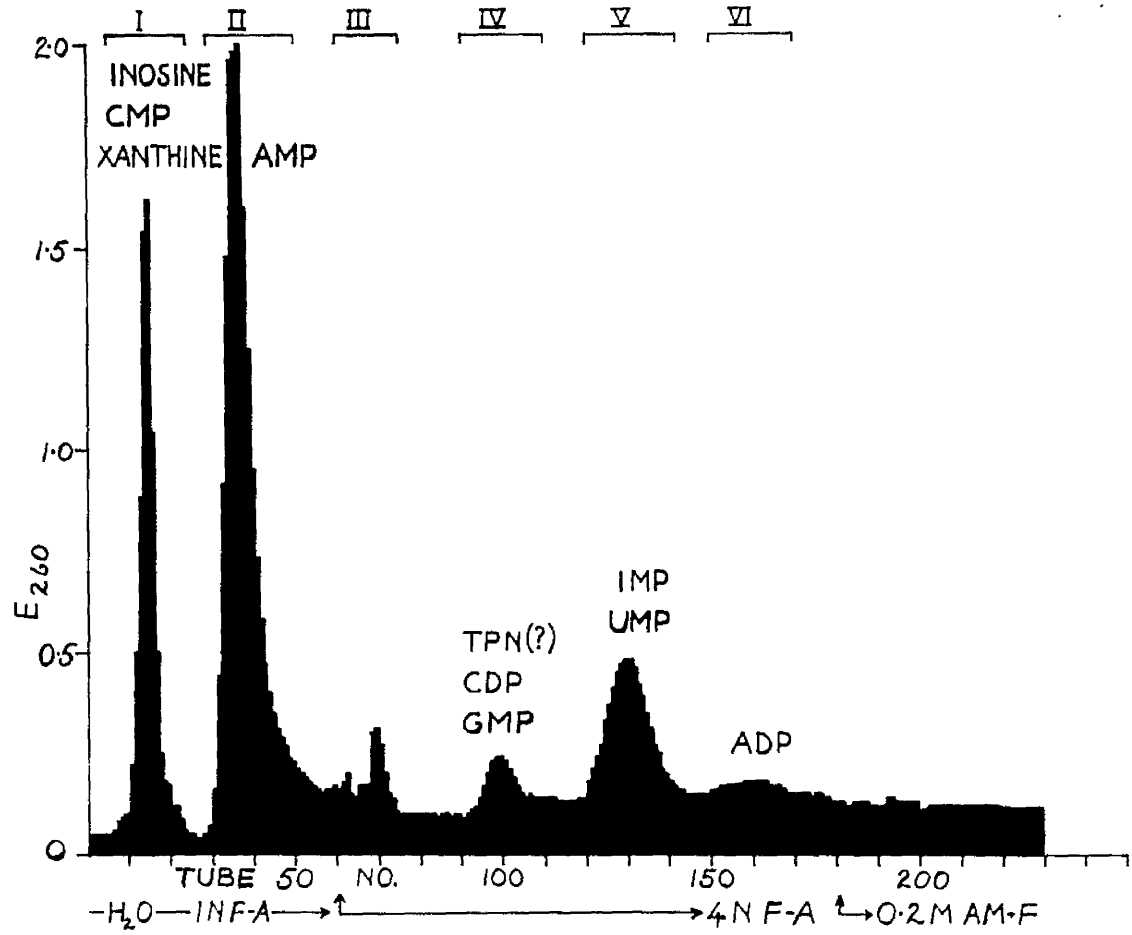
Column:- Dowex-1-formate; 26.0 cm. x 0.7 cm.

Mixing Volume:- 250 ml. Fractions:- 40 drops per tube about 2.5 ml. per tube.

Prior to application to the column, the sample was made slightly alkaline by the addition of a drop of ammonia solution.

Reading at 260 mμ which was too great to be plotted on the elution chart was peak II, 2.1.

The microsomes in this experiment were obtained from the same liver as the mitochondria of Chart 10. Both the mitochondria and the microsomes were stored in a deep-freeze cabinet for a few days prior to acid extraction.


I
II
III
IV
V
VI
2·0
1·5
1·0
0·5
0
E 260
INOSINE
CMP
XANTHINE
AMP
TPN(?)
CDP
GMP
IMP
UMP
ADP
TUBE 50 NO. 100 150 200
-H2O—INF-A→
→4N F-A
→0·2M AM-F

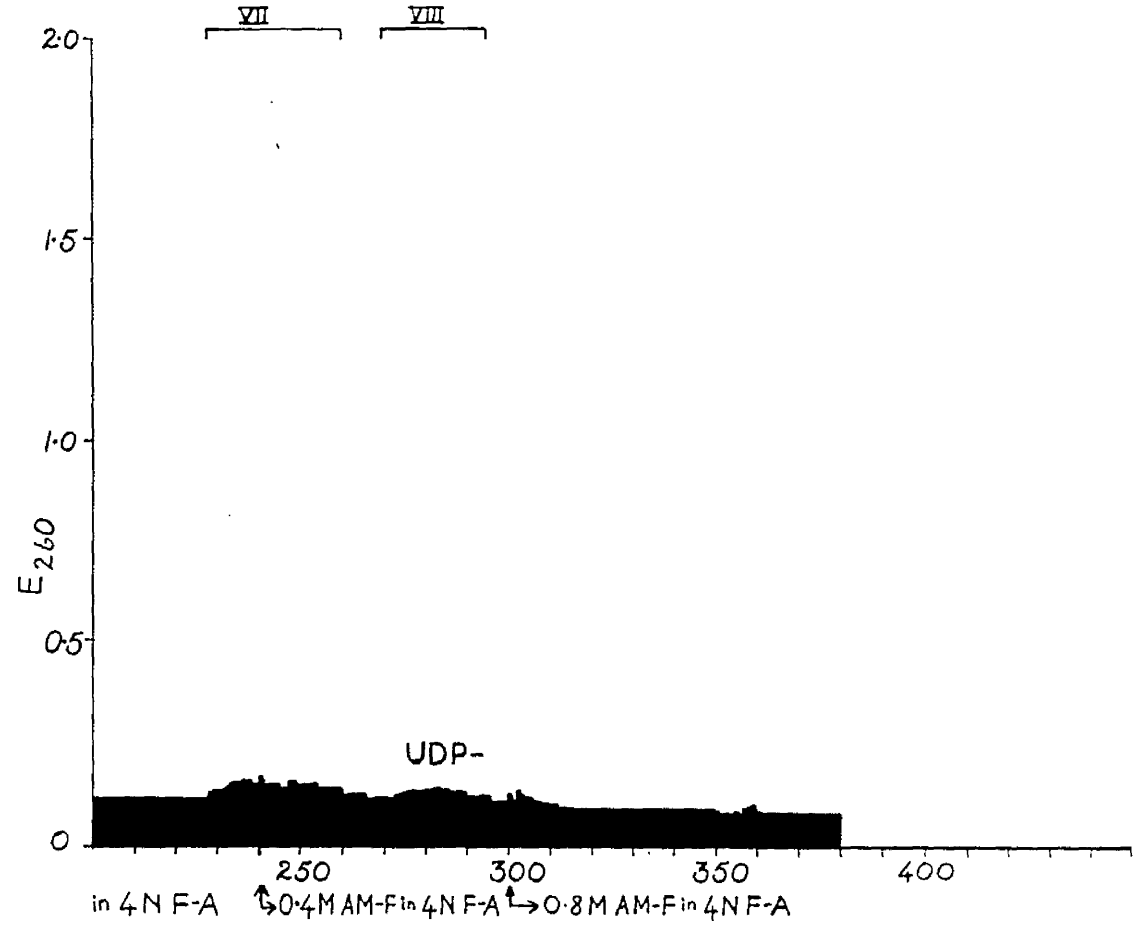

VII
VIII
2·0
1·5
1·0
0·5
0
E 260
UDP-
250 300 350 400
in 4N F-A
→0·4M AM-F in 4N F-A
→0·8M AM-F in 4N F-A

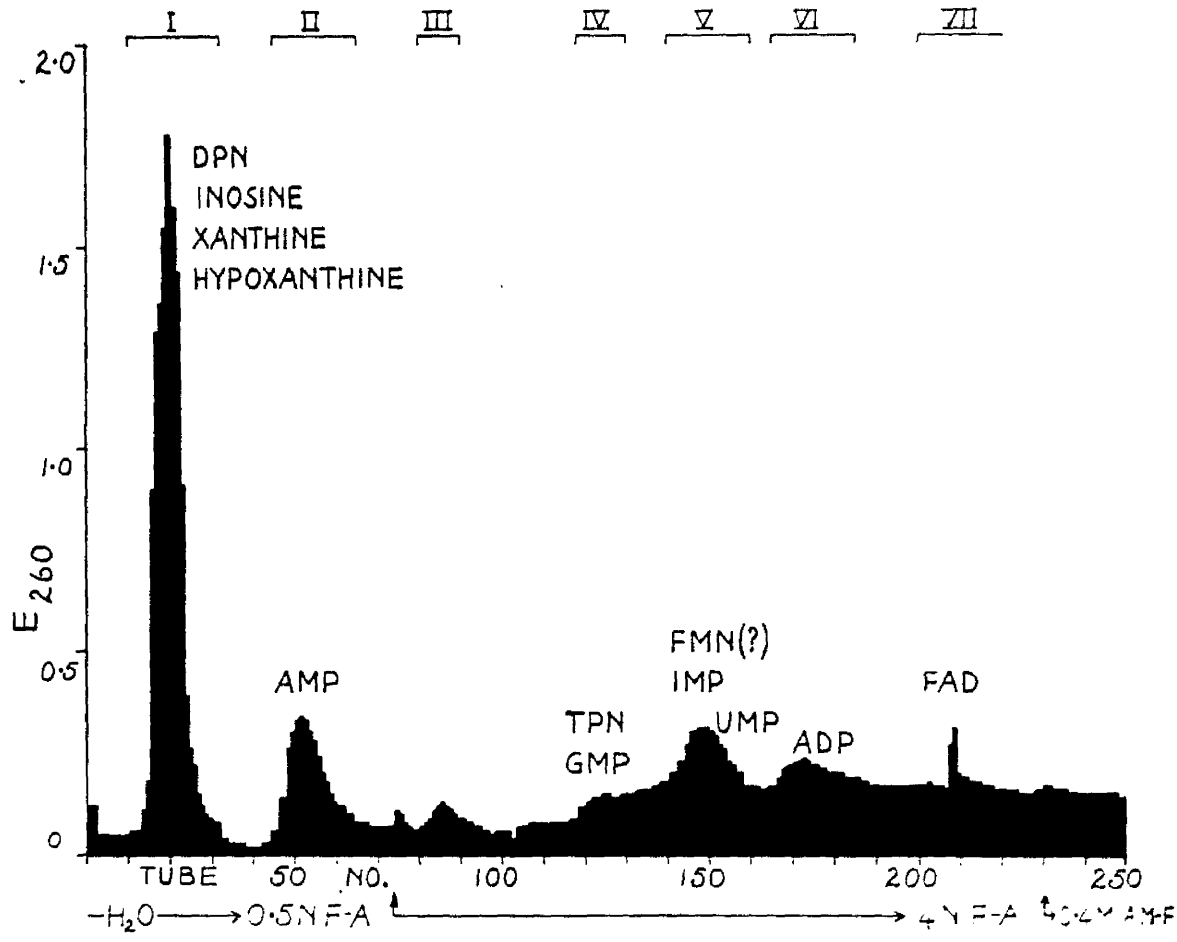
CHART 12.

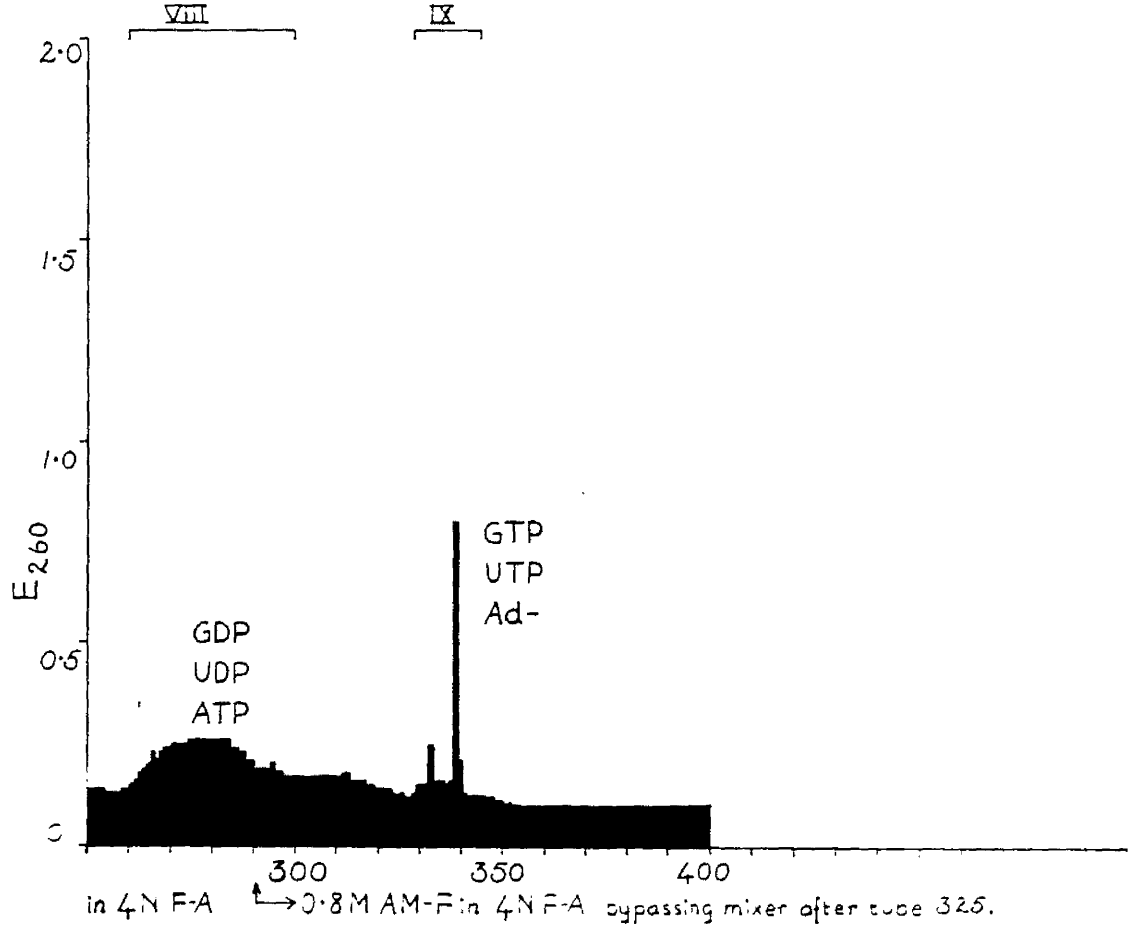
Elution chart from the ion exchange chromatography of acid-soluble compounds absorbing at 260 mμ, obtained from rabbit liver microsomes. The microsomes were prepared from 50 g. of liver tissue.

Column:- Dowex-1-formate; 26.0 cm. x 0.7 cm.
Mixing Volume:- 250 ml. Fractions:- 40 drops per tube about 2.5 ml. per tube.

Prior to application to the column, the sample was made slightly alkaline by the addition of a drop of ammonia solution.

The microsomes in this experiment were obtained from the same liver as the mitochondria of Chart 9. Both the mitochondria and the microsomes were treated with perchloric acid immediately after isolation.


I
II
III
IV
V
VI
VII
2·0
1·5
1·0
0·5
0
E 260
DPN
INOSINE
XANTHINE
HYPOXANTHINE
AMP
TPN
GMP
FMN(?)
IMP
UMP
ADP
FAD
TUBE
50
NO.
100
150
200
250
-H2O
0·5 N F-A
4 N F-A


VIII
IX
2·0
1·5
1·0
0·5
0
E 260
GTP
UTP
Ad-
GDP
UDP
ATP
300
350
400
in 4 N F-A
0·8 M AM-F in 4 N F-A bypassing mixer after tube 325.

days. However, in the present series of experiments, there was no indication of breakdown of nucleotides, particularly of tri- and diphosphates to monophosphates, in acid extracts stored in the frozen state at pH 7.

One of the most obvious differences between Charts 11 and 12 was the presence of a large amount of AMP in Chart 11 as compared with Chart 12. One cause of this could have been breakdown of the more highly phosphorylated adenosine compounds with resultant increase in the monophosphate concentration. In this connection, it is of interest to note that Chart 12 indicates the occurrence of larger amounts of the adenosine, uridine and guanosine di- and triphosphates than does Chart 11 in which the monophosphates occur in greater amounts with concomitant decrease in quantity of the more highly phosphorylated nucleosides.

The components of peak I in both charts were present in the relative amounts shown below.

Component	Relative Amount
Chart 11.	
AMP	Trace
Inosine	+ + +
Xanthosine (?)	+ +
Xanthine	+ + + + +

Component	Relative Amount
Chart 12.	
Hypoxanthine	+
Inosine	+ + + +
Xanthine	+
DFN	Trace

Although not shown on Chart 11 xanthine was possibly present both in the free state and in combination with ribose, as xanthosine. After elution from paper chromatograms run in the ethanol/ammonium acetate solvent the spot suspected to be xanthosine gave a spectrum, in acid solution, similar to that found for xanthosine by Beaven, Holliday and Johnson (1955). However, this xanthosine spectrum closely resembles the xanthine spectrum and the spot suspected to contain xanthosine may have in fact been a "tail" of the larger xanthine spot obtained on the same paper chromatogram. Both spots, after hydrolysis with perchloric acid, yielded xanthine.

A small peak was eluted from the column immediately after AMP in both Chart 11 and Chart 12. These peaks after paper chromatography in ethanol/ammonium acetate produced three spots, two of which gave spectra characteristic of adenosine nucleotides, while the third had a spectrum with a maximum absorption at 268 mμ. Further analysis of these peaks was not possible because of the shortage of material.

Peak V, Chart 12, after chromatography on paper in ethanol/ammonium acetate gave spots identified as IMP, UMP, ADP, possibly CDP, and two spots which gave a yellow fluorescence in ultraviolet light and another spot which gave a bright blue fluorescence. The bright blue spot travelled on paper in this solvent system with a high Radenosine value and absorbed ultraviolet light maximally at 257.5 mμ and at 330 mμ. The amounts of material were very small so that the ultraviolet absorption measurements could not be considered to be absolutely reliable. Silipxandi and Bianchi (1955) prepared FAD from yeast and analysed their preparations by electrophoresis on paper and by chromatography on paper. On these papers, yellow spots were produced together with a number of spots with a blue fluorescence when examined in ultraviolet light. Two of the yellow-fluorescing spots were identified as FAD and FMN respectively. In the present investigation it was found that acid hydrolysis of pure FAD produced adenine together with three spots, two of which gave a yellow fluorescence and the other a bright blue fluorescence in ultraviolet light (see Figure 7).

The possibility of there being FMN in peak V, Chart 12, is indicated on the flow chart.

Owing to the fact that conditions of low temperature and speed of preparation of the acid extract of Chart 12 were rigorously observed at all stages, the pattern shown in Chart 12 must be considered as being a more accurate picture

of the acid-soluble nucleotide content of liver microsomes than that shown in Chart 11.

The principal features of Chart 12 are (a) the large amount of inosine in peak I, and (b) the relative scarcity of the more highly phosphorylated nucleosides.

3.2d. The Acid-Soluble Nucleotides of Rabbit Liver Cell Sap.

Chart 13 is the elution chart from ion exchange chromatography of the acid-soluble ultraviolet-absorbing compounds of the rabbit liver cell sap fraction (Section 2.6b).

The cell sap fraction used in this experiment was obtained from the same batch of liver as the nuclei, mitochondria and microsomes of Charts 8, 9 and 12 respectively.

In another similar experiment with cell sap isolated from the same batch of liver as the mitochondria of Chart 10 and the microsomes of Chart 11, much more of the nucleotide material was in the mono-phosphate form.

