Certain Aspects of Polynucleotide Biosynthesis

by

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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
CMP	cytidine-5'-monophosphate
CTP	cytidine-5'-triphosphate
UMP	uridine-5'-monophosphate
UTP	uridine-5'-triphosphate
GMP	guanosine-5'-monophosphate
GTP	guanosine-5'-triphosphate
TMP	thymidine-5'-monophosphate
TDP	thymidine-5'-diphosphate
TTP	thymidine-5'-triphosphate
dCMP	deoxycytidine-5'-monophosphate
dCDP	deoxycytidine-5'-diphosphate
dCTP	deoxycytidine-5'-triphosphate
dGMP	deoxyguanosine-5'-monophosphate
dGDP	deoxyguanosine-5'-diphosphate
dGTP	deoxyguanosine-5'-triphosphate

dAMP	deoxyadenosine-5'-monophosphate
dADP	deoxyadenosine-5'-diphosphate
dATP	deoxyadenosine-5'-triphosphate
TMP^{32}	thymidine-5'-monophosphate containing a radioactive phosphorus atom
CEP	2-cyanoethyl phosphate
CEP^{32}	2-cyanoethyl phosphate containing a radioactive phosphorus atom
DCC	dicyclohexylcarbodiimide
DCU	dicyclohexylurea
TDR	thymidine
$TDR-H^3$	thymidine labelled with tritium
$DNA/TDR-H^3$	deoxyribonucleic acid labelled with tritiated thymidine
PP_i	inorganic pyrophosphate
tris	tris-(hydroxymethyl)-aminomethane
EDTA	ethylenediamine tetra-acetic acid

Contents

General Introduction

While working in Tübingen in 1868, as a pupil of Hoppe-Seyler, Friedrich Miescher became interested in the problem of isolating the chemical components of cell nuclei. He chose pus cells as his source of material, and from them, by digestion with pepsin-hydrochloric acid, followed by shaking with ether, was able to isolate the nuclei as a readily separable layer. From these nuclei he isolated a hitherto unknown acidic compound of high phosphorus content, which he called "nuclein", and which is now known to be nucleoprotein. After repeating Miescher's work, Hoppe-Seyler published an account of the findings in his own journal in 1871. (Miescher, F. Med. Chem. Unters. 1871, 4, 463). At Basel, Miescher continued his work using salmon sperm, and succeeded in isolating a high molecular weight nuclein, and a basic protein which he called "protamine". This nuclein gave analytical figures which correspond to what is now known as nucleic acid.

Miescher's work was taken up by others, notably, Altmann, who developed methods for the isolation of protein-free nuclein from yeast and animal tissues, and coined the name "nucleic acid" (Altmann, R. Arch. Anat. Physiol. Lpz. 1889, 524); Piccard, who discovered the

purine bases in nucleic acids (Piccard, J. Ber.d.d. Chem. Ges. 1874, 7, 1714); and Kossel, who isolated xanthine (Kossel, A. Zeits. F. physiol. Chem. 1883, 8, 404) and adenine (Kossel, A. Zeits. F. physiol. Chem. 1888, 12, 241). Thymine and cytosine were isolated and identified by various groups in the years around the turn of the century. Also about this time Neumann described a variation of the Altmann procedure for the isolation of nucleic acids (Neumann, A. Arch. Anat. Physiol., Lpz. 1899, Supplement: 552) which involved heating the minced tissue in a 3% solution of sodium hydroxide. This method, which set the pattern for the next thirty years, proved to be a mixed blessing, for although it led to an acceleration of the study of the hydrolysis products of the nucleic acids by the classical methods of organic chemistry, it was responsible for the acceptance of a highly erroneous value for the molecular weight of the nucleic acids. Thus while both Miescher and Kossel appreciated the complexity and lability of the nucleic acids, later workers did not. This misapprehension together with the discovery of four nucleotides in approximately equimolar proportions in a yeast nucleic acid hydrolysate, gave birth to the concept that the nucleic acids were tetranucleotides, or statistical tetranucleotides.

In spite of this, however, the hydrolytic studies,

although performed on partially degraded nucleic acids, yielded much useful information. So much so that by 1930, definite evidence had emerged for the existence of two types of nucleic acid. One type from yeast which on hydrolysis gave adenine, guanine, cytosine, uracil, phosphoric acid and ribose: and another which yielded adenine, cytosine, guanine, thymine, phosphoric acid and deoxyribose. No sugars other than D-ribose and 2-deoxy-D-ribose have been isolated from nucleic acids. These two forms of nucleic acid came to be called ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) respectively, and for some time it was believed by many that RNA occurred only in plants and DNA only in animals. Soon, however, the ever increasing volume of evidence for the presence of RNA in animal tissues (Brachet, J. Arch. Biol, 1933, 44, 519; Caspersson, T. and Schultz, J. Nature, 1939, 143, 602; Davidson, J.N. and Waymouth, C. Nature, 1943, 152, 47) rendered this belief untenable. As "animal RNA" had only been found in embryonic tissues, at this time it was postulated that RNA was characteristic of rapidly proliferating tissues, but this theory in its turn had to be abandoned, when in the early forties RNA was isolated from various adult tissues (Davidson, J.N. and Waymouth, C. Biochem. J. 1944,a, 38, 375; b, 38, 379).

In 1928 Griffith (Griffith, F.J. Hyg., Camb. 1928,

27, 113) described a "transforming principle" isolated from encapsulated pneumococci which, when added to a culture of an unencapsulated pneumococcus, caused the encapsulated type to be produced. In 1944 Avery et al. (Avery, O.T., Macleod, C.M. and McCarty, M. J. exp. Med. 1944, 79, 137) showed that this "transforming principle" was a highly polymerised deoxypentose nucleic acid. The significance of this discovery was not immediately recognised, as ideas concerning the structure of the nucleic acids were still prejudiced in favour of the erroneous tetranucleotide theory. It did, however, encourage a return to milder methods of nucleic acid extraction, which in turn made a more accurate estimate of the molecular weight possible.

With the development of paper and ion-exchange chromatography more precise analysis of the chemical constituents of nucleic acids became possible, and the tetranucleotide theory was shown to be fallacious.

With continued investigation using these new analytical techniques it was soon recognised that RNA occurs in the cell sap, the microsomes and the nucleus and is involved in protein synthesis, whereas DNA occurs almost entirely in the nucleus, and forms the genetic material of the cell ('The Nucleic Acids', Vols. I-III, Ed. Chargaff, E., Davidson, J.N., Academic Press, New York, 1955-60). If DNA is to convey genetic information

it must have a code incorporated in its chemical structure. As DNA is a linear co-polymer of the four deoxyribonucleotides, TMP, dAMP, dCMP and dGMP in 3'-5' phospho-diester linkages, it is likely that the code comprises the sequential variations of these compounds along the polymer chain. This simple concept of DNA as a single-stranded polynucleotide does not fully explain the role of DNA in genetic transfer, because any structural model must provide for, (a) the coding of genetic information, (b) reduplication at each cell division, and (c) translation of information into protein structures.

In 1953 Watson and Crick described a model which goes a long way towards fulfilling these requirements (Watson, J.D. and Crick, F.H.C. Nature, 1953, 171, 737). This structure, which is now widely accepted as the basic structure of DNA, consists of two complementary polynucleotide chains, which are coiled around each other to form a double helix, and are bound to each other by specific hydrogen bonds, such that adenine-thymine and guanine-cytosine are the only permissable pairings. Thus at cell division the strands unwind and each strand acts as a template for the formation of a new helix (See Fig. 1).

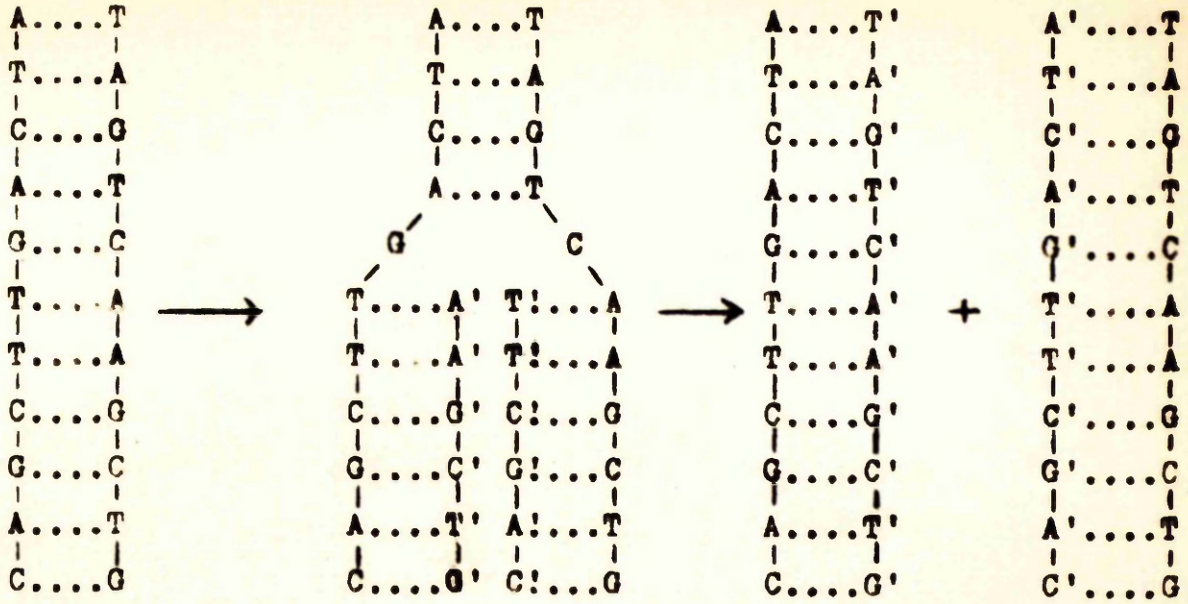
It is believed that the information contained in DNA is transferred to the nucleotide sequence of RNA, and that this secondary code controls the synthesis of protein.

Figure 1. The replication of DNA.

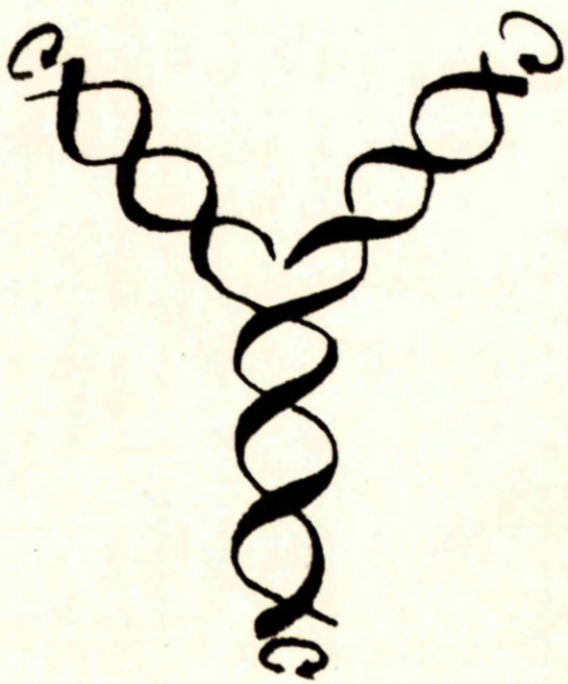
(a) shows how base pairing results in precise reduplication of the base sequence along the chains, and so perpetuation of the genetic code.

(b) attempts to give a more realistic picture of the simultaneous uncoiling and recoiling of the reduplicating DNA helices.

(From, Delbruck, M. and Stent, G.S. The Chemical Basis of Heredity. Ed. McElroy, W.D. and Glass, B., The John Hopkins Press, Baltimore, 1957, 704).



(a)



(b)

Crick has shown that it is possible, using only four nucleotides, to code the twenty amino acids found in normal proteins, if three successive bases on the chain are required to code each amino acid. This gives twenty - and only twenty - different and unequivocal codings (Crick, F.H.C. The Biological Replication of Macromolecules, Symp. Soc. Exptl. Biol., 12, 138)

The Watson and Crick model for DNA is supported by evidence from several fields of study. Hydrolytic studies have shown that the ratios of thymine to adenine, and guanine to cytosine, are unity as required by the theory. Investigations using X-ray crystallography (Jordan, D.O. The Chemistry of the Nucleic Acids, Butterworths, London, 1960, 156) and ultracentrifugation (Meselson, M. and Stahl, F.W. Proc. Nat. Acad. Sci., U.S. 1958, 44, 67) also support the double helix structure. Further support is given by the work of Benzer (Benzer, S. Proc. Nat. Acad. Sci., U.S. 1955, 41, 344; The Chemical Basis of Heridity, Ed. McElroy, W.D. and Glass, B., The John Hopkins Press, Baltimore, 1957, 70) who determined the number of genetic units in a bacteriophage by the classical methods of genetics and compared this with the number of nucleotide pairs available in the DNA of the organism. His calculation was very approximate but it indicated that there were only a few nucleotide pairs per

genetic unit.

During the last few years the investigations have been taken a step further by the study of the enzymes involved in the biosynthesis of DNA. The impetus for this work was the discovery (Grunberg-Manago, M. and Ochoa, S. J. Amer. Chem. Soc. 1955, 77, 3165) of an enzyme capable of in vitro synthesis of polyribonucleotide. This enzyme "polynucleotide phosphorylase", was capable, in the presence of magnesium ions, of catalysing the polymerisation of ribonucleoside diphosphates to give a polynucleotide plus inorganic ortho-phosphate, thus,

$$n.\ XRPP \rightleftharpoons (XRP)_n + n.P$$

where, X is a purine or pyrimidine, R is a ribose and P is phosphate.

In the following year Kornberg reported a similar system for polydeoxyribonucleotide synthesis in E. coli (Kornberg, A., Lehman, I.R., Bessman, M.J., Simms, E.S., Biochim. Biophys. Acta 1956, 21, 197). In this case the substrates were found to be the deoxyribonucleoside triphosphates and the reaction may be written,

$$n.XdRPPP \rightleftharpoons (XdRP)_n + n.PP$$

where, dR is deoxyribose and PP is inorganic pyrophosphate.

Apart from the four triphosphates and magnesium, this system requires the presence of a small amount of DNA to "prime" the reaction. Net synthesis of up to twenty times the amount of primer added, has been obtained.

It has been observed by Bollum (Bollum, F.J. J. Biol. Chem. 1959, 234, 2733) and others that the priming ability of many DNA samples can be enhanced by heating to 100°C. This treatment is known to bring about the collapse of the double helix structure; it therefore seems likely that single-stranded DNA may be the true priming agent.

During 1957-58, several groups found this enzyme, commonly termed "DNA polymerase", in cell-free extracts of mammalian tissues. It was found in regenerating rat liver (Bollum, F.J., Potter, V.R. J. Biol. Chem. 1958, 233, 479; Montenarino, R. and Canellakis, E.S. J. Biol. Chem. 1959, 234, 628) and also in Ehrlich ascites tumour of the mouse (Davidson, J.N., Smellie, R.M.S., Keir, H.M., McArdle, A.H. Nature, 1958, 182, 589).

Since that time Davidson and his co-workers have investigated the conditions required for *in vitro* DNA synthesis in this system by studying the uptake of labelled precursors. Thymidine triphosphate, and similar compounds, labelled with tritium, or radio-phosphorus, have been used (Smellie, R.M.S., Keir, H.M. & Davidson, J.N. Biochim. Biophys. Acta 1959, 35, 389; Keir, H.M. & Smellie, R.M.S. Biochim. Biophys. Acta 1959, 35, 405; Smellie, R.M.S., Grey, E.D., Keir, H.M., Richards, J. Bell, D. & Davidson, J.N. Biochim. Biophys. Acta 1960, 37, 243; Weissman, S.M. Smellie, R.M.S., Paul, J. Biochim. Biophys. Acta 1960, 45,

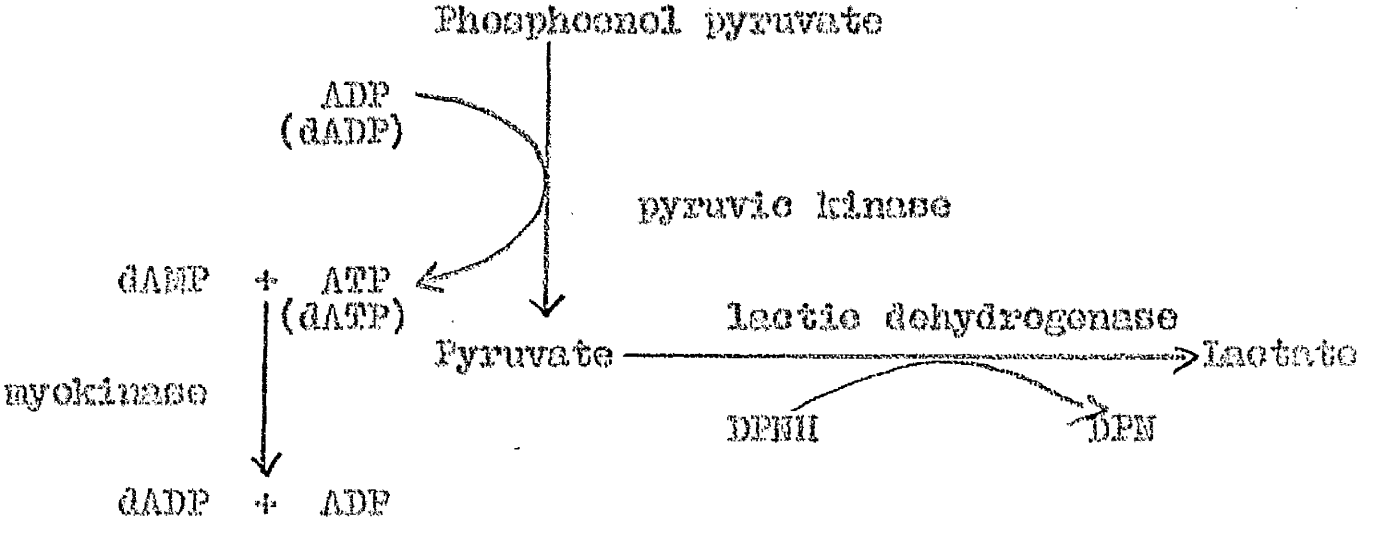
101; Gray, E.D., Weissman, S.M., Richards, J., Bell, D., Keir, H.M., Smollie, R.M.S. & Davidson, J.N. Biochim. Biophys. Acta 1960, 45, 111). The preparation of these precursors, by enzymic and chemical means is described in Parts I and II of this thesis. Part III describes a study of the reverse reaction of the polymerase, using both tritium labelled DNA and radioactive inorganic pyrophosphate.

Part I.

Enzymic Synthesis of Deoxyribonucleoside Triphosphates.

1. Introduction.

In 1954, during the course of an investigation into the metabolic role of deoxyribonucleotides, Sable et al, showed that purified muscle enzymes, in the presence of an energy donor, are capable of synthesising dADP and dATP from dAMP (Sable, H.Z., Wilber, P.B., Cohen, A.E. and Kano, M.R. Biochim. Biophys. Acta, 1954, 13, 150). The reaction may be written as follows



dATP may be formed by reversal of the myokinase step or by dADP acting as a phosphate acceptor in the pyruvic kinase reaction. The course of the overall reaction may be followed by measuring the conversion of DPNH to DPN by the lactic dehydrogenase reaction, as the pyruvate kinase

reaction is the rate determining step in the system.

When attempts were made to increase the amount of dAMP involved from 1μM to around 100μM the results were unsatisfactory, and experiments were therefore carried out using as a source of enzymes a mitochondrial system prepared from rabbit kidney cortex. The mitochondria were prepared by the method of Schneider (Schneider, W.C. J. Biol. Chem. 1948, 176, 259) and after washing were suspended in sucrose and fortified with appropriate amounts of ATP, DPN, cytochrome c, $MgCl_2$, NaF, and phosphate buffer. It was found that dAMP was almost as good a phosphate acceptor as AMP and by this method, it was possible to prepare up to 10μM of both dADP and dATP.

The observation that the di- and triphosphates of the ribonucleosides of cytosine, uracil, guanine and adenine occur (Schmitz, H., Hulbert, R.B. and Potter, V. R. J. Biol. Chem. 1954, 209, 41) in the acid-soluble fraction of rat tissues, posed the question of whether the deoxyribonucleotides also occur at the higher levels of phosphorylation. Hecht et al. tested for the presence of the enzymes necessary for deoxyribonucleotide synthesis (Hecht, L.I., Potter, V.R. and Herbert, E. Biochim. Biophys. Acta 1954, 15, 134) and found that the cytoplasmic fraction of regenerating rat liver homogenates, is capable of phosphorylating dCMP and TMP. They were able to isolate small amounts of dCTP and TTP, in

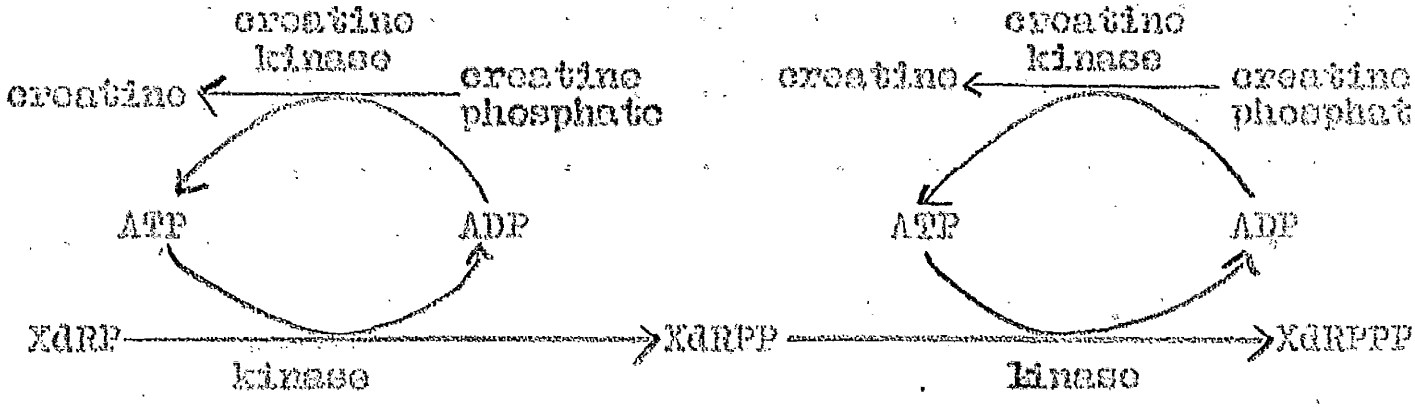
spite of the fact that the TTP reaction was inhibited at TMP concentrations above 800mμM/ml. R.L. Potter later demonstrated the presence of the pyrimidine deoxyribonucleotides in calf thymus extracts (Potter, R.L., Schlesinger, S.J. Amer. Chem. Soc. 1955, 77, 6714; Potter, R.L., Schlesinger, S., Buettner-Janusch, V. & Thompson, L. J. Biol. Chem. 1957, 226, 381).

In their paper on the enzymic synthesis of DNA, Kornberg and his collaborators (Kornberg et al. 1956) described the phosphorylation of thymidine to thymidine triphosphate by extracts of E. coli. At this time the phosphorylations were performed on a very small scale, and were designed to produce small amounts of labelled triphosphates for use in the DNA polymerase assay.

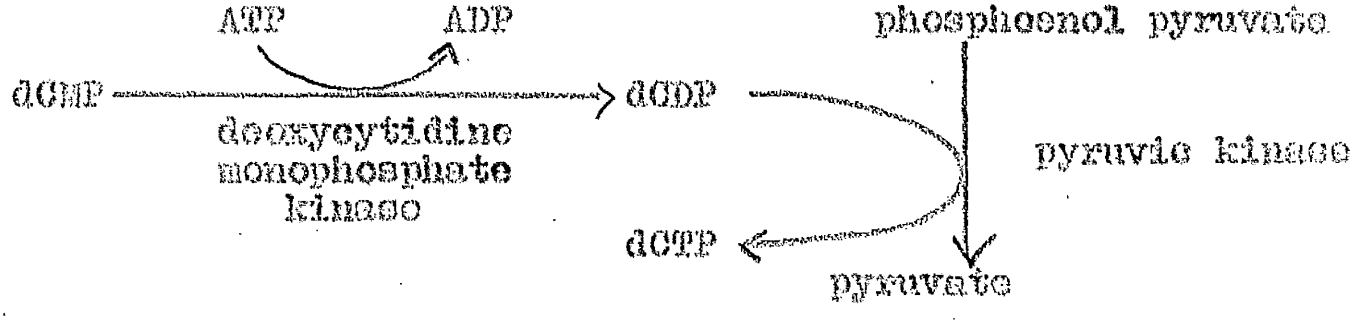
Klonow and Lichtler (Klonow, H., Lichtler, E. Biochim. Biophys. Acta 1957, 23, 6) demonstrated that aqueous extracts of red bone marrow and muscle contain enzymes which catalyse the phosphorylation of dAMP and dGMP and were able to isolate small quantities of dATP and dGTP.

Canellakis and Mantsavinos, using the high speed supernatant fluid from regenerating rat liver homogenate, achieved the synthesis of the triphosphates of thymidine, deoxycytidine, deoxyadenosine, deoxyguanosine, but once again, only on a very small scale (Canellakis, E.S.,

Mantsavinos, R. Biochim. Biophys. Acta 1958, 27, 643). An ATP generating system was used and the reaction may be summarised as follows



In the same year Maley and Ochoa described a method for the preparation of dCTP on a moderately large scale. This involved the use of a highly purified dCMP kinase from Azotobacter vinelandii and pyruvic kinase and quantities of dCTP of the order of 50 μmole were obtained. The reaction may be written as follows



The first system which could conveniently be used

The first system which could conveniently be used for the enzymic synthesis of all four deoxyribonucleosidesd by Kornberg and his co-workers in 1958. Using a partially purified enzyme preparation from E. coli they were able

to obtain quantities of the order of 50 μM of each triphosphate (Lehman, I.R., Bessman, M.J., Simms, E.S. and Kornberg, A. J. Biol. Chem. 1958, 233, 163).

At that time, similar experiments were in progress in this laboratory. In these the supernatant fraction obtained from osmotically disrupted Ehrlich ascites tumour cells, by centrifugation at 105,000 g. was used as enzyme source. These experiments form the subject matter of this part of the thesis.

Since the completion of this work in 1959, a paper has been published by Canellakis et al., describing an enzymic method of deoxyribonucleoside triphosphate synthesis, using enzyme fractions from E. coli and brewers' yeast (Canellakis, E.S., Gottesman, M.E. and Kammen, H. O. Biochim. Biophys. Acta 1960, 39, 82). The specificity and activity of the enzyme systems employed are wide enough to permit synthesis of both ribo- and deoxyribonucleoside triphosphates in relatively large amounts.

2. Methods.

2.1 Enzyme Source.

Ehrlich ascites carcinoma was used throughout. It was maintained by serial transplantation in mice of the departmental colony.

2.2 Preparation of the Tumour Extract.

Seven days after the inoculation of the tumour, the mice were killed by cervical dislocation under ether anaesthesia. Immediately after death a small incision was made in the abdomen, the tumour suspension drawn off with a Pasteur pipette, and collected at 0°C. These operations were carried out under aseptic conditions.

The pooled fluid from up to 24 mice was centrifuged at low speed (200 - 300 x g.) for 5 mins. to separate the cells from the plasma. The sediment was resuspended in 5 - 10 vols. of 0.1 M phosphate buffer, pH 8.1 and again centrifuged at 200 - 300 x g. for 5 mins. to separate the tumour cells from erythrocytes. This washing procedure was repeated several times until the sediment of tumour cells was free from contamination with erythrocytes. The cells were then resuspended and centrifuged at 600 x g. to pack them tightly into the centrifuge tube. 10 - 12 vol. ice-cold distilled water were added and the suspension gently homogenised in a Potter-type homogeniser. Three or four passes of the material were sufficient for adequate disruption. Micro-

scopic examination with the aid of crystal violet was used to control this process which ruptured most of the cells without destroying many nuclei. The resulting suspension was centrifuged in a Model L Spinco centrifuge at 105,000 x g. to yield a clear extract.

2.2c Dialysis of the extract.

In the early small scale experiments the extract was dialysed to remove intrinsic nucleotides, thereby facilitating the identification of deoxyribonucleoside polyphosphates on chromatograms of the deproteinised incubation mixtures.

The extract was dialysed against 4 x 1 litre changes of either 0.1M phosphate buffer pH 8.1, or 0.05M tris buffer pH 8.0. The resulting preparation contained 2 - 2.5 mg. protein/ml and was used as enzyme source in the subsequent incubations.

2.3 Storing the Tumour Extract.

The tumour extract retains its activity for about two weeks, if stored continuously at -10°C, but repeated thawing and freezing cause a decline in activity. A convenient method of storing the extract for many months is to freeze-dry it and keep the resulting powder at -10°C. An active extract can then be obtained by redissolving the powder in ice-cold 0.05M KCl solution (500 mg. powder in 25 ml solution). This preparation is less active than

the original extract but by increasing the protein concentration in the incubation mixture, satisfactory results can be obtained.

2.4 Estimation of Protein.

Protein estimation was carried out initially by the method of Gornall et al (Gornall, A.G., Bardawill, C.J. and David, M.M. J. Biol. Chem. 1949, 177, 751) but as this method consistently gave high blanks in the presence of 2-amino-2-hydroxymethylpropane-1:3 diol (tris) buffer, the method of Lowry et al (Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. J. Biol. Chem. 1951, 193, 265) was later adopted.

2.4a Gornall Method.

Reagents.