In Chart 13, the mono-, di- and triphosphates of adenosine, guanosine and uridine were present together with CMP and CDP. No CTP was detected.

The relative amounts of the components of peak I are given on the following page.

CHART 13.

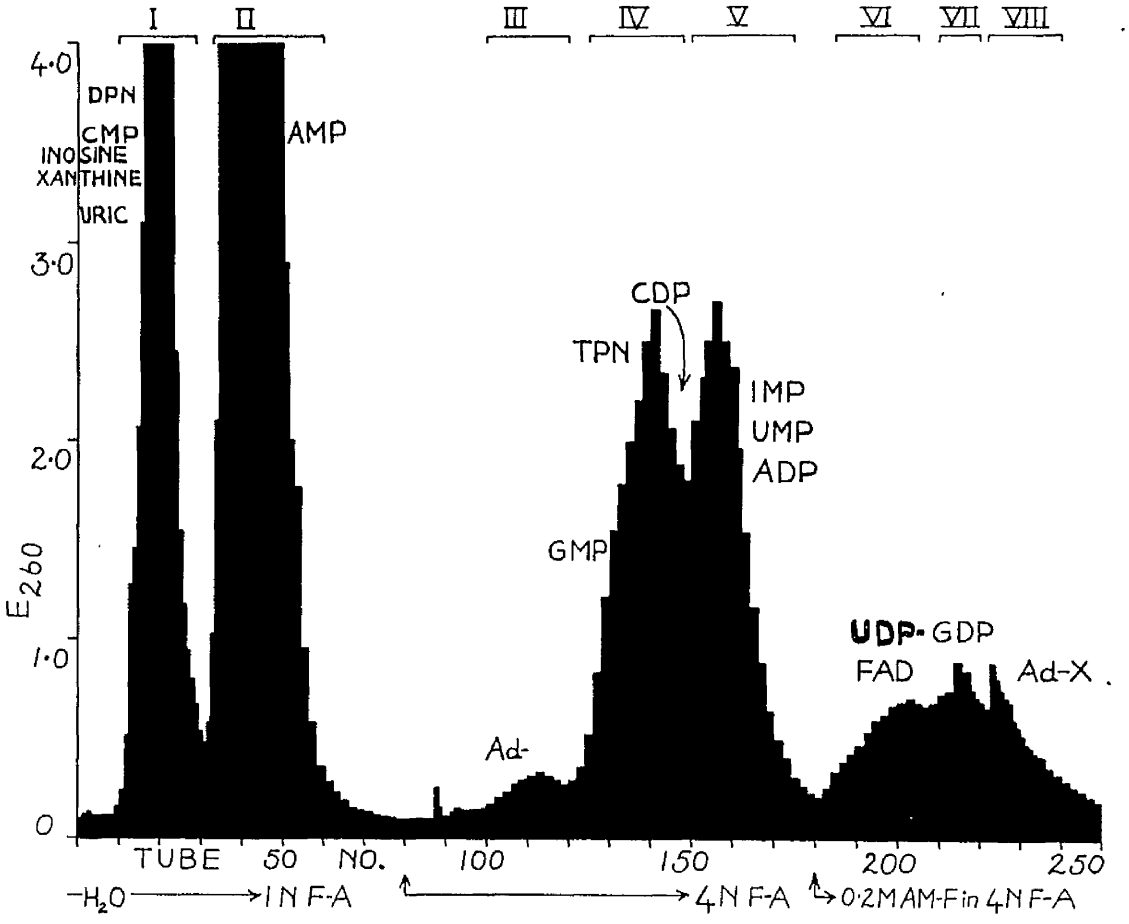
Elution chart from the ion exchange chromatography of acid-soluble compounds absorbing at 260 mμ, obtained from the cytoplasmic cell sap fraction of rabbit liver. The cell sap was prepared from 50 g. of liver tissue.

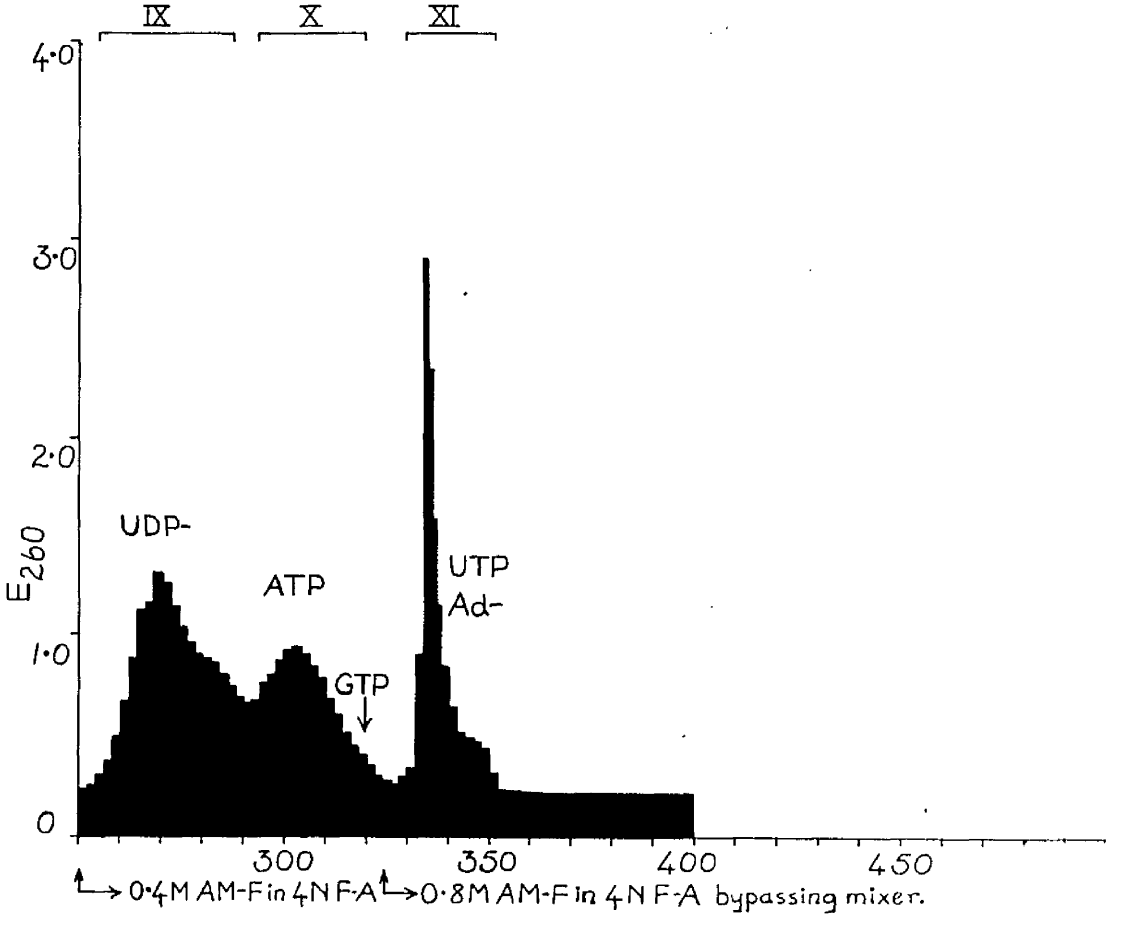
Column:- Dowex-1-formate; 20.0 cm. x 1.0 cm.
Mixing Volume:- 500 ml. Fractions:- 100 drops per tube, about 6.0 ml. per tube.

Prior to application to the column, the sample was made slightly alkaline by the addition of a drop of ammonia solution.

The cell sap fraction in this experiment was obtained from the same liver as the mitochondria of Chart 9 and the microsomes of Chart 12 and was treated with perchloric acid immediately after preparation.

Readings at 260 mμ which were too great to be plotted on the chart were peak I 5.1, 7.35, 12.0, 13.95, 12.0, 7.7, 4.0; peak II 4.35, 7.9, 13.0, 17.0, 33.0, 45.0, 45.0, 43.0, 35.0, 27.0, 27.0, 19.0, 13.9, 12.95, 6.6, 6.1.


I
II
III
IV
V
VI
VII
VIII
4·0
3·0
2·0
1·0
0
E_{260}
DPN
CMP
INOSINE
XANTHINE
URIC
AMP
CDP
TPN
IMP
UMP
ADP
GMP
Ad-
UDP-GDP
FAD
Ad-X
TUBE NO.
50
100
150
200
250
$-H_2O$
1 N F-A
4 N F-A
0·2 M AM-F in 4 N F-A


IX
X
XI
4·0
3·0
2·0
1·0
0
E_{260}
UDP-
ATP
GTP
UTP
Ad-
300
350
400
450
0·4 M AM-F in 4 N F-A
0·8 M AM-F in 4 N F-A bypassing mixer.

Component	Relative Amount
Xanthine	+ + + + +
Inosine	+ + + +
GMP	+
DPN	+
Uric Acid	+

Peak III contained a compound which, after acid hydrolysis yielded one base only, adenine. This peak had a high $E_{275}:E_{260}$ ratio, rather higher than that observed for the corresponding peaks on some elution charts described in previous sections, but in Chart 13, the peak in question is so close to GMP that some contamination of the peak III fractions with GMP might be expected. Such circumstances would raise the $E_{275}:E_{260}$ ratios of the fractions in peak III.

The amounts of TPN and GMP in peak IV were small. ADP was the nucleotide occurring in greatest quantity in this part of the chromatogram.

The amount of uracil obtained from peaks VI, VII and VIII after acid hydrolysis was small, constituting about one-fifth of the contents of these three peaks. In view of this, it is worth noting that the UDP- peak (peak IX) is large when compared with the other peaks eluted in the latter stages of the chromatogram. This may be the normal pattern for cell sap but it is quite possible that peak IX was increased in size beyond the normal limits by the production

of U/DP from breakdown of some of the UDP- material in peaks VI and VII.

An adenine compound together with UTP in approximately equal quantities were the only compounds eluted in the last elution range.

The monophosphates of cytidine, adenosine, guanosine and uridine were present in the cell sap fraction in great amounts and this obviously prevented the usual satisfactory separation of GMP and TPN from IMP and UMP and in fact brought ADP, which is usually eluted alone, into the IMP/UMP fraction. Nevertheless, there was an appreciable amount of each of the di-phosphates and of the triphosphates with the exception of CTP.

3.3. Ninhydrin-positive Components of the Acid-soluble Fraction of Liver.

In the course of preliminary studies on the acid extract of whole rabbit liver, it was observed that some of the fractions eluted from Dowex-1-formate before AMP gave positive reactions with the qualitative ninhydrin reagent when the latter was used to spray paper chromatograms which had been run in the ethanol/ammonium acetate solvent.

Since amino-acid derivatives of nucleotides are known to exist (Park, 1950; 1952), it was considered desirable to ascertain the nature of the ninhydrin-positive compounds in the liver acid extracts.

3.3a. Anion Exchange Chromatography of Whole Liver Acid Extracts.

Several experiments were carried out for the ion exchange separation of acid-soluble compounds of whole liver tissue. Most of these separations were stopped after AMP had been eluted.

Chart 14 refers to one of these experiments and shows the pattern of elution of compounds absorbing at 260 mμ. The elution of ninhydrin-positive compounds was also followed in this instance by taking a small aliquot from each tube for estimation of amino-nitrogen by the quantitative ninhydrin method of Cocking and Yemm (1954).

In all the experiments of this section, pooling of fractions from the column was carried out as far as possible according to the scheme shown on Chart 14. However, this scheme of pooling could not always be followed exactly since there were small variations in the elution pattern from experiment to experiment, so that, for example, pools III and IV of Chart 14 were sometimes obtained as one pool in certain runs.

The pooled fractions in all cases were dried from the frozen state and dissolved in a small volume of water. These solutions were stored at -10^{0} in the deep-freeze cabinet until required.

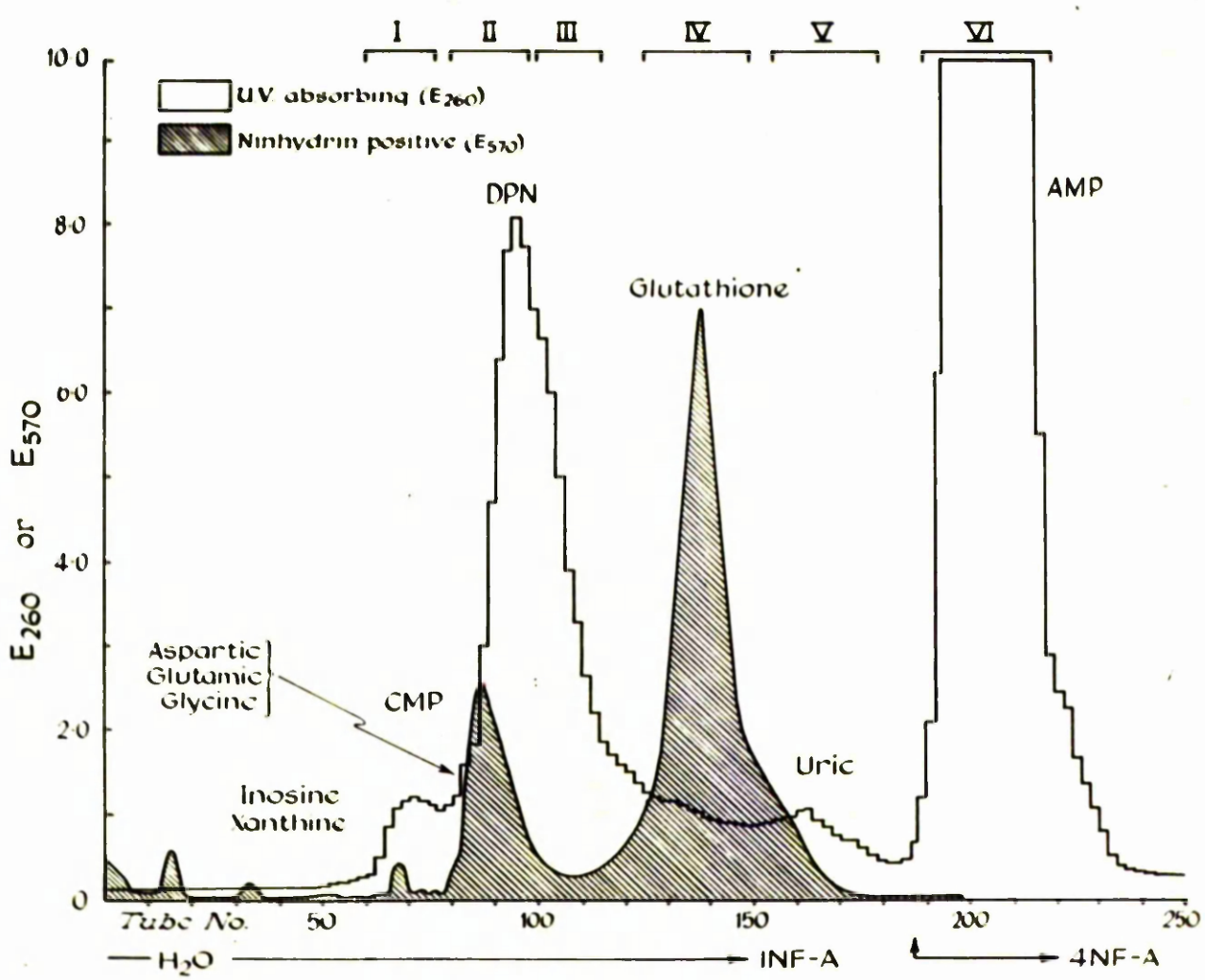
CHART 14.

Part of an ion exchange chromatogram of acid-soluble compounds absorbing at 260 mμ obtained from 134 gm. (wet weight) of rabbit liver. The liver tissue was obtained from two albino rabbits.

Column:- Dowex-1-formate, 16 cm. x 3.3 cm.
Mixing Volume:- 2 litres. Fractions:- 60 drops per tube from tubes 1 to 163; 100 drops per tube from tube 164.

The separation was stopped after the elution of AMP was complete. 0.2 ml. portions from each fraction were taken for the estimation of amino-nitrogen by the quantitative ninhydrin method of Cocking and Yemm (1954). The colour produced in these estimations was measured in the Unicam SP 600 spectrophotometer at 570 mμ, and the ninhydrin positive fractions were plotted on the chart as shown.

Prior to application to the column the acid extract (pH 6-7) was made slightly alkaline by the addition of a drop of NH_4OH.


I
II
III
IV
V
VI
UV absorbing (E_{260})
Ninhydrin positive (E_{570})
DPN
AMP
Glutathione
E_{260} or E_{570}
Aspartic
Glutamic
Glycine
CMP
Uric
Inosine
Xanthine
Tube No.
H_2O
INF-A
4NF-A

3.3b. Identification of the Ninhydrin-positive Components.

I. Ionophoresis on Paper.

By inspection of Chart 14, it would appear that the ninhydrin-positive peaks are separate from the ultraviolet-absorbing peaks. However, in earlier experiments in which ninhydrin-positive compounds were not plotted on elution charts as in Chart 14, the technique of ionophoresis on paper in a citrate buffer, pH 3.5 (Section 2.10), was used in order to separate the ninhydrin-positive material from the ultraviolet-absorbing material.

Figure 8 shows the location of some known compounds on ionophoretograms. It can be seen from this figure that uric acid was well separated from CMP, DPN and AMP on ionophoretograms. Although it moved a distance similar to that moved by xanthine and by inosine, it was well separated from these two compounds by the preceding ion exchange chromatography (Chart 14). Ionophoresis then, in the present investigation, was found to be a most reliable and efficient method of isolating uric acid for identification.

Figure 9 shows the ionophoretograms, ninhydrin spray strips (Section 2.10) and autoradiographs, after ionophoresis of the material contained in the fractions corresponding to peaks III-IV and IV-V on Chart 14. The acid extract was prepared from the liver of a rabbit which had received ^{32}P as inorganic phosphate. It can be seen from the diagrams

Location of some known compounds on ionophoretograms.

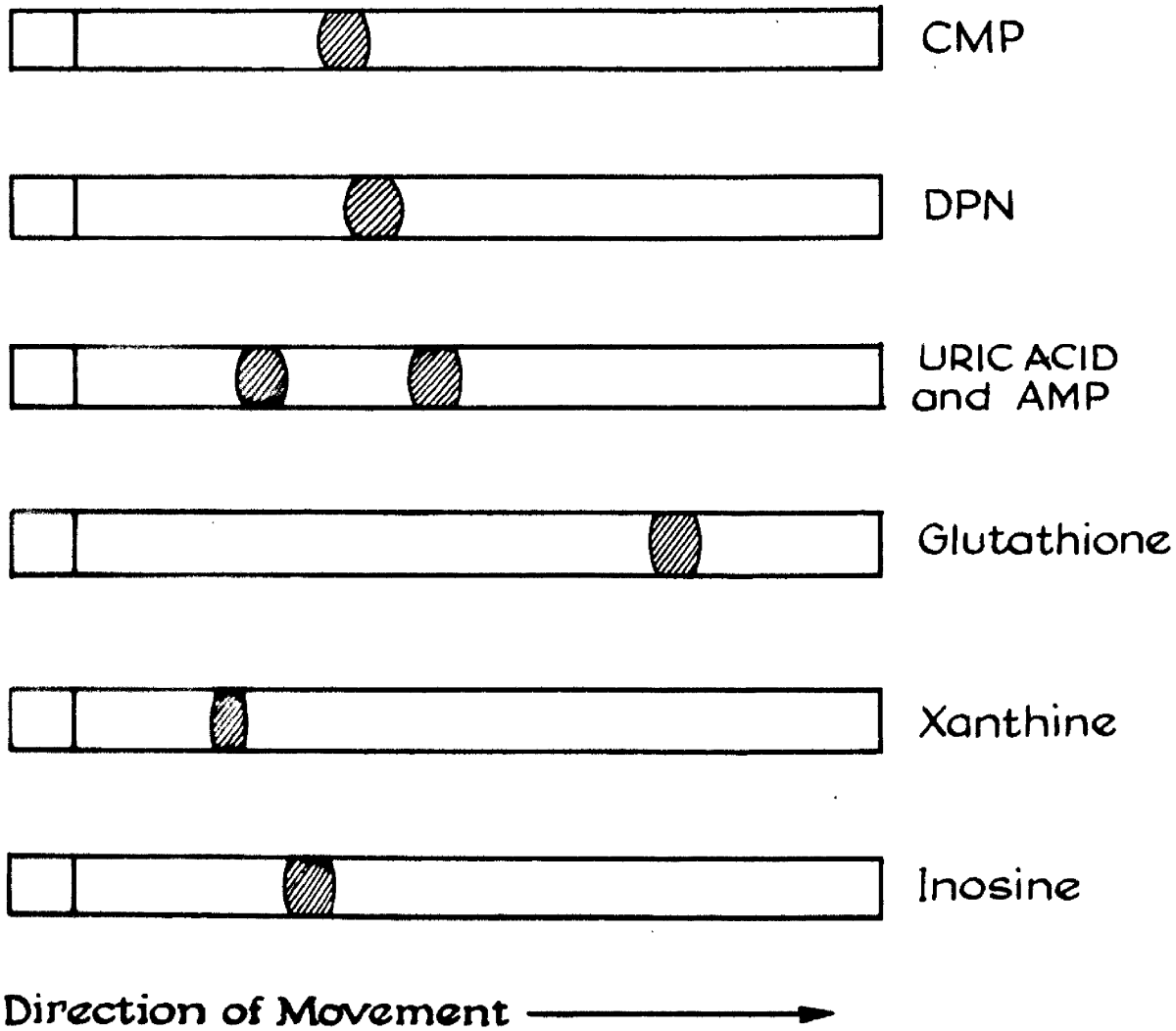


Figure 8.

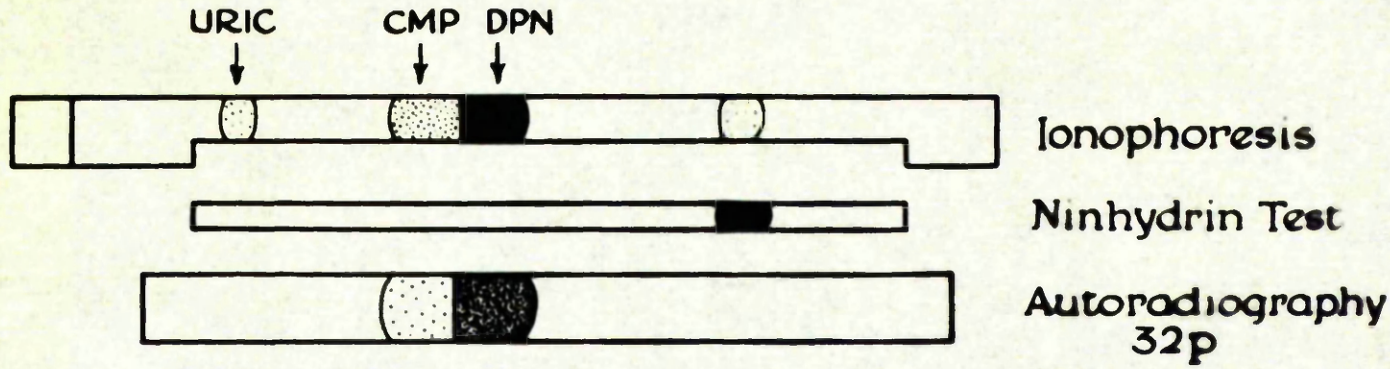
FIGURE 9.

Diagrams of the ionophoretograms of the ultraviolet-absorbing material present in some of the peaks of an ion exchange separation corresponding to that shown in Chart 14.

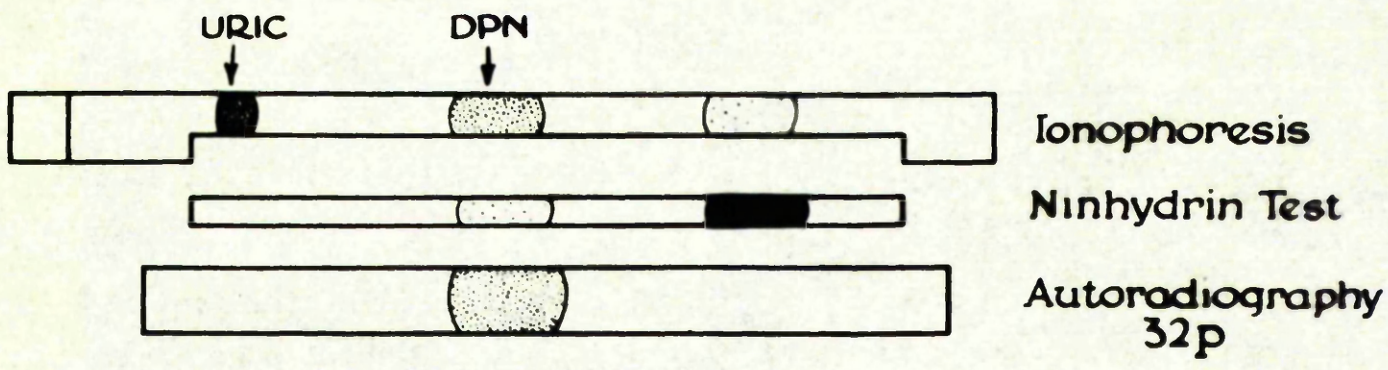
The tissue used was rabbit liver obtained from a rabbit which received an intramuscular injection of ^{32}P as inorganic phosphate.

Small strips were cut from one edge of each ionophoretogram and were sprayed with ninhydrin reagent to ascertain the behaviour of ninhydrin-positive material which was known by preliminary tests to be present in these peaks.

The Figure includes diagrams of autoradiographs corresponding to each ionophoretogram.



Peaks III & IV



Peaks IV & V

Figure 9.

that the ninhydrin-positive material moved well ahead of the ultraviolet-absorbing compounds known to occur in peaks III, IV and V, and that the ninhydrin-positive band absorbed ultraviolet light weakly. This ninhydrin-positive and ultraviolet-absorbing band will be referred to as the N.U.V. material. Autoradiography of the ionophoretograms indicated that the N.U.V. material did not contain exchangeable phosphorus. Further, phosphorus estimations (Section 2.13) on the N.U.V. material showed that phosphorus was at present in detectable amounts, although in a minority of experiments a trace could be detected. This variation among experiments was reflected in other analyses to be described in Section 3.3c.

Figure 10 gives the results from a similar set of experiments with the material from the liver of a rabbit which received 1 mc. ^{14}C-sodium formate 2 hours before killing. Incorporation of the isotope into uric acid was not detectable but both DPN and the N.U.V. band incorporated the isotope quite extensively.

In a third set of experiments (Figure 11) material was obtained from the livers of three rats, each of which received 1/3 mc. ^{35}S-methionine three hours before killing. The ultraviolet-absorbing components eluted before AMP were obtained as one peak. Autoradiography indicated that the N.U.V. band contained sulphur. Two other fainter bands were observed on the ninhydrin spray strip and on the autoradiograph.

FIGURE 10.

Diagrams of the ionophoretograms of the ultraviolet-absorbing material present in some of the peaks of an ion exchange chromatogram corresponding to that shown in Chart 14.

The tissue used was rabbit liver obtained from a rabbit which received an intramuscular injection of ^{14}C-sodium formate two hours prior to killing.

Small strips were cut from one edge of each ionophoretogram and were sprayed with ninhydrin reagent.

The Figure includes diagrams of autoradiographs corresponding to each ionophoretogram.

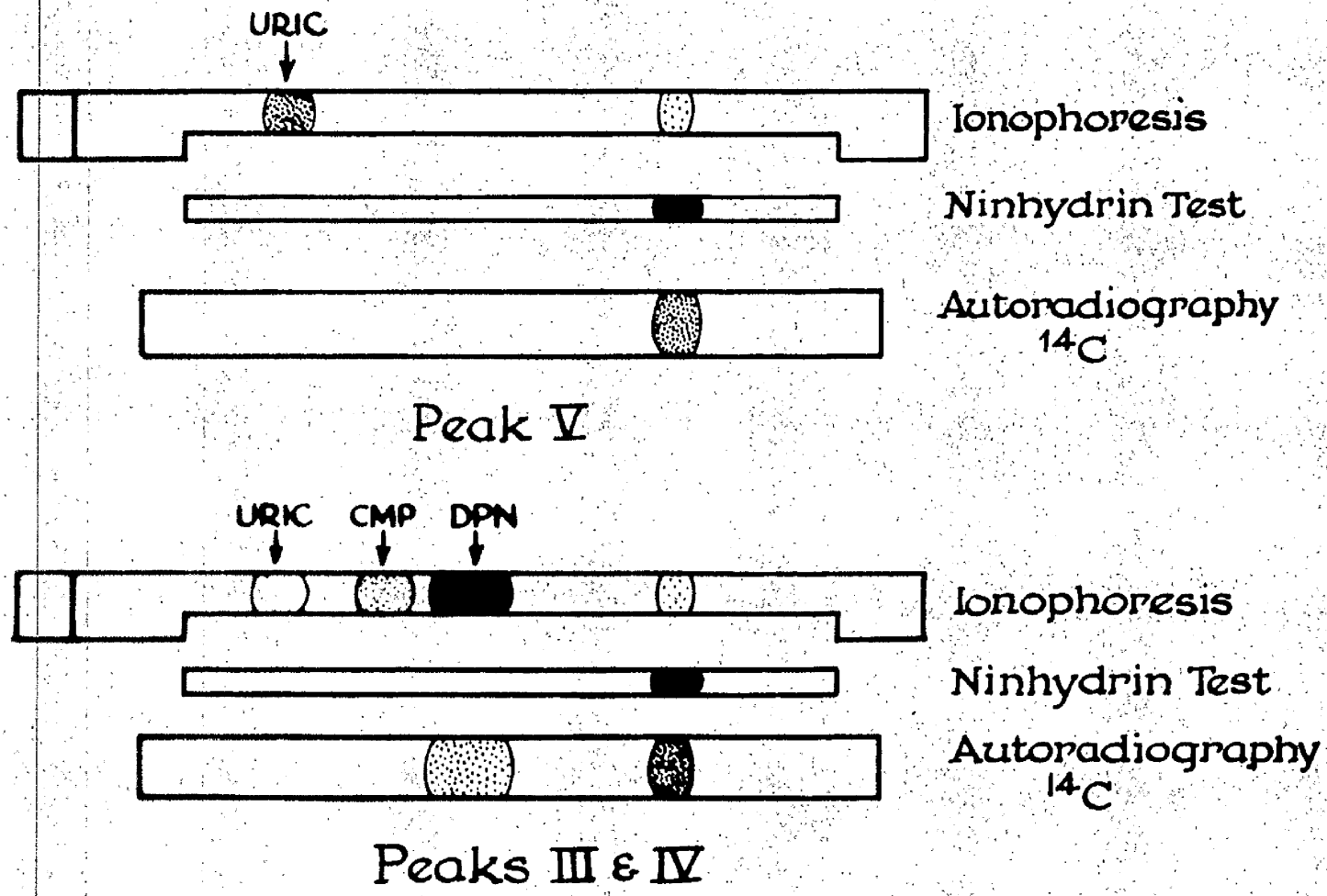

URIC
Ionophoresis
Ninhydrin Test
Autoradiography
14C
Peak V
URIC
CMP
DPN
Ionophoresis
Ninhydrin Test
Autoradiography
14C
Peaks III & IV

Figure 10.

FIGURE 11.

Diagram of an ionophoretogram of the ultraviolet-absorbing material present in the peaks preceding AMP in an ion-exchange separation similar to that shown in Chart 14.

The tissue used was rat liver obtained from three rats, each of which received an intramuscular injection of 3 mc. of ^{35}S-methionine three hours before killing.

A small strip was cut from one edge of the ionophoretogram and was sprayed with ninhydrin reagent.

The Figure includes a diagram of the autoradiograph corresponding to the ionophoretogram.

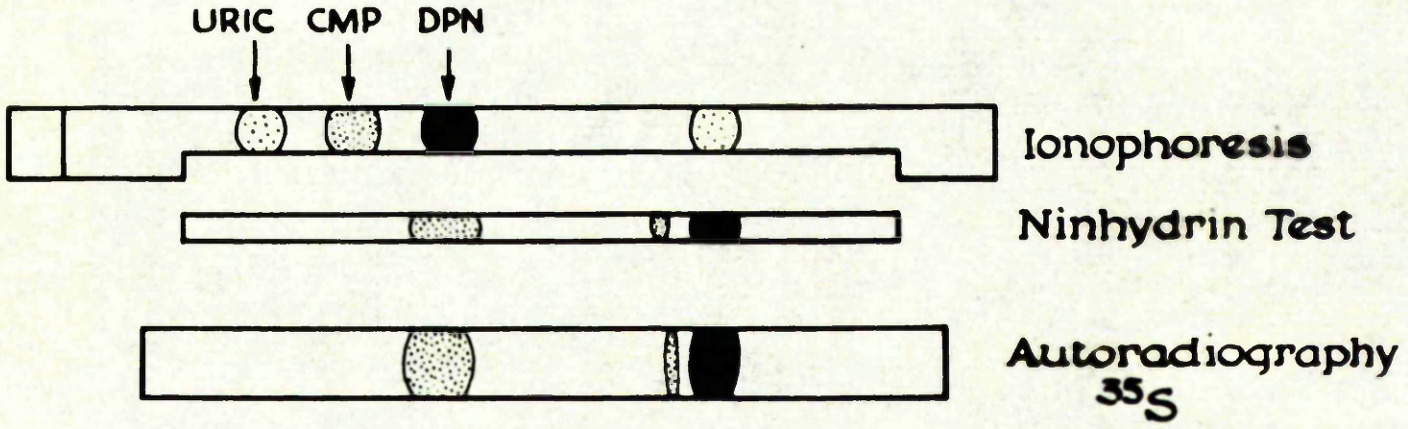

URIC
CMP
DPN
Ionophoresis
Ninhydrin Test
Autoradiography
^{35}S

Figure 11.

At this stage there was evidence that more than one ninhydrin-positive compound was retained on Dowex-1-formate, that the principal compound was sufficiently acidic to give it a high mobility on ionophoretograms at pH 3.5, and that this compound was faintly ultraviolet-absorbing. The N.U.V. material was known to contain carbon exchangeable with the carbon of administered ^{14}C-formate *in vivo*, sulphur exchangeable with the sulphur of methionine, and very little, if any, phosphorus.

Several ionophoretograms of the material in peaks III and IV, Chart 14, were prepared. The N.U.V. bands were eluted with water, and the eluates pooled and dried from the frozen state. The dry material was dissolved in a small volume of water and stored at -10^{0} until required for further analysis.

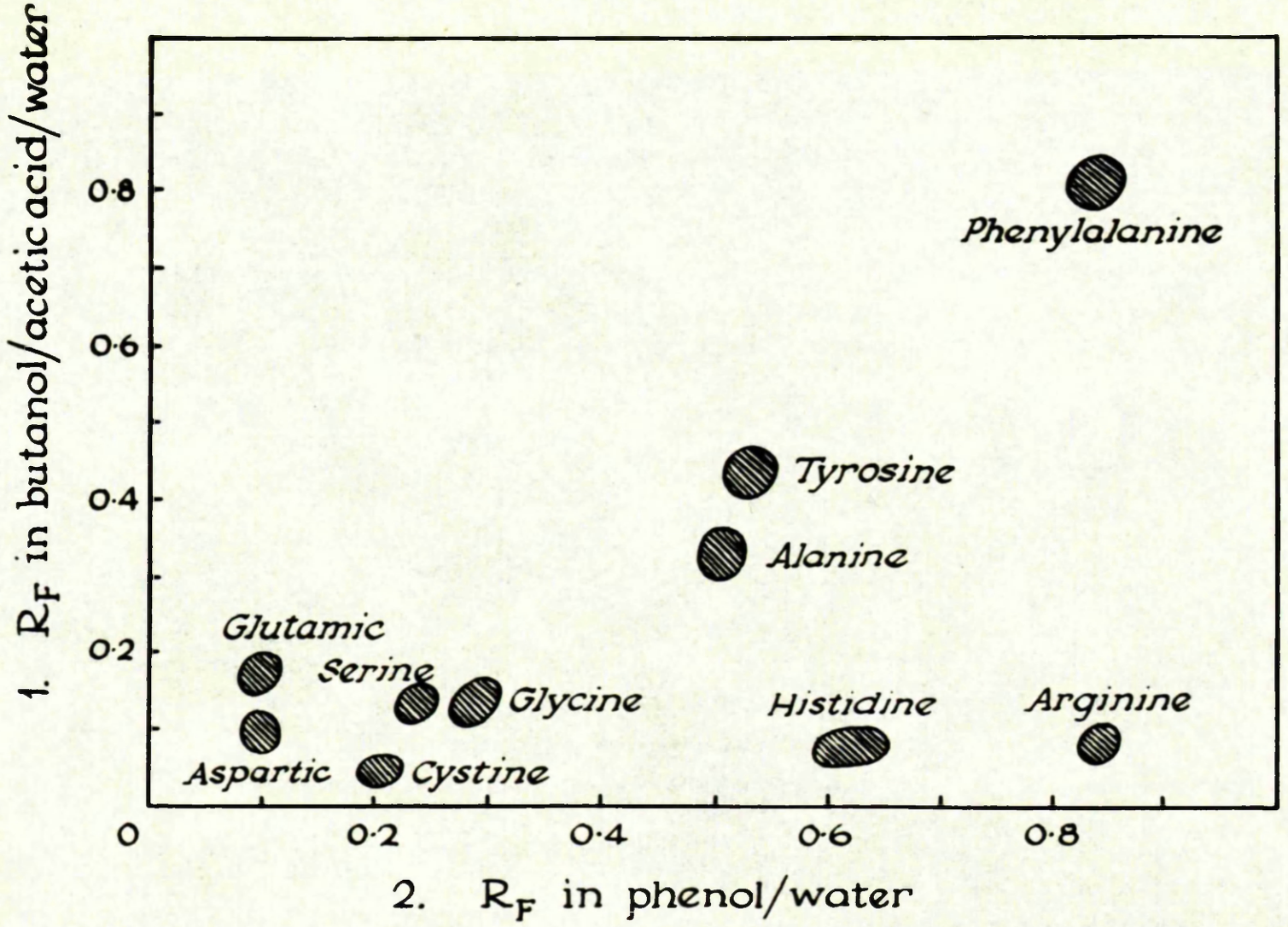
II. Paper Chromatography.

Figure 12 shows the location on paper of some marker amino-acids after two-dimensional chromatography in butanol/acetic acid/water and phenol/water. Such chromatograms of standard mixtures of amino-acids were always run in parallel with all test samples (Section 2.9d).

A portion of the concentrated solution of the material in peak II, Chart 14 (Section 3.3a) was used for two-dimensional chromatography in butanol/acetic acid/water and phenol/water. After spraying with ninhydrin the

Figure 12.

Location of some marker amino-acids after two-dimensional paper chromatography.



chromatogram showed three spots which lay in positions corresponding to those normally occupied by aspartic acid, glutamic acid and glycine. Another portion of the same concentrated solution after 6N HCl hydrolysis for 18 hours at 110° and subsequent two-dimensional paper chromatography in the same solvent system gave results identical with those obtained for the unhydrolysed material. Paper chromatography of the material in peak II (before and after 6N HCl treatment) in the pyridine/amyl alcohol/water and phenol/water solvent system, produced the same results. One dimensional paper chromatography in the ethanol/ammonium acetate solvent provided three spots after spraying the chromatogram with ninhydrin. These spots occupied the positions taken up by glutamic acid, aspartic acid and glycine when these amino-acids were run in this solvent. The $R_{adenosine}$ values are shown in Table I. The relative amounts of these amino-acids found in peak II, Chart 14, are given below.