Biuret reagent: 1.5g. copper sulphate ($CuSO_4.5H_2O$) and 6.0g. potassium tartrate ($NaKO_4H_4O_6.4H_2O$) are dissolved in about 500 ml. water and 300 ml. 10% sodium hydroxide solution (carbonate free) added, with constant swirling. The volume is made up to 1 litre with water. The reagent must be discarded if a black or red precipitate appears.

Sodium chloride: 0.85% solution.

Method.

To a sample of up to 0.5 ml., containing 0.5 mg. to 3 mg. of protein, 0.85% sodium chloride to bring the volume to 0.5 ml., and 2 ml. of the biuret reagent, are added. The solution is thoroughly mixed, allowed to stand at

room temperature for 30 minutes and the absorption at 540 mμ determined. A reagent blank is made by replacing the protein solution with 0.85% sodium chloride solution. The standard used was bovine serum albumin (Armour Pharmaceuticals, Eastbourne).

2.4b Lowry Method.

Reagent A: 2% sodium carbonate in 0.10N sodium hydroxide. Reagent B: 0.5% copper sulphate in 1% sodium or potassium tartrate. Reagent C: alkaline copper solution. 50 ml. of Reagent A is mixed with 1 ml. of Reagent B. This solution should only be kept for 1 day. Reagent D: Carbonate-copper solution, is the same as Reagent C except for the omission of sodium hydroxide. Reagent E: diluted Folin-Ciocalteau phenol reagent (Folin, O., Ciocalteau, V. J. Biol. Chem. 1927, 73, 627; supplied by British Drug Houses, Ltd.). The Folin-Ciocalteau reagent is titrated with sodium hydroxide to a phenolphthalein end-point. On the basis of this titration the Folin reagent is diluted to make it 1N with respect to acid. Bovine serum albumin was again used as standard.

Method.

To a sample of 5 to 100 μg of protein in 0.2 ml., or less, of solution, in a 3 ml. to 10 ml. test-tube, is added 1 ml. of Reagent C. The solution is mixed well and

allowed to stand for 10 min., or longer, at room temperature. 0.10 ml. of Reagent E is added very rapidly and mixed within a few seconds. After 30 mins., or longer, the colour intensity of the sample is read in a spectrophotometer. For the range 5 to 25 µg. of protein per ml. of final volume, it is desirable to take readings at 750 mµ, the absorption maximum. For stronger solutions, the readings may be kept in a workable range by reading near 500 mµ.

2.5 Incubation Procedure.

The normal components of an incubation mixture for the preliminary experiments were: deoxyribonucleoside-5'-monophosphate, 5 µmoles; ATP, 15 µmoles; $MgCl_2$, 15 µmoles; phosphate buffer at pH 8.1, 200 µmoles, containing 10 mg. protein from the tumour extract, or tris buffer at pH 8.0, 100 µmoles, containing 10 mg. protein; total volume, 5.0 ml. The incubation was conducted at 37°C for 2 hrs. with gentle shaking in stoppered conical flasks. In later experiments the quantities used were increased 20 - 100 times. At the end of the incubation period, the flasks were cooled to 0°C and ice-cold 10 N $HClO_4$ was added to the reaction mixture slowly with stirring until the final concentration of $HClO_4$ was N. The precipitated protein was removed quickly by centrifugation at 0°C and the protein free supernatant fluid was rapidly neutralised to pH 7 with 5 N KOH, the insoluble $KClO_4$

being removed by centrifugation. In some experiments the reaction was stopped by heating at 100°C for 2 min. followed by rapid cooling to 0°C, but it was generally found that the small amounts of protein remaining in solution interfered with subsequent paper chromatography.

2.6 Paper Chromatography.

The deproteinised incubation mixture was applied to sheets of Whatman No. 1 chromatography paper, 0.05 - 0.1 ml. being applied as a small spot, and 1.0 - 1.5 ml. as a band 8 cm. long. Marker spots of AMP, ADP, ATP and the appropriate deoxynucleoside monophosphate were also applied. The spots and bands were dried in cold air, and the paper developed for 18 hours as a descending chromatogram in the solvent of Krebs and Hems (Krebs, H.A. and Hems, R. Biochim. Biophys. Acta, 1953, 12, 172) modified to give a pH of 4.6. The solvent was made up thus; isobutyric acid, 100 ml.; water, 55.8 ml.; 35% (w/w) ammonia solution, 4.2 ml.; 0.1 M ethylene diamine- tetra-acetic acid (EDTA), 1.6 ml. The appearance of such chromatograms in U.V. light is shown in Fig. 2, and the R_F values of some nucleotides are shown in Table 1.

In some cases, the paper was developed as a descending chromatogram in a variation of the borate / ammonium acetate / ethanol solvent of Flenner (Flenner, P. Acta Chem. Scand., 1955, 9, 197; Klenow, H. and Lichtler, E.,

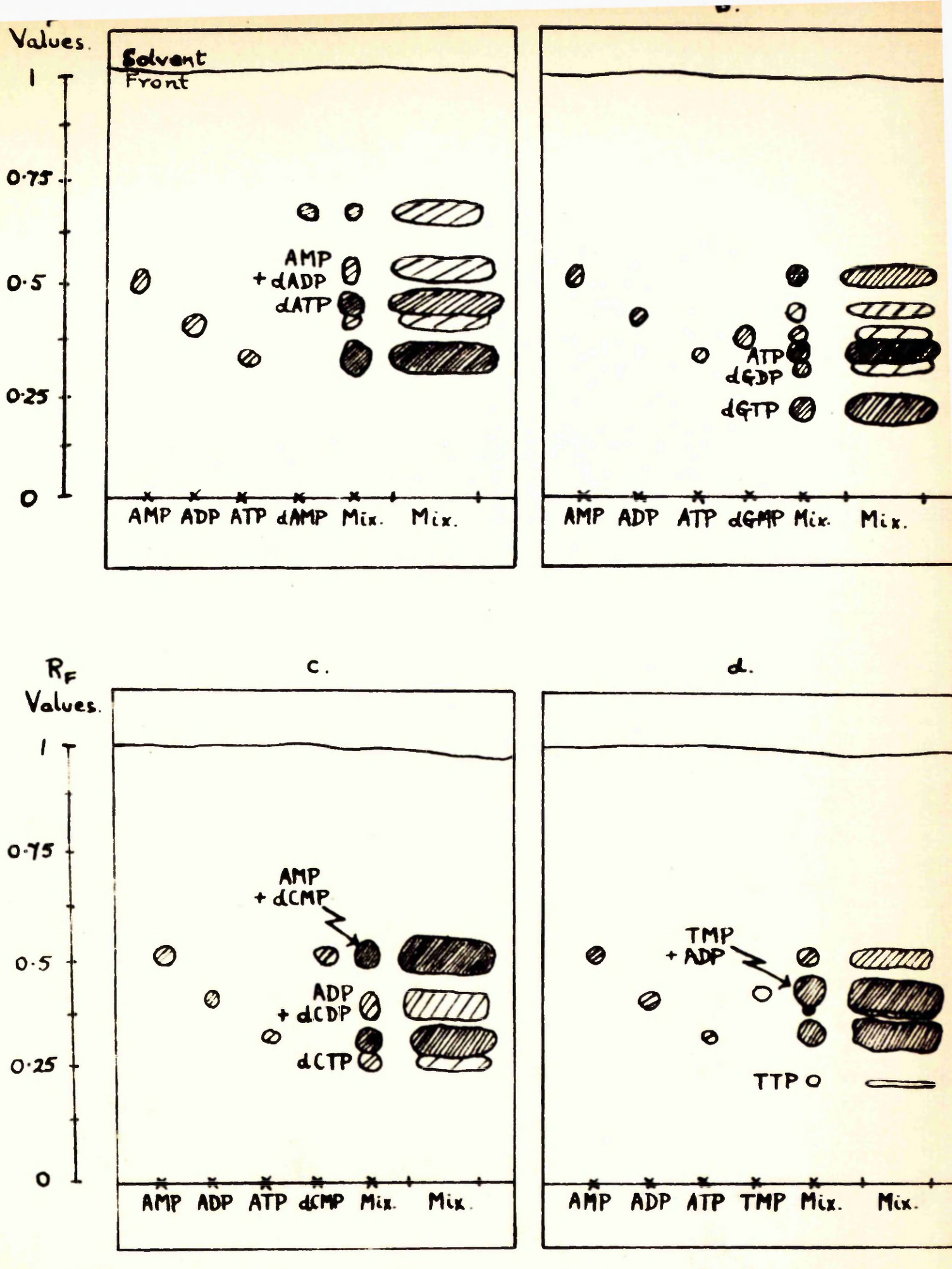
Figure 2.

The phosphorylation of deoxyribonucleoside monophosphates by extracts of Ehrlich ascites carcinoma.

Diagrams showing the location of U.V. absorbing spots obtained after paper chromatography of the deproteinised incubation mixtures, for (a) dAMP, (b) dGMP, (c) dCMP and (d) TMP.

The incubation mixtures contained: deoxyribonucleoside monophosphate (5 μmoles), ATP (15 μmoles), $MgCl_2$ (15 μmoles) phosphate buffer, pH 8.1 (200 μmoles), tumour extract (3 ml., 30 mg. of protein) in a total volume of 5 ml. The mixtures were incubated at 37° for 2 hrs. with shaking.

Solvents:- Isobutyric acid (300 ml.), water (55.8 ml.), 0.88 ammonia solution (4.2 ml.), 1 M EDTA (1.6 ml.).


Values.
1
0·75
0·5
0·25
0
Solvent Front
AMP
+ dADP
dATP
AMP ADP ATP dAMP Mix. Mix.
ATP
dGDP
dGTP
AMP ADP ATP dGMP Mix. Mix.
R_F Values.
c.
AMP
+ dCMP
ADP
+ dCDP
dCTP
AMP ADP ATP dCMP Mix. Mix.
d.
TMP
+ ADP
TTP
AMP ADP ATP TMP Mix. Mix.

1957). In this solvent ribose compounds form a highly polar complex with borate and move only very slightly from the origin. As the complex formation depends on the existence of cis - hydroxyl groupings, deoxyribose compounds do not form borate complexes. This solvent suffers from the disadvantage that development times of up to 60 hours are required for satisfactory deoxynucleotide separation. The composition of the solvent was: 6.5 parts ethanol to 3 parts of 1M ammonium acetate, which has been saturated with borate, adjusted to pH 9, and contains EDTA to a concentration of 10^{-2}M. It was found that this variation i.e. 6.5 parts, instead of 7 parts, of ethanol gave slightly better separations. The appearance of such chromatograms in U.V. light is shown in Fig. 3 and some relative distances moved for various nucleotides are shown in Table 2.

2.7 Detection of Deoxyribonucleotides on Paper Chromatograms.

After development the papers were dried in cold air, scanned in U.V. light and the positions of the absorbing areas marked, in pencil.

Three main dipping techniques, which enable the presence of ribose, or deoxyribose, or phosphate to be detected, were used (see Fig. 4).

a. The Detection of Ribose (Gordon, H.T., Thornburg, W. and Wozum, L.N. Anal. Chem. 1956, 28, 849).

Figure 3.

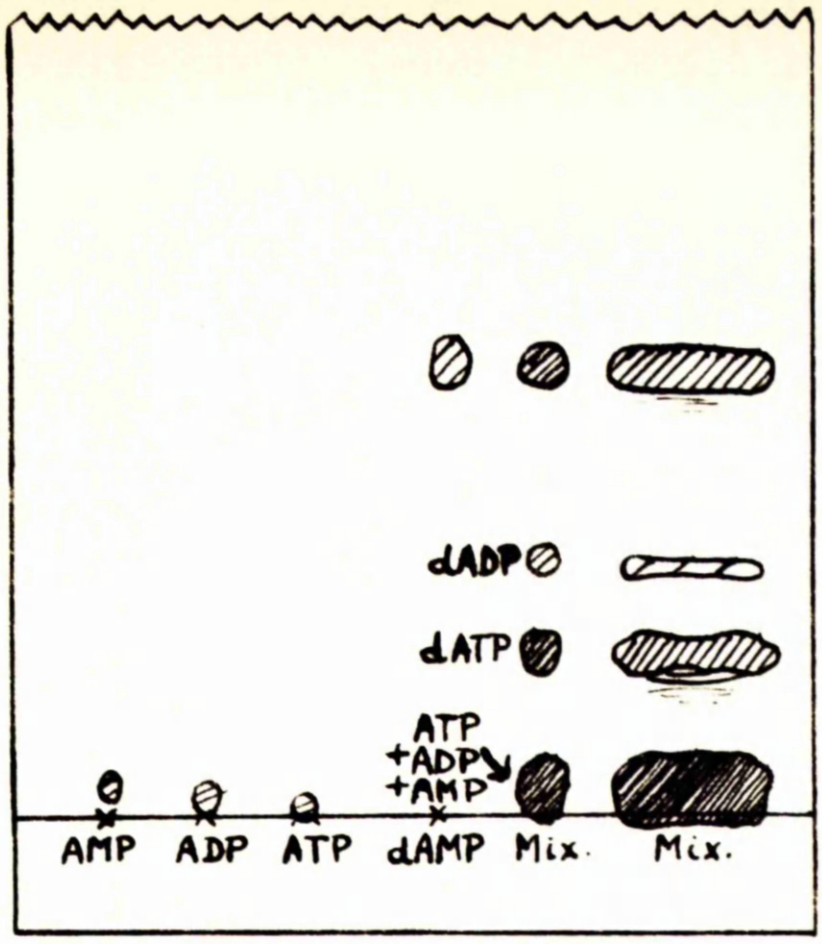
The phosphorylation of deoxyribonucleoside monophosphates by extracts of Ehrlich ascites carcinoma.

Diagrams showing the location of U.V. absorbing spots obtained after paper chromatography of the deproteinised incubation mixtures, for (a) dATP and (b) dGTP.

The incubation mixtures contained: deoxyribonucleoside monophosphate (5 μmoles), ATP (15 μmoles), $MgCl_2$ (15 μmoles) phosphate buffer, pH 8.2 (200 μmoles), tumour extract (3 ml., 10 mg. of protein) in a total volume of 5 ml. The mixtures were incubated at 37° for 2 hrs. with shaking.

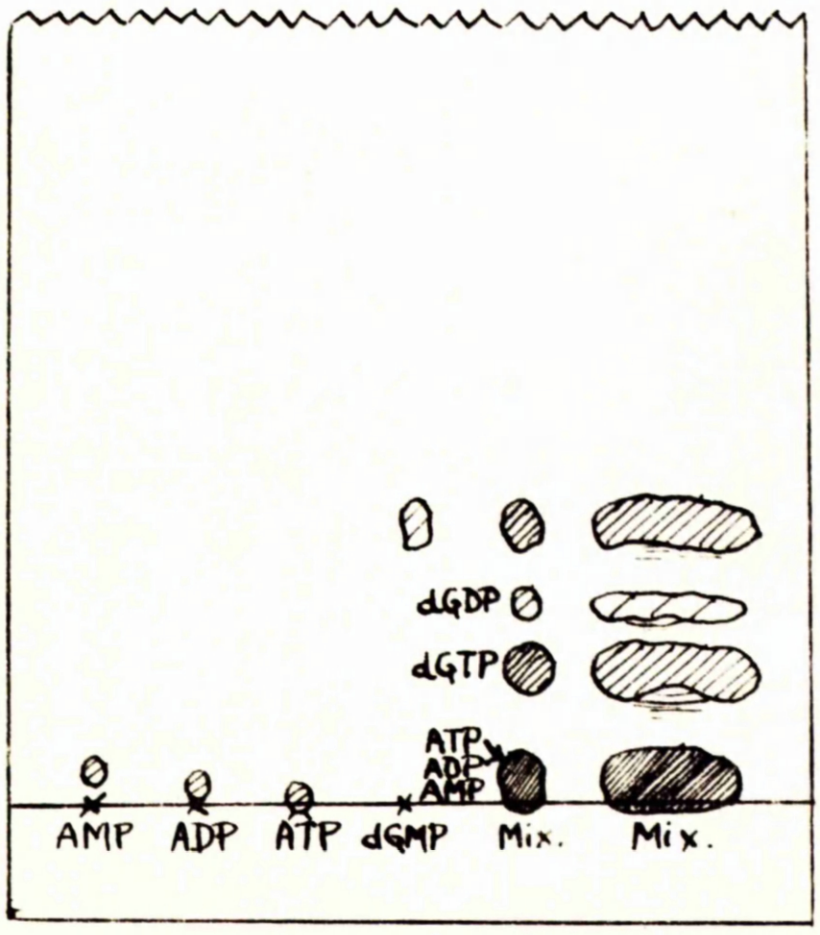
Solvents:- ethanol (135 ml.), 1 M ammonium acetate, saturated with borate, adjusted to pH 9 and containing EDTA at a concentration of 10^{-2} M (90 ml.),

a.



Direction of solvent flow.

b.



Direction of solvent flow.

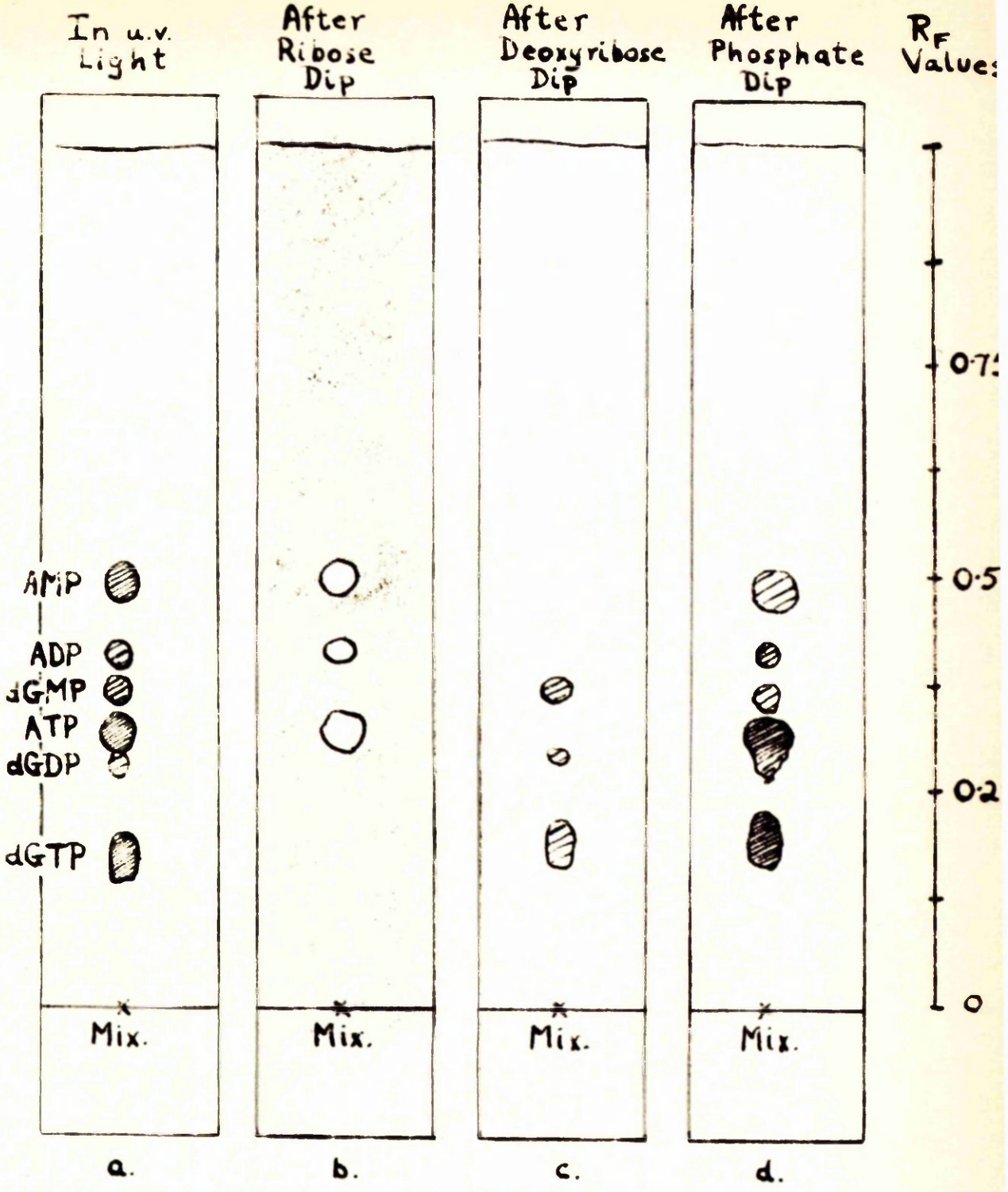
Figure 4.

The detection of deoxyribonucleotides on paper chromatograms, by use of diazine techniques.

Diagram showing a chromatogram obtained after chromatography of a deproteinised incubation mixture, as it appeared (a) in U.V. light (b) after ribose dip, (c) after deoxyribose dip and (d) after phosphate dip. (For details of the dips see section 2.7).

Incubation mixture contained, dGMP (5 μmoles), ATP (25 μmoles), $MgCl_2$ (35 μmoles), tris buffer, pH 8.0 (200 μmoles), tumour extract (3 ml., 9 mg. of protein) in a total volume of 5 ml. The mixture was incubated at 37° for 2 hrs. with shaking.

Solvent:- isobutyric acid (100 ml.), water (55.8 ml.), 0.88 ammonia solution (4.2 ml.), 1 M EDTA (1.6 ml.).


In u.v. Light
After Ribose Dip
After Deoxyribose Dip
After Phosphate Dip
R_F Values
AMP
ADP
dGMP
ATP
dGDP
dGTP
Mix.
Mix.
Mix.
Mix.
0·7
0·5
0·2
0
a.
b.
c.
d.

Reagents.

Reagent A: 228 mg. of sodium metaperiodate is dissolved in 10 ml. of water. 1 ml. of this solution is diluted with 19 ml. of acetone. This reagent is stable for 5 hr.

Reagent B: 184 mg. of benzidine is dissolved in a mixture of 0.6 ml. of glacial acetic acid, 4.4 ml. of water, and 95 ml. of acetone.

Method.

The chromatogram is dipped in Reagent A and dried for 3-4 min. It is then dipped in Reagent B and dried again. Spots containing a 2:3-diol structure show up as white areas on a blue ground.

2.7b The Detection of Deoxyribose (Dische, Z. in Thomson, J.N. Academic Press, New York, 1955; Buchanan, J.G. Nature, 1951, 168, 1091; Thomson, R.Y. in Chromatographic and Electrophoretic Techniques, Vol. 1. Ed. Smith, I. Heinemann, London, 1960).

Reagent.

10 ml. of cysteine hydrochloride (0.5% in 3N sulphuric acid) plus 90 ml. of acetone.

Method.

The reagent is made up fresh, the chromatogram is dipped and dried in the cold. It is then heated for

5-10 min. in an oven at 85 deg. C. Compounds containing deoxyribose show up as pink spots.

2.7c The Detection of Phosphate (Burrows, S., Grylls, F.G.H. and Harrison, J.S. Nature 1952, 170, 800).

Reagent.

1 g. of ammonium molybdate is dissolved in 8 ml. of water and to this 3 ml. of concentrated hydrochloric acid, 3 ml. of 12N perchloric acid and 86 ml. of acetone, are added.

The reagent should be freshly prepared.

Method.

The chromatogram is dipped in the reagent and allowed to dry. It is then exposed to U.V. light for at least 30 min., after which time phosphate containing spots show up as blue areas on a white ground. Enhanced sensitivity can be achieved by a longer exposure to U.V. light and subsequent treatment with ammonia fumes. The long exposure to U.V.light causes the whole paper to turn blue, but the ammonia fumes remove all the colour not associated with phosphate.

2.82.3 Anion Exchange Chromatography.

The resin used was Analytical Grade, Dowex-1 (AG-1-X8), 200-400 mesh (later 100-200 mesh) obtained from Bio-Rad Laboratories, Richmond, California, in the chloride form. The capacity of this resin is 1.4 milli-equivalents per ml. of resin bed. In all the separations

described at least a five-fold excess of resin was used.

2.2. Preparation of the resin.

When using the 200-400 mesh resin the very fine particles were removed by sedimentation in distilled water. The 100-200 mesh resin was ready for use and was poured, as a thin slurry, into a standard chromatography column (Quickfit and Quartz Ltd., England). The resin was stirred to remove trapped air bubbles, and to ensure satisfactory packing of the resin. After packing, the surface of the resin was stirred gently and allowed to settle until it was level. The surface of the resin, which was always kept under a short column of liquid, was covered with a small plug of glass wool, to prevent the resin being disturbed during chromatography. The column was then washed with about one column volume of distilled water and the pH, and U.V. absorption at 260 mμ, checked. If these tests were satisfactory the column was ready for use. If the pH was low the column was washed with more water, or if the optical density was high, it was washed with 1M HCl till the optical density of the effluent approached zero, and then the HCl removed by washing with distilled water. This procedure was also used to re-generate resin which had been used before.

2.8b Chromatography of a deproteinised incubation mixture.

The deproteinised incubation mixture was diluted to an ion molarity of less than 0.01, and applied to the column under slight pressure. The column was washed with distilled water to remove any material retained, but not adsorbed by the column. The effluent was collected as one fraction and retained for analysis.

In each preparation the material which had been adsorbed by the column was eluted with two concentrations of lithium chloride in 0.01N HCl, chosen on the basis of earlier gradient elution experiments by Dr. Keir of this department and the information published by Kornberg et al. in 1958.

For the preparation of dGTP the eluents were.

1) 0.1M LiCl in 0.01N HCl, to displace everything but dGTP.

2) 0.2M LiCl in 0.01N HCl to elute the dGTP (see Fig. 5).

For dATP they were

1) 0.08M LiCl in 0.01N HCl to displace everything but ATP and dATP

2) 0.2M LiCl in 0.01N HCl to elute the ATP/dATP mixture (see Fig. 5).

This latter procedure was necessary because ATP and dATP cannot be separated satisfactorily on Dowex-1. The ATP was removed by the method of Lohman et al. (see section 2.9).

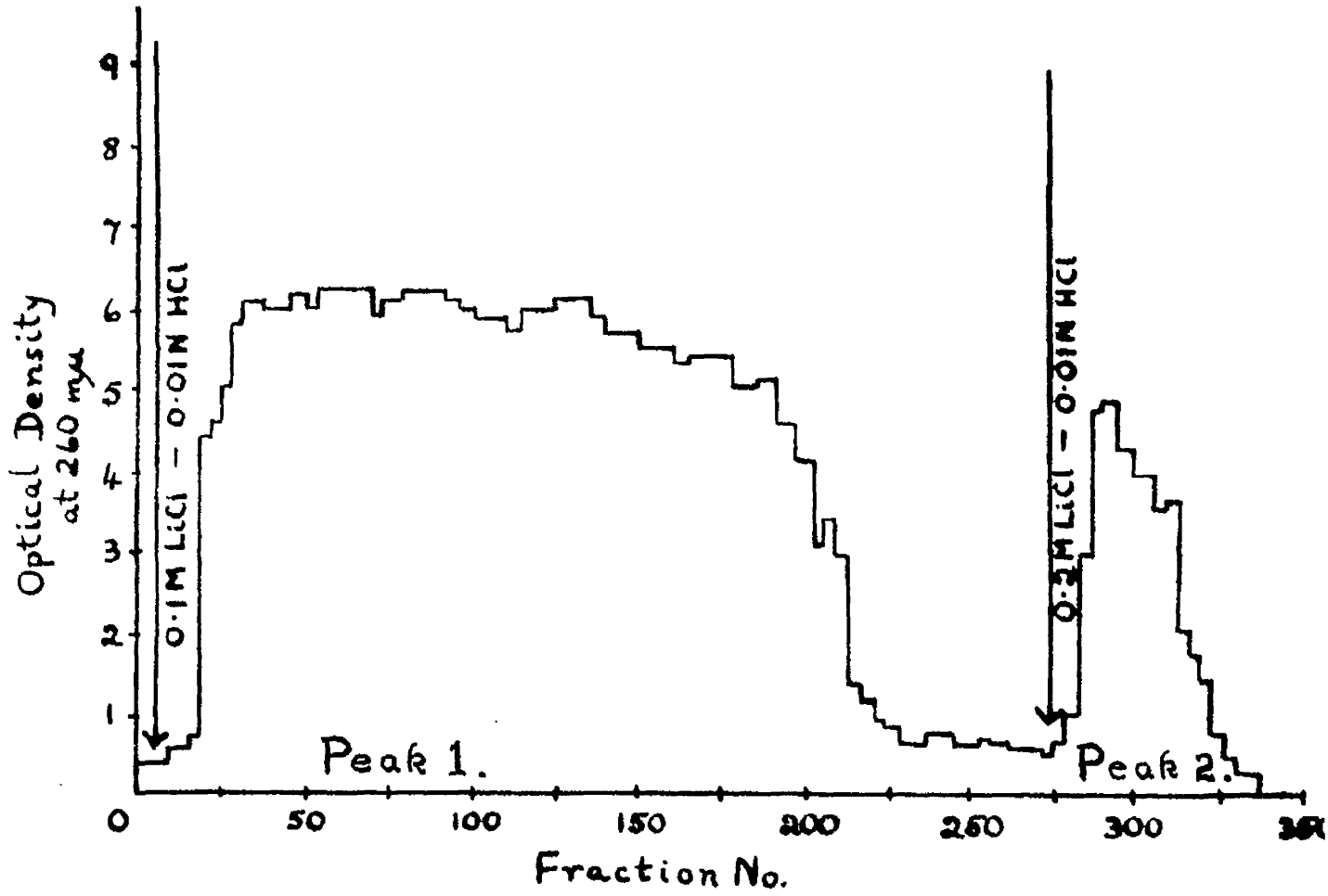
Figure 5.