Amino-acid	Relative Amount in Peak II.
Glutamic acid	+ + + + +
Aspartic acid	+ +
Glycine	+

On the above evidence, the ninhydrin-positive components of peak II, Chart 14, were identified as a mixture of glutamic acid, aspartic acid and glycine.

Similar two-dimensional paper chromatography of the material in peak IV, Chart 14, using the two solvent systems described above, and one dimensional chromatography in ethanol/ammonium acetate, revealed that the ninhydrin-positive material moved as one spot, had a very low Rp value in butanol/acetic acid/water and in pyridine/amyl alcohol/water and had an Rp value of 0.2 in phenol/water. The $R_{adenosine}$ value in ethanol/ammonium acetate was 0.15. After hydrolysis with 6N HCl, however, three ninhydrin-positive spots were found after two-dimensional chromatography in each of the two-dimensional solvent systems and in both cases the spots corresponded to glutamic acid, cystine and glycine when compared with standard chromatograms.

As these amino-acids are the constituent amino-acids of the tripeptide glutathione, the behaviour of this peptide was determined (without hydrolysis) in the two-dimensional paper chromatographic systems. It did not move appreciably in butanol/acetic acid/water, or in pyridine/amyl alcohol/water but had an Rp value of 0.21 in phenol/water. Racker and Krimsky (1952) reported that reduced glutathione and oxidised glutathione had Rp values in phenol/water of 0.50 and 0.22 respectively. It was highly probably that the glutathione used in the above chromatography was in the oxidised form as it had been stored in 1N $HClO_4$ solution for 18 hours before the perchlorate ion was precipitated as

potassium perchlorate by addition of potassium hydroxide. The same glutathione solution had an $R_{adenosine}$ value of 0.15 when run in the ethanol/ammonium acetate solvent.

On the above evidence, the material in peak IV, Chart 14, was identified as glutathione.

III. Cation Exchange Chromatography.

It was considered desirable to ascertain the relative proportions of glutamic acid, cystine and glycine in a 6N HCl hydrolysate of the material in peak IV, Chart 14, and to this end the technique of ion exchange chromatography was employed.

The material used for this experiment was the N.U.V. band eluate from ionophoresis, prepared as in Part I of this section. The material in this band corresponded to that in peak IV, Chart 14.

A portion of the N.U.V. band eluate was hydrolysed with 6N HCl. The HCl was removed from the hydrolysate and the remaining material was applied to the Dowex-50 resin (Section 2.8) in a small volume of citrate buffer, pH 3.41. Chart 15 shows the result of the ion exchange chromatography and confirms the results of the paper chromatography in Part II of this section. A small amount of aspartic acid was found on the chromatogram and probably appeared in the N.U.V. eluate as a contaminant from peak II

CHART 15.

Elution chart from the cation exchange chromatography of a 6N HCl hydrolysate of the material obtained from the ninhydrin-positive bands on ionophoretograms. The ionophoresis was conducted on the ninhydrin positive material eluted from a column of Dowex-1-formate in a position corresponding to peaks III and IV on Chart 14. The tissue used was rabbit liver.

Details of the preliminary anion exchange chromatography are given in the legend to Chart 14.

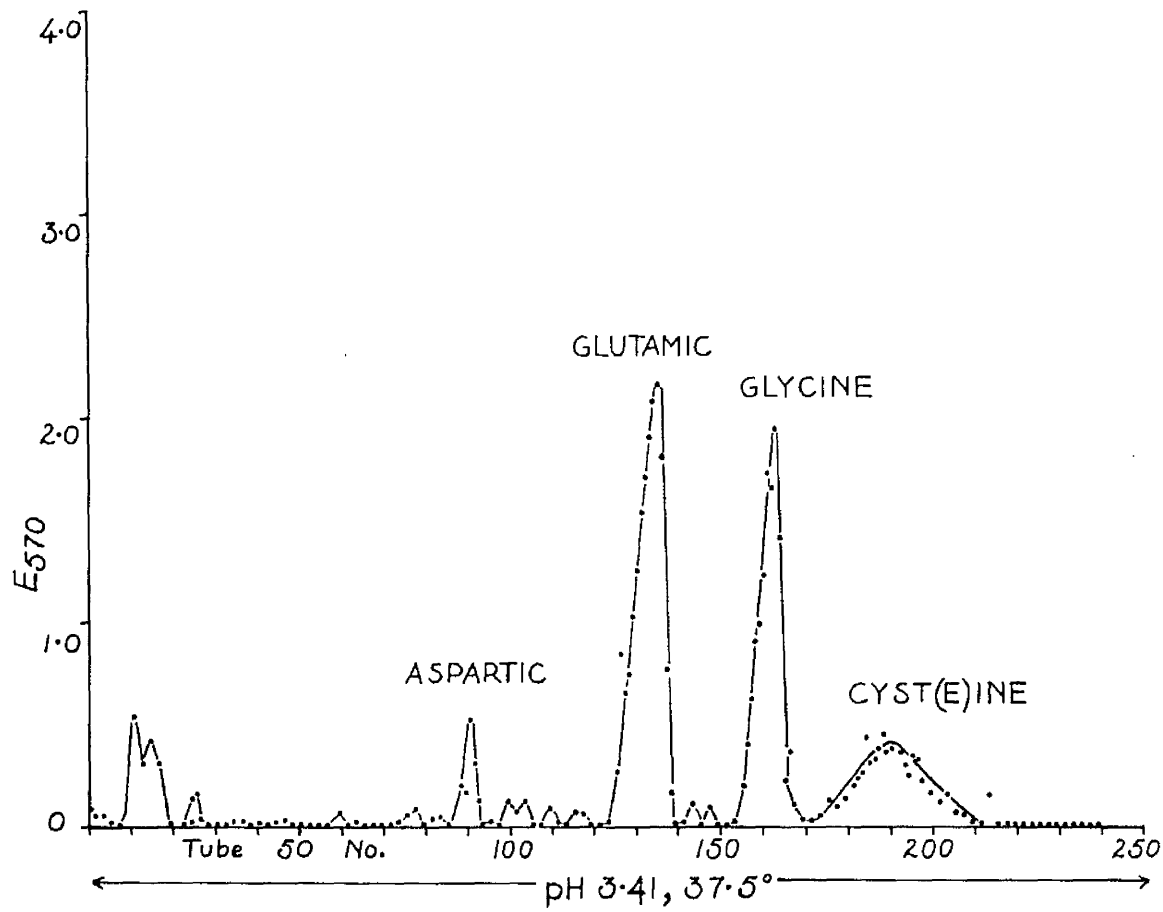
Column:- Dowex-50-Na^+ ; 100.0 cm. x 0.9 cm. with a temperature controlled water-jacket (57.5°).

Fractions:- 1 ml. per tube.

Eluent:- Citrate buffer pH 3.41.

Ordinate:-Optical density at 570 mμ, of colour produced by an aliquot of the solution from each tube after the quantitative ninhydrin reaction.

Abscissa:- Tube number.


4·0
3·0
2·0
1·0
0
E_{570}
GLUTAMIC
GLYCINE
ASPARTIC
CYST(E)INE
Tube No.
50
100
150
200
250
pH 3·41, 37·5°

of Chart 14. The amounts of the four amino-acids found after the ion exchange chromatography are shown in Table 9.

During the 6N HCl hydrolysis procedure and preparation of the extract for ion exchange chromatography, cysteine, if present as such at that stage, would be readily oxidised to cystine, so that it is in this form that the amino-acid appears on ion exchange chromatograms. (The cysteine residue of glutathione would, in fact, probably have been oxidised to cystine before the hydrolysis took place). Under the conditions of the experiment the yield of cystine is about 50% (Leaf, 1956). Glutamic acid from peak II, Chart 14, may have been present in the N.U.V. eluate as a contaminant. Taking these factors into account, it can be seen from Table 9 that glutamic acid, cystine and glycine were present in the N.U.V. eluate hydrolysate in molar concentrations consistent with the hypothesis that the original material was oxidised glutathione.

Stein and Moore (1954) provided unequivocal evidence for the decomposition or alteration during hydrolysis of cystine, which seemed to be oxidised largely to cysteic acid. In this connection it is of interest that on Chart 15 a small peak emerged (tubes 10 to 20) in the position occupied by cysteic acid when an authentic sample of the latter is chromatographed on Dowex-50 (Moore and Stein, 1954).

TABLE 9.

Amounts of amino acids found in the 6N HCl hydrolysate of the N.U.V. band eluate from ionophoretograms after ion exchange chromatography on Dowex-50 (Chart 15).

Amino Acid	Amount found (μ-moles of amino-nitrogen).
Aspartic acid	2.54
Glutamic acid	16.50
Glycine	13.25
Cystine	7.05

On the above evidence, peak IV on Chart 14 was identified as glutathione. In previous parts of this section the evidence has pointed to glutathione existing in the oxidised state after the extraction procedure from liver and subsequent anion exchange chromatography.

3.3c. The Ultraviolet Light-absorbing Character of the N.U.V. Material.

Although the ninhydrin-positive components were identified at this stage, the ultraviolet-absorbing nature of the N.U.V. material was not accounted for and this section describes some experiments which were carried out to investigate this problem.

I. Ultraviolet-absorbing Properties of Glutathione.

According to Anslow and Lyman (1941) glutathione has at about 280 mμ a weak double absorption band with separation about 10 mμ in water ethanol and alkali. However, in the present investigation an authentic sample of glutathione, both before and after two dimensional chromatography in isopropanol/HCl and n-butanol/NH_3, gave the ultraviolet absorption spectrum in water shown in Figure 13, Chart A. A similar spectrum was found for pure glutathione after ionophoresis on paper and elution with water.

FIGURE 13.

CHART A. Ultraviolet absorption spectrum in water of an authentic sample of glutathione after two-dimensional paper chromatography in isopropanol/HCl and n-butanol/NH_3. The spectrum before chromatography was similar.

CHART B. Ultraviolet absorption spectrum in water, of material which moved with R_F 0.5 in isopropanol/HCl and R_F 0.05 in n-butanol/NH_3 obtained from two-dimensional chromatography of glutathione pretreated with 1N $HClO_4$ (by standing in the acid for 18 hours at 4°). The perchlorate ion was removed by addition of KOH to pH 7.

CHART C. Ultraviolet absorption spectrum in water of the N.U.V. spot after chromatography in acetone/formic acid.

CHART D. Ultraviolet absorption spectrum in 0.1N HCl of a spot found on chromatograms of a 12N $HClO_4$ hydrolysate of the N.U.V. material from iono-phoretograms, after chromatography in isopropanol/HCl and n-butanol/NH_3. The spot had the same chromatographic properties as cytosine in this solvent system.

CHART E. Same material as in Chart D but at pH 13.

CHART F. Same procedure as in Chart D but the N.U.V. material was obtained from the acetone/formic acid chromatograms.

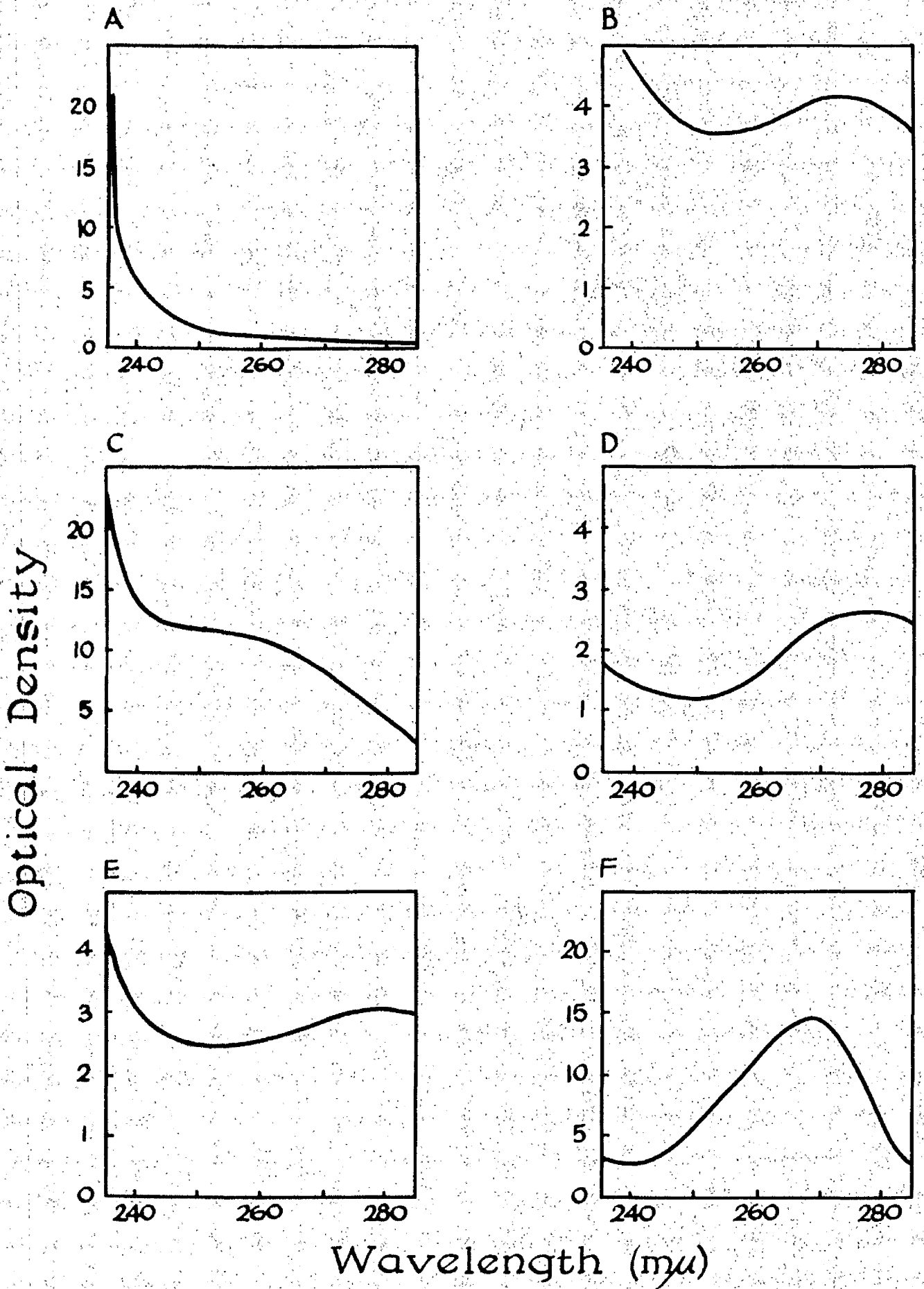


Figure 13.

After an authentic sample of pure glutathione had been treated with 1N perchloric acid followed by precipitation of the perchlorate ion by addition of potassium hydroxide, two-dimensional chromatography in isopropanol/HCl and n-butanol/NH_3 produced two ultraviolet absorbing spots. Neither spot moved appreciably in n-butanol/NH_3, but one, which was very faint, had an R_F value of 0.3 in isopropanol/HCl, while the second spot had a value of R_F in isopropanol/HCl, about 0.5. Figure 13, Chart B, gives the ultraviolet-absorption spectrum of the latter spot. The same glutathione sample had R_F values of 0.21 in phenol/water and 0.15 in ethanol/ammonium acetate. The behaviour of this glutathione sample in the acetone/formic acid solvent is shown in Figure 4. In the ethanol/ammonium acetate and acetone/formic acid solvents, it was possible to locate the glutathione on paper by its weak ultraviolet absorption.

II. The Ultraviolet-absorbing Properties of the N.U.V. Material.

The N.U.V. material was obtained from two main sources, (i) ionophoresis on paper of the material in peak IV, Chart 14, and (ii) paper chromatography of the material in peak IV. The ionophoresis procedure has been described in Section 3.3b, part I.

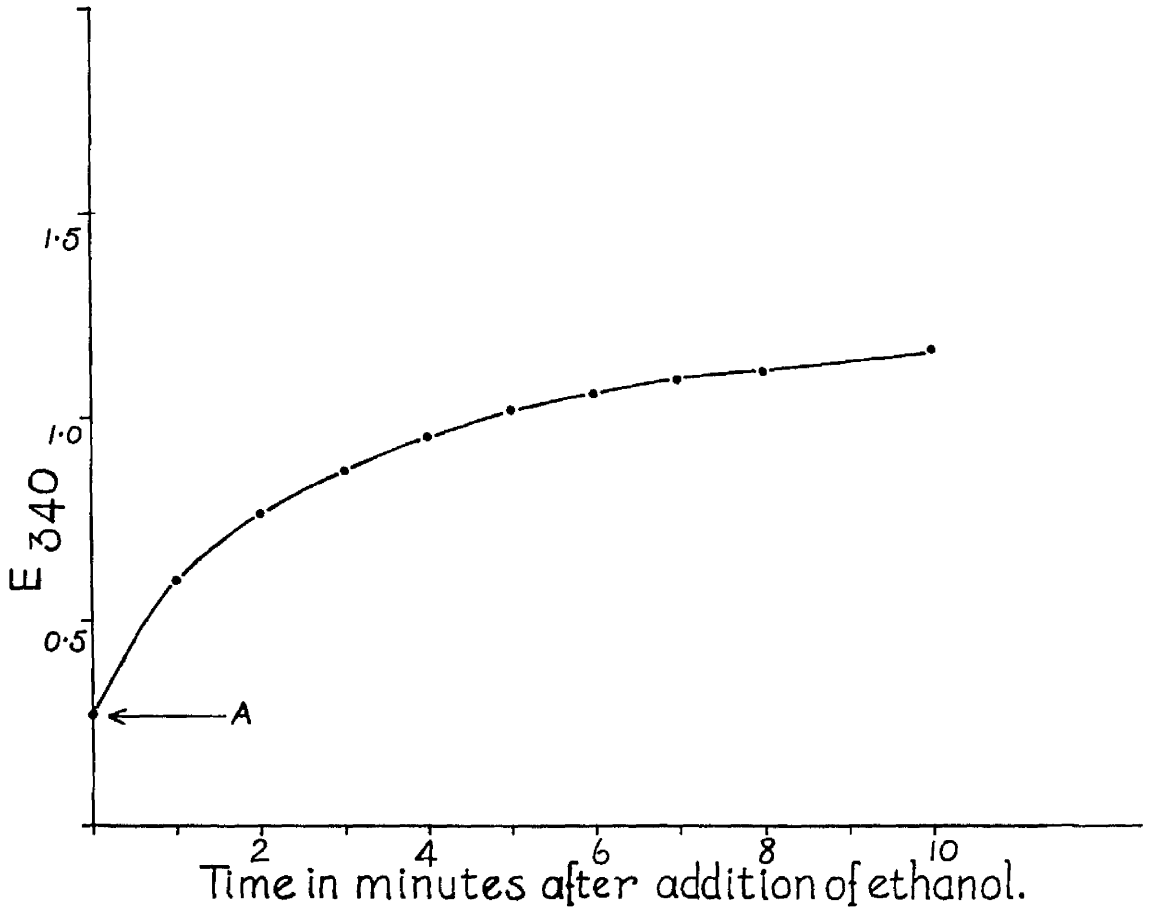
As ionophoresis and ion exchange chromatography are both techniques which involve separation of compounds by

difference in charge, it was decided that the technique of chromatography on paper might be used in an attempt to separate completely the ultraviolet-absorbing principle from the ninhydrin-positive material of peak IV, Chart 14. Accordingly, portions of the material in peak IV were submitted to paper chromatography in the ethanol/ammonium acetate solvent. Chromatograms were run for 50 hours and even after this time the ninhydrin-positive spot was still inseparable from the ultraviolet-absorbing principle. It moved 3.5 cms. on the paper, the same distance moved by the authentic sample of glutathione applied to the paper after treatment with 1N $HClO_4$. The pure glutathione (Part I of this section) was faintly ultraviolet-absorbing. There was, however, evidence of alteration or breakdown of glutathione as more than one ninhydrin-positive spot was found on the paper after spraying. In this solvent system, the DPN and uric acid contaminating peak IV of Chart 14 moved well ahead of the N.U.V. material. To confirm this, the N.U.V. spot and the DPN spot were eluted in water and assayed enzymically for DPN (Section 2.15). In both cases the optical density of the eluate was measured at 260 mμ and a volume of eluate equivalent to 0.1 μ-mole of DPN was taken for assay. The course of enzymic reduction of the DPN eluate (Figure 14) by alcohol dehydrogenase in the presence of ethanol was followed by measurement of the appearance of a peak at 340 mμ. No change in optical density at 340 mμ was observed in the

FIGURE 14.

Enzymic Reduction of DPN.

The DPN was obtained from peak III, Chart 14, by paper chromatography in ethanol/ammonium acetate of a concentrated solution of the material. The resulting ultraviolet-absorbing spot was cut out and the DPN eluted with water. The optical density of the eluate was measured at 260 mμ, and a volume of eluate equivalent to 0.1 μ-mole of DPN was taken for assay (0.2 ml. DPN eluate). The calculations were based on the observation that 0.056 μ-mole of pure DPN solution gives an optical density of 1.0 per ml. at 260 mμ. In addition to the 0.2 ml. DPN solution, the reaction cell contained 0.75 ml. 0.1M tris buffer pH 9.1, 0.05 ml. alcohol dehydrogenase solution, and 0.01 ml. 5.75% aqueous ethanol was added at point A. The presence of DPN in the solution is demonstrated by the appearance of an ultraviolet-absorbing peak at 340 mμ as the DPN is reduced under the action of alcohol dehydrogenase on the ethanol.


1·5
1·0
0·5
E 340
A
2
4
6
8
10
Time in minutes after addition of ethanol.

reaction mixture when the N.U.V. eluate was assayed for DPN. In both cases prior to addition of ethanol to the reaction mixture, the optical density at 340 mμ was about 0.270, showing that the solutions did not contain DPN which was already reduced. The optical density of 0.270 before addition of ethanol to the reaction mixture was due to absorption of light by the enzyme protein in the solution.

Several ethanol/ammonium acetate chromatograms were run to prepare a large amount of the N.U.V. material. The N.U.V. spots were cut out, eluted in water and the eluates pooled and concentrated by partial lyophilisation. Several aliquots of this concentrated solution were run as one-dimensional chromatograms in the acetone/formic acid solvent. Again the ninhydrin-positive material was not separable from the ultraviolet-absorbing principle and moved on paper with an Rp value of 0.70. Glutathione (Part I of this section) behaved similarly in the acetone/formic acid solvent but in this case there was evidence of a small amount of breakdown or alteration of the glutathione as a faitnly ninhydrin-positive area was observed moving ahead of the main spot with an Rp 0/0.85-0.90. The glutathione spot was faitnly ultra-violet-absorbing. The N.U.V. test spots showed well defined ultraviolet-absorption. The ultraviolet absorption spectra of the N.U.V. spots after chromatography in ethanol/ ammonium acetate were similar to those found after the further chromatography in acetone/formic acid. The spectrum

in water of one of these spots is shown in Figure 13, Chart C. The spectrum at pH 13 was similar.

The ultraviolet absorption spectra, in water and in alkali, of the N.U.V. bands from ionophoresis were identical to those found for the N.U.V. spots from paper chromatography.

At this stage it appeared that the ultraviolet-absorbing character of the ninhydrin-positive material of peak IV, Chart 14, was due to alteration of glutathione during chemical operations.

In a final set of experiments, the N.U.V. material was submitted to hydrolysis with 12N $HClO_4$ and run in the isopropanol/HCl and n-butanol/NH_3 solvent system -

(a) N.U.V. material from ionophoretograms:- the two-dimensional chromatograms showed an ultraviolet-absorbing spot which behaved similarly to cytosine on paper (see Figure 5). The ultraviolet absorption spectra of this spot are shown in Figure 13, Charts D and E. These spectra are similar to, but not identical with those of cytosine.

(b) N.U.V. material from acetone/formic acid chromatograms:- the two-dimensional chromatograms showed an ultraviolet-absorbing spot which had the paper chromatographic properties of uracil but which did not give a uracil spectrum in acid (Figure 13, Chart F).

(c) 6N HCl hydrolysate of the material in peak IV, Chart 14:- two-dimensional chromatography of the 12N $HClO_4$ hydrolysate

of this material produced results identical to those described in (b) on the previous page.

(d) material in peak IV, Chart 14:-

two-dimensional chromatography of the 12N $HClO_4$ hydrolysate of this material produced results identical with those described in (b) on the previous page.

In some cases, $HClO_4$ hydrolysis did not give rise to any ultraviolet-absorbing areas on the subsequent two-dimensional chromatograms although the same material as in (a) and (b) on the previous page and (c) and (d) above was used.

The results suggest that peak IV, Chart 14, contains glutathione only, and that in the course of the various chemical operations the glutathione may undergo some chemical change to give rise to a substance which absorbs ultraviolet light, and which does not possess the stability in 12N $HClO_4$ of purines and pyrimidines.

III. Estimations of Ribose on the N.U.V. Material.

Ribose estimations (Section 2.14) on aliquots of the N.U.V. eluates from ethanol/ammonium acetate chromatograms of the material in peak IV, Chart 14, indicated that ribose was absent from these eluates. After ionophoresis of the material in peak IV and elution of the N.U.V. bands, ribose was again found to be absent.

3.3d. Identification of the Ultraviolet-absorbing Components of Chart 14.

Inosine and Xanthine.

These compounds were identified by their mobility on ethanol/ammonium acetate chromatograms after comparison with the behaviour of authentic samples; by their spectra in acid and in alkali (Beaven et al., 1955); and by identification of the base produced after perchloric acid hydrolysis (Beaven et al., 1955). In addition, the spot suspected to be xanthine contained neither ribose nor phosphorus, while the spot suspected to be inosine contained ribose and hypoxanthine in the ratio 1.00 μ-mole ribose : 0.83 μ-mole hypoxanthine. Phosphorus was absent. Probably some hypoxanthine was lost during the procedure for isolation of this base.

CMP and AMP.

These nucleotides were identified by their mobility in the ethanol/ammonium acetate solvent and the acetone/formic acid solvent on paper after comparison with the behaviour of authentic samples under identical conditions. The spectra at pH 7 were identical with those given for CMP and AMP by Pabst Laboratories (1956). Approximately equimolar proportions of base, ribose and phosphorus were found in the paper eluates but again, the amount of base was rather lower than would be expected, but this was probably due to loss of bases during the isolation procedure.

DPN.

DPN was identified enzymically using alcohol dehydrogenase (Figure 14).

Uric Acid.

Ionophoresis on paper has already been stated (Section 3.3b) to be a most reliable method of obtaining uric acid free from contaminants. Its spectra in acid and in alkali corresponded exactly to those described for uric acid in the literature.

SECTION IV.

DISCUSSION.

DISCUSSION.

4.1 Ion Exchange Chromatography of Nucleotides - Comments on Practical Aspects.

In the present investigation, the acid-soluble nucleotide distribution in the appendix, intestinal mucosa and liver of the rabbit was studied using ion exchange procedures similar to that described in 1954 by Hurlbert et al. However, in the early experiments with appendix and intestinal mucosa illustrated in Charts 1 and 4 respectively, it was clear that a better separation of compounds eluted from the Dowex-1-formate in the 4N formic acid range was desirable. By including a preliminary elution range with 1N formic acid as the reservoir eluent, a considerable improvement in the separation of the more weakly retained compounds was effected. The collection of smaller fractions throughout the 1N and 4N formic acid ranges resulted in further improvements in the separation. These points are well illustrated in Chart 6 and in Chart 14. In the latter experiment, a two litre mixing volume was used to extend the separation by bringing about a more gradual increase in concentration of eluent entering the column. However, increase in mixing volume is not always desirable as it is accompanied by a corresponding increase in effluent volume, and for this reason it is not of great value when relatively small amounts of nucleotides are being fractionated.

The gradient elution system employed here was such that the concentration of eluting fluid increased to approach asymptotically the concentration of the eluent in the reservoir as shown in Figure 15, curve A. Cherkin et al. (1953) have evolved mathematical expressions for calculating the increase in concentration of eluent in a gradient elution system of this type. Lakshmanan and Lieberman (1953) devised a modification of this apparatus so that it could be used to alter the shape of the concentration curve to that shown in Figure 15, curve B. Their apparatus for mixing the concentrated reservoir eluent with the dilute eluent in the mixing vessel was not airtight, as shown in Figure 2, but was open so that the mixing volume would decrease as the reservoir volume decreased. The shape of their concentration curve was then a function of the surface area of the liquid in the mixing vessel and this could be altered to give the shape of concentration curve desired. From curve A in Figure 15 it is obvious that compounds eluted in the first part of the 4N formic acid elution range will emerge from the column in quick succession, while in the second part of the elution range, where the increase in eluent concentration is more gradual, the compounds eluted will tend to be more effectively separated from one another (see Charts 4 and 6 in which CMP, DPN and AMP are poorly separated, while ADP, the last compound in the same

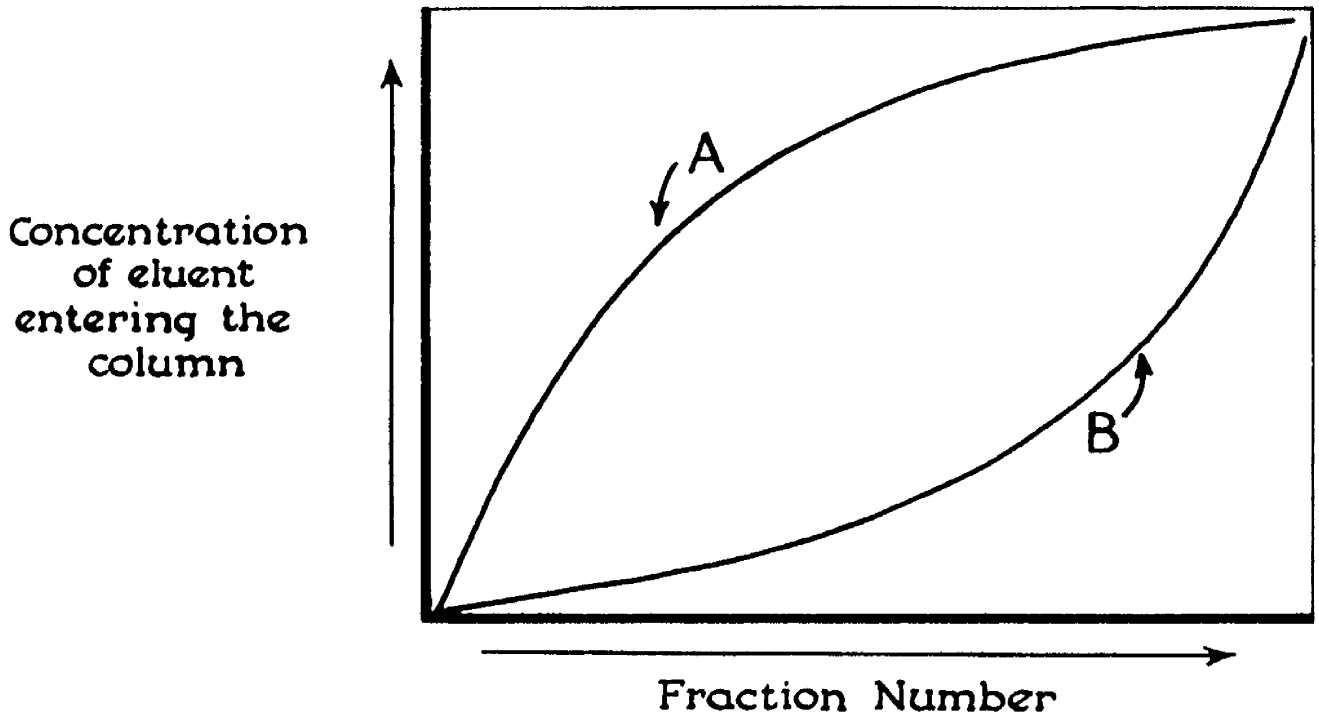


Figure 15.

Concentration curves during gradient elution chromatography, using a closed system (A) and an open system (B).

range, is clearly separated from the neighbouring nucleotides). Curve B in Figure 15 represents a reversal of this situation and it would appear that conditions giving rise to a concentration curve of this type would be eminently suitable in the separation of CMP, DPN, AMP, GMP, TPN, IMP and UMP on Dowex-1-formate. A trial experiment employing these conditions was carried out. Although the results are not recorded here, it was clear that the system of Lakshmanan and Lieberman was promising at least for the separation of the nucleotides mentioned above. However, in the present series, the concentration of eluent leaving the mixing vessel was controlled by alteration of the reservoir concentration and/or by changing the mixing volume. For example, when small amounts of material were to be fractionated on Dowex-1-formate, the 1N formic acid range was used and the mixing volume was reduced (Charts 3 and 7).

Although standard columns were always used, it was found that replication of ion exchange patterns was difficult and probably depended on rigorous adherence to rigidly standardised conditions and techniques. For this reason, the reservoir solutions were not always changed at a predetermined fraction number according to the practice adopted by Hurlbert et al. (1954). It was found practicable to follow the elution of compounds by measurement of the optical density of each fraction at 260 mμ and to change the

reservoir solution only when the elution of the last compound in each elution range was complete (see Chart 4).

In these experiments, the head of pressure above the column was kept constant, so that the rate of flow of eluent through the resin varied among experiments only if the resin particle size varied. In general, it was found that a decrease in flow rate by using a smaller particle size tended to improve the separation of nucleotides by providing sharper peaks. Charts 1, 4 and 5 illustrate experiments in which the finer resin particles were not removed by decantation during preparation of the resin. In such experiments, however, the time taken to complete a run was long and it was therefore found convenient in other experiments to hasten the ion exchange chromatography by removing the finer resin particles prior to preparation of the resin. Although the resulting elution patterns were not so well-defined, the procedure did not adversely affect the final results, as the ensuing paper chromatography effectively completed the separations. Hurlbert et al. (1954) removed the extremes of fine and coarse resin particles by repeated sedimentation and decantation, and regulated the rate of flow of eluent through the column by air pressure up to several pounds per square inch to give a constant flow rate of 0.6 ml. of eluent/minute/sq. cm. of cross-section of the column.

After elution had started it was found that the best results of ion exchange chromatography were obtained by carrying the elution through to completion without any interruptions in the flow of eluent through the column. By stopping the flow of eluent, even for periods as short as two hours, the normal appearance of the elution pattern was altered, particularly if the stoppage took place at a stage when several compounds were in the course of being eluted (see peak VI, Chart 7).

A column of dimensions 26.0 cm. x 1.0 cm. could deal with the acid extract from as much as 37 g. (wet weight) of intestinal mucosa, but for a column of this size, the best results were obtained when about 20 g. (wet weight) was the amount of tissue used. Larger amounts of material could be fractionated by increasing the column dimensions, e.g. the acid extract from 134 g. of liver was satisfactorily dealt with on a column of dimensions 16.0 cm. x 3.3 cm. However, the separation of compounds eluted beyond ADP on such a column tended to become ill-defined with low peaks extending over 50 to 70 tubes. It was clear that the critical factor was not simply the amount of resin but rather the length of the column. Columns of Dowex-1-formate of length up to 100 cm. have been used recently in successful attempts to improve ion exchange separations, (Greenberg, 1957). Bergkvist (1956) used a column 40.0 cm. x 1.6 cm. in order

to separate the soluble nucleotides from wheat plants and found that he could clearly separate mixtures of 2'-CMP, 3'-CMP, 2'-AMP, 3'-AMP, 5'-AMP, 2'-UMP, 5'-UMP, 2'GMP and 3'-GMP using a stepwise elution scheme. In addition UDP, the UDP derivatives, UTP, ADP, ATP, GDP, GTP and CTP emerged from the column as separate peaks. Baddiley, Buchanan, Carss, Mathias and Sanderson (1956) used a column of Dowex-2-formate of dimensions 50 cm. x 2.5 cm. when investigating the nature of some CDP derivatives in extracts of Lactobacillus arabinosus.

In many experiments in which the pH of the tissue extract was adjusted to about pH 8.0 with ammonium hydroxide solution prior to application to the column, inosine was detected in the first peak eluted from the resin (e.g. Chart 12). When the pH of the extract was 6.0 to 7.0, inosine was not detected in the first peak eluted from the resin (e.g. Charts 1 and 4). The minimum pH at which inosine (also hypoxanthine) is retained on the resin has not been determined but as inosine (and hypoxanthine) has a weakly dissociable H^+, it probably would not be retained at pH 6.0-7.0, thus accounting for the fact that it was not detected in some experiments (Charts 1,4,5 and 7), while relatively large amounts were found to be present in other experiments using the same tissues (Charts 3 and 5). Albert (1953) found that the hydroxyl groups of hypoxanthine and inosine were anionic in alkaline solution with pK values

of 8.8. Chart 10 shows that hypoxanthine and inosine emerged from the column at the beginning of the 1N formic acid elution range but that inosine was rather more strongly held on the resin. This difference in two substances with the same pK value can probably be attributed to a non-polar attraction for the resin. Such differences in elution of nucleotides with pK values of the same order have been observed by Cohn (1950). Xanthine was also found to be retained in many experiments in which the pH of the extract on application to the column was about 8.0. Ogston (1935) found a pK value of 7.7 for xanthine. Uric acid with three anionic groups in alkaline solution was retained more strongly than either hypoxanthine or xanthine, as would be expected.

4.2 The Acid-soluble Nucleotides of Whole Tissue.

The nucleotide composition of the acid-soluble fractions of whole rabbit appendix and intestinal mucosa are similar and closely resemble the pattern reported by Schmitz et al. (1954) for rat liver. The whole tissue extracts contain the mono-, di- and triphosphates of cytidine, adenosine, guanosine and uridine together with certain other ultraviolet-absorbing compounds. These were the nucleotide coenzymes DPN and TPN, several UDP derivatives, and unidentified adenine nucleotide (ADP-X), inosine and uric

acid. There were indications also of the existence of other ultraviolet-absorbing substances, for example FAD, in the extracts studied. The nucleoside-free base fraction was not examined in detail but in acid hydrolysates of some NFB fractions, the bases, hypoxanthine, guanine, adenine, cytosine and uracil were identified.

The occurrence of DPN and TPN in these extracts is not surprising as they would be expected to be present for the normal oxidative processes of the cell.

The exact nature of the UDP derivatives was not determined but presumably they are identical with the UDP derivatives found by Hurlbert and Potter (1954) after ion exchange chromatography of rat liver acid extracts and of extracts of Flexner-Jobling carcinoma. Hurlbert and Potter tentatively identified UDPAG and UDPG (containing a mixture of glucose and galactose) in a chromatographic position corresponding that occupied by the fractions associated with and immediately preceding GDP on Charts 1,2,4,5 and 6, and UDPGA and UDP in a position occupied by the peak immediately preceding ATP on the same Charts. Smith and Mills (1954a) observed that acid extracts of guinea pig liver contain UMP, UDP, UTP, UDPAG, UDPG and UDPGA.

The peak designated Ad-X on elution charts is probably the same as the ADP-X peak recognised by Schmitz et al. (1954) in rat liver extracts. It was analysed in part by Hurlbert et al. (1954) and was shown to contain a

group of adenosine polyphosphates. Manson (1956) reported the presence of the ADP-X material in goat mammary gland.

Inosine was identified in intestinal mucosa (Chart 6) and in appendix, although it was detected only in the appendix NAN preparation (Chart 3). Uric acid was found in liver, and in smaller amounts in the intestinal mucosa and appendix extracts. Recognition of its presence was greatly facilitated by its high $E_{275}:E_{260}$ ratio in fractions emerging from the column.