(a) Elution chart from the ion-exchange chromatography of a deproteinised incubation mixture, containing AMP, ADP, ATP, dGMP, dGDP and dGTP. Pool 1 contained AMP, dGMP, ADP, dGDP and ATP. Pool 2 contained dGTP.

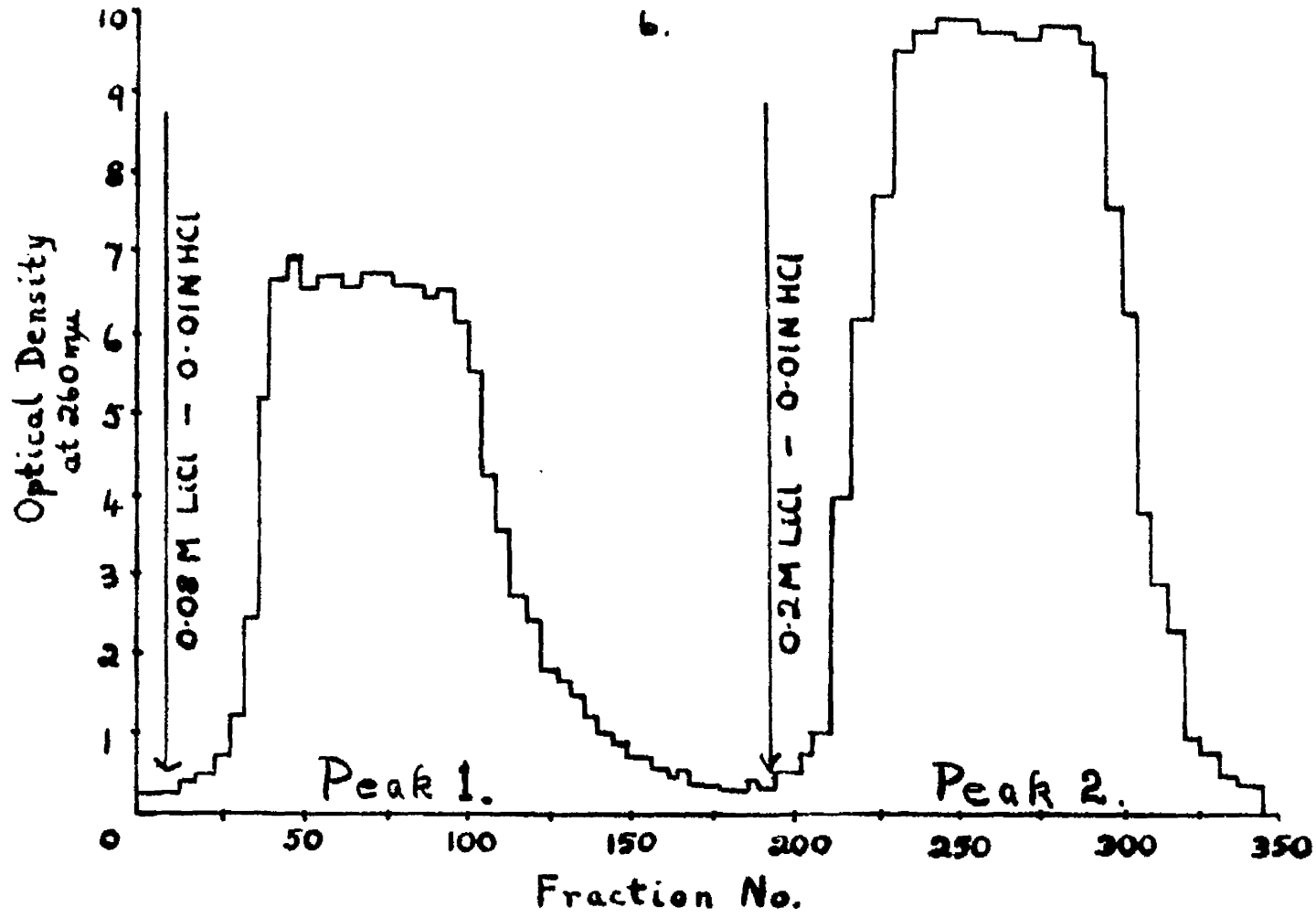
(b) Elution chart from the ion-exchange chromatography of a deproteinised incubation mixture, containing AMP, ADP, ATP, dAMP, dADP and dATP. Pool 1 contained AMP, dAMP, ADP and dADP. Pool 2 contained ATP and dATP.

Columns: Dowex-1-chloride, 35 cm x 2 cm.

Fractions: 20 ml. per tube.


Optical Density at 260 mμ
0·1M LiCl – 0·01N HCl
0·2M LiCl – 0·01N HCl
Peak 1.
Peak 2.
Fraction No.

b.


Optical Density at 260 mμ
0·08M LiCl – 0·01N HCl
0·2M LiCl – 0·01N HCl
Peak 1.
Peak 2.
Fraction No.

The effluent from the column was collected in fractions (usually 20 ml. in volume) using a Locarte automatic fraction collector.

2.8c Analysis of column elution.

The optical density of each fraction, obtained from the column was read at 250 mμ, 260 mμ and 280 mμ in a Beckman Model DU spectrophotometer, using water in the blank cell. As the ratios of the optical densities at 250 mμ/260 mμ and 280 mμ/260 mμ are characteristic of the various nucleosides, the U.V. absorbing component of a fraction could often be identified. The optical density at 260 mμ was plotted against fraction number to give an elution chart for each separation. Typical charts are shown in Fig. 5.

From these elution charts the appropriate fractions were pooled so that each pool contained only one discernable peak. These pools were neutralised with lithium hydroxide, dried from the frozen state and then redissolved in a small amount of ice-cold water. To each of these solutions an equal volume of methanol was added and then ten times the combined volume of acetone. The resulting mixture was allowed to stand at 0° for about 2 hr., during which time the nucleotide material precipitated, and the lithium chloride remained in solution. These nucleotide precipitates were removed by centrifugation, dried and redissolved in small amounts of water. A

sample was taken from each pool concentrate, for chromatography in the modified Krebs and Hems iso-butyrate solvent, together with dAMP, AMP, ADP and ATP marker spots. The chromatogram was developed and scanned as before (see sections 2.6 and 2.7), and the pool containing the required triphosphate selected for cation exchange chromatography (see section 2.10).

2.9 Removal of ATP from ATP/dATP mixture. (Whitfield, P.R. Biochem. J. 1954, 58, 390; Lehman, I.R. et al. 1958).

Lehman et al. have shown that ATP can be selectively destroyed in the presence of deoxyribonucleotides by treatment with periodate followed by alkali, as previously described by Whitfield for oligonucleotides. This procedure was used to remove the ATP from the unresolved ATP/dATP mixture obtained after column chromatography on Dowex-1-Cl (see section 2.6).

0.1M Glycylglycine buffer (pH 7.4) was added to the mixture till it was 0.03M with respect to the buffer, and the resulting solution incubated with sodium periodate (5µm/µMATP; the ATP being estimated roughly from U.V. and chromatographic data) for 30 min. at 25°C.

The excess periodate was destroyed by incubation with glucose for a further 30 min. at 25°C, then the mixture was adjusted to pH 10 with glycine buffer (pH 10.2)

and incubated for 14 hr. at 37°C. It was then diluted about ten-fold with distilled water, applied to a Dowex-1-Cl column and subjected to stepwise elution as shown in Fig. 6.

The fractions containing the peaks were pooled, neutralised, lyophilised and freed of lithium chloride and chromatographed on paper as before (see section 2.8) and the pool containing the dATP selected.

2.10 Cation Exchange Chromatography.

The resin used was Dowex-50 (AG-50-X8), 200-400 mesh obtained from Bio-Rad Laboratories, in the hydrogen form. The resin was converted to the sodium form with 2N sodium hydroxide, then washed with water till it was neutral. The capacity of this resin is 1.7 milli-equivalents per ml. of resin bed.

The Lithium-dATP (or, dATP) obtained after anion exchange chromatography and removal of lithium chloride, was converted to the sodium form by passage through a column of Dowex-50-sodium. The column was washed with 1-2 column-volumes of water to ensure that none of the triphosphate was retained in the resin.

2.11 Final analysis of the product.

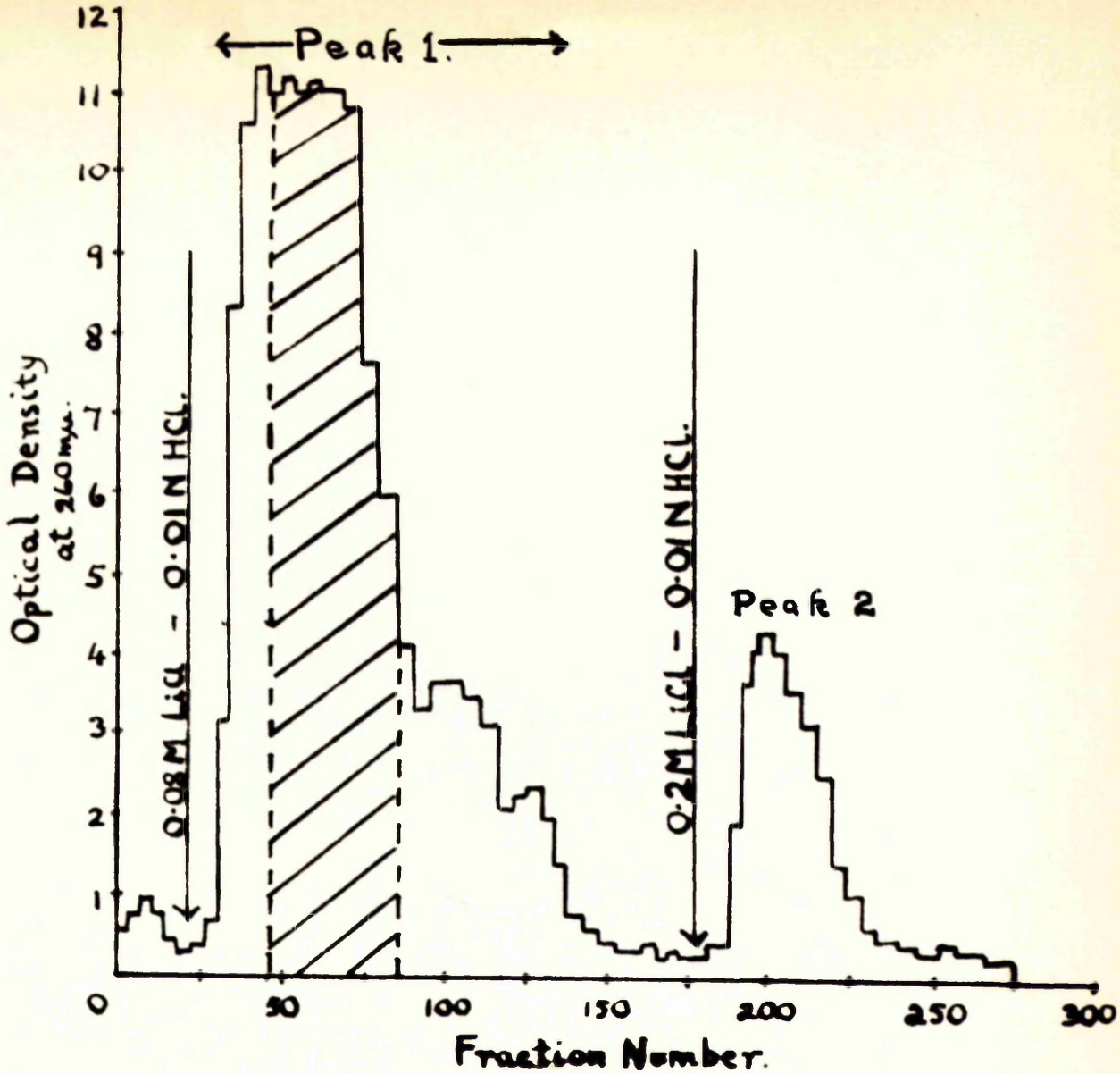
The triphosphate solution was spotted on paper as a series of spots of increasing deoxyribonucleotide content, (i.e. spots of 0.1 ml., 0.2 ml., 0.3 ml. etc.)

Figure 6.

Elution chart from the rechromatography of the ATP/ADP mixture obtained as shown in Figure 5.6 after periodate treatment (see section 2.9). Peak 1 contained ATP dehris, and Peak 2 contained ADP.

Column: Dowex-1-chloride, 35 cm. x 2 cm.

Fractions: 20 ml. per tube.


Peak 1
Peak 2
0·08M LiCl - 0·01N HCl.
0·2M LiCl - 0·01N HCl.
Optical Density at 260mμ.
Fraction Number.
0
50
100
150
200
250
300
1
2
3
4
5
6
7
8
9
10
11
12

and the chromatogram developed as before in the Krebs and Hems solvent. The resulting chromatogram was scanned in U.V. light and the absorbing spots marked. Those spots which showed the maximum amount of material but no signs of overloading or tailing, were cut out with matching blanks and eluted with water, by capillary flow. The base, deoxyribose and phosphorus contents of the eluates were determined. (Typical analyses are shown in Table 3.).

2.11a Estimation of Base. (Smellie, R.M.S., Thomson, R.Y. and Davidson, J.N. Biochem. Biophys. Acta 1958, 29, 59).

A sample of the solution to be analysed was freeze-dried into a Pyrex centrifuge tube and treated with 0.1 ml of 12 N $HClO_4$ at 100°C for 1 hr. The digest was then neutralised to about pH 8 with 7 N KOH, acidified to about pH 1 with 6 N HCl, and centrifuged. A portion of the hydrolysate was put on Whatman No. 1 paper for two-dimensional chromatography. The spots were applied 12 cm. from the edge on the long axis and 4 cm. from the edge on the short axis. The solvents employed were isopropanol/HCl (descending: isopropanol, 170 ml.; conc. HCl, 41 ml.; water to 250 ml.; Wyatt, G.R. Biochem. J. 1951, 48, 584) and butanol/NH_3 (ascending; n-butanol saturated with water at about 23°, 100 ml.; 15N ammonium hydroxide, 1 ml.; MacNutt, W.S. 1952). The chromatograms were run

in both directions so that the solvent reached about 10 cm. from the end of the paper. After the runs the spots were cut out after location by inspection in U.V. light. Pieces of No. 1 paper were stapled to either end of each spot and the composite strip thus obtained was run for 5 to 6 hr. (descending) in distilled water to remove contaminating material. Before the guanine spots were run in this way, they were moistened with 1.6N HCl, dried thoroughly in a current of warm air, exposed to NH_3 vapour for 2 min. and the excess NH_3 removed by storing in a dessicator for 1 to 2 hr. over sulphuric acid. The bases were once again located in U.V. light and cut out along with corresponding areas from blank papers. Elution was carried out by capillary flow, using 0.1N HCl for adenine and cytosine, and 1.6N HCl for guanine, 0.3 to 0.5 ml. of eluate being collected. 0.1 or 0.2 ml. was then diluted to 4 ml. with the appropriate concentration of acid for estimation in the Beckman Model DU spectrophotometer.

Adenine was read in 0.1N HCl at 263 mμ and the molar extinction coeff't. used = 13,000

Guanine was read in 1.6N HCl at 249 mμ and the molar extinction coeff't. used = 10,900

Cytosine was read in 0.1N HCl at 275 mμ and the molar extinction coeff't. used = 11,000

2.11b Estimation of Deoxyribose (Brody, S. Acta Chem. Scand. 1953, 7, 502).

For this adaptation of the method of Brody, calibration curves were constructed using pure samples of the deoxyribonucleoside monophosphates, the most satisfactory results in this estimation being obtained using 87.86% (w/v) sulphuric acid. To 0.2 ml. of the deoxyribonucleotide solution (maintained at a low temperature in a test-tube surrounded by a solid CO_2/alcohol mixture), 5 ml. of cold 87.86% sulphuric acid were added slowly, with vigorous shaking in the freezing mixture, to give a final concentration of 85.75% (w/v). The partially frozen contents of the reaction tubes were allowed to thaw slowly with continued shaking and were then incubated at 25 deg. for exactly 25 min., quickly cooled to 0 deg. and the optical densities read at 474 mμ.

When the amount of deoxyribose was not thus determined, the evidence that the nucleotide was of the deoxyribose type was based on the positive result of the cysteine/sulphuric acid dip technique (see section 2.7b), performed on a portion of the nucleotide run on a paper chromatogram.

2.11c Estimation of Phosphorus (Allen, R.J.L. Biochem. J. 1940, 34, 858).

For the purpose of this work the quantities used were

one fifth of those used by Allen. A calibration curve was constructed using standard solutions of inorganic orthophosphate.

To the nucleotide solution to be estimated, 10N sulphuric acid (0.24 ml.) was added, and the resulting solution heated in a sand-bath at about 130 deg. until the volume had dropped to around 0.1 ml. The solution was cooled and 2 drops of hydrogen peroxide (100 volume) added. The solution was then heated for a further 30 min. after which it was again cooled and water (5 ml.) added. Ammonium molybdate (0.2 ml. of an 8.3% solution) and then 2:4 diamino-phenol hydrochloride (Amidol: 0.4 ml. of a 1% (w/v) solution in 20% (w/v) sodium metabisulphite solution) were added, and the mixture allowed to stand for 10 min. at room temperature, before the optical density was read at 640 mμ. The readings were completed between 10 and 30 min. after the addition of the final reagent.

In most of the later experiments the final nature of the reaction products was confirmed by relating the phosphorus content of the deoxyribonucleotides to the deoxyribose content, as determined by U.V. absorption measurements. The molar extinction coefficients used in these calculations were, at pH 7.0,

deoxycytidine, 8900 (Beaven, G.H., Holiday, E.R., & Johnson, E.A. The Nucleic Acids, Vol. I. Ed. Chargaff, E. & Davidson, J.N., Academic Press, New York, 1955; MacNutt, W.S. Biochem. J. 1952, 50, 384).
deoxyguanosine, 13,600 (MacNutt, W.S. 1952).
deoxyadenosine, 15,800 (Gulland, J.M. & Story, L.F. J. Chem. Soc. 1938, 259). (Typical analyses are shown in Table 4).

2.12 Storage of Deoxyribonucleoside Triphosphates.

After the final analysis, the deoxyribonucleoside triphosphates were freeze-dried into phials, in 10 to 20 µM quantities and stored at -10°, or later at -75°. If stored dry at -75° the triphosphates showed little, or no, sign of breakdown after many months

3. Results and Discussion.

3.1 Preliminary small scale experiments.

Early experiments were carried out in conjunction with Dr. Keir of this department, to determine the ability of Ehrlich ascites tumour extracts to phosphorylate the deoxyribonucleoside monophosphates.

The monophosphates of thymidine, deoxycytidine, deoxyadenosine and deoxyguanosine were each incubated with ATP and $MgCl_2$, in the presence and absence of the dialysed high-speed ascites extract, in phosphate or tris buffer for 2 hours at 37°. The deproteinised (see section 2.5)

incubation mixture, with ATP, ADP, AMP and deoxyribonucleoside monophosphate markers was chromatographed on paper in the ammonium isobutyrate solvent (see section 2.6) and the resulting chromatogram examined for the presence of the higher phosphates of the deoxyribonucleosides. The positions of the U.V. absorbing spots were noted and sections of the chromatograms were dipped to test for the presence of ribose, deoxyribose and phosphate (see sections 2.7a, b and c). The results of a typical series of experiments are shown in Fig. 2.

The bands, corresponding to those spots giving positive U.V., deoxyribose and phosphate reactions, were cut out and eluted with water by capillary flow. They were then reapplied to paper and run for about 36 hours in the ammonium isobutyrate solvent, to effect a satisfactory separation from the adenosine compounds which in most cases contaminated the bands eluted. The chromatogram was dried and the position and nature of the spots ascertained as before. The deoxyribonucleotide spots were cut out, together with corresponding areas of blank paper and eluted as before. When there was sufficient material, the base, deoxyribose and phosphate contents were determined (see sections 2.11a, b and c). Allowance was made for contaminating material derived from the solvent or paper

during chromatography by carrying out the same series of estimations on the blank paper eluates. The results of typical analyses are shown in Table 3.

When less material was available, analyses were restricted to relating the phosphorus, to the deoxyribose content of the compound (see Table 4).

It was evident from these experiments that, in the presence of the dialysed extract, dAMP, dGMP, dCMP and TMP could all be converted to the corresponding triphosphates. The percentage conversions were dAMP, 60-80%; dGMP, 50-60%; dCMP, about 15% and TMP, about 1%.

Several different concentrations of ATP and $MgCl_2$ were tried but the yields were not improved. Even in the presence of a vast excess of ATP the amount of triphosphate did not increase. The concentrations used initially were those which had been found by Smellie, Keir and Davidson (Smellie et al, 1959) to give the best incorporation of TDR into DNA.

From these results it is clear that this system provides a worthwhile preparative route to dGTP and dATP only, and large scale preparations of them were undertaken.

3.2 Large scale experiments.

Incubation mixtures were made up as follows.

Deoxyribonucleoside monophosphate 500μM in 8 ml. at pH 7.

ATP 1500μM in 6 ml. at pH 7.

$MgCl_2$	1500µM in 6 ml. at pH 7.
tris/HCl buffer, pH 8.0	15mM in 150 ml.
Tumour extract (see 2.2)	100 ml. containing about 300 - 350 mg. protein.
or dialysed extract (see 2.2a)	120 ml. containing about 300 - 350 mg. protein
or reconstituted extract (see 2.3)	500 mg. in 25 ml., 0.05M KCl solution.
Total volume	270 ml.

and incubated at 37° for 2 hours, with shaking.

The mixture was deprotoinised as before and a sample taken for chromatography, in the ammonium isobutyrate solvent. The resulting chromatogram was examined (see sections 2.7a, b and c), for the presence of the deoxyribonucleoside triphosphate. If more than 50% of the monophosphate appeared to have been converted to the triphosphate, the deproteinised incubation mixture was chromatographed on a Dowex-1-chloride column (35 x 2 cm.) as described in section 2.8a, b and c.

By this procedure about 45% of the dGMP incubated could be isolated as dGTP. In the case of dATP, in spite of the higher percentage conversion to the triphosphate, the loss incurred during the destruction of the contaminating ATP and the subsequent rechromatography (see section 2.9), combined to reduce the yield of dATP to around 35% of the dAMP incubated. The main source

of loss during the purification procedure, in both cases, was the removal of lithium chloride by precipitation of the triphosphate with methanol and acetone. Improvements in this method were later introduced, when this method was used in the chemical preparations of the triphosphates, and are discussed in Part II. The recovery of deoxyribonucleoside derivatives from Dowex-1 is about 90%, under the conditions described, and virtually 100% from Dowex-50 as used here.

The method described provides a simple preparative route to dGTP, and a useful, if slightly more tedious, route to dATP. As a general method of deoxyribonucleoside triphosphate synthesis it is greatly inferior to the method described by Kornberg, (Kornberg, et al. 1958), by means of which yields of between 60-75% can be obtained for the four deoxyribonucleoside triphosphates. The method described can, however, be useful, especially if the Ehrlich tumour is readily available, as a supplement to the more general chemical method described in Part II.

Table 1.

Compound	R_F Value
AMP	0.53
ADP	0.42
ATP	0.34
dAMP	0.62
dADP	0.53
dATP	0.44
dGMP	0.53
dGDP	0.41
dGTP	0.29
dCMP	0.38
dCDP	0.30
dCTP	0.19
TMP	0.44
TDP	0.31
TTP	0.24
TDR	0.66
Orthophosphate	0.38
Pyrophosphate	0.23
Tripolyphosphate	0.17

Table 2.

Compound	Distance travelled after 90 hr.
AMP	3.6 cm.
ADP	2.7 cm.
ATP	1.3 cm.
3'-AMP	17.9 cm.
dAMP	19.3 cm.
dADP	11.1 cm.
dATP	7.3 cm.
dCMP	19.8 cm.
dCTP	8.0 cm.
dGMP	11.7 cm.
dGTP	6.5 cm.
TMP	29.7 cm.
TTP	14.0 cm.
Orthophosphate	13.8 cm.
Pyrophosphate	5.1 cm.
Tripolyphosphate	3.0 cm.

Table 3.

Complete analyses of the two purine deoxyribonucleotides.

Base	Adenine
Deoxyribose	0.87 μmoles/μmole of base
Total phosphorus	2.82 μmoles/μmole of base
Compound is,	dATP

Base	Guanine
Deoxyribose	0.97 μmoles/μmole of base
Total phosphorus	2.87 μmoles/μmole of base
Compound is,	dGTP.

Table 4.

Analyses relating the phosphorus and deoxyribonucleoside contents of deoxyribonucleotides.

Wavelength of maximum absorption.	Total phosphorus/µmole of deoxyribonucleoside	Compound
259 mµ	2.04	dADP
259 mµ	3.00	dATP
253 mµ	2.15	dGDP
254 mµ	2.89	dGTP
272 mµ	2.97	dCTP
267 mµ	2.78	TTP

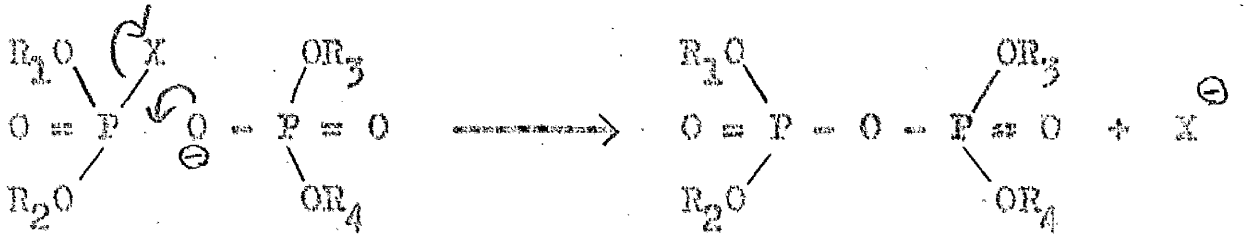
The amount of deoxyribonucleoside was calculated from the spectrum of the solution of the deoxyribonucleotide at pH 7.0. Total phosphorus in the same solution was estimated and expressed as µmoles/µmole of deoxyribonucleoside.

Part II

The Chemical Synthesis of Deoxyribonucleoside Triphosphates.

4. Introduction.

Methods for the chemical synthesis of nucleotides were first developed by Todd and his colleagues as an extension of their work on new routes for the phosphorylation of alcohols and phenols and on general methods for the synthesis of pyrophosphates. These and later methods were nearly all based on one fundamental principle, the activation of a phosphoric ester, followed by reaction with a phosphoric ester, as in equation (1)

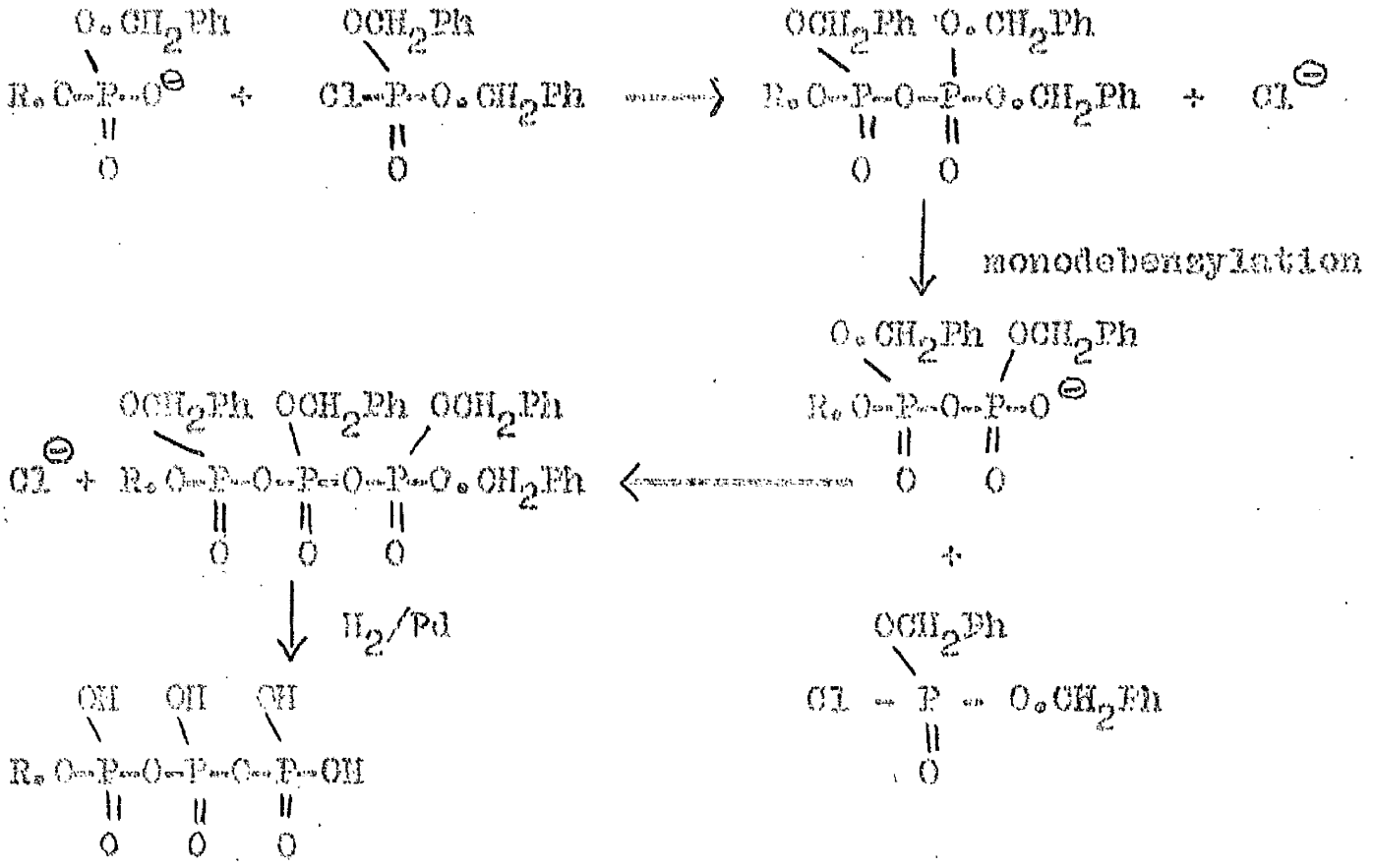


There have been three main methods of activation used, (a) the phosphorochloridate method, (b) phosphoramidate method and (c) the carbodiimide method.

4.1 The phosphorochloridate method.

This method utilises the well-known reaction between an anhydride, AB and an acid C, where A is a stronger acid than B or C, (Corby, N.S., Kenner, G.W. and Todd, A.R. J. Chem. Soc. 1952, 1234). The anhydride BC is formed and A is released as the free acid. In nucleoside polyphosphate synthesis A is most commonly hydrochloric

acid and AB a phosphorochloridate. If a specific product, or set of products, is to be formed e.g., the nucleoside-5'-polyphosphates, the other reactive groups must be protected. For example in the preparation of ATP the reaction scheme was, where R = adenosine



The benzyl group was chosen as blocking agent because of its ready removal by mild methods. Benzyl groups can be removed completely by hydrogenolysis in dioxan or ethanol using a nickel or palladium catalyst (Atherton, F.R., Howard, H.T. and Todd, A.R. J. Chem. Soc. 1948, 1106) or in a controlled manner in the presence of triethylamine

(Clark, V.M., Kirby, G.W. and Todd, A.R. J. Chem. Soc. 1958, 3039). Alternatively in sulphur containing compounds where ordinary catalytic hydrogenolysis is precluded, benzyl groups can be removed by treatment with sodium in liquid ammonia, (Arris, J., Baddiley, J., Buchanan, J.G. and Thain, E.M. J. Chem. Soc. 1956, 4968). One benzyl group can be selectively removed from a triester of phosphoric acid by nucleophilic displacement using either tertiary bases (Baddiley, J., Clark, V.M., Michalski, J.J. and Todd, A.R. J. Chem. Soc., 1949, 815) or suitable anions such as chloride or iodide (Clark, V.M. and Todd, A.R. J. chem. Soc. 1950, 2023; Cremlyn, R.J., Kenner, G.W., Mather, J. and Todd, A.R. J. Chem. Soc. 1958, 528).

The preparation of the dibenzyl phosphorochloridates and nucleoside-5'-benzyl phosphorochloridates is best achieved by chlorination of the corresponding phosphites with N-chlorosuccinimide (Kenner, G.W., Todd, A.R. and Weymouth, F.J. J. Chem. Soc. 1952, 3675).

The first nucleoside triphosphate to be synthesised by chemical methods was ATP, which was synthesised by Baddiley et al. in 1949 (Baddiley, J., Michelson, A.M. and Todd, A.R. J. Chem. Soc. 1949, 582). It was prepared by treating the silver salt of adenosine-5'-benzyl phosphate with dibenzyl phosphorochloridate to give adenosine-5'-tribenzyl pyrophosphate. Monodebenzylation gave adenosine-

5'-dibenzyl pyrophosphate which after treatment with dibenzyl phosphorochloridate and total debenzylation gave adenosine-5'-triphosphate.

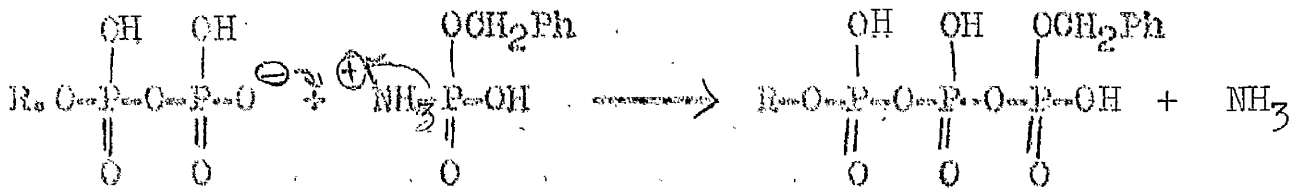
Uridine (Kenner, G.W., Todd, A.R., Webb, R.F. and Weymouth, F.J. J. Chem. Soc. 1954, 2288) and thymidine triphosphates have also been synthesised by this procedure (Griffin, B.E. and Todd, A.R., J. Chem. Soc. 1958, 1389) but there have been no published syntheses of cytosine, guanosine, deoxycytosine, deoxyguanosine or deoxyadenosine triphosphates by this method.

Michelson has recently developed an improved phosphorochloridate method (personal communication) of triphosphate synthesis in which yelds before purification of between 75 - 85% have been obtained.

4.2 The phosphoramidate method.

In early experiments by Todd et al (Clark, V.M., Kirby, G.W. and Todd, A.R. J. Chem. Soc. 1957, 1497) in which they reacted benzyl phosphoramidate with adenosine-5'-phosphate the main product was a monobenzyl ester of adenosine-5'-pyrophosphate, little or no higher phosphate being obtained. When, however, a salt of adenosine-5'-pyrophosphate was treated with benzyl phosphoramidate the product was a benzyl ester of adenosine-5'-triphosphate. It is believed that the reacting species of the phosphoramidate is one in which the nitrogen

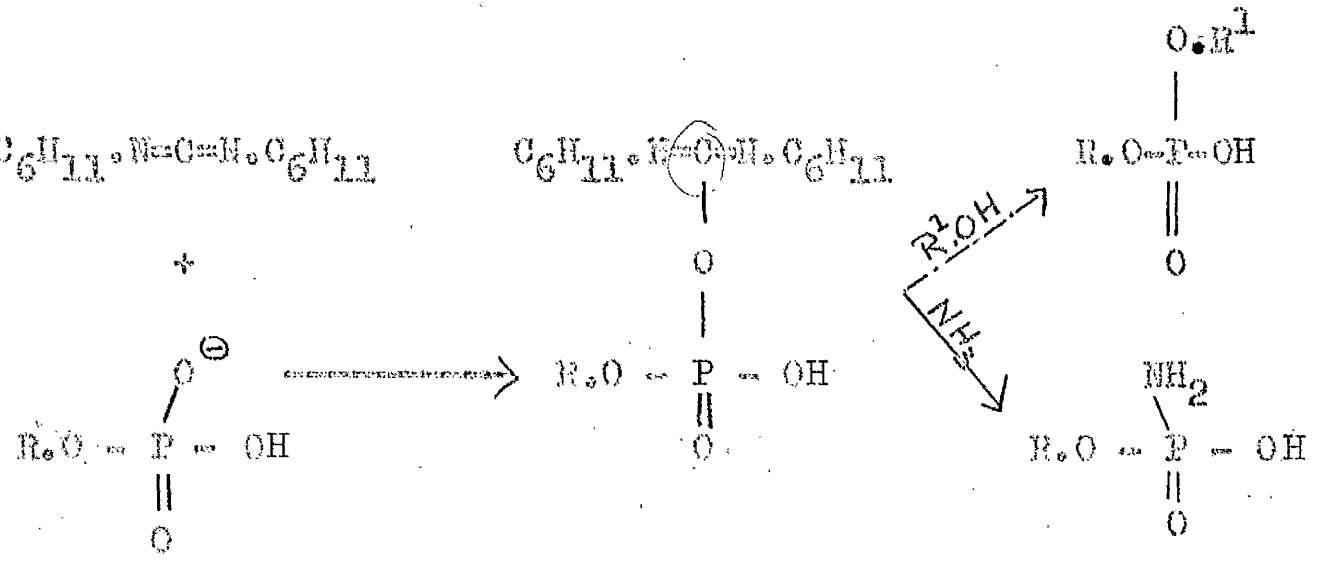
bears a positive charge. The reaction can be written as follows



In contrast to the findings of Todd et al. with benzyl phosphoramidate, Chamber & Khorana (Chambers, R.W. and Khorana, H. G. J. Amer. Chem. Soc. 1958, 80, 3749) using potassium hydrogen phosphoramidate and adenosine-5'-phosphate, obtained a mixture of adenosine-5'-polyphosphates. This difference in products is probably due to the lability of benzyl esters of adenosine polyphosphates compared to the free adenosine polyphosphates. This indicates one of the main advantages of the phosphoramidate method over the phosphorochloridate method. In the latter the fully substituted phosphorochloridate is necessary, and the triphosphates are obtained as tetrabenzyl esters. In the phosphoramidate method, these substitutions are unnecessary. A further advantage of this method is the relative lack of reactivity of the phosphoramidates towards alcoholic hydroxyl groups. This means that nucleoside 2'- and 3'-hydroxyl groups do not need to be protected.

Nucleoside phosphoramidates were first synthesised using monophenyl phosphorochloridate to phosphorylate the protected nucleoside, treatment with ammonia and

alkaline removal of the protective groups (Chambers, R.W. and Khorana, H.G. J. Amer. Chem. Soc. 1958, 80, 3749). A superior one-step synthesis of adenosine-5' and uridine-5'phosphoramidates was described by Chambers and Moffat (Chambers, R.W. and Moffat, J.G. J. Amer. Chem. Soc. 1958, 80, 3753). This method consisted of reacting the nucleoside monophosphate with dicyclohexylcarbodiimide and aqueous ammonia in t-butanol and formamide at 80°, and gave yields of up to 90%

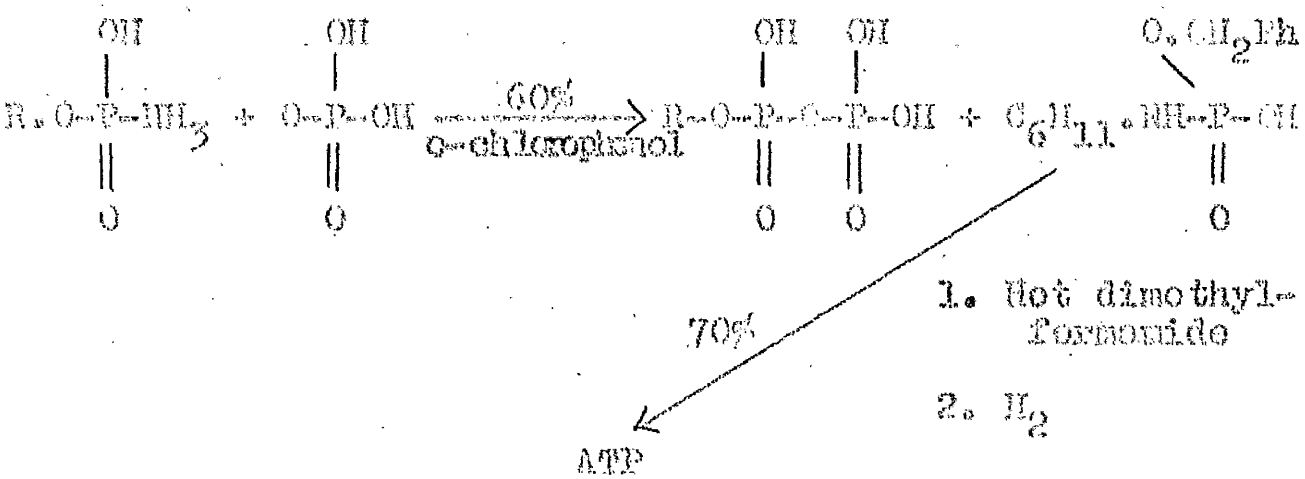


where R = nucleoside and R^1 = alkyl group.

To prevent esterification of the phosphate complex by the solvent alcohol (dotted arrow) a sterically hindered alcohol must be used. t-Butanol was found to be very suitable.

The N-substituted phosphoramidates are usually more reactive and may be prepared by substituting the appropriate amine e.g., morpholine, cyclohexylamine or benzyl-

amine for ammonia. ADP can be prepared from AMP in 60% yield by treating dicyclohexylguanidinium adenosine-5'-phosphoramidate with phosphoric acid in o-chlorophenol (Chambers, R.W. and Khorana, H.G. J. Amer. Chem. Soc., 1958, 80, 3749). Treatment of the tri-n-butylammonium salt of ADP with benzyl N-cyclohexylphosphoramidate gives ATP in 70% yield (Clark, V.M., Kirby, G.W. and Todd, A.R. J. Chem. Soc. 1957, 3035). This means that by this method an overall yield of ATP from AMP of about 40% can be achieved, thus, where R = adenosine



4.3 The Carbodiimide Method.

In 1953 Khorana and Todd (Khorana, H.G. and Todd, A.R. J. Chem. Soc. 1953, 2257) investigated the suitability of carbodiimides as condensing agents in nucleoside polyphosphate synthesis. It was then known that carbodiimides reacted with acids to give either the acid anhydride and the urea, or to give the N-acylurea (Zetsche, F., Mescher,

E. and Meyer, H.E. Ber. 1958, 71, 1088). Aromatic acids usually followed the former pathway and aliphatic acids the latter. Preliminary reactions with dicyclohexylcarbodiimide and dibenzyl phosphate gave high yields of tetrabenzyl pyrophosphate, indicating that carbodiimides were likely to be effective in nucleoside polyphosphate synthesis. It was found that a large variety of solvents could be used for the reaction and that the reaction could tolerate fairly large amounts of water. The effect of organic bases on the reaction was studied and it was found that although pyridine did not affect the yield, triethylamine proved to be completely inhibitory. As the nucleotides and orthophosphoric acid are insoluble in anhydrous pyridine, experiments designed to produce the nucleoside-5'-polyphosphates, by condensation of the nucleoside monophosphate with phosphoric acid, were carried out in aqueous pyridine. Mixtures of the mono, di-, tri- and higher phosphates were obtained, the yields of triphosphate being around 10-20% of the original monophosphate. (Chambers, R.W. and Khorana, H.G. J.Amer. Chem. Soc. 1957, 79, 3752; Hall, R.H. and Khorana, H.G. J. Amer. Chem. Soc. 1954, 76, 5056; Khorana, H.G. J. Amer. Chem. Soc. 1954, 76, 3517). At this time it was believed that it would be futile to try to improve the solubility of the nucleotides and

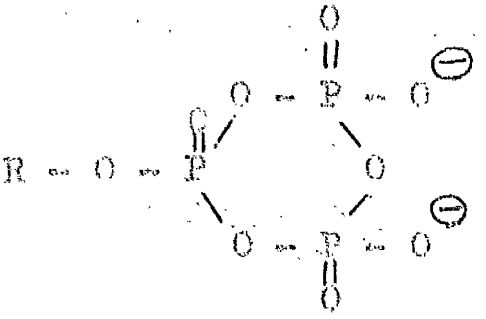
phosphoric acid, in anhydrous pyridine, by using their aliphatic amine salts, because of the known inhibitory effect of triethylamine on the carbodiimide condensation of dibenzyl phosphate. Khorana and his co-workers tried to overcome the problem of the difference in carbodiimide and nucleotide solubilities by synthesising the following water-soluble carbodiimide,

$$(C_2H_5)_2 \cdot \overset{\oplus}{\underset{\underset{CH_3}{|}}{N}} \cdot (CH_2)_3 \cdot N = C = N \cdot C_6H_{11}$$

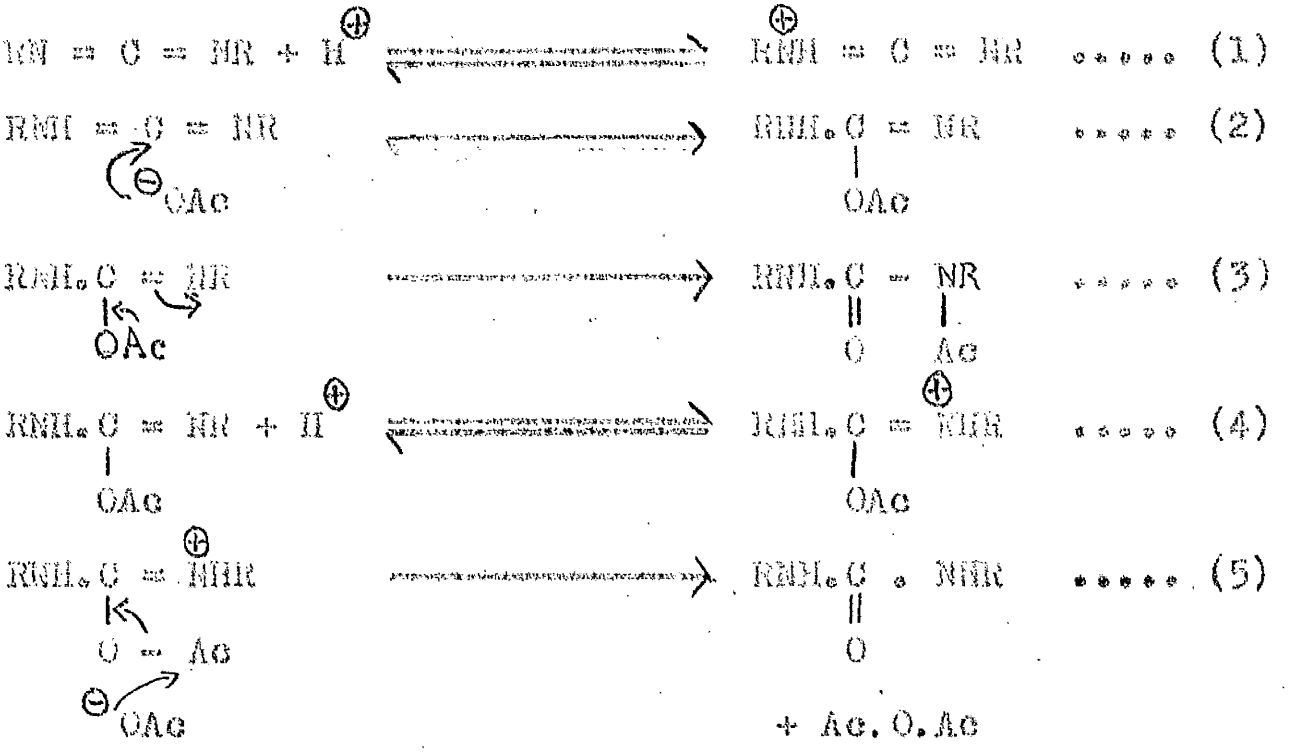
In homogeneous aqueous pyridine solutions this reagent rapidly effected the condensation of AMP and phosphoric acid. However the recovery of the nucleotide material from solutions containing large quantities of quaternary ammonium salts was unsatisfactory, and so this approach was abandoned.

Attention was next turned to the use of trialkylammonium salts in order to enhance the solubilities of phosphoric esters in anhydrous solvents. They found that the inhibitory effect that trialkylamines display in phosphoric diester condensations, is negligible in condensations involving monoesterified, or unsubstituted phosphoric acids. It was also found that in condensations between nucleoside-5'-monophosphates and phosphoric acid, a large excess of dicyclohexyl-carbo-

at the expense of the di- and higher phosphates. This might be explained if a cyclic metaphosphate



were formed, as this might be unusually stable during the reaction but would hydrolyse to the linear triphosphate when the products were being isolated (Smith, M. and Khorana, H.G. J. Amer. Chem. Soc. 1958, 80, 1141). A detailed study of the reaction was published later (Smith, M., Moffatt, J.G. and Khorana, H.G. J. Amer. Chem. Soc. 1958, 80, 6204) in which the reaction between an acid and a carbodiimide was formulated thus,



It can be seen from equations (1) and (4) that the trialkylamines exert their inhibitory effect by competing with the carbodiimide for protons. From equations (2) and (5) it is obvious that the nucleophilic power of the attacking acid anion is also an important factor. Thus, because benzyl phosphate is more nucleophilic than dibenzyl phosphate, it will condense in the presence of trialkylamines, whereas dibenzyl phosphate will not.

On the basis of these findings Smith and Khorana (Smith, M. and Khorana, H.G. J. Amer. Chem. Soc. 1958, 80, 1141) described a general method of nucleoside-5'-triphosphate synthesis, and isolated about 100 µM of pure ATP; a 36% yield.

4.4 Choice of synthetic method.

As deoxyribonucleoside triphosphates were regularly required for DNA polymerase assays, a preparative method which was capable of synthesising all four deoxyribonucleoside triphosphates in good yield, and which involved the minimum number of steps was required. A comparison of the various methods as seen from this viewpoint was undertaken, and the results may be summarised in this way.

(a) The phosphorochloridate method. Preparations usually involve several steps and yields are fairly

low, and no details of preparations of deoxycytosine or deoxyguanosine triphosphates are available. The unpublished results of Michelson were not known to us until the end of 1960, but even these methods involve several steps and also changes in solvent composition for various nucleotides.

(b) The phosphoramidate method. This method is capable of producing ATP from dicyclohexylguanidinium adenosine-5'-phosphoramidate and phosphoric acid in about 40% yield. The salt of adenosine-5'phosphoramidate was obtained in about 90% yield from AMP. This procedure again involves several steps and as no details are available for preparations involving other ribonucleoside or deoxyribonucleoside triphosphates, a considerable amount of preliminary work would be necessary.

(c) The carbodiimide method. This method appeared to fulfill most completely the requirements of general applicability and simplicity of procedure. By a one-step reaction the monophosphates could be converted to the triphosphates in yields of up to 65%. There was considerable loss during the subsequent purification procedure but pure ATP had been obtained in 36% yield. This method was selected as the one which could best be turned into a routine preparative procedure for the

triphosphates. Initial experiments were therefore undertaken on the basis of the method of Smith and Khorana (Smith, M. and Khorana, H.G. J. Amer. Chem. Soc. 1958, 80, 1141).

5. Methods.

5.1 The reaction.

a) Reaction mixture

Mixture A: (The proportions of the reactants are the same as those used by Smith and Khorana for the preparation of ATP; Smith, M. and Khorana, H.G. J. Amer. Chem. Soc. 1958, 80, 1141).

Deoxyribonucleoside-5'-monophosphate (2 mmoles); 85% phosphoric acid (1.3 ml., 20 mmoles); pyridine (40 ml.); tri-n-butylamine (10 ml., 20 mmoles) and dicyclohexylcarbodiimide (20 gm., 100 mmols).

Mixture B: Deoxyribonucleoside-5'-monophosphate (2.0 mmoles); 85% phosphoric acid (3.9ml., 60 mmoles); pyridine (120 ml.); tri-n-butylamine (30 ml., 60 mmoles and dicyclohexylcarbodiimide (1.0, 60 gm., 300 mmoles).

Mixture C: As for B, but the deoxyribonucleoside-5'-monophosphate was reacted in the form of its tri-n-butylammonium salt. The monophosphate (usually as the sodium salt) was dissolved in water (200 µmoles in about 25 ml.) and passed through a column (3 x 2 cm., for 200 µmoles) of Dowex-50-tri-n-butylammonium (prepared by treating Dowex-50-hydrogen with tri-n-butylammonium chloride). The column was washed with a little water and the optical density of the effluent and washings at

260 mμ determined. The number of μmoles of nucleotide present was calculated and the requisite amount of solution dried into the vessel to be used for the phosphorylation to the triphosphate.

b) Reaction procedure

When using mixture A or B, the deoxyribonucleoside 5'-monophosphate and the phosphoric acid were dissolved in the pyridine/tri-n-butylamine mixture and then the DCC was added.

When mixture C was used the solution of the tri-n-butylammonium deoxyribonucleoside-5'-monophosphate was dried into the reaction vessel and to the resulting powder, or clear gum, the pyridine, tri-n-butylamine and phosphoric acid were added. When everything had dissolved the DCC was added.

In every case the mixture was shaken vigorously at room temperature for around 48 hrs.

5.2 The isolation procedure of Smith and Khorana.

The purification method described by Smith and Khorana for the preparation of ATP from 2 moles of ADP (mixture A) is as follows:-

(a) Removal of dicyclohexylurea (DCU)

At the end of the reaction time the precipitated DCU was filtered off and washed with water.

(b) Removal of pyridine.

The combined filtrate and washings were further diluted with water (total volume, 100 ml.) and extracted three times with ether (total volume, 200 ml.). The ether solution was washed with a little water and the combined aqueous solutions concentrated in a flash evaporator, at a temperature not exceeding 20° (when this step was repeated a freeze-drier was used). Water was added to the concentrate (volume around 20 ml.) and the solution re-evaporated. The resulting concentrate which did not smell of pyridine and was neutral was made up to 50 ml. with water.

(c) Removal of inorganic phosphates

Twenty-five millilitres of this solution was made up to 175 ml. with water. Acid-washed Norite A (10 gm.) was added with stirring to the solution over a 2-minute period. The charcoal was spun down and a further amount of Norite A (1 gm.) added to adsorb some U.V. absorbing material remaining in the supernatant liquid. After collection on a Celite filter-bed the total charcoal was washed several times with water (total volume, 1 litre). The ribonucleotides were then eluted with 50% aqueous ethanol containing 2% of concentrated ammonia solution (specific gravity 0.9; total volume of eluent 800 ml.). Concentration of the eluate in a flash

evaporator (bath temperature 30° - 35°) gave a neutral solution which was freed from a trace of charcoal by filtration through Celite. The solution was made up to 40 ml. and adjusted to pH 3 with 1.0 N sodium hydroxide solution.

(d) The separation of ATP from AMP, ADP and higher adenosine polyphosphates.

The total solution was adsorbed onto a Dowex-2-chloride column (7 x 2.2 cm.; Dowex-1-chloride was used in this work). After a water wash, elution was begun with 0.003 N hydrochloric acid containing the following amounts of lithium chloride (1) 0.003N HCl + 0.02 N LiCl (1465 ml.) (2) 0.003 N HCl + 0.05 M LiCl (790 ml.) (3) 0.003 N HCl + 0.075 M LiCl (2000 ml.). A flow-rate of 4 ml./min. was maintained. These eluents removed the AMP and ADP from the column and the eluates were discarded. Subsequent elution with 0.003 N HCl + 0.15 M LiCl gave the ATP, the higher polyphosphates remaining on the column. This solution was neutralised with 2 N lithium hydroxide and stored at 0°.

(e) Removal of lithium chloride

Four hundred millilitres of this solution were concentrated to a syrup using a flash evaporator and a bath temperature of 30° - 40°. The syrup was diluted with an equal volume of methanol (around 10 ml.) and then

with acetone (about 200 ml.) to precipitate the tetralithium salt of ATP. The precipitate was collected by centrifugation, washed with acetone containing some methanol, then with ether and dried over phosphorus pentoxide, at 20° and 1 mm of mercury, to give the salt as the octahydrate

5.3 General methods of deoxyribonucleoside triphosphate isolation.

The quantities mentioned are those required for preparations involving 2 mmoles of deoxyribonucleoside monophosphate.

Method I.

(a) Removal of DCU.

At the end of the reaction time the reaction mixture was shaken with an equal volume of water and allowed to stand at 0°C for 30 mins. The DCU was then filtered off as before.

(b) Removal of pyridine.

As described in Section 5.2 (b).

(c) Removal of tri-n-butylamine.

The aqueous solution obtained after (b) was passed through a Dowex-50-sodium column (15 x 4 cm.) and the column washed with one column volume of water.

(d) Removal of inorganic phosphates.

The Norite A (V.A. Howe & Co. Ltd., London) was

activated by refluxing with 3 N hydrochloric acid for 6 hr., washed with water till neutral and dried at 85° overnight. The nucleotide solution was adjusted to pH 2 - 3 and the Norite stirred in slowly (about 10 gm./ mmole of nucleotides). The charcoal was filtered off, through three layers of Whatman No. 1 paper, and washed with water. The nucleotides were re-eluted with 70% aqueous ethanol/0.14 M ammonia solution (Hecht, L.I., Zameenik, P.C., Stephenson, M.L. and Scott, J.F. J. Biol. Chem. 1958, 233, 954). The eluate was concentrated as before and the concentrate diluted and adjusted to pH 8.

(c) The separation of the deoxyribonucleoside triphosphate from the mono-, di- and higher deoxyribonucleoside phosphates.

Separation was by anion exchange column chromatography on Dowex-1-chloride (AG-1-X8, 100-200 mesh) resin. A column of resin about 20 cm. x 2 cm. was used for the products arising from 2 mmoles of starting material. The nucleotide solution was poured onto the column and the column washed with a little water. The separation was effected by step-wise elution with hydrochloric acid/ lithium chloride mixtures.

The concentrations used were,

for TTP, (1) 0.01 N HCl + 0.1 M LiCl; (2) 0.01 N HCl + 0.2 M LiCl

for dCTP, (1) 0.01 N HCl + 0.05 M LiCl; (2) 0.01 N HCl + 0.08 M LiCl

for dATP, (1) 0.01 N HCl + 0.08 M LiCl; (2) 0.01 N HCl + 0.2 M LiCl

and for dGTP, (1) 0.01 N HCl + 0.01 M LiCl; (2) 0.01 N HCl + 0.2 M LiCl.

The first eluent in each case displaced the mono- and di-phosphates and the second eluent displaced the triphosphate. The solution containing the triphosphate was neutralised with lithium hydroxide, at 0°.

(f) Removal of lithium chloride.

This neutral solution was concentrated to a thin syrup and then diluted with 10 - 12 times its own volume of absolute alcohol. The lithium salt of the deoxyribonucleoside triphosphate was precipitated while the lithium chloride stayed in solution. The precipitate was collected, and washed as before.

(g) Conversion of the deoxyribonucleoside triphosphate from the lithium to the sodium salt.

The lithium deoxyribonucleoside triphosphate was redissolved in water and passed through a Dowex-50-sodium column. The column was washed with about one column volume of water and the combined eluates freeze-dried into phials and stored at -75°.