In liver, appendix and intestinal mucosa, the adenosine nucleotides accounted for about half of the total soluble nucleotide fraction. The uridine nucleotides were next most abundant, with smaller amounts of guanosine nucleotides. The cytidine nucleotides, although present only in very small amounts, were easily detected on ion exchange chromatograms by the high $E_{275}:E_{260}$ ratios of fractions containing these nucleotides. These results are in agreement with those reported by Kay and Davidson (1955) who estimated the nucleotides bases from the acid-soluble fractions of various rabbit tissues including intestinal mucosa and appendix. The relative amounts of the adenosine, uridine, guanosine and cytidine nucleotides in these tissues are similar to those reported by Hurlbert et al. (1954) for rat liver. Schmitz, Hurlbert and Potter (1954) emphasised the difficulty of isolating the cytidine nucleotides from

rat liver in quantities large enough for conducting analyses of the fractions for purposes of identification.

The quantities of monophosphates in most experiments were greater than those of the di-phosphates, which were in turn more abundant than the triphosphates (e.g. Chart 4), but in one experiment (Chart 2) in which appendix tissue was used, a reversal of the above pattern was observed, the triphosphates predominating. In this experiment, appendix was the only tissue excised, so that all attention could be focussed on the strictest possible observance of cold experimental conditions and on the maximum reduction of the time interval between the killing of the animal and homogenisation of the tissue in ice-cold perchloric acid. It was possible to keep this time interval very short in the case of appendix, but when intestinal mucosa was the tissue under examination the time lag was inevitably much larger, as the mucosal layer had to be removed from the muscle layers of the intestine prior to lyophilisation and treatment with perchloric acid. All the charts which illustrate ion exchange chromatography of intestinal mucosa acid extracts exhibit a predominance of mono-phosphates over tri-phosphates (Charts 4,5,6 and 7), presumably due to enzymic degradation of the more highly phosphorylated nucleotides during the interval between excision of the small intestine and lyophilisation of the mucosa tissue. Allfrey and Mirsky (1955) fractionated the cold acid extract of calf thymus

tissue and of thymus nuclei prepared by their modification of the Behrens' technique (Allfrey, Stern, Mirsky and Saetren, 1952) using ion exchange chromatography (Hurlbert et al., 1954). They found that these nuclei contain mononucleotides of adenine, cytosine, guanine and uracil together with several UDP derivatives, the adenine nucleotides predominating in amount. However, they emphasised that the predominance of the nucleoside monophosphates in these extracts should not be taken to indicate the actual conditions in vivo and that the predominance of the monophosphates was probably a reflection of anoxia resulting from death of the animal. They provided experimental evidence for this conclusion by carrying out nucleotide analyses on rat thymus tissue which was frozen in liquid nitrogen immediately after excision. In this instance the triphosphates predominated over the di- and monophosphates. This makes it clear that in order to obtain results approximating to nucleotide pattern prevailing in vivo, the tissues under examination must be excised very quickly and, if possible, frozen in liquid nitrogen, liquid air (Hurlbert et al., 1954) or a solid CO_2/ethanol mixture prior to extraction with perchloric acid.

The order of elution of the nucleotides from the column corresponds almost exactly to the order shown by Schmitz et al. (1954) for the 5'-nucleotides in rat liver

and by Manson (1956) for the nucleotides in goat mammary gland. Although Grégoire et al. (1957) fractionating the soluble nucleotides from Micrococcus lysodeikticus and Ballio et al. (1956) studying the soluble nucleotide composition of Penicillium chrysogenum used the ion exchange system employed in the present series of experiments, they reported elution orders slightly different from that found by Schmitz et al. (1954) and by Manson (1956). Both groups of workers found that DPN preceded CMP, but while Grégoire et al. found that CMP was eluted before TPN and UMP before IMP, Ballio and his collaborators observed a reversal of this elution order for CMP, TPN, UMP and IMP. These differences in elution order have not been accounted for but may be related to batch differences in the Dowex-1 resin used and to differences in experimental conditions such as environmental temperature. Cohn (1955) has pointed out that non-polar affinities exhibit a greater temperature dependency than the polar attractions, which are influenced only by pH and complex formation which in turn affect the sign and degree of charge.

In Charts 1,2,4,5 and 6, which illustrate the elution patterns of soluble nucleotides from whole appendix and intestinal mucosa, some of the fractions eluted between AMP and GMP had $E_{275}:E_{260}$ ratios greater than the ratio to be expected from AMP (see Tables 3 and 6). After hydrolysis

with acid, these fractions yielded the base adenine. In Charts 1 and 5, the material (which will be referred to as the A1 material) contained in these fractions appeared as well-defined peaks. The nature of the A1 material was not investigated further, but it should be mentioned that Ballio et al. (1956) in their work on the soluble nucleotides of P. chrysogenum found two substances in the material eluted immediately after AMP. One showed a pyridine nucleotide spectrum but was unable to act as coenzyme for either alcohol dehydrogenase or glucose-6-phosphate dehydrogenase; the other had a spectrum with maximum at 267 mμ in acid solution and at 270 mμ in neutral and alkaline solution and was phosphorus-free. Baddiley et al. (1956) found CDP-ribitol and CDP-glycerol in extracts of Lactobacillus Arabinosus. They used Dowex-2-formate and found these CDP-derivatives eluted between AMP and GMP on their ion exchange chromatograms. Although the compounds mentioned above have not been reported to be present in animal tissues, it is possible that small amounts of them (or of related compounds) may exist in intestinal mucosa and appendix, and that the presence of such substances together with an adenine-containing compound may account for the $E_{275}:E_{260}$ ratio of 0.5 to 0.55 in the A1 material. CDP-choline and CDP-ethanolamine have been identified in rat and hen liver by Kennedy and Weiss (1956) who used ion exchange chromatography on Dowex-1-formate to isolate these compounds from ethanol extracts of the tissues.

However, they did not give a complete description of the ion exchange chromatography so that it is not possible to relate the position of elution of these CDP derivatives to that of AMP, although it is probable that the compounds would emerge from the column before AMP, as both possess cationic groups unlike CDP-ribitol and CDP-glycerol. Nevertheless the existence in living organisms of these CDP-derivatives described by Baddiley and his collaborators and by Kennedy and Weiss may bear some relation to the unidentified A1 material, despite the fact that adenine was the only base detected in acid hydrolysates of the fractions concerned.

Charts 1, 2, 4, 5 and 6 revealed that several compounds were eluted after ADP in the 4N formic acid range. These compounds will be referred to as the A2 material. They had $E_{275}:E_{260}$ ratios higher than ADP (Tables 3 and 7), and contained adenine and phosphorus. In ion exchange chromatography of extracts of M. lysodeikticus Gregoire et al. (1957) found an ADP compound different from ADP eluted at the end of the ADP peak, and Ballio et al. (1956), using extracts of P. chrysogenum, reported that a compound with an ultraviolet absorption maximum at 267 mμ in acid was eluted in the fractions immediately after ADP. The latter compound contained phosphorus and was stable to mild acid hydrolysis. Joklik (1956) in studies on the nature of ultraviolet

absorbing substances present in extracts of mouse and rabbit liver has briefly reported the occurrence of adenyl-succinic acid and adenine-succinic acid in these tissues. Using the continuous gradient elution technique with the formic acid system described in Section 2.7b, he found a high $E_{275}:E_{260}$ ratio in some of the fractions associated with ADP. It was from this part of the chromatogram that he isolated and identified adenyl-succinic acid and adenine-succinic acid. It is of considerable interest that despite the difference in chemical structure both compounds emerged from his column at the same point. While continuing their investigations into the nature of the ultraviolet-absorbing compounds in P. chrysogenum, Ballio and Serlupi-Crescenzi (1957) identified adenyl-succinic acid in ethanol extracts of the mould. Adenyl-succinic acid was probably the compound reported earlier (Ballio et al., 1956) to be eluted immediately after ADP. These compounds may be present in all living organisms and may constitute some of the A2 material in intestinal mucosa and appendix. The formation of adenyl-succinic acid from AMP and fumaric acid in the presence of a yeast enzyme fraction (Carter and Cohen, 1956) and from inosinic acid and aspartic acid by a purified enzyme from Escherichia coli (Lieberman, 1956) has been described. Schmitz et al. (1954), working with rat liver, and Manson (1956), with goat mammary gland, also found several compounds

corresponding to the A2 material on their ion exchange chromatograms, but they did not come to any definite conclusions as to the nature of the compounds in question. Several compounds containing ADP are known to exist in tissue extracts and may account in part for some of the A2 material. One of these compounds isolated by Hansen, Hageman, Freedland and Wilkin (1956) from rat, chick and guinea pig liver, was identified as an adenosine nucleotide containing adenine : ribose : phosphorus in the ratio of 1 : 2: 2. A suggested name for this compound was adenosine-diphosphate-ribose. Hansen and Hageman (1956) also found ADP-amino acid complexes in extracts of liver and lactating mammary gland. The compounds were identified as ADP-glutamic acid and ADP-aspartic acid, probably with the β-carboxyl group of aspartic acid and the γ-carboxyl group of glutamic acid linked to the terminal phosphate of ADP by an anhydride bond.

In some experiments in which the full eluting power of the last elution range was put into effect by omitting the mixing vessel from the elution system, adenine was detected in acid hydrolysates of the last peaks which usually contained GTP and UTP (Chart 3). The nature of this adenine-containing material (the A3 material) was not determined, but in order to be retained on the resin until the last elution stage, the material would have to possess a

group or groups with a high negative charge. This is of interest in view of the fact that Marrian (1954) and Sacks (1955) found adenosine tetraphosphate in commercial ATP preparations. Moreover, Sacks (1955) showed that these preparations contained a small amount of adenosine penta-phosphate. Hilz and Lipmann (1955) in studies on the enzymic activation of sulphate gave a tentative formulation of "active" sulphate. The "active" sulphate was subsequently shown by Robbins and Lipmann (1956) to be adenosine-3'-phosphate-5'-sulphate. It was eluted from their Dowex-1-formate columns with 1M ammonium formate in 5N formic acid after all other nucleotides had been eluted with 0.3M ammonium formate in 4N formic acid. These adenosine compounds, then, may be present in small amounts in tissue extracts such as those studied here and may account for the adenine in the A3 material.

In addition to the mono-, di- and triphosphates of cytidine, adenosine, guanosine and uridine it is clear that many other ultraviolet-absorbing compounds are to be found in tissue extracts, quite apart from the non-ultraviolet-absorbing materials present. Hurlbert et al. (1954) and Daoust and Cantero (1955) carried out a series of trial experiments fractionating known nucleotides on Dowex-1. It was found by comparison of these standard chromatograms

with chromatograms of tissue extracts that the naturally occurring soluble nucleotides are in the 5'- form as the 2'- and 3'- ribonucleotides on the standard chromatograms had no corresponding peaks on the test chromatograms. Further, the nucleotides were all of the ribonucleotide type and no definite evidence for the existence of deoxyribonucleotides was obtained, so that if the latter are present at all they must constitute a very small proportion of the nucleotide fractions. Both groups of workers stressed the complexity of the ultraviolet-absorbing compounds in tissue extracts. Daoust and Cantero (1955) used Dowex-1-chloride columns with a stepwise elution system. They fractionated the soluble nucleotides from rat liver and intestinal mucosa and their results, although incomplete, are in general agreement with those found in the present investigation, providing further evidence suggesting that the acid-soluble nucleotide composition of appendix, intestinal mucosa, liver and several other tissues resemble one another closely, qualitatively at least.

4.3 The Acid-soluble Nucleotides of Cell Nuclei Isolated in Non-aqueous Media.

It was considered desirable at the outset of the present series of experiments to investigate the soluble nucleotides in the cell nucleus. Many observations from this and other laboratories had led to the conclusion that the most suitable method to be used for the isolation of cell nuclei prior to the investigation of their acid-soluble constituents was the technique of isolation in non-aqueous solvents. This method was originally devised by Behrens (1932; 1938) and modified by Allfrey, Stern, Mirsky and Saetren (1952). The technique was further modified and improved in this department by Kay, Smellie, Humphrey and Davidson (1956), and it was this modification that was used throughout the present series of experiments for the study of the free nucleotide composition of the cell nucleus.

Kay et al. (1956) compared cell nuclei isolated from rabbit tissues by aqueous and non-aqueous procedures. The former nuclei were isolated from dilute citric acid solution as described by Smellie, Humphrey, Kay and Davidson (1955) and will be referred to as citric nuclei (CN). When Kay et al. (1956) treated preparations of NAN with citric acid under conditions similar to those obtaining in the preparation of CN, the citric acid extract was found to contain appreciable

amounts of phosphorus and nitrogen. On treatment of the extract with trichloroacetic acid an abundant precipitate consisting mainly of protein appeared. The supernatant fluids from such precipitates showed negligible amounts of materials reacting as deoxypentose but contained appreciable amounts of phosphorus and pentose-reacting substances consisting mainly of simple nucleotides. Indeed, when pentose estimations were carried out on trichloroacetic acid extracts of NAN and ON, Kay et al. found that 85-90% of the acid-soluble pentose-reacting materials in the NAN had been removed by citric acid. Stern and Mirsky (1952) had found that DPN was present in considerable concentration in nuclei isolated in non-aqueous media but this coenzyme was found to be generally scarce in or absent from nuclei isolated in aqueous media (Dounce, 1955). It was clear, then, that NAN contained the bulk of the acid-soluble constituents of the cell nucleus and were suitable for studies on the soluble nucleotide composition of the nucleus.

Further work by Kay and Davidson (1955) on the acid-soluble fraction from the NAN prepared from various rabbit tissues showed that the nucleotide bases in these acid extracts included adenine, which was most abundant, hypoxanthine, guanine, uracil and cytosine. In 1954, Naora and Tukeda isolated rat liver NAN and showed that they contained labile phosphate derived possibly from ATP.

In the present investigation the yield of clean nuclei was usually low and so the ensuing ion exchange chromatography was scaled down in order that satisfactory separations might be obtained (see Figures 1 and 3).

The patterns of acid-soluble nucleotides in rabbit appendix NAN (Chart 3) and in rabbit intestinal mucosa NAN (Chart 7) were found to be similar and to resemble closely the patterns for the corresponding whole tissue extracts (Charts 1,2,4,5 and 6) with a few minor exceptions. The mono-, di- and triphosphates of adenosine, cytidine, guanosine and uridine were found to be present along with IMP, some UDP derivatives, DPN and small amounts of TPN.

Nuclei are known to contain enzymes which can synthesise nucleotides. The synthesis of DPN from nicotinamide mononucleotide and ATP takes place in nuclei (Hogeboom and Schneider, 1952) and UTP can be formed from UDPG and inorganic pyrophosphate in isolated liver nuclei by the action of a uridyl transferase (Smith and Mills, 1954a; Mills, Ondarza and Smith, 1954); so the existence of acid-soluble nucleotides in nuclei might be expected.

The A1 material which was eluted between AMP and GMP in the whole tissue experiments (Section 4.3) was probably absent from NAN as the $E_{275}:E_{260}$ ratios of fractions eluted immediately after AMP were low and entirely characteristic of AMP. However, at least some of the A2

and A3 material found in the whole tissue extracts was present in the corresponding NAN extracts (Charts 3 and 7). The fact that the A1 material was not evident on chromatograms of the NAN acid extracts may be due to the smaller amounts of starting material used in these experiments. In the experiment with appendix NAN (Chart 3) guanine was found in peak I. This was probably a consequence of applying the NAN extract to the resin at a higher pH than in other experiments. Cavalieri, Kerr and Angeles (1951) found that guanine was anionic in alkaline solution, with a pK value of 9.36.

The NAN then, appear to be very satisfactory for studies on the acid-soluble nucleotide composition of the cell nucleus. In the removal of the mucosal layer from the excised gut the inevitable time lag before freezing remains and, as mentioned in Section 4.2, this probably permits a certain amount of autolysis to take place. However, despite this disadvantage, substantial amounts of the triphosphates were found in the intestinal mucosa NAN extracts (Chart 7). Even during the isolation procedure from the dry mucosa tissue, the strictest precautions were taken and the material was always kept cold while the tissue was being disintegrated in the organic solvents. It was found that the original mucosa or appendix tissue (or the corresponding NAN preparations) could be stored in the dry state for at least three months without any apparent degradation of the

nucleotides, whereas experiments on tissue which was stored at -10° without prior lyophilisation revealed that a certain amount of breakdown of triphosphates to monophosphates took place (Sections 4.2 and 4.5).

Since the completion of this work a paper by Osawa, Allfrey and Mirsky (1957) has appeared, comparing the acid-soluble nucleotide composition of calf thymus nuclei with that of the corresponding whole tissue. The results are in close agreement with those obtained by the present author for rabbit appendix and intestinal mucosa. Osawa et al. prepared NAN from calf thymus by a modification of the Behrens' procedure (Allfrey et al., 1952), and used identical methods to those described here for the ion exchange chromatography of acid extracts. The elution patterns which they obtained for nuclei and for whole tissue were quantitatively and qualitatively much the same, the relative amounts of individual nucleotides being similar to those found in the present investigation for appendix and thymus. Moreover, since they found similar results with calf liver and chicken erythrocytes it would appear that the acid-soluble nucleotides occur generally in all tissues, being equally distributed between the nucleus and cytoplasm of the cell. Osawa et al. also made some remarkable observations on the acid-soluble nucleotide composition of nuclei isolated in sucrose/$CaCl_2$ media. These results will be discussed in Section 4.5, together

with the results obtained in the present investigation for liver nuclei isolated in sucrose/$CaCl_2$ solution and with some observations made by Edmonds and Lepage (1955) on the soluble nucleotide composition of SN from Flexner-Jobling carcinoma.

4.4 Incorporation of ^{14}C-formate into the Acid-soluble Purine Nucleotide Bases from Rabbit Appendix and Intestinal Mucosa.

In general, two hours after the administration of ^{14}C-formate, the acid-soluble purine nucleotide bases from appendix, appendix NAN, intestinal mucosa and intestinal mucosa NAN were found to have specific activities of the same order, viz. about 7,000, (Tables 5 and 8). There were, however, certain deviations from this value particularly in the case of the guanine nucleotides of whole appendix and appendix NAN (Table 5). In these experiments, the incorporation of formate into GDP and GTP was about half that into the other purine nucleotides including GMP. This difference in specific activity need not be regarded as of particular significance at the present time for the following reasons: (i) The final modification of the technique for purification of bases after hydrolysis and two-dimensional paper chromatography (Section 2.9c) had not been perfected in the early experiments (e.g. whole

appendix, Table 5); (ii) The amounts of guanine available from GDP and GTP were usually small, particularly when experiments with NAN were being carried out (e.g. appendix NAN, Table 5); (iii) In the early experiments, some of the GMP and GTP material had to be taken for identification purposes, leaving very small amounts for specific activity determinations. For these reasons, the specific activities of the guanine of GDP and GTP in Table 5 would be subject to error and are not regarded as being of particular significance. The guanine from GMP, which was always available in much larger amounts in these experiments, would not be so susceptible to such errors in specific activity determinations.

Such differences between the purine nucleotide bases were not observed in the experiments with intestinal mucosa whole tissue and NAN two hours after ^{14}C-formate administration (Table 8). In these experiments the purine nucleotide bases all had specific activities of the same order.

The specific activities of the adenines from the various adenine nucleotides from appendix and intestinal mucosa whole tissue and NAN were not subject to the same error as applied to the bases from the guanine nucleotides (even before perfection of the technique for further chromatography of bases) since adenine from the adenine nucleotides was always available in fairly substantial quantities.

The adenines of AMP, ADP and ATP from appendix NAN had specific activities slightly higher than those for the corresponding bases in the whole tissue experiment (Table 5) but the NAN were obtained from a single rabbit appendix while the whole tissue extract was prepared from the pooled appendix tissue from four rabbits (Section 5.1b) and so direct comparisons cannot be legitimately made. In the whole intestinal mucosa and intestinal mucosa NAN experiments (Table 8) the specific activities of the purine nucleotide bases after two hours were essentially of the same order. Edmonds and Lepage (1955) fractionated acid soluble nucleotides from the nuclear and cytoplasmic fractions of Flexner-Jobling carcinoma in rats which had received 2-^{14}C-glycine. The incorporation of this precursor into the corresponding purine nucleotides of each fraction showed small differences but only at early time intervals (8 minutes). At later times after administration of 2-^{14}C-glycine the specific activities of the corresponding nucleotides tended to be similar.

Overall, the results laid out in Tables 5 and 8 indicate that after two hours the specific activities of the purine nucleotide bases are of the same order showing that there has been a fairly rapid equilibration among the nucleotides after this time, and that the specific activities are much the same whether the tissue used is

appendix, appendix NAN, intestinal mucosa or intestinal mucosa NAN.

Smellie, Humphrey, Kay and Davidson (1955) carried out time/activity studies on the incorporation of ^{32}P into the nucleic acids of different rabbit tissues *in vivo* and found that the tissues could be divided into three main groups according to the turnover of the tissue DNA. In kidney, where mitotic activity is negligible, the DNA turnover was very low. In tissues of moderate mitotic activity, such as intestinal mucosa, spleen and thymus, the renewal of DNA was fairly rapid, while in bone marrow, where cell division is brisk, the DNA turnover was very high. The pattern of activity for appendix fitted into the latter category as it was comparable with that of bone marrow and had a DNA turnover much more rapid than is found in the other lymphoid tissues. A continuation of this work (Smellie and Davidson, 1956) involving the incorporation *in vivo* of ^{14}C-formate (also 8-^{14}C-adenine and 2-^{14}C-glycine) into the nucleic acid bases of different rabbit tissues two hours after administration of the isotope, provided results similar to those found by Smellie et al. (1955) for ^{32}P. The incorporation of ^{14}C-formate into appendix DNA bases was much higher than that into the intestinal mucosa DNA bases after two hours. The uptake of the isotope by the RNA bases in both tissues did not

show such large differences. All specific activities at 2 hours were very much lower than those found for the acid-soluble purine nucleotide bases in the present series of experiments.

It can be seen from Tables 5 and 8 that the differences between appendix and intestinal mucosa nucleic acids mentioned above are not reflected at all in the acid-soluble fractions of these tissues after two hours, or in the acid-soluble fractions of the NAN. In some comparable experiments with rabbit liver it was found by the present author that the specific activities of the acid-soluble purine nucleotide bases were low and of the order of 600. Smellie, McIndoe, Logan, Davidson and Dawson (1953), using ^{32}P, found that liver, unlike appendix and intestinal mucosa, had a very low DNA turnover similar to that of kidney (Smellie et al., 1955), and Smellie and Davidson (1956) found that the incorporation of ^{14}C-formate into the nucleic acid bases of rabbit liver after two hours was very low indeed. The low activities of the purine nucleotide bases from the liver acid-soluble fraction relative to the activities of the corresponding purines from the appendix and intestinal mucosa acid-soluble fractions may be due in part to larger pools of soluble nucleotides in liver or to a greater variety of reactions involving formate incorporation in the latter organ.

In the experiment (Chart 6) carried out to ascertain whether any large differences in specific activities of purine nucleotide bases could be demonstrated at a very short time interval between administration of ^{14}C-formate and the killing of the animal the animal was sacrificed 15 minutes after administration of the isotope. The specific activities of the purine nucleotide bases of the intestinal mucosa acid extract are shown in Table 8, Column III. The most striking feature of this experiment was the very high specific activity of hypoxanthine from IMP relative to the other purine nucleotide bases, but there was still a rapid equilibration among the mono-, di- and triphosphates of adenosine and of guanosine. This observation stresses the importance of working at very short time intervals after administration of the isotope when studying the metabolic interrelationships of the acid-soluble nucleotides.

The rapid incorporation of ^{14}C-formate into IMP is in agreement with the work of Greenberg (1950; 1951b) and Schulman and Buchanan (1952), who carried out *in vitro* studies on the incorporation of the isotope into IMP. Greenberg (1951b) conducted a time/activity study of the incorporation of ^{14}C-formate into IMP, inosine and hypoxanthine. Measurements of the specific activities of these compounds at early stages showed that the highest activity was located in the IMP while inosine had a specific

activity half that of IMP but twice that of hypoxanthine. Greenberg's results made it clear that IMP was the first purine compound formed in pigeon liver systems and that inosine was not a precursor of IMP but an intermediate in the conversion of IMP to hypoxanthine. The results shown in Table 8 are in agreement with these observations.

The results shown in Table 8 also suggest that IMP is a precursor at least in part of the adenine and guanine nucleotides, although it should be borne in mind that only two time points have been studies, 15 minutes and 2 hours. Abrams and Bentley (1955a; 1955b) working with soluble extracts of rabbit bone marrow showed that these preparations could convert inosine and IMP to derivatives of adenine and guanine. Gehring and Magasanik (1955) observed that extracts of Aerobacter aerogenes were capable of converting IMP to XMP in the presence of DPN. Further work (Abrams and Bentley, 1955c and Bentley and Abrams, 1956) proved that there were at least three reaction sequences involved in the biosynthesis of adenine and guanine nucleotides from IMP in dialysed preparations of their rabbit bone marrow extracts. These sequences are shown below:-

(1) $$\text{IMP} \xrightarrow[\text{DPN}]{\text{inosinic acid dehydrogenase}} \text{XMP}$$

(2) $$\text{XMP} \xrightarrow[\text{ATP} + \text{Mg}^{++}]{\text{L-glutamine}} \text{GMP}$$

(3) $$\text{IMP} \xrightarrow[\text{high energy phosphate source}]{\text{l-aspartic acid}} \text{AMP}$$

Lagerkvist (1955) in studies on the metabolism of IMP in pigeon liver extracts made observations identical with those obtained by Abrams and Bentley (1955c) for the formation of GMP from IMP (reactions 1 and 2 on the previous page).

In 1955, Carter and Cohen reported the formation of adenylsuccinic acid from AMP and fumaric acid in the presence of an enzyme purified from yeast autolysates, and more recently Lieberman (1956) reported the synthesis of adenylsuccinic acid from IMP and aspartic acid by a purified enzyme from Escherichia coli. The enzyme catalysed the following reaction:-

IMP + aspartic acid + GTP → adenylsuccinic acid + GDP + inorganic phosphate

It seems likely, in view of the observations of Abrams and Bentley (1955c) who found the conversion of IMP to AMP to be specifically dependent on aspartic acid, that adenylsuccinic acid is an intermediate in the biosynthesis of AMP from IMP. It is possible that the corresponding but hypothetical guanylglutaric acid might be an intermediate in the formation of GMP from XMP, but these suggested functions for adenylsuccinic acid and guanylglutaric acid in purine biosynthesis as the immediate precursors of

AMP and GMP respectively, have not yet been established.

The results from Table 8, Column III, are in agreement with the general scheme of reactions described above, which establish IMP as a key compound in purine biosynthesis. These reaction sequences, then, may also be of significance *in vivo* in mammalian tissues.

The specific activities (Table 8, Column III) of the adenines from AMP, ADP and ATP are much lower than those of the guanines from GMP, GDP and GTP which in turn have specific activities about half that of the hypoxanthine from IMP. The difference between the adenosine and guanosine phosphate activities is only to be expected in view of the finding that the pool sizes of the adenine nucleotides are much greater than those of the guanine nucleotides (see Charts 4,5 and 6), but as the pool sizes of the former are in fact at least five times the pool sizes of the latter nucleotides (Charts 4, 5 and 6) it is surprising that the ratio of the specific activity of the guanine derivatives to that of the adenine derivatives is only 2:1. This finding implies that there is a difference in rates of production of AMP and GMP from IMP and/or that quite apart from *de novo* synthesis, there may be an exchange of ^{14}C-formate with carbon of the purine ring in the adenine nucleotides and that this exchange is more pronounced than

any similar exchange which may take place in the purine ring of the guanine nucleotides. Buchanan and Schulman (1953), working with pigeon liver systems, reported that when de novo synthesis of IMP from glycine is low an "enzymatic exchange reaction" can be demonstrated in which ^{14}C-formate is preferentially incorporated into position 2 of the hypoxanthine moiety of IMP.

It might be supposed that AMP could serve as a precursor of GMP in the light of the finding that ^{14}C-adenine can be converted to nucleic acid guanine (Brown, Roll, Plentl and Cavalieri, 1948; Smellie and Davidson, 1956) but the specific activity relationships in Table 8 do not lend support to this hypothesis, since after 15 minutes the bases from the guanosine phosphates have specific activities higher than those of the adenine bases derived from the adenosine phosphates. After two hours the specific activities of all these purine nucleotide bases are similar and much higher than at 15 minutes.

Edmonds and Lepage (1955) conducted a short-term time study of the in vivo incorporation of 2-^{14}C-glycine into the acid-soluble purine nucleotides and into the nucleic acids of rat liver and Flexner-Jobling carcinoma. Their data did not support the assumption that acid-soluble AMP is a precursor of the acid-soluble guanine nucleotides, since in the tumour the guanine nucleotides had a higher specific activity than the adenine nucleotides.

Moreover, they found that the guanine nucleotides did not appear to be obligatory intermediates in the biosynthesis of the adenine nucleotides, since 8 to 10 minutes after administration of the radioactive glycine the adenine nucleotides were observed to become labelled before the guanine nucleotides. The results of Edmonds and Lepage did not lend support to the hypothesis that IMP is the precursor of the guanine nucleotides as, 8 minutes after administration of the isotope, the guanine nucleotides had higher specific activities and a higher total radioactivity than the IMP. Further, this relationship between IMP and the guanine nucleotides continued to exist for the next 30 minutes. This may, however, be due in part to peculiarities in purine biosynthesis in the tumour or to the possibility that purine nucleotide biosynthesis is more complex than suggested by the work of Abrams and Bentley (1955c), Lieberman (1956), and Carter and Cohen (1955). In fact, these results of Edmonds and Lepage (1955) offer evidence supporting an alternative hypothesis for purine nucleotide biosynthesis, viz. that IMP, the guanine nucleotides and the adenine nucleotides are formed from a common precursor at some early stage of nucleotide synthesis after which each nucleotide is synthesized along an independent pathway.

The data in Table 8, Column III, indicate that the adenine of DPN and of TPN is not highly labelled after 15 minutes, an observation which is not surprising, as these

nucleotide coenzymes are synthesised from preformed ATP. DPN is synthesised from nicotinamide mononucleotide and ATP (Hogeboom and Schneider, 1952), and the phosphorylation of DPN by ATP and a purified pigeon liver enzyme (DPN kinase) results in the formation of TPN (Wang and Kaplan, 1954).

4.5 The Acid-soluble Nucleotides of the Subcellular Fractions of Rabbit Liver.

Most of the data concerning the biochemical structure of the cell cytoplasmic fractions have been obtained from studies on mammalian liver. This tissue is particularly convenient for cell fractionation experiments because, in terms of total mass, it is composed largely of what appears to be a single type of cell bearing a preponderance of cytoplasm in relation to nuclei. Moreover, the tissue can be readily disrupted by mechanical means without undue damage to the intracellular elements (Hogeboom and Schneider, 1955).

The technique which was used in the present series of experiments for the investigation of the acid-soluble nucleotide composition of cell nuclei from appendix and intestinal mucosa (see Section 2.5) is not suitable when applied to liver tissue. Kay et al. (1956) could not obtain satisfactory preparations of NAN from adult rabbit liver although they did find that it was possible to do

so in the case of embryo liver. Allfrey et al. (1952) encountered similar difficulties with rat liver but found that these difficulties were fairly successfully overcome if the rats were subjected to a fast before sacrifice.

The technique of isolation of liver cell nuclei from a sucrose medium, however, appeared to offer a very convenient means of obtaining liver nuclei, for although it was recognised that the method had the important limitation that possibly low molecular weight compounds would be extracted in the course of the isolation procedure (Section 2.6a), it was nevertheless considered worth while to explore the possibilities of the technique for studies on the acid-soluble nucleotide content of the cell nucleus.

4.5a. The Acid-soluble Nucleotides of Rabbit Liver Cell Nuclei Isolated in a Sucrose Medium.

Liver cell nuclei can be isolated in isotonic sucrose solution as the suspending medium (Arnesen, Goldsmith and Dulaney, 1949) but when they are isolated in isotonic solutions of sucrose containing a low concentration of calcium chloride, superior preparations can be obtained. The use of calcium chloride, which apparently inhibits autolytic degradation (Dounce, 1955),

was originally introduced by Schneider and Peterman (1950) and further developed by Hogeboom, Schneider and Striebich (1952).

A modification of the procedure used by Hogeboom et al. (1952) was followed in the present investigation (Section 2.6a) and during the isolation the nuclear residue was washed twice with the sucrose/calcium chloride solution. It would appear that these washes removed soluble substances from the cell nuclei for certainly the elution chart (Chart 8) from ion exchange chromatography of the ultraviolet-absorbing compounds in the acid extract of the nuclei indicated the presence of only small amounts of acid-soluble nucleotide material. Stern and Mirsky (1952) reported that liver cell nuclei isolated in non-aqueous media by their modification of the Behrens' procedure (Allfrey et al., 1952) contained DPN in considerable concentration, but on the other hand, liver cell nuclei isolated in aqueous media were found to contain little, if any, DPN (Dounce, 1955). The quantity of DPN (Chart 8) obtained from liver nuclei by the present author was very small indeed in relation to the amount of liver tissue (20 g.) taken for isolation of nuclei. DPN would be expected to occur in nuclei in considerable amounts as Hogeboom and Schneider (1952) found that the enzyme catalysing the synthesis of DPN in liver tissue is localised exclusively in the nucleus.

Edmonds and Lepage (1955) explored the intracellular distribution of acid-soluble nucleotide by examining the nuclear and cytoplasmic fractions of the Flexner-Jobling carcinoma. They recognised that in order to obtain significant results, it was necessary to exclude, as far as possible, contamination of nuclei by cytoplasmic materials and that this requirement, which is normally met by washing the nuclei several times, would be likely to result in loss of acid-soluble nuclear components. Their nuclei, therefore, were isolated in isotonic sucrose in high dilution without washing, a 20% homogenate in isotonic sucrose being prepared from the minced tissue. The 20% homogenate was immediately diluted to 5% with the sucrose solution before centrifugation. This high dilution was reported to insure a minimum of mechanical contamination with non-nuclear, non-particulate acid-soluble nucleotides. The unwashed nuclei so prepared contained 12% of the total units absorbing ultraviolet light at 260 mμ. A single sucrose wash of these nuclei resulted in a 50% loss of the ultraviolet absorbing material which appeared to be mainly of authentic nuclear origin rather than merely cytoplasmic contamination. The results of their ion exchange chromatography indicated that the nucleotide composition

of the acid extract of nuclei resembled that of the cytoplasm in the Flexner-Jobling carcinoma, but that extensive degradation of the di- and tri-phosphates normally present in extracts of the whole tissue (Schmitz et al., 1954) frozen in liquid air, was evident. This, of course, was to be expected, since enzymic degradation was likely to occur during the time interval required for the separation of the nuclei from the homogenate (see Sections 3.1a, 3.2b, 3.2c and 4.2).

In Section 4.3 it was mentioned that Osawa et al. (1957) found that NAN prepared from calf thymus, calf liver and chicken erythrocytes possessed quantitativel and qualitatively the same free nucleotide patterns as the corresponding whole tissues, although nuclei from metabolically active tissues like calf thymus or calf liver had a higher content of nucleotides than nuclei of such metabolically inert cells as chicken erythrocytes. When the same authors carried out isolation of calf thymus nuclei in 0.25M sucrose containing 0.002-0.003M $CaCl_2$, they found that these nuclear preparations retained 60-80% of the acid-soluble nucleotides, and that such nucleotides remained in the nuclei even after repeated washing with the cold sucrose/$CaCl_2$ solution. Thorough microscopic examination showed that the calf thymus nuclei were quite free from adhering cytoplasm. These

observations are in direct contrast to similar work on calf liver nuclei isolated in sucrose/$CaCl_2$ solutions in the same laboratory. Such nuclei were ordinarily grossly contaminated with cell debris and much of their acid-soluble contents had been washed away (Mirsky, 1955). The extent to which the acid-soluble nucleotides were retained by SN from calf thymus in the work of Osawa et al. (1957) is indeed surprising, for the nuclear membrane of nuclei isolated in sucrose solutions will allow the passage of much larger molecules. Watson (1954) examined the nuclear membrane in very thin sections of mouse pancreas using the electron microscope and demonstrated the presence of pore-like structures in the membrane. The pores were sufficiently large to permit the passage of the largest nucleic acid and protein molecules. Osawa et al. (1957) suggest that the nuclear nucleotides are held in some complex structure within the nucleus in calf thymus tissue, and this would account for the retention of the nuclear nucleotides even after repeated washing with sucrose/$CaCl_2$ solution.

Another surprising observation came to light in the experiments of Osawa et al. (1957) on thymus nuclei. Calf thymus NAN and SN had similar free nucleotide patterns (disregarding, for the present, the loss of 20-40% of the soluble nucleotides from the SN) resembling closely the

patterns found by the present author for rabbit appendix and intestinal mucosa NAN (Charts 5 and 7). The two nuclear preparations of Osawa et al. from calf thymus showed the predominance of the monophosphates over the di- and triphosphates, but when the SN dispersion was stirred for 60 minutes at 4° prior to centrifugation and acid extraction, a large proportion of the monophosphates was converted back to the triphosphate form, which predominates in the living tissue (Allfrey and Mirsky, 1955). This synthesis of high energy phosphate bonds was probably the result of aeration of the SN suspension during the stirring procedure. The phosphorylation was subsequently found to occur exclusively in the nucleus and to involve only nuclear nucleotides. These observations of Osawa et al. (1957) show that the phosphorylation which occurs in nuclei must differ in some respects from that which occurs in mitochondria, for it is known that in preparations of the latter from rat liver considerable amounts of exogenous nucleotides can be added and become phosphorylated (Herbert, Potter and Takagi, 1955; Herbert and Potter, 1956). These differences between nuclei and mitochondria may perhaps be related to the observations that mitochondria, but not nuclei, contain the biochemical components for ordinary terminal oxidation such as cytochrome oxidase, cytochrome-c and flavoproteins including cytochrome-c-

reductases (Hogeboom et al., 1952; Dounce, 1955).

The present evidence then shows that nuclei contain a soluble nucleotide complement similar to that of the cytoplasm but that the intranuclear nucleotides are metabolically distinct from the cytoplasmic nucleotides (at least under the experimental conditions chosen). In the experience of the present author SN isolated from rabbit liver as described in Section 2.6a do not provide a suitable preparation for studies on the acid-soluble nucleotide composition of the liver cell nucleus as the washing procedure appears to result in considerable loss of nucleotides.

4.5b. The Acid-soluble Nucleotides of Rabbit Liver Mitochondria.

In the experiments carried out on the acid extracts of liver mitochondria (Charts 9 and 10) several of the acid-soluble nucleotides observed in whole liver extracts (Hurlbert et al., 1954) were not detected or were found to be present only in very small amounts, e.g. GDP and the UDP-derivatives. However, these observations cannot be regarded as being of serious importance owing to the small amounts of starting material used in these experiments with the consequent possibility that some

constituents present in small quantities would escape detection.

The pattern of soluble nucleotides obtained from rat liver mitochondria by Siekevitz and Potter (1955a) is essentially the same as that shown in Chart 9 for rabbit liver mitochondria, but Siekevitz and Potter used a stepwise elution system involving a large number of solutions of increasing formate concentration and did not carry out a complete identification of all compounds eluted from the column.

It is clear from Chart 9 that adenine nucleotides are present in mitochondria in very large amounts in relation to the other nucleotides. A compound of adenine, which was not ADP, was eluted from the Dowex-1 column along with UMP. Siekevitz and Potter (1955a) also found a compound of adenine eluted immediately before ADP in their ion exchange chromatograms of the acid-soluble nucleotides from rat liver mitochondria. The chemical nature of this compound is unknown but may be related in some way to one of the compounds found by various authors, as mentioned in Section 4.2, to be associated with this part of ion exchange chromatograms of the acid extracts from whole tissue.