Method II.

The only difference between this method and Method I is that the removal of lithium chloride and inorganic phosphates was achieved in one step, using a charcoal column procedure.

Preparation of the charcoal column.

The charcoal used was "20/60 grist" obtained from British Charcoals and MacDonalds, Greenock. This charcoal was ground and sieved to 60 - 100 mesh and stirred with 5N hydrochloric acid until evolution of hydrogen sulphide ceased. It was then poured into a wide chromatography column, washed with more hydrochloric acid (about 5 column volumes) and then with water until the effluent was neutral. A large excess (20 - 30 column volumes) of 0.01 M sodium bicarbonate was then passed through the column and then water until the effluent was neutral again. The charcoal was then washed with 70% ethanol/0.14 M ammonia solution (about 50 column volumes) and water once more until the effluent was neutral. The column was then washed with 1N hydrochloric acid and then with water until the effluent was neutral. The charcoal was then ready for use.

Regeneration of the charcoal.

After use the charcoal was regenerated by washing with 70% ethanol/0.14 M ammonia solution (about 5 column

volumes), water until the effluent was neutral, 1 N hydrochloric acid (about 5 column volumes) and water until the effluent was neutral again.

Method II.

(a) Removal of DCU. As in method I (a).

(b) Removal of pyridine. As in Method I (b).

(c) Removal of tri-n-butylamine. As in Method I (c).

(d) Separation of the deoxyribonucleoside triphosphate from the mono-di- and higher phosphates.

As in method I (c) but instead of neutralising the triphosphate solution thus obtained, it was adjusted to pH 2 - 3 with lithium hydroxide.

(e) Removal of lithium chloride and inorganic phosphates.

The deoxyribonucleoside triphosphate solution at pH 2 - 3 was poured onto a column of charcoal (about 10 ml. wet bed volume/100 µmoles of nucleotide) prepared as described above. The charcoal was washed with water until no chloride ions could be detected in the effluent (by the silver nitrate test) and then with 0.01 M sodium bicarbonate (about 10 - 15 column volumes) to remove the inorganic polyphosphates which adhere to this charcoal under these conditions. One to two column volumes of water were then passed through the column to remove any remaining bicarbonate, and then 70% ethanol/0.14 M ammonia solution to displace the deoxyribonucleoside tri-

phosphate. The solution obtained in this way was concentrated in a rotary evaporator at about 35°, and then diluted (to about 100 ml.) with water.

(f) Conversion of the deoxyribonucleoside triphosphate from the lithium to the sodium salt. As in Method I.

5.4 Paper chromatography.

Paper chromatography was conducted very much as described in Part I, Section 2.6. Chromatograms were usually run (in the isobutyrate solvent) after steps (c) and (e) of the Smith and Khorana procedure, step (d) of Method I and step (d) of Method II. A sample of the final product was always chromatographed in the isobutyrate solvent and a section of the chromatogram dipped as described in Part I, Section 2.7c, to test for the presence of inorganic phosphates and other impurities.

5.5 Investigation of the inability of Norite A to adsorb nucleotides under the conditions of the Smith and Khorana procedure.

As Norite A, used as described by Smith and Khorana had failed to adsorb more than 60 - 70% of the deoxyribonucleotides present in the reaction mixture it was decided to test the adsorptivity of the charcoal, using AMP as a model compound. The effect of pH on the adsorptive capacity of the charcoal was studied and it was observed that the capacity was highest at low pH. However, as

highly acid conditions cause degradation of the deoxyribonucleotide polyphosphates, it was decided that pH 2 - 3 provided the best conditions for this work.

Solutions of AMP were adjusted to pH 2 - 3 and stirred with varying amounts of Norite A (prepared as in Section 5.3, Method I (d)). The charcoal was removed by centrifugation and the optical density of the supernatant fluids at 260 mμ determined. The results are summarised in Table 5.

It was obvious from these figures that this charcoal had an adsorptive capacity similar to that used by Smith and Khorana and was not responsible for the low percentage adsorption. It therefore seemed likely that the charcoal was being desorbed by something in the reaction solution. The compound most likely to show this desorptive effect was tri-n-butylamine which would be present as tri-n-butylammonium phosphate. It was decided to study the adsorptive capacity of the Norite A in the presence of tri-n-butylammonium phosphate at the concentration found in the reaction solution. The results are shown in Table 6.

It was clear from these findings that the tri-n-butylamine was exerting a desorptive effect on the charcoal, but this effect was not large enough to account for the 30 - 40% non-adsorption encountered with the reaction

Table 5.

Tube No.	1	2	3	4
µmoles AMP	100	100	100	100
Volume of solution	20ml.	20ml.	20ml.	20ml.
Amount of charcoal added	0.5gm.	1.0gm.	1.5gm.	2.0gm.
µmoles AMP unadsorbed	0.25	0.05	-	-

Table 6.

Tube No.	1	2
μmoles AMP	100	100
Volume of solution	20 ml.	20 ml.
μmoles of tri-n-butylammonium phosphate added	0	1
Amount of charcoal added	1 gm.	1 gm.
μmoles AMP unadsorbed	< 0.05	20

mixture. Believing that the remainder of the non-adsorption was due to the presence of traces of pyridine it was decided to pass the reaction solution through a column of Dowex-50-sodium as described in Method I (c), thereby removing the tri-n-butylamine and any pyridine that might be present.

5.6 The preparation of thymidine triphosphate labelled with ^{32}P in the proximal position.

(1) The preparation of ^{32}P-labelled thymidine monophosphate.

Method A (after the method of Hurwitz, J. J. Biol. Chem. 1959, 234, 2351, for the preparation of CMP^{32}).

100 mC of carrier free orthophosphate (^{32}P; P.B.S. I Radiochemical Centre, Amersham) were transferred to a test-tube and dried in a vacuum dessicator containing phosphorus pentoxide and solid potassium hydroxide for about 18 hr. Analar orthophosphoric acid (88% w/w; 225 mg., 0.13 ml.) was added and the mixture dried as before. The resulting yellowish oil was heated at 250° for 20 min. to convert it to polyphosphoric acid, cooled to room temperature and 500 μmoles of thymidine added. The mixture was stirred thoroughly and incubated at 60° for 4 hrs., with intermittent stirring.

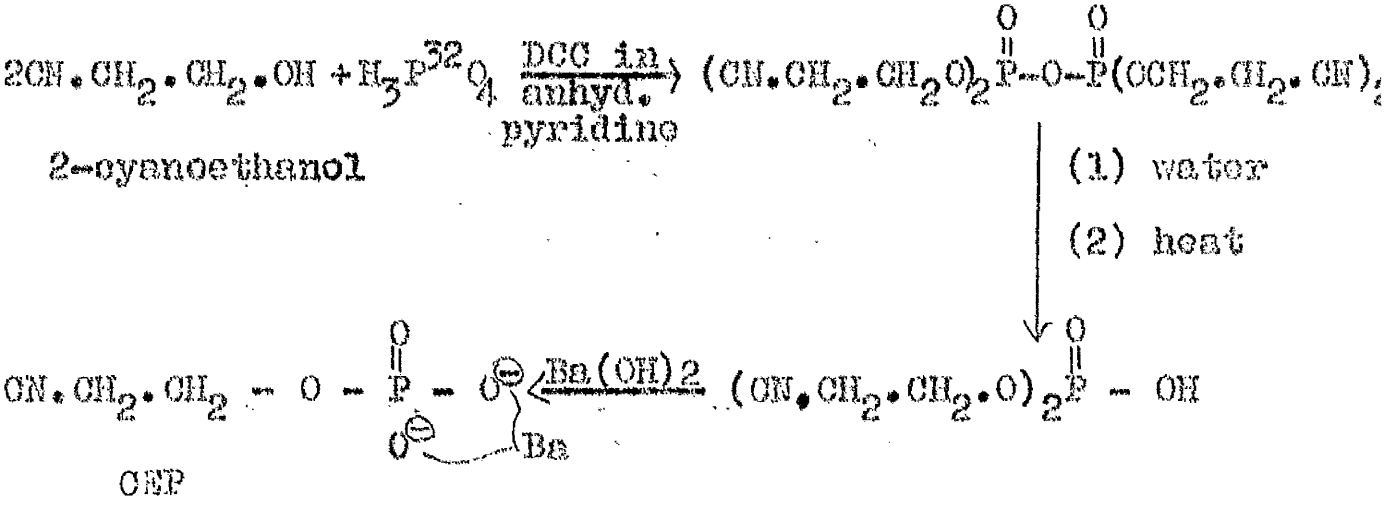
In the method of Hurwitz the CMP^{32} was separated from the unreacted phosphate by chromatography on Dowex-

freeze-dried into the reaction vessel for the preparation of TTP. A yield of TMP^{32} (of which over 90% was TMP^{32}-5') of just 20%, as based on thymidine, was obtained. The percentage of the phosphoric acid used was, of course, very small and consequently a great deal of radioactivity was wasted. The specific activity of the product was about 2×10^6 cpm/μmole as determined in a windowless gas flow counter.

Method B. (Tener, G.M. J. Amer. Chem. Soc. 1961, 83, 159)

(a) The preparation of P^{32}-labelled 2-cyanoethyl phosphate (CEP).

The reactions involved can be outlined as follows:



A solution of P^{32}-labelled orthophosphoric acid (1 ml. of 1M) was concentrated to dryness *in vacuo* at 40° to remove traces of hydrochloric acid. Anhydrous pyridine (10 ml., dried with CaH_2) and 2-cyanoethanol

(1.0 ml.) were added and the solution concentrated *in vacuo* at 40° to an oil, (the crystalline pyridinium phosphate which sometimes separated redissolved as the solution became more concentrated). A second portion of anhydrous pyridine (10 ml.) was added and the solution again concentrated to an oil. Anhydrous pyridine (5 ml.) and DCC (2.1 gm.) were added and the reaction set aside overnight at room temperature. Water (5 ml.) was added to stop the reaction and the resulting solution heated in a boiling water bath for 30 minutes. The solution was then concentrated to dryness *in vacuo* and water (10 ml.) and saturated barium hydroxide (10 ml.) added to the residue. After 5 minutes at room temperature the solution was adjusted to pH 7.5 with acetic acid (5 drops of glacial acetic acid) and filtered to remove dicyclohexyl urea and barium phosphate. Two volumes of ethanol were added to precipitate the barium 2-cyanoethyl phosphate which after standing for 1 hour, was collected by centrifugation. The crystals were redissolved in water (5 ml. + a trace of acetic acid to help solution), centrifuged to remove a trace of insoluble material and recrystallised by adding ethanol (10 ml.). The product was collected by centrifugation, washed with ethanol, then acetone and finally ether. Yield after air drying 190 mg. (60%). For use in

phosphorylations the product was dissolved in water (5 ml. + a trace of acetic acid) and passed through a Dowex-50-hydrogen column (3 x 0.8 cm.). The column was washed until all the activity was removed and the effluent and washings plus pyridine (5 ml.) were taken down to dryness in the reaction vessel to be used for the phosphorylation of the thymidine.

(b) The phosphorylation of thymidine to TMP^{32} with CEP^{32}.

Thymidine (2 mmoles, 484 mg.) was dissolved in pyridine (10 ml.) and P^{32}-labelled 2-cyanoethyl phosphate (1 mmole, as the pyridinium salt, prepared as described above) was added. The solution was concentrated to an oil *in vacuo* at 40° and then anhydrous pyridine (10 ml.) was added and the solution again concentrated to dryness. The process was repeated once more and the residue was dissolved in anhydrous pyridine (5 ml.) and DCC (620 mg.) added. After 30 minutes at room temperature, concentrated ammonium hydroxide (10 ml.) was added and the solution warmed at 60° for 1 hour. The reaction mixture was concentrated to dryness *in vacuo* and water (10 ml.) added to the residue. The resultant solution was filtered to remove dicyclohexylurea and the filtrate (25 ml. including washings) was applied to a column (2.2 x 16 cm.) of Dowex-1-chloride resin. The

column was washed with water to remove thymidine, 0.01 N hydrochloric acid to displace orthophosphate (P^{32}) and then with 0.05 N hydrochloric acid to bring off the TMP^{32}. About 30% of the thymidine was converted to TMP^{32} i.e. a 60% yield based on CEP^{32}. More than 90% of the TMP^{32} produced by this method was the 5'-derivative.

(2) Preparation of thymidine triphosphate from TMP^{32}.

Phosphorylations were carried out using mixture B (see section 5.1) and the products purified by Method II (see section 5.3). Yields of pure thymidine triphosphate of up to 60% have been obtained. The specific activity of the triphosphate was about 1.0×10^6 cpm/μmole as determined in a windowless gas flow counter.

5.7 Modification of the CEP Preparation.

In order to obtain CEP of higher specific activity the labelled phosphoric acid used in the preparation (see section 5.6, Method B (a)) was replaced by a mixture of very highly radioactive, carrier free disodium hydrogen phosphate (PBS. I, from The Radiochemical Centre, Amersham) and unlabelled phosphoric acid. In this way phosphate with a specific activity four times that obtainable as phosphoric acid

was obtained. By using this technique CEP of very high activity was obtained and thymidine triphosphate with a specific activity of 3.5×10^6 cpm/μmole (windowless gas flow counter) synthesised.

5.8 Modification of the method for the separation of deoxyribonucleoside triphosphates from their mono-, di- and higher phosphates, on Dowex-1-chloride resin.

In recent preparations of labelled thymidine triphosphate the step-wise elution system described in Method I (e) was replaced by a single mixing chamber gradient elution system. In this system a 0.2 M lithium chloride solution was run into a mixing chamber containing 0.01 N hydrochloric acid (1 litre for about 1 mmole of nucleotide material).

6. Results and Discussion.

6.1 Preparation of TTP by the method described by Smith & Khorana for ATP (Smith, M. and Khorana, H.G. J. Amer. Chem. Soc. 1958, 80, 1141; See sections 5.1 and 5.2).

When an attempt was made to prepare TTP by this method several difficulties were encountered.

TMP in Mixture A was shaken for 48 hrs. and put through steps (a) and (b) of the Smith & Khorana procedure. When an attempt was made to adsorb the deoxyribonucleotides onto Norite A, as described in step (a), it was found that only 60% - 70% of the material was adsorbed. The addition of further small amounts of charcoal did not improve appreciably the amount of deoxyribonucleotide material adsorbed. It was decided to carry on with the purification despite this loss of material and the Norite was treated with 50% ethanol/2% ammonia solution to elute the deoxyribonucleotides. At this stage trouble arose due to the formation of colloidal charcoal which could not be removed by centrifugation or filtration, even through Celite. The colloid was precipitated by blowing air through the suspension for 5-10 minutes, but having to resort to this repeatedly throughout the elution made the method very tedious. The deoxyribonucleotides were eventually eluted in about 80% yield and concentrated to remove ethanol and ammonia. When the solution became concentrated a

precipitate was obtained, which did not redissolve on the addition of more water. The material was filtered off and identified as DCU. The filtrate was chromatographed on Dowex-1-chloride as described in step (e) of Method I (see section 5.3) and the TTP thus isolated subjected to step (e) of the method of Smith & Khorana (see section 5.2) to remove the contaminating lithium chloride. During this procedure, however, it was discovered that 30-40% of the triphosphate was not precipitated, but remained in the water/methanol/acetone mixture. The precipitate obtained was washed and dried as described, to give a yield of TTP of 18.5%.

In view of the difficultion encountered it was decided to examine several steps of the purification procedure to see if they could be improved.

6.2 The Norite A procedure.

The charcoal used in this work was commercial Norite A which had been refluxed for 6 hr. with 3N hydrochloric acid, washed with water till it was neutral then dried at 85° overnight. Using AMP as a model compound the adsorptive capacity of this Norite was determined as described in section 5.5. It was observed that pH 2-3 provided the best conditions for adsorption of nucleotides. The capacity of the charcoal under these conditions was found to be almost identical to that used by Smith & Khorana and so the low percentage adsorpt-

lon observed during the purification procedure remained unexplained. Since the tri-n-butylammonium phosphate, present in the solution from which the nucleotides had to be adsorbed, might be responsible for deactivitating the charcoal, the adsorptive capacity of the charcoal was determined in the presence of tri-n-butylammonium phosphate at the concentration at which it occurred in the reaction mixture (see section 5.5). The adsorptive capacity was found to be reduced by 20% but although this is a significant decrease it does not fully explain the 30-40% decrease in capacity experienced in practice. This latter decrease was probably due to the combined effect of the tri-n-butylammonium phosphate and traces of pyridine which had not been removed by step (b).

This difficulty could be overcome by carrying out the Norite step after the isolation of the triphosphate on Dowex-1-chloride and removal of LiCl, or by passing the reaction solution obtained after step (b) through a Dowex-50-sodium column and performing the Norite step on the effluent. The former method seemed to be the simpler but when this was tried the separation obtained on Dowex-1 was not good, and so the latter procedure was adopted. Under these new conditions the adsorption of the deoxyribonucleotides onto Norite was virtually 100%.

The elution of the deoxyribonucleotides from the charcoal was also studied. No improvement in the percentage return (about 80%) could be achieved, but it was found that the eluting solution of Hecht et al (Hecht, L.I., Zamecnik, P.C., Stephenson, M.L. and Scott, J.F. J. Biol. Chem. 1958, 233, 954) which is 70% aqueous ethanol/0.14 M ammonia did not give colloidal charcoal and so was preferred to the 50% aqueous ethanol/2% ammonia formerly used.

6.3 The removal of lithium chloride.

On studying this procedure it was found that the percentage of the total deoxyribonucleoside triphosphate that was precipitated, varied greatly with the amount of water present. If the lithium chloride/triphosphate solution was not concentrated to a very thick syrup as much as 50% of the triphosphate would remain in the water/methanol/acetone solution. Even under ideal conditions only 70% of the total triphosphate was precipitated.

Several other organic solvents were tried in place of the methanol/acetone mixture and the best of these was found to be absolute alcohol. By concentrating the lithium chloride/triphosphate mixture to even a thin syrup and adding 12 volumes of absolute alcohol, 75-80% of the triphosphate could be precipitated.

Alcohol had the added advantage over the methanol/acetone, of being non-U.V. absorbent. This meant that the nucleotide content of the alcohol after a precipitation could be checked directly.

In view of the above findings the method of Smith & Khorana was modified to give Method I.

6.4 Method I (see section 5.3).

(a) At the end of the reaction time the mixture was shaken with an equal volume of water and left for 30 minutes at 0^{o}, to ensure the complete removal of DCU.

(b) As for Smith and Khorana procedure.

(c) Tri-n-butylamine and pyridine were removed by passage of the solution, obtained from (b), through a Dowex-50-sodium column, and washing with water.

(d) Removal of inorganic phosphates by adsorption of the deoxyribonucleotides on Norite A and re-elution with 70% aqueous ethanol/0.14 M ammonia.

(e) Chromatography on Dowex-1-chloride as in the Smith & Khorana procedure but with the appropriate change in eluting solvents (see section 5.3e).

(f) Lithium chloride was removed by precipitation of the lithium salt of the triphosphate in 80% yield with absolute alcohol.

(g) The lithium salt of the triphosphate was converted to the sodium salt by passage through Dowex-50-sodium. This step was necessary because lithium ions had been shown to have an adverse effect on the "DNA polymerase" assay in which the triphosphates were to be used.

With this method several successful preparations were performed and tritium-labelled TTP was obtained in 36% yield. Since TMP was supplied commercially as the disodium salt it was necessary before each preparation to convert it to the free acid by passing it through a Dowex-50-hydrogen column. dAMP, dCMP and dGMP were also supplied as the disodium salts, but because of the presence of ring amino-groups they adhere to Dowex-50-hydrogen and so were less readily converted to the free acids (the free acids could be obtained by converting the sodium to the barium salts on Dowex-50-barium and addition of equimolar amounts of sulphuric acid. The barium sulphate formed is insoluble and was removed by centrifugation). Because of this, attempts were made to react these nucleotides as the sodium salts but their solubility in the reaction mixture was so low that yields were very poor. By increasing the quantities of the reactants to double the

amounts used by Smith & Khorana, while keeping the amount of deoxyribonucleoside monophosphate (as the disodium salt) the same, low, but worthwhile yields of the triphosphates could be obtained. That is to say yields of pure triphosphate of between 10 - 20% were obtained.

In view of this it was thought that an investigation of the reaction mixture might be worthwhile and so various trial experiments were undertaken.

6.5 Investigation of the reaction.

In these small scale trial experiments the mixtures were incubated for between 42 and 48 hrs., put through steps (a), (b) and (c) of Method I (see section 5.5) and then spotted on chromatography paper, together with the appropriate markers, and run in the isobutyrate solvent for 18 hrs. The papers were dried, and the nucleotide spots located in U.V. light. Half of the paper was dipped to detect phosphorus containing spots (see section 2.7c) and in the other half the U.V. spots were cut out and eluted with 0.01N hydrochloric acid. In some experiments areas corresponding to spots containing inorganic tripolyphosphate were also cut out and eluted. Only small amounts of inorganic ortho- and pyrophosphates were detected.

6.5c The effect on the yield of nucleoside triphosphate of decreasing the ratio of the nucleoside monophosphate to the other reactants.

AMP (200 μmoles) was shaken for 45 hrs. in mixtures containing one, two and three times the quantities of the reactants used in the Smith and Khorana mixture, and the resulting products analysed as described in Section 6.5. The results are shown in Table 7.

It was clear from these figures that by doubling the amounts of the reactants while keeping the amount of nucleotide the same an improved yield of the triphosphate could be obtained. The further small increase obtained by using three times the reactant quantities did not seem worthwhile in view of the increased inorganic tripolyphosphate formation.

This experiment was repeated using TMP in place of AMP and the results are shown in Table 8. Once again double the amounts of the reactants gave the most satisfactory results.

This experiment was repeated yet again with dCMP and the results are shown in Table 9. The figures indicated again, although less decisively, that a reaction mixture containing double the amounts of the

Table 7.

The effect on the yield of ATP of decreasing the ratio of AMP to the other reactants.

Reaction mixtures.	(1)	(2)	(3)
AMP	200 μmoles	200 μmoles	200 μmoles
tri-n-butylamine	1.0 ml.	2.0 ml.	3.0 ml.
pyridine	4.0 ml.	6.0 ml.	8.0 ml.
85% phosphoric acid	0.13 ml.	0.26 ml.	0.39 ml.
DCC	2.0 gm.	4.0 gm.	6.0 gm.

were shaken for 45 hrs. at room temperature. On analysis (see section 6.5) the following results were obtained

	per cent total adenosine nucleotide recovered			
	AMP	ADP	ATP	Higher adenosine phosphates
(1)	18	11	62	9
(2)	10	7	72	11
(3)	7	5	74	14

	(1)	(2)	(3)
μmoles adenosine nucleotide recovered	190	185	193
μmoles inorganic tripolyphosphate formed	610	1080	1640

Table 8.

The effect on the yield of TTP of decreasing the ratio of TMP to the other reactants.

Reaction mixtures, as in Table 7 with TMP in place of AMP, were shaken for 45 hrs. at room temperature and analysed (see section 6.5) to give the following results

	per cent of total thymidine nucleotide recovered			
	TMP	TDP*	TTP	higher thymidine phosphates
(1)	9	20	71	-
(2)	9	15	76	-
(3)	9	12	79	-

	(1)	(2)	(3)
μmoles thymidine nucleotide recovered	190	195	187
μmoles inorganic tripolyphosphate formed	630	1150	1570

* This spot had an R_p of 0.36 as compared with R_p's of 0.31 for TDP and 0.44 for TMP and was taken as TDP

N.B. In this and succeeding tables - signifies that no U.V. absorbing material was detected.

Table 9.

The effect on the yield of dCTP of decreasing the ratio of dCMP to the other reactants.

Reaction mixtures, as in Table 7, with dCMP in place of AMP, were shaken for 46 hrs. at room temperature and analysed (see section 6.5) to give the following results

	per cent of total deoxycytidine nucleotide recovered.			
	dCMP	dCDP	dCTP	higher deoxycytidine phosphates
(1)	33	13	45	9
(2)	27	5	58	10
(3)	16	-	64	20

	(1)	(2)	(3)
µmoles deoxycytidine nucleotide recovered	166	135	187

No analysis for inorganic tripolyphosphate was performed, but the phosphate dip showed the expected increase from (1) to (3).

reactants gave the best results.

6.5b The effect on the yield of deoxyribonucleoside triphosphate of using the tri-n-butylammonium salt of the deoxyribonucleoside monophosphate.

Since the conversion of the disodium salt of dCMP to the free acid was time-consuming, it was decided to repeat the above experiment using dCMP in the form of its tri-n-butylammonium salt (see section 5.1a) in the hope that it would prove to be a good and readily available substitute for the free acid. The results are shown in Table 10. As can be seen from these results tri-n-butylammonium-dCMP gave better yields of the triphosphate than the free acid, in all three mixtures. Because of this, experiments with tri-n-butylammonium-AMP and tri-n-butylammonium-TMP were carried out to determine whether improved yields of ATP and TTP could also be obtained. The results appear in Tables 11 and 12.

The yields of ATP obtained from tri-n-butylammonium-AMP were only slightly better than those obtained from the free acid, but the yields of TTP produced from tri-n-butylammonium-TMP showed worthwhile improvements over those obtained from the free acid, especially in mixture (3).

Table 10.

The effect on the yield of dCTP of decreasing the ratio of tri-n-butylammonium-dCMP to the other reactants.

Reaction mixtures, as in Table 7, with tri-n-butylammonium salt of dCMP in place of AMP, were shaken for 45 hrs. at room temperature, and analysed to give the following results

	Per cent of total deoxycytidine recovered			
	dCTP	dCDP	dCTP	higher deoxycytidine phosphates
(1)	30	15	55	-
(2)	28	8	64	-
(3)	27	-	73	-

	(1)	(2)	(3)
μmoles deoxycytidine nucleotide recovered	183	187	181

No analysis for inorganic tripolyphosphate was performed, but the phosphate dip showed the expected increase from (1) to (3).

Table 11.

The effect on the yield of ATP of decreasing the ratio of tri-n-butylammonium-AMP to the other reactants.

Reaction mixtures as in Table 7, with tri-n-butylammonium salt of AMP in place of AMP, were shaken for 44 hrs. at room temperature, and analysed to give the following results

	per cent of total adenosine nucleotide recovered			
	AMP	ADP	ATP	Higher adenosine phosphates
(1)	20	7.5	65	7.5
(2)	15	8	67	10
(3)	8.5	3.5	76	12

	(1)	(2)	(3)
μmoles adenosine nucleotide recovered	130	178	184

No analysis for inorganic tripolyphosphate was performed, but the phosphate dip showed the expected increase from (1) to (3).

Table 12.

The effect on the yield of TTP of decreasing the ratio of tri-n-butylammonium-TMP to the other reactants.

Reaction mixtures, as in Table 7 with tri-n-butylammonium salt of TMP in place of AMP, were shaken for 46 hrs. at room temperature, and analysed to give the following results

	per cent of total thymidine nucleotides recovered			
	TMP	TDP*	TTP	higher thymidine phosphates
(1)	3	23	74	-
(2)	-	20	80	-
(3)	-	8	92	-

	(1)	(2)	(3)
μmoles thymidine nucleotide recovered	193	195	190

No analysis for inorganic tripolyphosphate was performed, but the phosphate dip showed the expected increase from (1) to (3).

* as in Table 8.

In view of these results it was decided that for the purpose of this work, i.e. to prepare deoxyribonucleoside triphosphates (especially labelled TTP) in high yield, the best reaction mixture was that which contained the tri-n-butylammonium salt of the deoxyribonucleotide and three times the reaction quantities used by Smith & Khorana (The yields obtained by Smith & Khorana from various nucleotides are shown in Table 14). This was termed mixture C and is detailed in section 5.1a.

Two further experiments, in which tri-n-butylammonium-dAMP and tri-n-butylammonium-dCMP were reacted in mixture C, were performed to ascertain whether dATP and dCTP could also be obtained in high yield by this method. The results are shown in Table 13. As these results were highly satisfactory it was decided to adopt this reaction mixture for large scale preparations.

6.6 The charcoal column procedure (see section 5.3, Method II).

Before these preparations were undertaken, however, a survey of the readily available charcoals was carried out to see if there was amongst them a charcoal which could be used to separate the deoxyribonucleoside

Table 13.

The phosphorylation of tri-n-butylammonium-dAMP and tri-n-butylammonium-dGMP in Mixture C.

Reaction mixtures corresponding to Mixture C of section 5.1a were shaken for 46 hrs. at room temperature, and analysed (see section 6.5) to give the following results

	per cent of total deoxyribonucleotide recovered			
	mono-	di-	tri-	higher deoxynucleoside phosphates
dAMP-tri-n-butylammonium	7	-	93	-
dGMP tri-n-butylammonium	-	8	92	-
		deoxyadenosine nucleotide		deoxyguanosine nucleotide
µmoles recovered		185		192

Table 14.

The yields of mono-, di-, tri-, and higher nucleoside phosphates obtained by Smith and Khorana (Smith, M. and Khorana, H.G. J. Amer. Chem. Soc. 1958, 80, 1141).

	per cent of total nucleotide recovered			
	mono-	di-	tri-	higher nucleoside phosphates
AMP	2	28*	60	10
UMP	5	21.5	64.5	9
CMP	26	14	39	21
GMP	5	15	71	9
dCMP	34	14	43	9
dGMP	3	17.5	79.5	-

N.B. The mono-, di-, tri- and higher phosphates were separated by ion-exchange chromatography. * signifies that two peaks were present.

triphosphate from lithium chloride and the inorganic polyphosphates in a column procedure. It was realised that if this type of procedure was possible worthwhile economies could be made in the amount of triphosphate lost, and time wasted, during procedures (d) and (f) of Method I.