Beyer, Glomset and Beyer (1955) also examined the nucleotide composition of isolated mitochondria using

rat liver. Their elution chart, which was similar to Chart 9, revealed the presence of large amounts of AMP, ADP and ATP in their mitochondrial preparations, but they did not come to definite conclusions as to the exact nature of some of the other peaks; this was possibly due to the scarcity of material emerging from the column. They did not state what weight of liver tissue was used for isolation of mitochondria. Apart from AMP, ADP and ATP the only other nucleotides conclusively identified were DPN and UMP. The possible occurrence of a fairly large amount of FAD was indicated.

Chart 12, which shows a good separation of compounds eluted before AMP, demonstrates that DPN is present in mitochondria in appreciable quantity, and the finding that FAD also is abundant in mitochondria is in agreement with the observations of Schneider and Hogeboom (1956) who found that this nucleotide is located mainly in the mitochondria of the liver cell. Beyer et al. (1955) reported that the peak, which corresponded to the FAD fractions on Chart 9, was suspected to be FAD but did not correspond to either FAD or FMN when submitted to paper chromatography. Siekevitz and Potter (1955) identified this peak tentatively as being FAD. It is possible then that the peak contains not pure FAD

but FAD combined with an amino-acid or peptide, that the FAD in the mitochondrion is intimately bound to apoenzyme protein and that under the conditions of isolation of the mitochondria and subsequent treatment with acid a compound of FAD with an amino acid or a peptide appears in the acid-soluble fraction. This hypothesis is supported by the observations of Kearney, Massey and Singer (1956) working with highly purified beef heart succinic dehydrogenase. They treated the latter protein with a mixture of trypsin and chymotrypsin and found that this procedure released a number of flavin peptides which were purified by column chromatography. One of these flavin peptides was found to be as fully active as FAD in functioning as the prosthetic group for the apoenzyme of D-amino acid oxidase. In developing a chromatographic procedure for the isolation of FAD from animal tissues, Dimant, Samadi and Huennekens (1952) located a new dinucleotide containing riboflavin (FAD-X). The existence of such FAD compounds then may account for the difficulty encountered by Beyer et al. (1955) in identifying the material in the peak which was suspected to contain FAD.

Although the mitochondria were isolated in 0.25M sucrose solution, it appears from Charts 9 and 10 that they retain a large proportion of their acid-soluble

nucleotides. However, they were not washed with sucrose solution as were the corresponding nuclei, which apparently lost much of their soluble nucleotide material. There are several other lines of evidence which suggest that mitochondria prepared in isotonic sucrose, without washing procedures, would tend to retain their soluble components. (a) Electron microscopy has shown that mitochondria possess a surface membrane (Sjöstrand and Rhodin, 1953), which was suggested by Tedeschi and Harris (1955) to be semi-permeable and lipid in nature; (b) Nucleotides were readily released from isolated mitochondria when the latter were incubated at 30° in isotonic sucrose solution (Siekevitz and Potter, 1954a); (c) The endogenous citrate of the liver cell was found by Schneider, Striebich and Hogeboom (1956) to be localised in the isolated mitochondria and could be released by suspending the mitochondria in distilled water.

The probability of enzymic degradation of nucleotides taking place in tissues during the time interval between death of the animal and treatment of the tissue with perchloric acid has been discussed in Section 4.2. The likelihood of such autolytic changes taking place is most certainly increased in the case of mitochondria

during the time interval required for their isolation from the liver homogenate, and the predominance of AMP over ATP in the mitochondrial elution chart (Chart 9) indicates that such changes have probably occurred (see also Section 3.2b).

The presence of nucleotides of cytosine, guanine and uracil in mitochondrial extracts is not surprising in view of the recent work of Herbert, Potter and Takagi (1955) and Herbert and Potter (1956) who reported that isolated rat liver mitochondria are capable of phosphorylating UDP to UTP by a transfer of phosphate from the terminal phosphate of ATP, and that they can phosphorylate both CDP and GDP to the corresponding triphosphates.

The mitochondria then appear to possess a fairly complete complement of acid-soluble nucleotides particularly rich in adenosine phosphates and FAD. This is not surprising, as oxidative phosphorylation is probably an exclusive function of mitochondria (Siekevitz, 1952; Siekevitz and Potter, 1955a, 1955b).

4.5c. The Acid-soluble Nucleotides of Rabbit Liver Microsomes.

Charts 11 and 12 show the elution patterns from ion exchange chromatography of the acid-soluble compounds absorbing light at 260 mμ obtained from microsomes. For

reasons which were pointed out in Section 3.2c, Chart 12 is regarded as representing a more accurate picture of the *in vivo* nucleotide composition of microsomes, but owing to the time required for the isolation of microsomes from the liver homogenate, it is recognised that some enzymic degradation of nucleoside polyphosphates may well have occurred in the microsomes. This point has been discussed in previous sections (4.2, 4.5a, 4.5b).

The most striking features of the microsome elution chart (Chart 12) are the occurrence of a large amount of inosine in peak I and the relative scarcity of nucleotide material. Siekevitz (1955) recently found that rat liver microsomes contain only inosine in appreciable amounts but he used only 12 g. of rat liver as the starting material in his experiment, so that other compounds eluted from his Dowex-1 column would be present in very small amounts. In the present experiment the microsomes were isolated from 50 g. of rabbit liver and in addition to the large amount of inosine, small amounts of AMP, GMP, UMP and ADP were found. It was also possible to infer the presence of other nucleotides by isolation and identification of purine and pyrimidine bases from acid hydrolysates of the various fractions (Chart 12) of the ion exchange chromatogram. It is emphasised that, apart from inosine, most of the ultraviolet-absorbing compounds

were present in very small amounts and may possibly have been due to contamination from other cell fractions.

The scarcity of high energy nucleoside polyphosphates in microsomes might have been predicted, since microsomes are known to be lacking in the factors associated with the oxidative phosphorylation system (Hogeboom and Schneider, 1955). The work of Siekevitz (1952) showed that incorporation of labelled alanine into liver protein occurred in homogenates of that tissue and that the incorporation was greater into microsomal protein than into any other cell fraction. Siekevitz also demonstrated that the incorporation did not occur when the microsomes were incubated alone but that it took place when the microsomes were incubated along with the mitochondria. His work suggests that the microsomes possess the enzyme system which is responsible for the incorporation of the ^{14}C-alanine, but lack the energy-generating systems required to carry out the incorporation reaction so that they must derive the necessary energy, possibly in the form of ATP, (which is apparently scarce in microsomes as shown in Charts 11 and 12) from the oxidative phosphorylation systems of the mitochondria. Keller and Zamecnik (1955) found that there was also a requirement for GTP in the enzymically catalysed

incorporation of labelled amino-acid into the microsome protein fraction of a purified liver preparation.

4.5d. The Acid-soluble Nucleotides of Rabbit Liver Cell Sap.

Little importance can be attached to the soluble nucleotide pattern (Chart 13) found for this cell fraction as it probably contains appreciable amounts of nucleotide material which was initially located in the particulate fractions of the cell *in vivo*. This is strongly suggested by the appearance (Chart 13) of a fairly complete free nucleotide pattern showing the presence of massive amounts of the low energy phosphate forms, particularly of AMP. Nevertheless, in view of the evidence presented in Sections 4.5a and 4.5b that some nucleotide material was not extracted from nuclei and mitochondria, and in view of the fact that a large amount of inosine, a very soluble substance, was obtained in the microsome fraction, it must be assumed that some of the nucleotide material shown on Chart 13 had its origin in the cell sap as it exists *in vivo*.

Sidelevitz and Potter (1955b) arrived at the conclusion that although mitochondria could absorb added AMP or ADP, they could not accumulate either of these

nucleotides or retain the ATP which they synthesised. No doubt the mitochondria function as ATP-manufacturing centres and distribute the ATP to extra mitochondrial sites including cell sap for synthetic and other purposes.

4.6 Ninhydrin-positive Components of the Acid-soluble Fraction of Rabbit Liver.

The observation that certain fractions eluted before AMP on ion exchange chromatograms of the acid-soluble fraction of rabbit liver gave a positive reaction with ninhydrin was interesting in view of the work of Park (1950; 1952) who isolated and characterised three compounds accumulated by Staphylococcus aureus grown in a medium containing penicillin. Each compound contained UDP linked to an N-acetyl amino-sugar acid. In addition, one of the compounds was found to contain alanine and another a peptide of D-glutamic acid, L-lysine and three alanine residues. Cantoni (1953) reported that an intermediate formed in the "activation" of methionine by ATP and a liver enzyme was a sulphonium compound composed of the 5'-deoxyadenosine moiety attached to the sulphur of methionine. There have been recent reports that amino-substituted acyl-AMP compounds (e.g. L-leucyl-AMP) may be possible intermediates in protein synthesis (De Moss, Genuth and Novelli, 1956; Hoagland, Keller and Zamecnik, 1956). Hansen and Hageman (1956) identified ADP-glutamic acid and ADP-aspartic acid in extracts of liver or lactating mammary tissue. In the light of these reports it was considered desirable to ascertain the nature of the ninhydrin-positive compounds eluted from

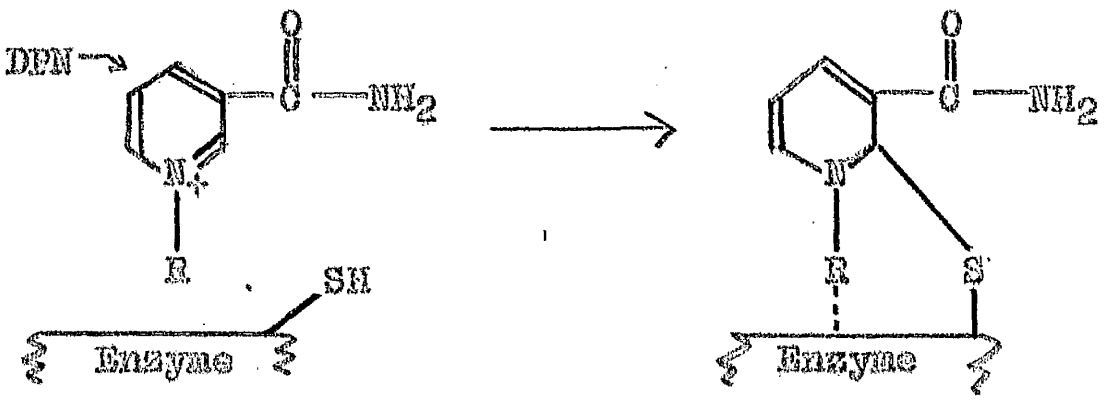
Dowex-1-formate columns with 1N formic acid during ion exchange chromatography of rabbit liver acid extracts.

It was clear from the work described in Section 3.3 that the ninhydrin-positive constituents of the acid-soluble fraction of rabbit liver, which were retained by Dowex-1 and eluted with 1N formic acid, were not combined in any way with ultraviolet-absorbing compounds such as those mentioned in the previous paragraph. The ninhydrin-positive compounds identified were aspartic acid, glutamic acid, glycine and glutathione (Chart 14). The existence of glutathione in animal tissues has been known for many years. Hopkins(1921) isolated it from yeast and from mammalian liver and muscle tissue and more recently Tallan, Moore and Stein (1954) found it together with aspartic acid, glutamic acid glycine and a variety of other amino acids and peptides in acid extracts of several cat tissues including liver.

However, in the present experiment the surprising factor was the ultraviolet-absorbing property associated with the glutathione (the N.U.V. material - Section 3.3) as found on ionophoretograms and paper chromatograms. Pure glutathione did not absorb ultraviolet light (Figure 13, Chart A) while the N.U.V. material and glutathione

treated with $HClO_4$ in the same way as the excised liver tissue both exhibited an absorption maximum at approximately 265 mμ. Anslow and Lyman (1941) reported that glutathione has a weak double absorption band at 280 mμ in water but no such absorption spectrum was found by the present author for pure glutathione (Figure 13, Chart A).

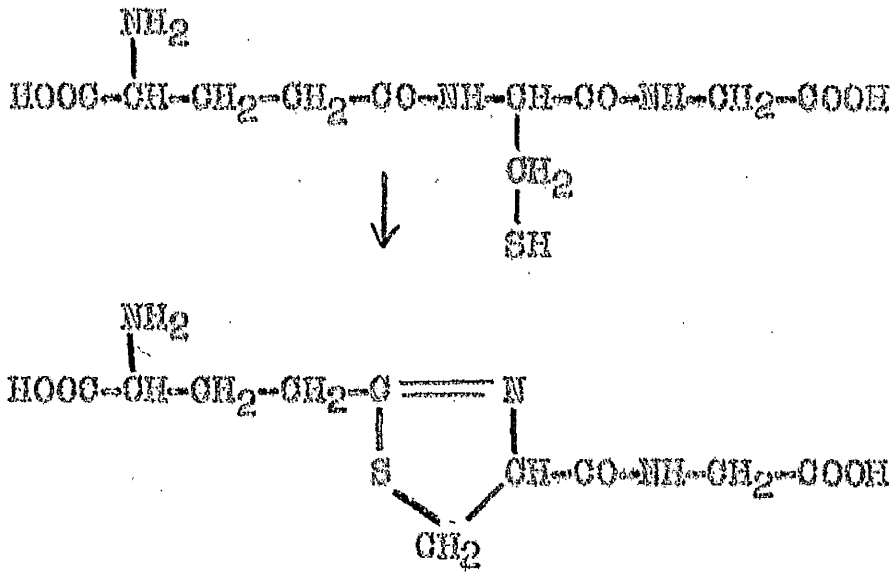
In the early stages of the investigation several possibilities which could account for the ultraviolet-absorption of the N.U.V. material were considered. One of these stemmed from the interesting observation made by Velick, Hayes and Harting (1953) that glyceraldehyde-3-phosphate dehydrogenase isolated from rabbit skeletal muscle is combined with DPN. The recrystallised enzyme-DPN complex contained two molecules of DPN per molecule of protein. Velick et al. also found that the enzyme contained -SH groups and was inactivated by p-chloro-mercuribenzoate and reactivated by cysteine. Racker (1954) suggested that the nicotinamide moiety of DPN was bound to a glutathione residue of the enzyme protein as shown below:



Since both DPN and glutathione are eluted from Dowex-1-formate at the beginning of the ion exchange chromatogram (Chart 14) the possibility was considered that the peak of glutathione might contain some glutathione which had become detached from glyceraldehyde-3-phosphate dehydrogenase and which was still bound to DPN. Further examination, however, revealed that this was unlikely, as Chart 14 showed that there was no ultraviolet-absorbing peak coincident with the glutathione peak (peak IV, Chart 14) and that the DPN peak was not associated with glutathione. Moreover, the N.U.V. material isolated from peak IV, Chart 14, by ionophoresis and paper chromatography was unable to act as coenzyme for alcohol dehydrogenase and did not exhibit a pyridine nucleotide spectrum. Acid hydrolysis of the N.U.V. material did not yield either adenine or nicotinamide as would have been expected had the material contained DPN. It was therefore concluded that the glutathione was free and not combined in any way with DPN.

Glutathione apparently exists in the tissues in the reduced form (Fujita and Numata, 1938) but can readily be oxidised to the corresponding disulphide; the latter is readily reduced again to glutathione. Even if the glutathione exists in the N.U.V. material in the oxidised state the ultraviolet absorption would not be

accounted for, but it seems reasonable to postulate that under the influence of the various chemical manipulations involved (all of which were in acid conditions) ring closure took place with the formation of a thiazoline-type structure



which would absorb ultraviolet light. Glutathione is known to possess a spectrum with an absorption maximum at 268 mμ in 12N HCl (Calvin, 1954) similar to that of a thiazolinium ion. Calvin also assumed ring formation in 0.01N HCl, but this has been very recently questioned by Préaux and Lontie (1957) who found that 2-methylthiazoline is hydrolysed in dilute acid solution with liberation of a sulphydryl group, but not in concentrated acid solution, e.g. 6-18N HCl and H_2SO_4 (as shown by the constancy of the extinction at 260 mμ). With glutathione, Préaux and Lontie (1957)

found that the extinction band at 265 mμ developed appreciably only when the glutathione was treated with HCl or H_2SO_4 in concentrations higher than 2N. The extinction increased with increase in acid concentration and remained constant only for the higher concentrations, i.e. 16N, 20N and 26N H_2SO_4. It seems justifiable to conclude that in the present investigation ring closure took place in the glutathione with production of an ultraviolet absorption band at about 268 mμ (Figure 13, Charts B and C) and that after treatment of the glutathione with 12N $HClO_4$ for 60 minutes at 100° ring closure was further encouraged with liberation of a product showing an absorption spectrum with maximum at about 268 mμ (Figure 13, Chart F).

SUMMARY.

1. The acid-soluble nucleotide compositions of certain rabbit tissues and of the corresponding cell nuclei have been studied with the aid of ion exchange chromatography on columns of Dowex-1-formate using an extended gradient elution system.

2. Cell nuclei prepared in non-aqueous media were found to be eminently suitable for studies on the soluble nucleotides of the cell nucleus as they retained the bulk of their acid-soluble nucleotides during the isolation procedure.

3. Whole appendix, whole intestinal mucosa and the corresponding cell nuclei (prepared in non-aqueous media) were found to have qualitatively and quantitatively similar soluble nucleotide patterns. It therefore seems probable that there is a uniform distribution of nucleotides between the cytoplasm and the nucleus of the cell.

4. The acid extract of each tissue and of the non-aqueous-type nuclei contained the mono-, di- and tri-phosphates of adenosine, guanosine, uridine and cytidine together with inosine-5'-phosphate, DPN, TPN and several derivatives of uridine-5'-diphosphate. No thymine was

detected either combined or in the free state, in any of the extracts examined.

5. The adenosine-5'-phosphates were by far the most abundant, comprising more than 50% of the soluble nucleotide fraction. The cytosine nucleotides were present only in very small amounts.

6. The presence of other nucleotides was also detected and their possible identities are discussed.

7. Evidence is presented which strongly suggests that soluble nucleotides exist *in vivo* chiefly as nucleoside-5'-triphosphates.

8. Fractionation of the acid extracts from several animals which had received ^{14}C-formate two hours before killing, revealed that the specific activities of the purine nucleotide bases were of the same order of magnitude whether the nucleotides were obtained from whole appendix, whole mucosa or from the nuclei of either of these tissues.

9. When the ^{14}C-formate incorporation time was reduced to 15 minutes, determination of the specific activities of the purine nucleotide bases from whole intestinal mucosa revealed that the isotope was incorporated more extensively into inosine-5'-phosphate than into any other nucleotide. The specific activity of the

hypoxanthine from inosine-5'-phosphate was twice that from each of the guanine moieties of the guanine nucleotides and four times that of the bases from the adenine nucleotides. The difference in specific activity between the guanine nucleotides and the adenine nucleotides was accounted for, at least in part, by the observation that the pool size of the latter nucleotide group was some five times as great as that of the former group. It was concluded that inosine-5'-phosphate occupies a key position in purine nucleotide synthesis in vivo.

10. The nuclear, mitochondrial, microsomal and cell sap fractions from rabbit liver homogenates were obtained by differential centrifugation in isotonic sucrose solutions, and the acid extract from each fraction was submitted to ion exchange chromatography on Dowex-1.

11. It was demonstrated that such nuclear preparations are not suitable for studies on the acid-soluble nucleotide composition of the nucleus, owing to serious losses of the nucleotides during the isolation procedure.

12. Mitochondria retained most of their soluble nucleotides which consisted predominantly of adenosine derivatives.

13. Inosine was the principal ultra-violet-absorbing compound retained by Dowex-1 during fractionation of

the acid extract from microsomes. Only small amounts of other ultra-violet-absorbing compounds were present.

14. The cell sap fraction contained large quantities of nucleotide material, the monophosphates predominating.

17. During ion exchange chromatography of the acid extract from whole rabbit liver, aspartic acid, glutamic acid, glycine and glutathione were eluted from Dowex-1. In the course of the ensuing analytical procedures, the glutathione gave rise to a substance which absorbed ultra-violet light. It was concluded that the ultra-violet absorption could be accounted for by the occurrence of ring closure in the glutathione molecule with the formation of a thiazoline-type compound.

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