The most suitable charcoal found was that supplied by British Charcoals and MacDonalds of Greenock, as 20/60 grist". This is a very coarse, impure charcoal which had to be ground (to between 60-100 mesh), purified and activated before it could be used (see section 5.3, Method II). Trial experiments with AMP demonstrated that this charcoal had the following advantages (a) a high flow rate, well suited to column procedures (b) a high ion tolerance i.e. it was not strongly desorbed by lithium chloride and (c) a high return of nucleotides (about 90-95%). It had the disadvantages of (a) requiring a sodium bicarbonate wash to remove all the inorganic polyphosphates and (b) releasing contaminating impurities when washed with ethanolic ammonia.

In spite of the disadvantages associated with this technique it was decided to adopt it in preference to the Norite A and ethanol precipitation steps of Method I.

The biggest disadvantage of this method was the release of impurities during the ethanolic ammonia wash. This could not be overcome by washing the charcoal with ethanol/ammonia and was probably due to the continual breakdown of the charcoal grains and the concomittant release of the impurities trapped within the grains. Prolonged washing merely caused increased breakdown and the granular nature of the charcoal was destroyed. The impurity (or impurities) released in this way had no effect on the DNA polymerase assays in which the deoxyribonucleoside triphosphates were used. Only very small amounts of the triphosphates were used in each assay and so the concentration of the impurity in the assay mixture was very low indeed. If necessary the impurity can be removed from the charcoal eluates by evaporating to dryness (at 30° in vacuo) and extracting with absolute ethanol. The impurity is ethanol soluble and can be reduced to a very low level by this treatment.

6.7 Method II (see section 5.3).

Method II can be summarised as follows:

(a) As in Method I.

(b) As in Method I.

(c) As in Method I.

(d) As for step (c) of Method I.

(e) The removal of lithium chloride and inorganic phosphates by passage of the solution obtained from (d) (adjusted to pH 2-3) through a charcoal column. The column was washed with 0.01M sodium bicarbonate and the deoxyribonucleoside triphosphate eluted with 70% ethanol/0.14 M ammonia solution.

(f) The removal of impurities (from charcoal) and the conversion of the lithium salt of the deoxyribonucleoside triphosphate to the sodium salt. The solution obtained from (e) was concentrated at 30° in vacuo either to (1) small bulk or (2) dryness.

(1) The solution thus obtained was diluted with water and passed through a Dowex-50-sodium column as described in Method I (g).

(2) The solid thus formed was extracted with absolute ethanol to remove the charcoal impurities and the residual material re-dissolved in water and treated as in (1).

In practice (2) was rarely performed, for the reasons mentioned above. (see section 6.6).

By this method TTP, dATP and dGTP can be prepared pure in about 60% yield. (If step f(2) is omitted the yields are nearer 70%). Pure dCTP can

be obtained in around 40% yield.

6.8 The preparation of TTP labelled with ^{32}P in the proximal position (see section 5.6).

In order that proximally labelled TTP might be prepared methods of preparing ^{32}P-labelled nucleoside monophosphates were sought.

^{32}P-labelled nucleotides can be prepared biologically (eg. Eggleston, L.V. Biochem. J. 1954, 58, 503; Heidelberger, C. Harbers, E., Liebman, K., Takagi, Y. and Potter, V.R. Biochim. Biophys. Acta 1956, 20, 445; Lehman, I.R., Bessman, M.J., Simms, E.S. and Kornberg, A. J. Biol. Chem. 1958, 233, 163) but the quantities and specific activities that can be obtained are limited. Chemical means are not subject to these limitations and at the time that this work was undertaken two reagents had been used to prepare ^{32}P-labelled nucleotides. Barker (Barker, G.R. J. Chem. Soc. 1954, 3396) had prepared adenosine 2'- and 3'monophosphates using ^{32}P-phosphorus oxychloride as phosphorylating agent. In 1959 both Chambers (Chambers, R.W. J. Amer. Chem. Soc. 1959, 81, 3032) and Hurwitz (Hurwitz, J. J. Biol. Chem. 1959, 234, 2351) described methods of preparing ^{32}P-labelled nucleoside monophosphates involving the use of

$H_3P^{32}O_4$. Chambers prepared UMP^{32} using a mixture of $H_3P^{32}O_4$ and P_2O_5 and Hurwitz prepared CMP^{32} by a method involving ^{32}P-labelled polyphosphoric acid.

As adequate facilities for the handling of radioactive materials were not available at that time, it was decided not to use the method of Barker which involved the use of a volatile ^{32}P-labelled compound.

The methods of Chambers and Hurwitz were very similar but as the method of Hurwitz gave products of higher specific activity it was chosen for use in this work (see section 5.6, Method A). This method suffers from the disadvantagethat very large amounts of radioactivity (about 100 mC) are required because of the very low yield (based on the polyphosphoric acid).

Using this method thymidine was converted in 20% yield to TMP^{32} of specific activity 2 x 10^6 cpm/μmole (as determined in a windowless gas flow counter).

Also in 1959 Gilham and Tener published a new method of phosphorylating nucleosides utilising 2-cyanoethylphosphate (CEP) as the phosphorylating agent. (Gilham, P.T. and Tener, G.M. Chem. and Ind. 1959, 542) This method was not suited at that time to the preparation of ^{32}P-labelled nucleotides as the CEP could only be prepared by the phosphorylation of 2-cyanoethanol with

polyphosphoric acid in very low yield (Chorbulicz, E. and Rabinowitz, J. Helv. Chem. Acta 1956, 39, 1461). However when Tener later developed a method of synthesising CTP from 2-cyanoethanol and phosphoric acid in 60% yield the method became well suited to the preparation of ^{32}P-labelled nucleotides, and TMP^{32} was prepared from thymidine in 60% yield. This method (see section 5.6, method B. Private communication, later published Tener, G.M. J. Amer. Chem. Soc. 1961, 83, 159) was much more efficient that that of Hurwitz and was adopted in its place. By using this method much smaller amounts of radioactivity were necessary and TMP^{32} of specific activity 1.5 - 2.0 x 10^6 cpm/μmole (windowless gas flow counter) was obtained from 25 m C of $H_3P^{32}O_4$.

TMP^{32} of even greater specific activity was prepared (see section 5.7) by substituting a mixture of carrier-free $NaHP^{32}O_4$ and H_3PO_4 for the $H_3P^{32}O_4$ used by Tener, as $NaHP^{32}O_4$ is obtainable with a specific activity four times as high as that of $H_3P^{32}O_4$.

TTP labelled with ^{32}P in the proximal position was prepared from the TMP^{32} obtained by the above methods by the use of Mixture C (see section 5.1a) and Method II (see section 5.3) in yields of up to 70% and with specific activities between 1.0 and 3.5 x 10^6 cpm/μmole

(windowless gas flow counter).

It has recently been found to be more convenient to replace the step-wise elution system used in Method II for the separation of TTP on Dowex-1-chloride resin, by a continuous single mixing chamber gradient elution system. This system gives greater reproducibility and has the added advantage that the separation can be carried out overnight.

Part III

The Pyrophosphorolysis of DNA.

7. Introduction

Using the highly purified "DNA polymerase" which they had isolated from extracts of E. coli, Bessman et al. (Bessman, M.J., Lehman, I.R., Simms, E.S. and Kornberg, A. J. Biol. Chem. 1958, 233, 171) demonstrated the ability of inorganic pyrophosphate to bring about the reversal of the DNA forming polymerase reaction. This pyrophosphorolysis was best achieved in the presence of DNA, the deoxyribonucleoside triphosphates, magnesium ions and 5×10^{-3}M pyrophosphate.

The reaction was studied by incubating the polymerase, DNA and ^{32}P-labelled pyrophosphate, with the appropriate additions in tris buffer at pH 7.5. After incubation at 37° for 30 minutes the mixture was chilled and cold solutions of albumin, perchloric acid and "carrier" pyrophosphate were added. The mixture was centrifuged, the precipitate discarded and the supernatant fluid treated with acid-washed Norite. The Norite precipitate was collected by centrifugation and washed three times with water containing a little perchloric acid to remove unreacted pyrophosphate. It was then suspended in 50% ethanol/0.03N ammonia solution, trans-

ferred to a planchet, dried and counted. The radioactivity present was then indicative of the extent to which pyrophosphate had been transferred to the triphosphates.

It was found that there was no transfer in the absence of the enzyme, DNA or pyrophosphate, and only very limited transfer in the absence of the four triphosphates. However, the omission of one, two or even three of the triphosphates from the incubation mixture did not greatly affect the reaction.

An interesting feature of the work was the observation that when ^{32}P-labelled DNA and cold pyrophosphate were used, little transfer could be demonstrated in presence or absence of the four triphosphates, whereas the same DNA, with ^{32}P-labelled pyrophosphate and the four triphosphates produced the usual amount of transfer.

Similar, though less exhaustive studies, on the reversal of the DNA polymerase reaction, were carried out by Bollum (Bollum, F.J. J. Biol. Chem. 1960, 235, 2399) using a partially purified fraction from calf thymus glands. The results obtained were very similar to those of Bessman but no experiments using labelled DNA were performed.

As the DNA polymerase, which had been demonstrated in Ehrlich ascites tumour, in this department (see

General Introduction, page 8), exhibited properties in its forward reaction which were very similar to those found by Kornberg, for E. coli, and Bollum, for calf thymus, it was of interest to know if this similarity extended to the back reaction and so this work was undertaken.

8. Methods.

8.1 Enzyme source.

Three sources of enzyme were used, Ehrlich ascites carcinoma, rabbit thymus and calf thymus. The tumour was maintained by serial transplantation in mice of the departmental colony. The rabbit thymus glands were obtained from young rabbits of the departmental colony weighing about 1500 gms. Calf thymus glands were obtained fresh from the slaughterhouse.

8.2 Preparation of extracts.

a) Tumour extract.

The tumour extract was prepared as described in Part I, 2.2.

b) Rabbit thymus homogenate.

Chopped rabbit thymus glands were homogenised for 2 mins. in 10 volumes of ice-cold distilled water using a Potter-type homogeniser. The resulting suspension was centrifuged at 105,000 x g. for 1 hour, to obtain a clear extract.

c) Calf thymus homogenate. (Bollum, F.J. J. Biol. Chem. 1960, 235, 2399).

Calf thymus glands were obtained within 30 minutes after death of the animal, packed in ice and transported to the laboratory. All subsequent operations were per-

formed in a cold room or in refrigerated centrifuges at 0 - 4°. After the extraneous tissue had been removed the glands were cut into ½ to 1 inch cubes, placed in a gauze bag, and washed in 0.15 M sodium chloride. When the excess liquid had drained, 1 kg. portions of the cubed glands were blended for 60 seconds (at alternate 15 second periods of low and high speeds in a Waring Blendor) in 3 litres of a buffered sucrose homogenisation medium. This medium consisted of 1 litre of 1.0 M sucrose; 48 ml. of 1.0 M Tris buffer, pH 8.1; and 24 ml. of 1 M potassium chloride diluted to 4 litres with glass distilled water. The macerated tissue was then transferred to 250 ml. cups and centrifuged for 20 minutes at 15,000 x g. To avoid release of tissue nucleases, only enough tissue to fill the available centrifuge was blended at one time. The supernatant fraction from the homogenate was collected in large aspirator bottles or separatory funnels to allow fatty material to float to the surface. It was then distributed in Spinco tubes and centrifuged at 78,000 x g. for 60 minutes in a No. 30 rotor of the Spinco preparative centrifuge. The supernatant fluid so obtained was used as a source of enzyme.

8.3 Fractionation of the tumour extract.

The crude extract of Ehrlich ascites tumour was

adjusted to pH 4.5 by careful addition of N acetic acid at 0° (final adjustment with N/10 acetic acid). The precipitate was collected by centrifugation, suspended in 50 ml. of water and quickly neutralised with 0.3 N sodium hydroxide, to a final pH between 7.2 and 7.4 to give Fraction 1, containing about 5 mg. of protein/ml.

Fraction 1 was further fractionated by the addition of solid ammonium sulphate until the solution was 30% saturated. The resulting precipitate was allowed to stand for 30 minutes at 0° before it was removed by centrifugation at 3000 x g., in the cold, for 30 minutes. The supernatant fluid was brought to 40% saturation by the further addition of solid ammonium sulphate and allowed to stand for 30 minutes at 0°. The precipitate, which was centrifuged down as before, was redissolved in about 15 ml. of water and dialysed against 0.01M tris containing 10^{-4}M mercaptoethanol. This dialysed material was termed Fraction 2 and contained about 5 mg. of protein/ml.

Fraction 1 was also fractionated to obtain the protein fraction precipitable between 25 - 50% ammonium sulphate saturation in the manner described for Fraction 2. This fraction was termed Fraction 3, and contained about 5 mg. of protein/ml.

8.4 Estimation of protein.

Protein was estimated by the method of Lowry et al. (see section 2.4b).

8.5 Preparation of highly polymerised DNA (Kay, E.R.M., Simmons, N.S. and Dounce, A.L. J. Amer. Chem. Soc. 1952, 74, 1724).

100 ml. of Ehrlich ascites carcinoma cell suspension was centrifuged at 1500 x g. for 10 minutes to pack the cells, and the supernatant fluid was discarded. The cells were washed repeatedly with 0.1M phosphate buffer, pH 8.0, to remove contaminating red cells and were lysed by the addition of 12 times the packed cell volume of distilled water followed by homogenisation in a Potter-type homogeniser. The resulting material was centrifuged at 4,500 x g. for 30 minutes. The supernatant fluid was discarded and the residue was homogenised with 0.9% sodium chloride/0.01M sodium citrate solution and made up to about 200 ml. with the same solution. The resulting material was centrifuged at 900 x g. for 10 minutes and the supernatant fluid discarded. This was repeated three times in all. The sediment finally obtained was suspended in 0.9% sodium chloride solution, homogenised in a Potter-type homogeniser and made up to 300 ml. with more 0.9% sodium chloride solution. 27 ml. of Duponal (5% sodium dodecyl sulphate in 45% ethanol) were added with stirring and stirring

continued for 30 minutes, then 16.5 gms. of sodium chloride were added to give a 1 M solution. Stirring was continued until all the sodium chloride had dissolved and then the mixture was centrifuged at 20,000 x g. for 30 minutes. The supernatant liquid was decanted and 2 volumes of ethanol added with swirling. The DNA, which precipitated in thread-like strands, was collected on a glass rod and washed 3 times in ethanol and 3 times in acetone and allowed to dry. The DNA thus obtained was further purified as follows. The DNA was chopped into small pieces and dissolved in 300 ml. of water at room temperature using a Vibromix stirrer. 27 ml. of Duponal were added and the mixture stirred for 30 minutes before 20-25 gm. of sodium chloride were added. The stirring was continued until the sodium chloride had dissolved and then the mixture was centrifuged at 20,000 x g. for 30 minutes. The DNA was precipitated, washed and dried as before. The DNA obtained in this way was redissolved again, this time in 200 ml. of water and 1.8 gm. of sodium chloride added and dissolved. The resulting mixture was centrifuged as before and 10 gms. of sodium chloride dissolved in the supernatant fluid. 2 volumes of ethanol were added to precipitate the DNA which was collected, washed and dried as before. The yield of DNA was 120 mg.

8.5a Preparation of highly polymerised DNA, labelled with tritiated thymidine (^{3}H-TDR).

Ehrlich ascites carcinoma supplemented by the addition of glucose (5 μmole/ml.) and ^{3}H-TDR (10 μC/ml.) was incubated in air (5 ml. portions in 25 ml. conical flasks) at 37° for 4 hrs. The DNA was isolated as described above and contained about 0.7 - 2.0 x 10^6 cpm/mg. as determined in a windowless gas flow counter.

8.5b Preparation of "heated" DNA.

The term "heated" DNA, when used in this work refers to DNA, prepared as above, which was heated at 100° for 15 minutes at a concentration of about 1 mg./ml. in water.

8.6 Incubation procedure.

Incubation of the enzyme solution with the appropriate additions was carried out in air in stoppered 25 ml. conical flasks at 37° with shaking. The total volume of the reaction mixture was 5 ml. All reactions were terminated by immersion of the flasks in a mixture of solid carbon dioxide and ethanol.

8.7 Analytical procedures.

To each of the thawed incubation mixtures (volume, 5 ml.) 2.5 ml. of ice-cold 2.1 N perchloric acid was added to precipitate the proteins and nucleic acids. The mixture was stirred thoroughly and centrifuged at

600 x g. for 5 minutes at 0^{o}, and the supernatant fluid decanted into a clean tube. This "acid soluble fraction" was then subjected to one of three analytical procedures. Procedures A and C were used when the labelled compound used in the incubations was ^{32}P-inorganic pyrophosphate, whereas B was used in experiments with DNA labelled with titrated thymidine.

Procedure A.

The acid soluble fraction was passed through a column of activated charcoal (prepared as described in Part II, section 5.3) and the effluent collected. The charcoal was then washed with 0.01 M sodium bicarbonate until the radioactivity of the washings had fallen to a consistently low level. The combined effluent and washings were diluted 1 in 50 and a 5 ml. sample counted in an M.6 liquid counter. The charcoal was further washed with 70% ethanol/0.14 M ammonia solution until the activity of the eluate had fallen to a low level, and the total eluate concentrated *in vacuo* and counted. When the eluate contained sufficient radioactivity, it was spotted on paper and run for 65 hrs. in the ethanol/ammonium acetate/borate solvent described in Part I, section 2.6. The chromatogram was sectioned in 2 cm. strips which were digested with sulphuric acid,

and counted.

The charcoal was regenerated as described in section 5.3.

Procedure B.

The acid soluble fraction was neutralised at 0° with 7 N KOH (final adjustments with 1 N KOH) and a sample chromatographed, with markers of TDR, TMP and TTP, in the isobutyrate solvent described in Part I, section 2.6. The resulting spots were located in U.V. light, cut out, eluted, and samples counted in a windowless gas flow counter.

Procedure C.

The neutralised acid soluble fraction was poured onto a small column (3 x 1 cm.) of Dowex-1-chloride resin and the effluent collected. The column was then washed with 0.1 M sodium carbonate until the activity of the eluate was very low (about 100 column volumes). The column was then washed with 1 M ammonium bicarbonate until the eluate activity again reached a very low level. The total ammonium bicarbonate eluate was counted in a liquid counter, or where more sensitive determination was necessary it was concentrated and a sample counted in a windowless gas flow counter.

The column was regenerated by the passage of 1 M ammonium chloride solution, then washing with water.

The resin was packed in columns of the type shown in Figure 14, thereby facilitating the washing etc. of numerous small columns with relatively large amounts of eluent.

9. Results and Discussion.

9.1 The method of Bessman et al. (see section 7).

It was decided to study the pyrophosphorolysis of DNA in Ehrlich ascites tumour extracts by observing the transfer of ^{32}P-labelled inorganic pyrophosphate to the deoxyribonucleoside triphosphates and trial experiments were carried out, in which attempts were made to separate inorganic pyrophosphate from the deoxyribonucleoside triphosphates, by the method of Bessman et al. It was found, however, that between 0.3 and 0.5% of the

inorganic pyrophosphate remained on the Norite even after twice the prescribed number of washings. This difference in behaviour between the Norite used by Bessman et al. and the Norite used in this work is not surprising since different batches have been shown (by Dr. Mills and other in this department) to have widely differing properties.

Since the reaction demonstrated by Bessman et al. with the highly purified DNA polymerase from E. coli only occurred to the extent of 0.35% it seemed likely that the amount of activity retained on the charcoal would be sufficient to mask any reaction that might occur in crude extracts of Ehrlich tumour, and it was therefore decided to try another separation procedure.

9.2 Procedure A (see section 8.7).

In an attempt to improve on the Norite procedure this charcoal column technique was developed from the method used in the purification of deoxyribonucleoside triphosphates which have been prepared by chemical means. Trial experiments indicated that with increased washing with 0.01M sodium bicarbonate the inorganic pyrophosphate remaining could be reduced to about 0.1% of the amount applied. Unfortunately when this procedure was used in attempts to demonstrate pyrophosphorolysis two difficulties arose, (a) poor duplication between columns, i.e., the volume of bicarbonate solution required to

remove a given amount of pyrophosphate varied from column to column, so that control values did not always agree, and (b) radioactivity continued to be released from some 'test' columns even after prolonged washing with bicarbonate.

A few experiments were carried out using this system (a typical set of results are shown in Table 15) and although the data obtained were not completely reliable there seemed to be evidence for the occurrence of very limited pyrophosphorolysis of DNA in crude extracts of the tumour. The extent of the reaction appeared to be unaffected by the addition of small amounts of the deoxyribonucleoside triphosphates. The method was so variable, however, that it was abandoned in favour of procedure B.

2.3 Procedure B (see section 8.7).

In this method DNA labelled with tritrated thymidine (DNA/TDR-H^3, see section 8.5a) was incubated with unlabelled inorganic pyrophosphate and the appropriate additions in extracts of Ehrlich ascites tumour. Samples of the acid soluble fraction (neutralised) after incubation were chromatographed on paper with TTP, TMP and TDR markers in the ammonium isobutyrate solvent (see section 2.6). The spots thus obtained were excised, eluted and samples of the eluates counted in a windowless gas flow counter.

Table 15.

Evidence for the pyrophosphorolysis of DNA in crude extracts of Ehrlich ascites carcinoma.

System	^{32}P-nucleoside triphosphate* mμmole
Complete system	0.89
" "	0.71
Omit TTP, dATP, dGTP & dCTP	0.95
" " " " " "	0.80
Omit inorganic pyrophosphate‡	0.76
" " "	0.68
Omit extract	0.63
" "	0.66

The complete system contained: TTP, dATP, dGTP and dCTP (5 μmoles/ml. each), DNA (Ehrlich ascites, 50 μg./ml.), $MgCl_2$ (5 μmoles/ml.), tris buffer, pH 7.7 (100 μmoles/ml.), 2-mercapto-ethanol (1μmole/ml.), inorganic pyrophosphate (6 μmoles/ml., 5×10^5 cpm/μmole) and crude Ehrlich ascites tumour extract (3 ml., 10 mg. of protein) in a final volume of 5 ml.

* This material was shown by chromatography in the ethanol/ammonium acetate/borate solvent (see section 2.6), to contain a large amount of ribonucleotide, about 55%.

Table 15 (contd.)

‡ Inorganic pyrophosphate was omitted from the incubation mixture but added to the cold incubation mixture after incubation and immediately before the mixture was deproteinised.

By this means it was hoped to detect pyrophosphorolysis in an increase in the radioactivity of the TTP spot.

Experiment (1) The effect of incubating heated (see section 8.5b) and unheated labelled DNA with Ehrlich ascites tumour extract in the presence and absence of inorganic pyrophosphate was studied. The results, which are shown in Figure 7, were difficult to interpret. In the case of unheated DNA the total radioactivity released in the presence of inorganic pyrophosphate, was more than double the control value, but there was no evidence of increased TTP formation. When heated DNA (which has been shown by Bollum and others to be a better primer than unheated DNA) was used the total radioactivity released in the presence of inorganic pyrophosphate was slightly less than the control value, which had risen to more than three times the control value for unheated DNA. There was some evidence, however, of a slight increase in TTP formation after 60 minutes incubation, although by 120 minutes the TTP activity had dropped almost to zero.

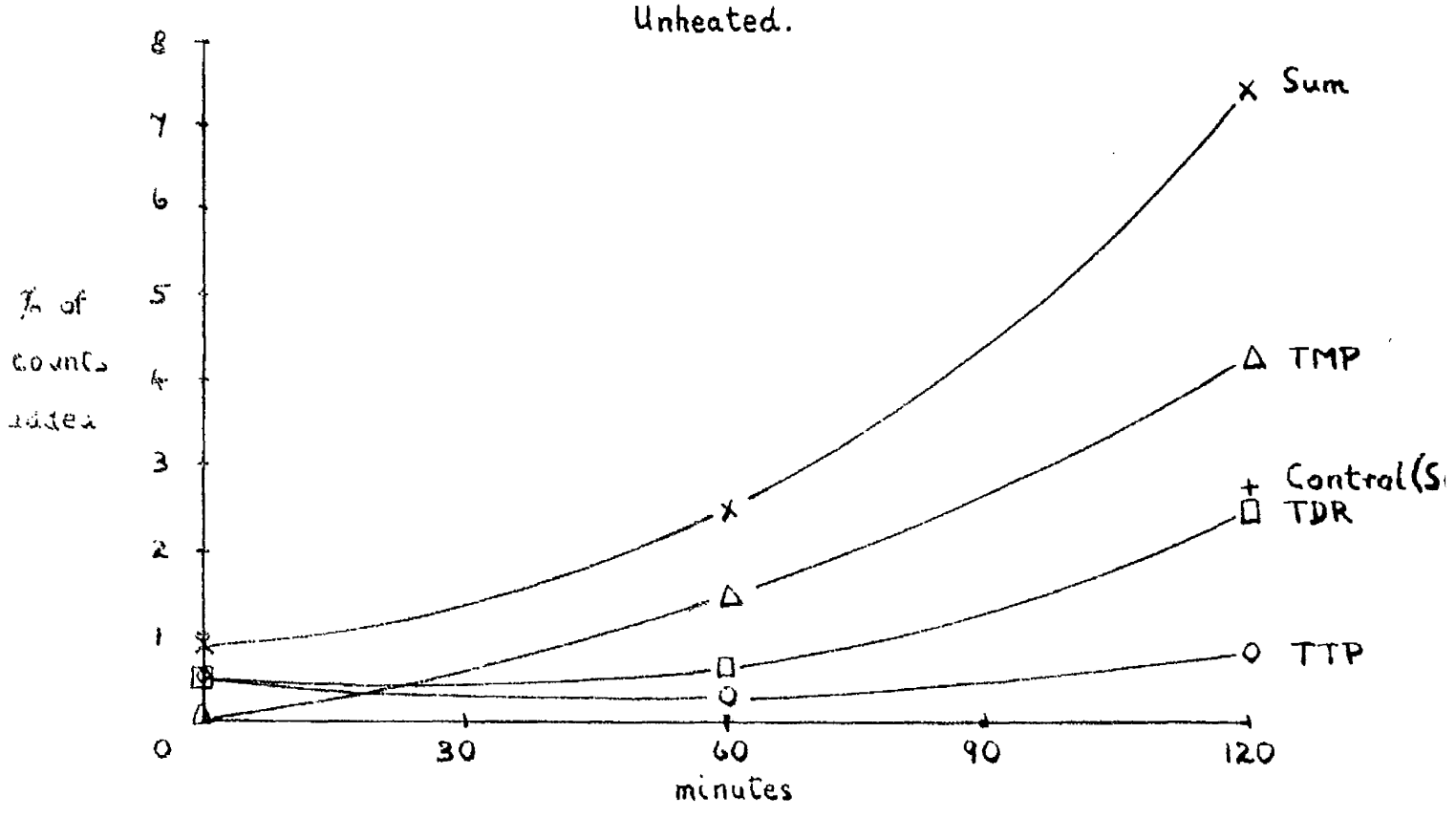
Experiment (2) In order to assess the significance of these results the effect of variation in inorganic pyrophosphate concentration on the release of radioactivity from heated and unheated DNA in the presence of the tumour extract was studied. The results (see

Figure 7.

The effect of incubation time on the breakdown of heated and unheated DNA in the presence of crude extracts of Ehrlich ascites carcinoma and inorganic pyrophosphate.

Incubation mixtures contained: DNA/^{32}P-II (200 µg./ml., 0.7 x 10^6 cpm/µg.), $MgCl_2$ (5 µmoles/ml.), tris buffer, pH 7.7 (300 µmoles/ml.), 2-mercaptoethanol (1 µmole/ml.), inorganic pyrophosphate (10 µmoles/ml.) and crude extract of Ehrlich ascites carcinoma (3 ml., 9 mg. of protein) in a final volume of 5 ml. The mixtures were incubated at 37°.

Inorganic pyrophosphate was omitted from the controls.


Unheated.
% of counts added
8
7
6
5
4
3
2
1
0
30
60
90
120
minutes
Sum
TMP
Control (S
TDR
TTP

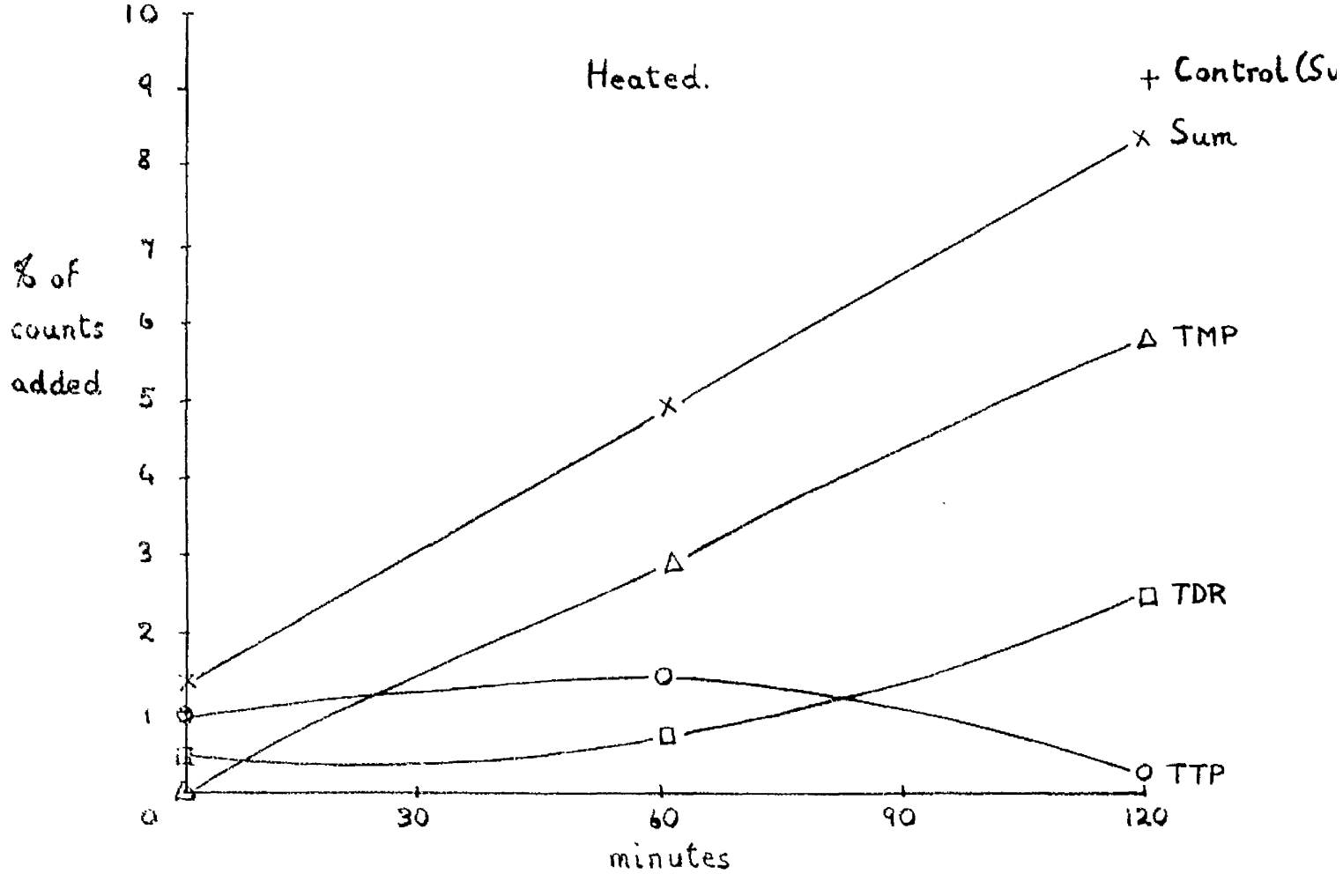

Heated.
% of counts added
10
9
8
7
6
5
4
3
2
1
0
30
60
90
120
minutes
+ Control (Su
Sum
TMP
TDR
TTP

Figure 8) contained several points of interest. In the case of unheated DNA the total radioactivity released after 2 hrs. incubation in the absence of inorganic pyrophosphate differed considerably from the value obtained in the previous experiment (see Figure 7). Most of the radioactivity, however, was associated with TDR and the value dropped sharply on the addition of small amounts of inorganic pyrophosphate. A repeat of experiment (2) gave the same high value for the total radioactivity released in the absence of inorganic pyrophosphate. At no concentration of inorganic pyrophosphate was there evidence for increased TTP formation, or an increase in the total radioactivity released, as compared with controls containing no pyrophosphate.

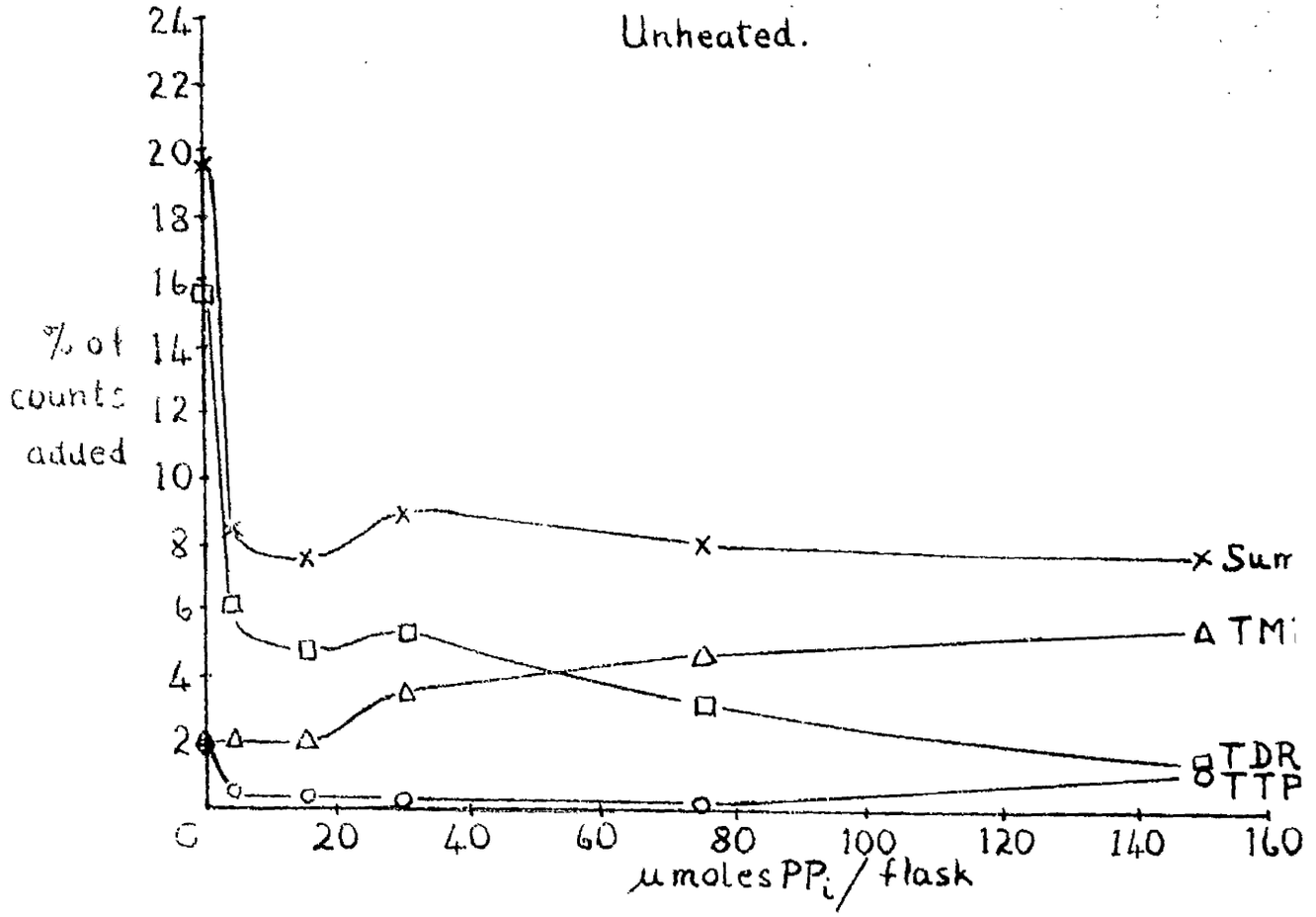
Heated DNA in the absence of inorganic pyrophosphate gave the same high TDR value but the total radioactivity released was considerably higher, but in contrast to unheated DNA it showed increased TTP formation at an inorganic pyrophosphate concentration of 3 μmoles/flask and an increase in the total activity released at 30 μmoles/flask.

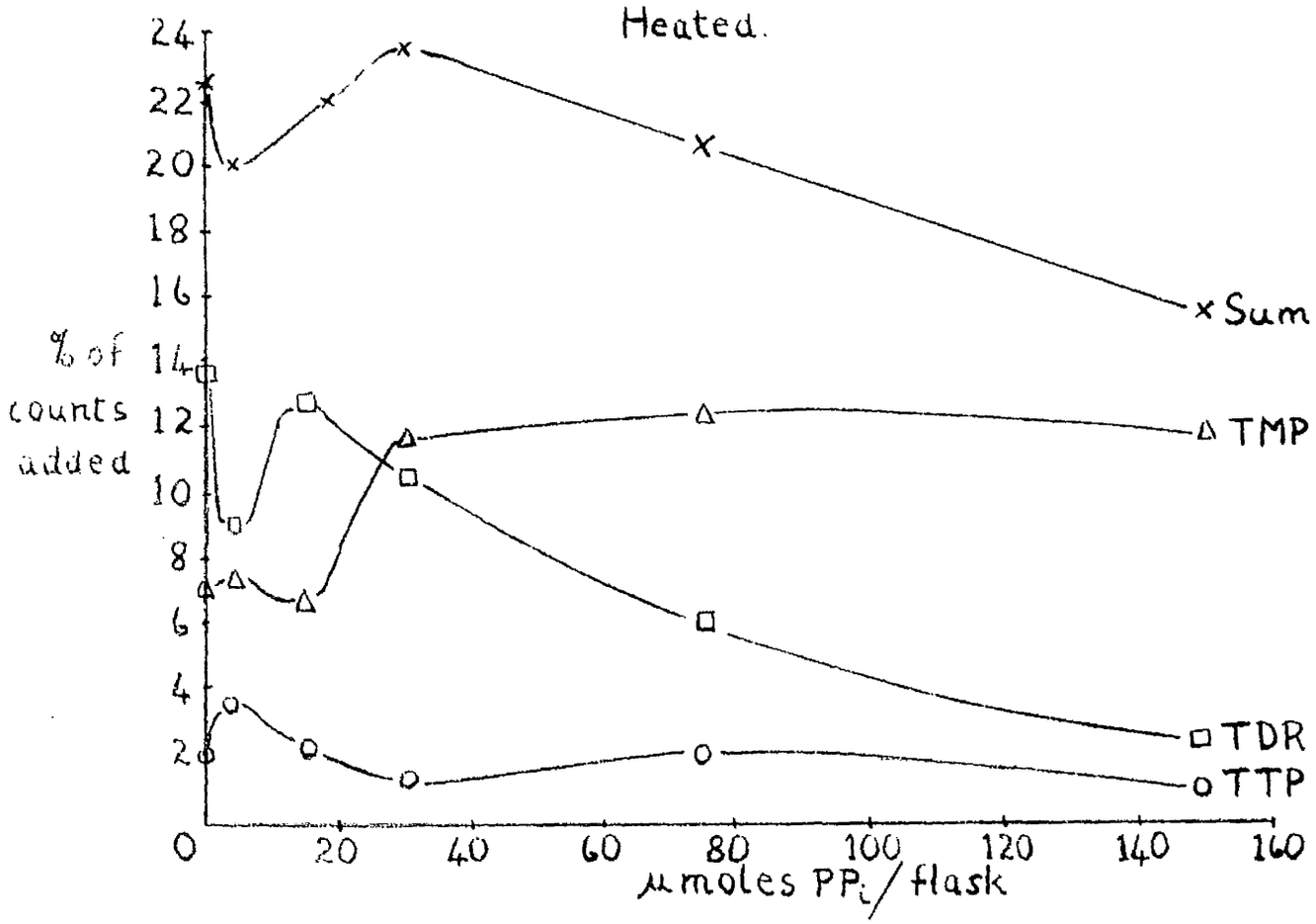
Experiment (3) On the basis of these findings it was decided to study the effect of incubation time on the

Figure 8.

The effect of inorganic pyrophosphate concentration on the breakdown of heated and unheated DNA in the presence of crude extracts of Ehrlich ascites carcinoma.

Incubation mixtures contained: DNA/TDR-H^3 (200 µg/ml., 0.7×10^6 cpm/µg.), $MgCl_2$ (5 µmoles/ml.), tris buffer, pH 7.7 (100 µmoles/ml.), 2-mercaptoethanol (1 µmole/ml.), inorganic pyrophosphate (various) and Ehrlich ascites carcinoma, crude extract (5 ml., 9.5 mg. of protein) in a final volume of 5 ml. The mixtures were incubated at 37° for 2 hrs.


Unheated.
% of counts added
24
22
20
18
16
14
12
10
8
6
4
2
0
20
40
60
80
100
120
140
160
μmoles PPi / flask
Sum
TMP
TDR
TTP


Heated.
% of counts added
24
22
20
18
16
14
12
10
8
6
4
2
0
20
40
60
80
100
120
140
160
μmoles PPi / flask
Sum
TMP
TDR
TTP

release of radioactivity from heated DNA in the presence of Ehrlich tumour extract plus 3 μmoles and 30 μmoles of inorganic pyrophosphate per flask. The results are shown in Figure 9. The most striking feature of these was the smaller total release of radioactivity in the presence of inorganic pyrophosphate as compared with the previous experiment. The control value for the total release appeared to be almost identical to that obtained in experiment (2) but this was due to an increased contribution from TDR, the TTP and TCP values being lower than in experiment (2).

Experiment (4) In view of these findings an experiment was performed in which control, and test reactions containing 30 μmoles of inorganic pyrophosphate per flask, were compared at different time intervals. The results are shown in Figure 10. No evidence was obtained for the occurrence of pyrophosphorolysis at any time during the incubation. In fact the total amount of activity released and of radioactivity in TTP were lower in the presence of inorganic pyrophosphate.

The experiment was repeated and almost identical results were obtained.

From the results thus far discussed it seemed clear that if any pyrophosphorolysis was occurring it was being masked by further enzyme activity, and so it

Figure 9.

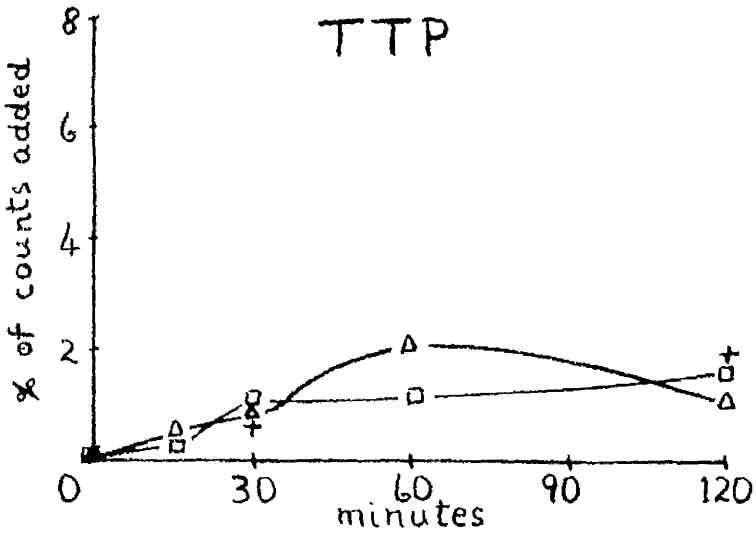
The effect of incubation time on the breakdown of heated DNA in the presence of crude extracts of Ehrlich ascites carcinoma and two concentrations of inorganic pyrophosphate.

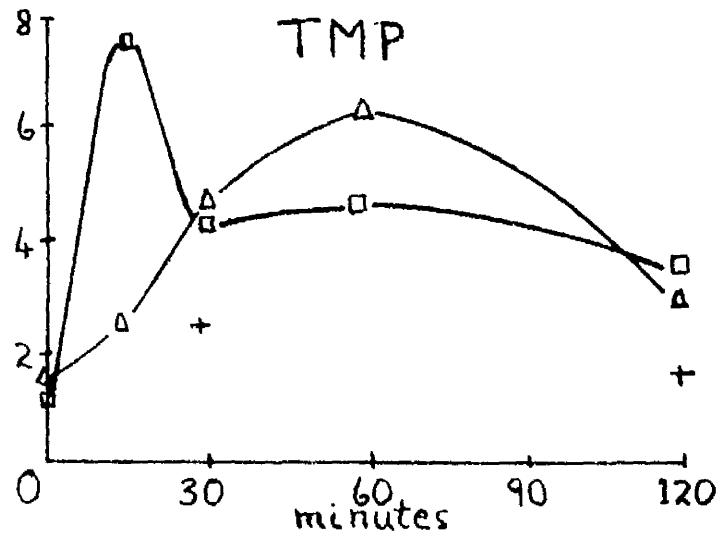
Incubation mixtures contained: DNA/TDR-H^{3} (heated; 200 µg/ml., 0.7 x 10^{6} cpm/µg.), $MgCl_2$ (5 µmoles/ml.), tris buffer pH 7.7 (10^{2} µmoles/ml.), 2-mercaptoethanol (1 µmole/ml.), inorganic pyrophosphate (3 µmoles/flask, or, 30 µmoles/flask) and crude extract of Ehrlich ascites carcinoma (5 ml., 9 mg. of protein) in a final volume of 5 ml. The mixtures were incubated at 37°.

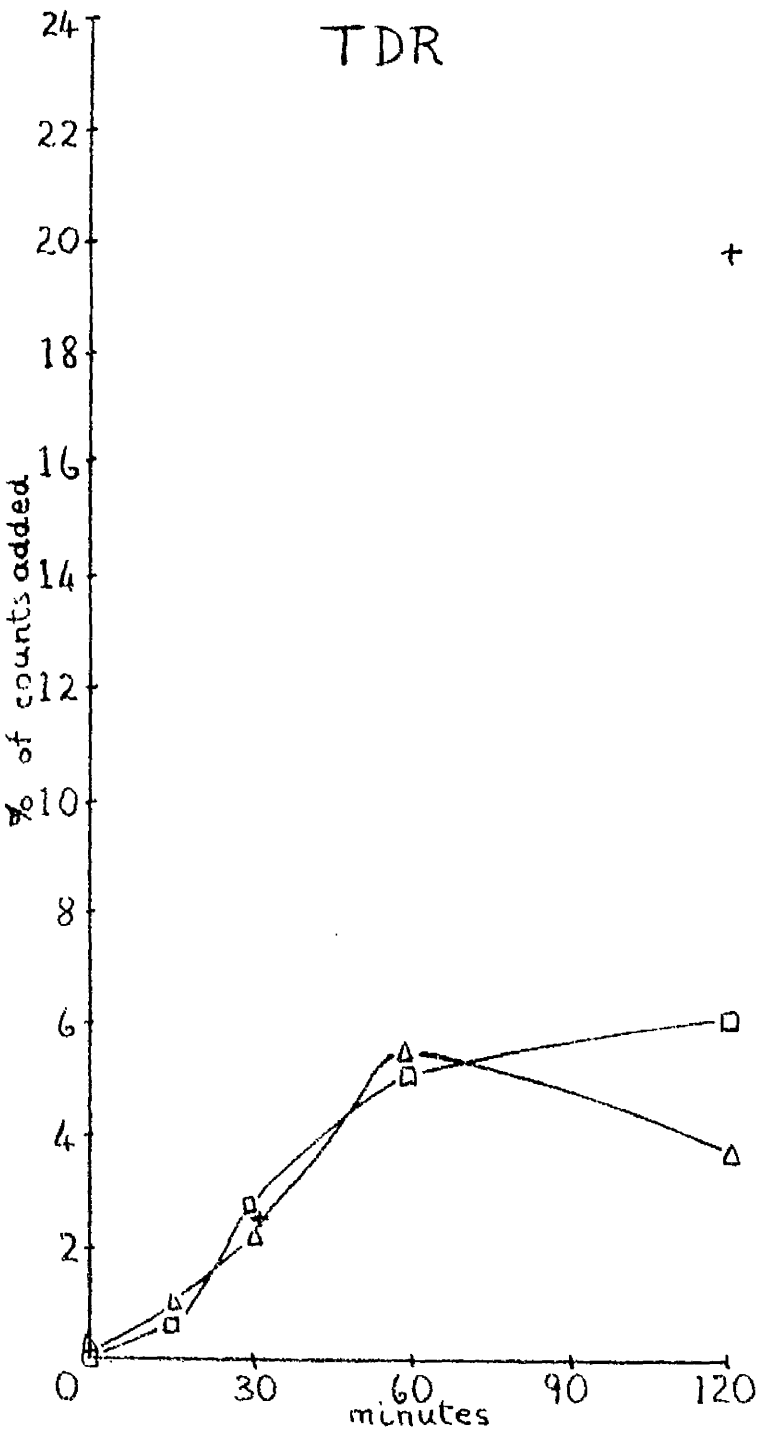
Inorganic pyrophosphate was omitted from the controls.

per flask

per flask







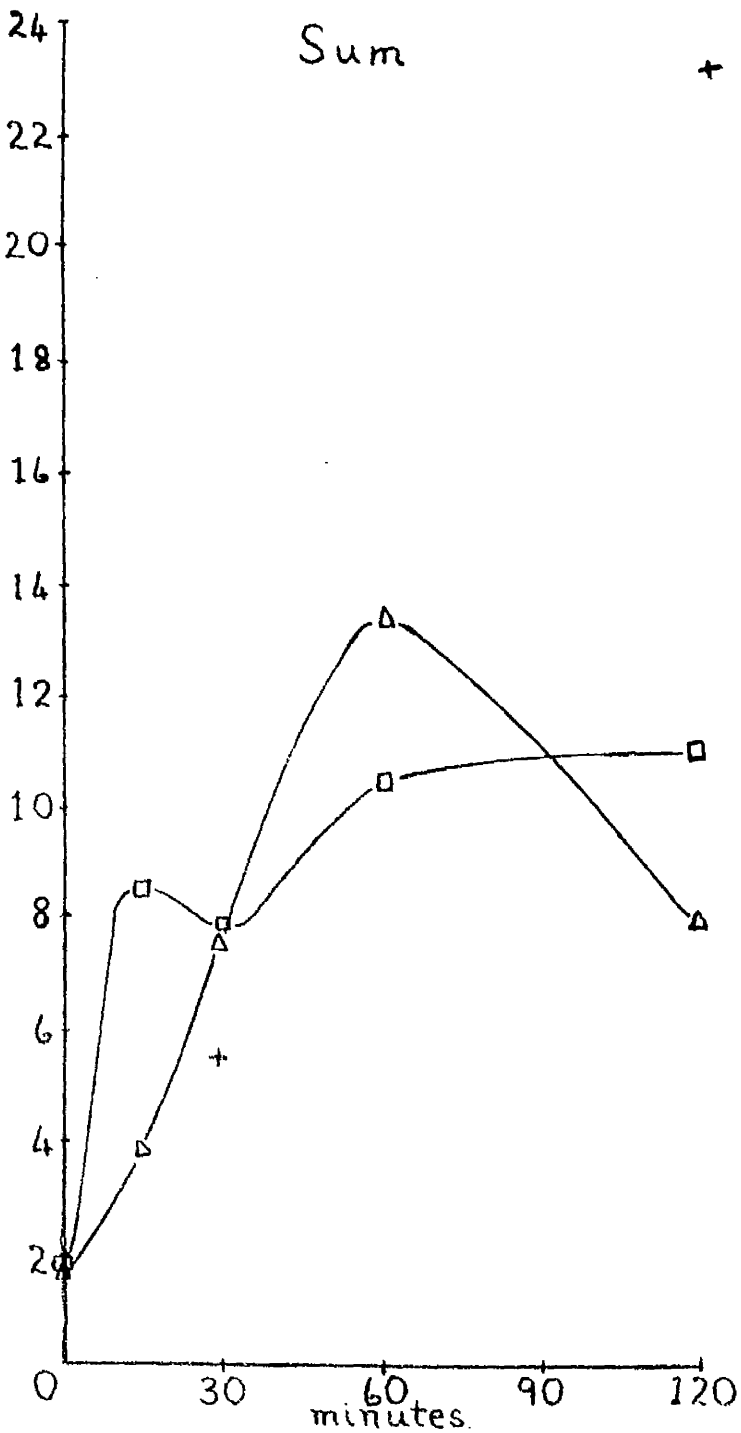
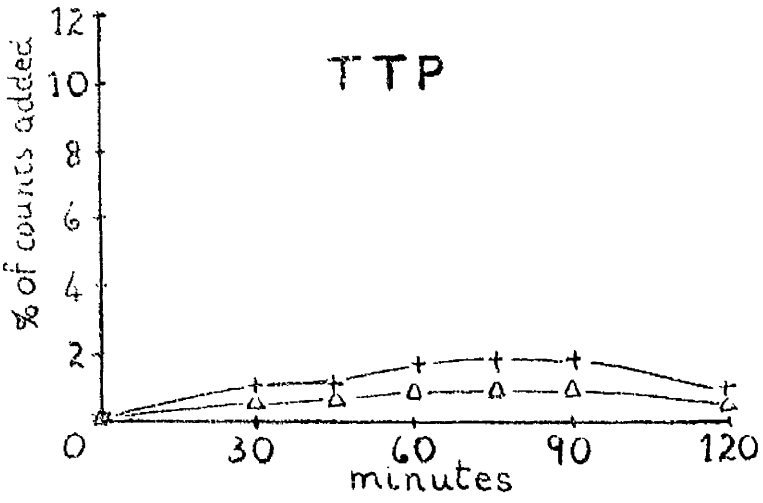


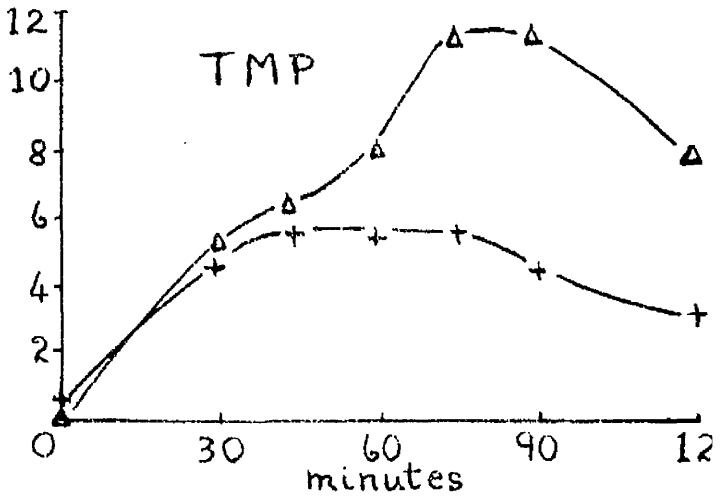
Figure 10.

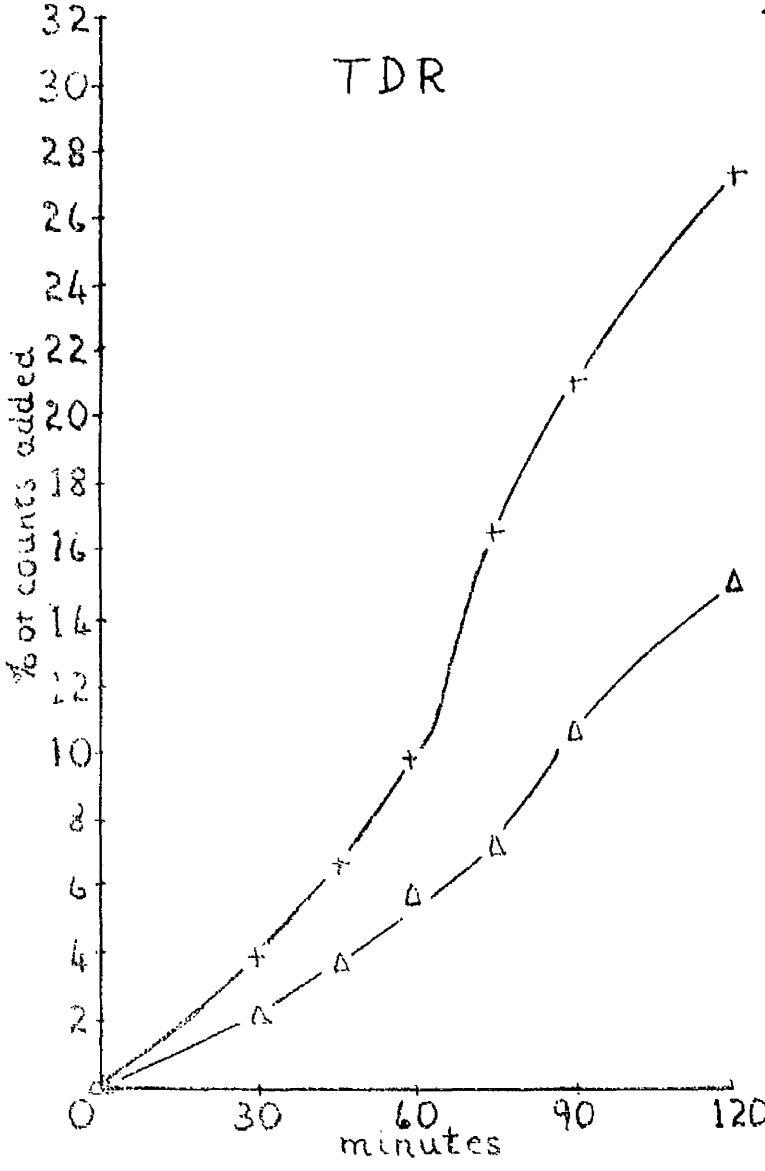
The effect of incubation time on the breakdown of heated DNA in the presence of crude extracts of Ehrlich ascites carcinoma and inorganic pyrophosphate.

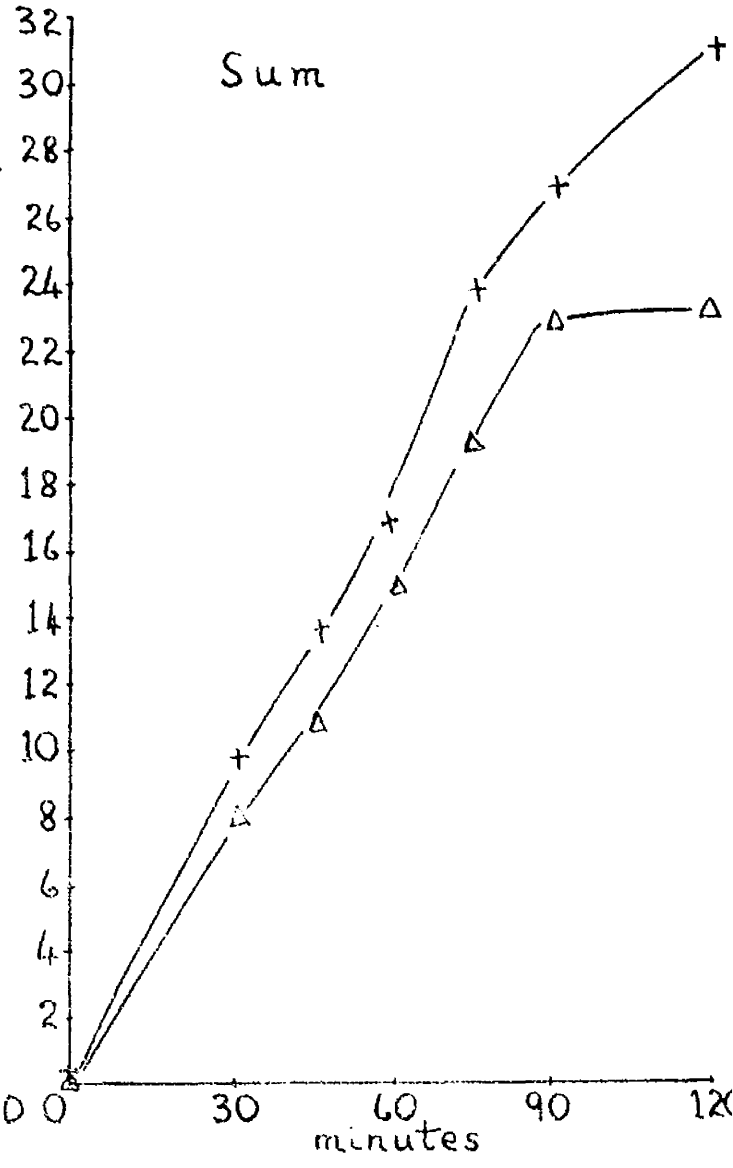
Incubation mixtures contained: DNA/TDR-H^3 (heated 200 µg./ml.; 1.25 x 10^6 cpm/mg.), $MgCl_2$ (5 µmoles/ml.), tris buffer pH 7.7 (100 µmoles/ml.), 2-mercaptoethanol (1 µmole/ml.), inorganic pyrophosphate (30 µmoles/flask) and crude extract of Ehrlich ascites carcinoma (3 ml., 8.5 mg. of protein) in a final volume of 5 ml. The mixtures were incubated at 37°. Inorganic pyrophosphate was omitted from the controls.

△ 30 μmoles PP_i per flask + Control









was decided to use a partially purified enzyme source. The purification was carried out (see section 8.3) according to the methods of Dr. E.D. Gray (Gray, E.D., Weissman, S.M., Richards, J., Bell, D., Keir, H.M., Smellie, R.M.S. and Davidson, J.N. Biochim. Biophys. Acta 1960, 45, 111; and private communication).

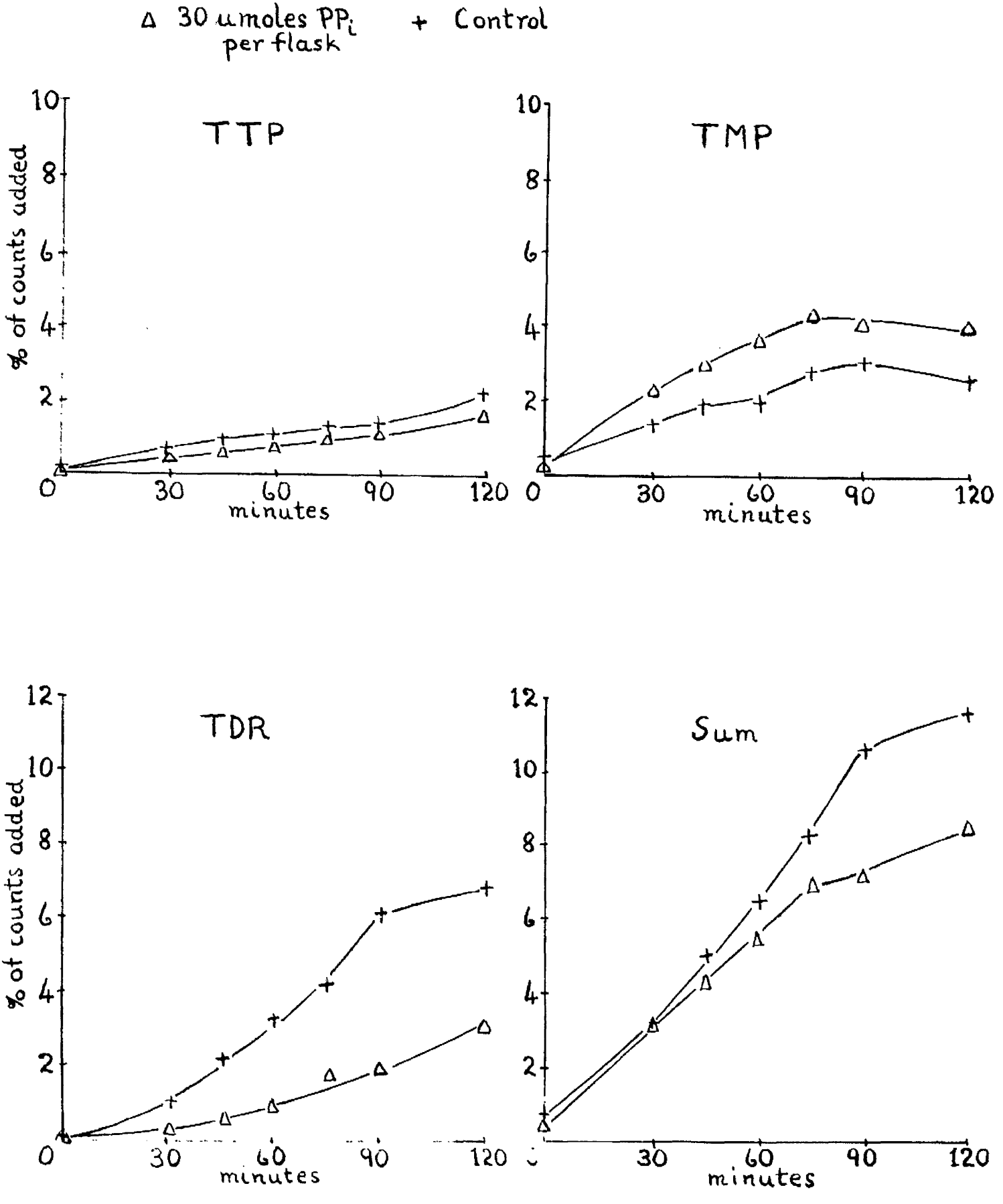
Experiment (5) Labelled DNA was incubated with the partially purified Ehrlich ascites polymerase (fraction 1, see section 8.3) and the appropriate additions in the presence and absence of 30 μmoles of inorganic pyrophosphate for various times at 37°. The results which are shown in Figure 11, indicated that the fractionation had greatly reduced the ability of the extract to release activity as TDR. The formation of TMP had also been reduced whereas the proportion of TTP formed appeared to have increased. The relationship between the control and the test remained the same, however, i.e. there was no evidence for pyrophosphorolysis. Due to the variations in activity which the tumour extract had shown in previous experiments the apparent increase in TTP formation was not necessarily real. In spite of this it was decided to try a further fractionation step using ammonium sulphate precipitation. The DNA polymerase had been shown (Dr. E.D. Gray, private communication) to be

Figure 11.

The effect of incubation time on the breakdown of heated DNA in the presence of a partially purified polymerase fraction (fraction 1) from Ehrlich ascites carcinoma and inorganic pyrophosphate.

Incubation mixtures contained: DNA/TDR-H^3 (heated 200 µg./ml.; 1.25 x 10^6 cpm/mg.), $MgCl_2$ (5 µmoles/ml.), tris buffer pH 7.7 (100 µmoles/ml.), 2-mercaptoethanol (1 µmole/ml.), inorganic pyrophosphate (30 µmoles/flask) and fraction 1 of the Ehrlich ascites tumour extract (2 ml., 10 mg. of protein. See section 8.3) in a final volume of 5 ml.

The mixtures were incubated at 37°. Inorganic pyrophosphate was omitted from the controls.


Δ 30 μmoles PPi per flask
+ Control
TTP
TMP
TDR
Sum
% of counts added
minutes
0
30
60
90
120
2
4
6
8
10
12

precipitated between 30% - 40% saturation with ammonium sulphate. This fraction was prepared (see section 8.3) from fraction 1 and termed fraction 2.

Experiment (6) Fraction 2 was assayed for the ability to bring about pyrophosphorolysis of DNA at an inorganic pyrophosphate concentration of 30 μmoles/flask, and the results are shown in Figure 12. Although the total radioactivity released by this fraction in the presence of inorganic pyrophosphate was higher than in the control, most of the activity was present as TMP and not as TTP. The amount of TMP formed was even lower than in experiment (5).

In an attempt to obtain a more active enzyme preparation fraction 1 was further treated to give a fraction between 25% - 50% saturation with ammonium sulphate, termed fraction 3.

Experiment (7) Fraction 3 was assayed in the same manner as fraction 2 and the results, which are shown in Figure 13, indicated that there had been an overall reduction in enzyme activity and a return to the pattern of release of radioactivity obtained in experiment (5). A repeat of this experiment gave the same results.

This variation in results may have been due to variations in the tumour or to enzyme damage during

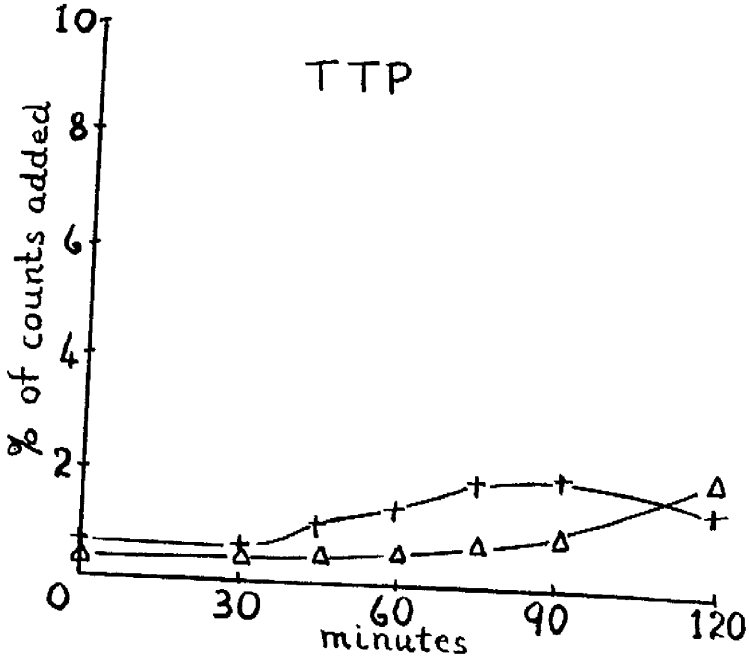
Figure 12.

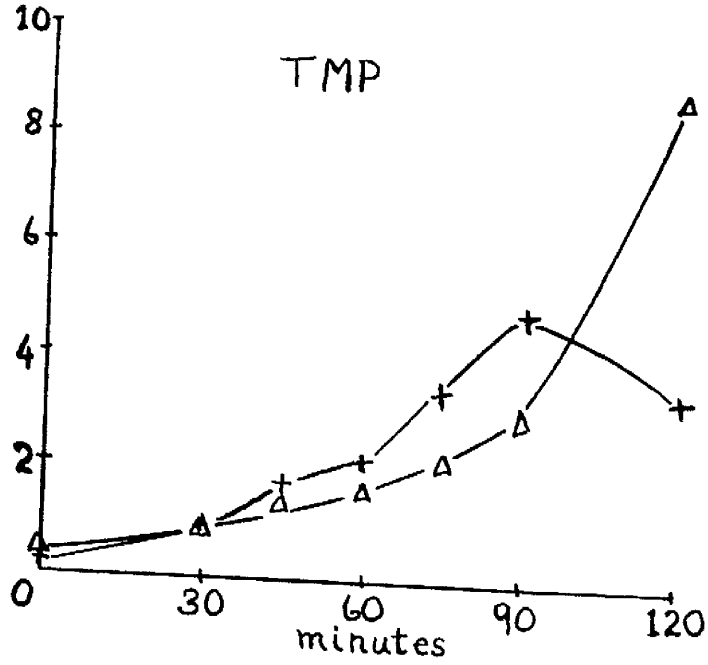
The effect of incubation time on the breakdown of heated DNA in the presence of partially purified polymerase fraction (fraction 2) from Ehrlich ascites carcinoma and inorganic pyrophosphate.

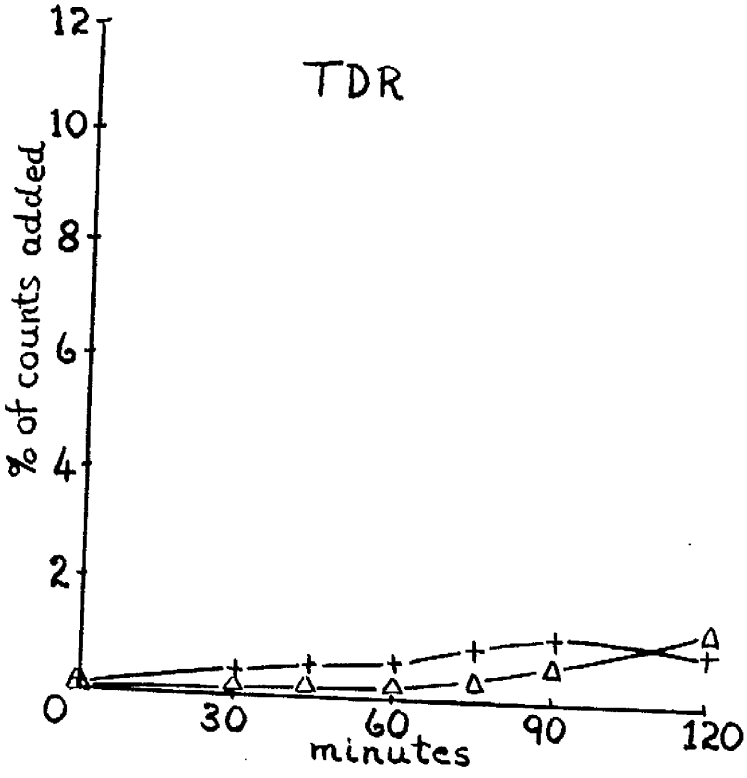
Incubation mixtures contained: DNA/EDR-H^3 (heated 200 µg./ml.; 1.25×10^6 cpm/mg.), $MgCl_2$ (5 µmoles/ml.), tris buffer pH 7.7 (100 µmoles/ml.), 2-mercaptoethanol (1 µmole/ml.), inorganic pyrophosphate (30 µmoles/flask) and fraction 2 of the Ehrlich tumour extract (2 ml., 10 mg. of protein. See section 8.3) in a final volume of 5 ml.

The mixtures were incubated at 37°. Inorganic pyrophosphate was omitted from the controls.

Δ 30 μmoles PP_i per flask + Control







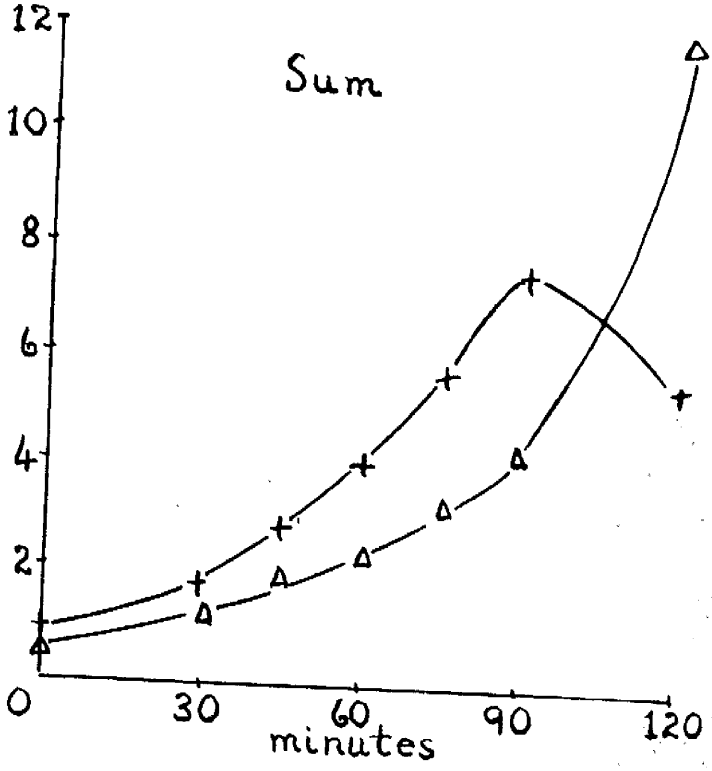
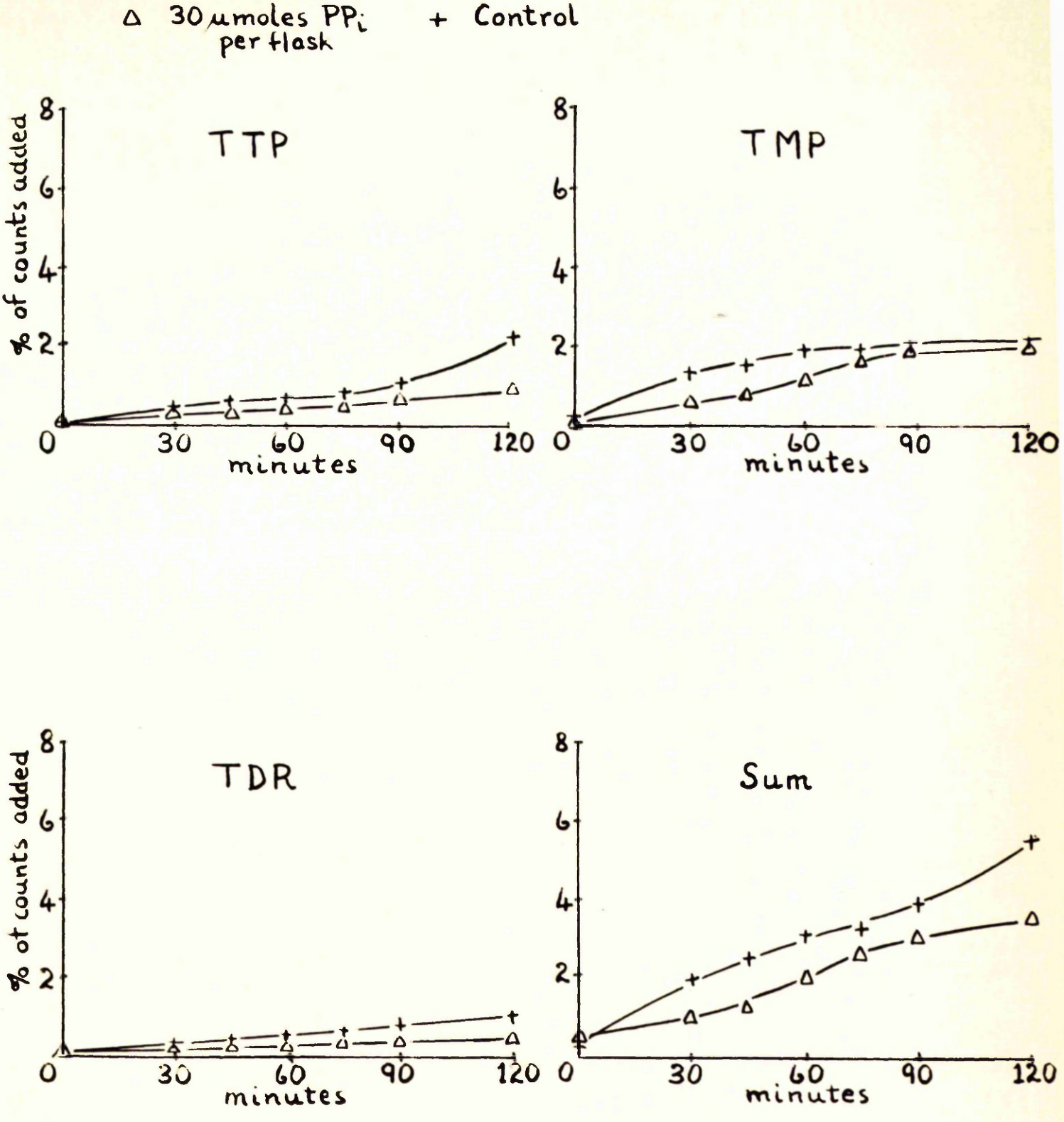


Figure 13.

The effect of incubation time on the breakdown of heated DNA in the presence of a partially purified polymerase fraction (fraction 3) from Ehrlich ascites carcinoma and inorganic pyrophosphate.

Incubation mixtures contained: DNA/TDR-H^3 (heated 200 µg./ml.; 1.25×10^6 cpm/mg.), $MgCl_2$ (5 µmoles/ml.), tris buffer pH 7.7 (100 µmoles/ml.), 2-mercaptoethanol (1 µmole/ml.), inorganic pyrophosphate (30 µmoles/flask) and fraction 3 of the Ehrlich tumour extract (2 ml., 9.5 mg. of protein. See section 8.3) in a final volume of 5 ml.

The mixtures were incubated at 37°. Inorganic pyrophosphate was omitted from the controls.


Δ 30 μmoles PPi per flask
+ Control
TTP
TMP
TDR
Sum
% of counts added
minutes
0
30
60
90
120
2
4
6
8

fractionation, but whatever the reason it seemed clear that this assay method suffered from the disadvantage of reflecting the activity of numerous enzymes and so being subject to considerable variation from one experiment to another.

In view of this it was decided to revert to using labelled inorganic pyrophosphate and to try and develop a new method of separating it from the deoxyribonucleoside triphosphates.

9.4 Procedure C (see section 8.7).

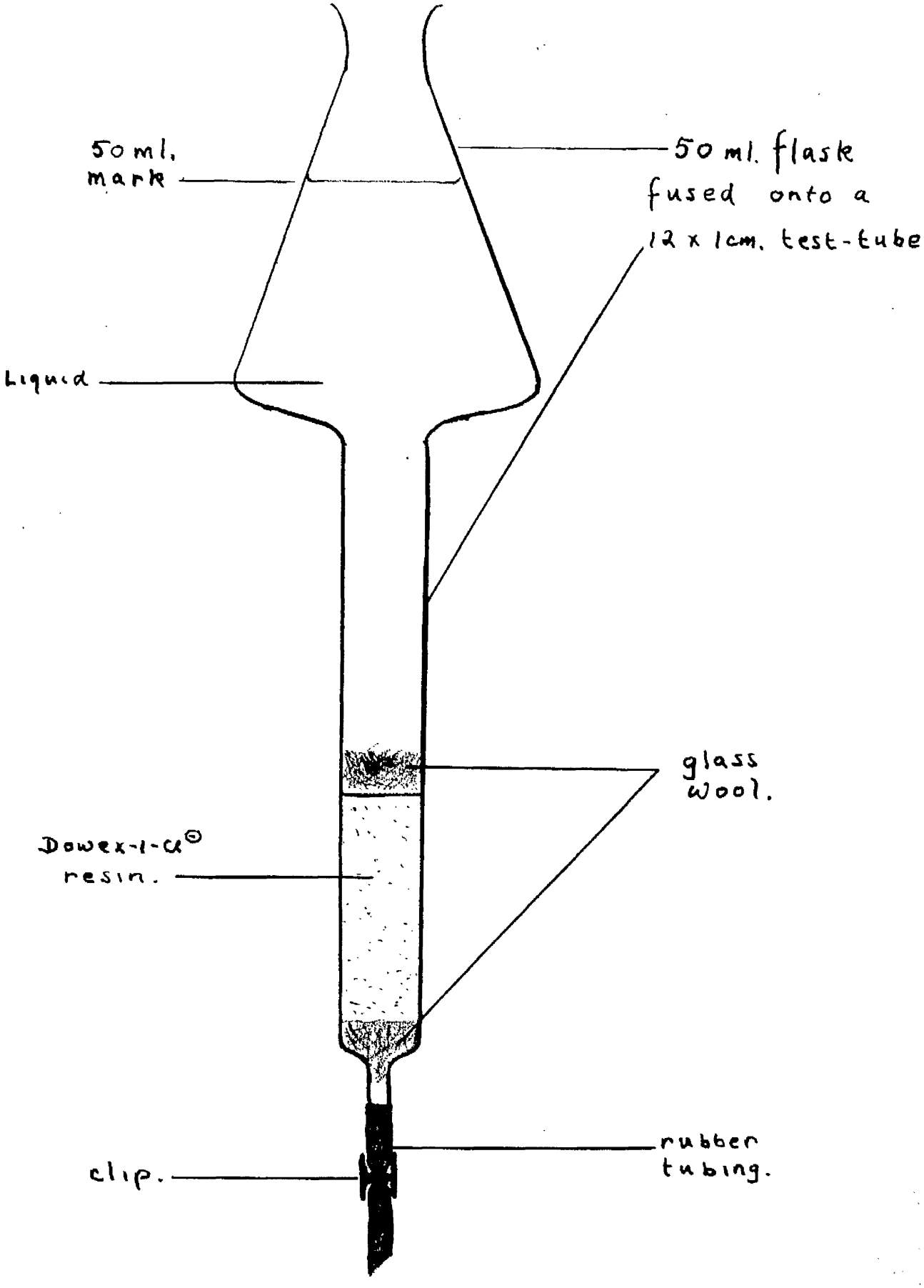
During the purification of pseudouridylic acid Waldo Cohn (Cohn, W.J. Biol. Chem. 1960, 235, 1488) discovered that nucleotides (such as AMP and CMP) not possessing an acid dissociation in the alkaline region in addition to the secondary phosphate dissociation, can be eluted from Dowex-1-formate by 0.1 M sodium carbonate solution. Pseudouridylate having this additional dissociation remained on the resin and was eluted with 0.2 - 0.4 M ammonium bicarbonate solution.

In the hope that inorganic pyrophosphate could similarly be eluted with 0.1 M sodium carbonate, leaving the deoxyribonucleoside triphosphates untouched, the following experiment was carried out.

A mixture of ^{32}P-labelled inorganic pyrophosphate,

Figure 14.

Diagram of the type of column used for the separation of inorganic pyrophosphate from the deoxyribonucleoside triphosphates on Dowex-1-chloride (Procedure C, see section 8.7).


50 ml. mark
50 ml. flask fused onto a 12 x 1cm. test-tube
Liquid
glass wool.
Dowex-1-X8 resin.
rubber tubing.
clip.

TTP, dATP, dGTP and dCTP was applied to a column of Dowex-1-chloride. The column was washed with 0.1 M sodium carbonate solution until no further activity was eluted and then with increasing concentrations of ammonium bicarbonate solution till all the U.V. absorbing material had been eluted. The 0.1 M carbonate solution removed all but 0.02% of the inorganic pyrophosphate activity and 1 M ammonium bicarbonate eluted all the triphosphates. Ammonium bicarbonate was used as the final eluting solvent because of the ease with which it could be removed from solutions of the triphosphates prior to paper chromatography, by vacuum distillation. This technique was termed Procedure C and is described in section 8.7.

At this time Bollum, using ^{32}P-labelled inorganic pyrophosphate (Bollum, F.J. J. Biol. Chem., 1960, 235, 2399) demonstrated the pyrophosphorolysis of DNA in the presence of a partially purified (about 50 fold) DNA polymerase fraction from calf thymus glands. Under optimal conditions only 0.08% of the radioactivity added as inorganic pyrophosphate was recovered in the deoxyribonucleoside triphosphates. This suggested that it might be impossible to detect pyrophosphorolysis in crude extracts.

Experiment (8) An experiment was carried out, using procedure C, however, in which the Ehrlich tumour extract was assayed at 60 and 120 minutes for the ability to pyrophosphorolyse heated DNA. Incubation mixtures contained: DNA (heated; 200 µg./ml.), $MgCl_2$ (5 µmoles/ml.), tris buffer, pH 7.7 (100 µmoles/ml.), 2-mercaptoethanol (1 µmole/ml.), inorganic pyrophosphate (30 µmoles/flask) and crude Ehrlich ascites tumour extract (3 ml., 10 mg. of protein) in a final volume of 5 ml. The mixtures were incubated at 37°. Inorganic pyrophosphate was omitted from the controls. No evidence for the occurrence of pyrophosphorolysis was obtained. The controls which represented about 0.02% of the radioactivity added were about five times as high as the test values.

Due to an infection in the departmental mouse colony no tumour was available at this time and the experiment was repeated using rabbit thymus homogenate (see section 8.2b) in which DNA polymerase activity had also been demonstrated (Smellie, R.M.S., Keir, H.M. and Davidson, J.N. Biochim. Biophys. Acta 1959, 35, 589). Incubation mixtures were made on the basis of the mixture used by Bollum and contained; DNA (heated; 500 µg./ml.), $MgCl_2$ (4 µmoles/ml.), tris buffer, pH 7.7 (40 µmoles/ml.), inorganic pyrophosphate (5 µmoles/ml.) and rabbit thymus

homogenate (0.5 ml.) in a final volume of 1 ml. The mixtures were incubated at 37°. Inorganic pyrophosphate was omitted from the controls. Identical results were obtained, in this experiment (Experiment (9)).

It was thought that this discrepancy between the tests and controls might be due to conversion of inorganic pyrophosphate to orthophosphate during the incubation. The orthophosphate thus formed would be more easily removed from Dowex-1-chloride than the inorganic pyrophosphate of the controls and so very low test values would be obtained.

Experiment (10) Experiment (9) was repeated at an inorganic pyrophosphate concentration of 30 μmoles/ml. i.e. six times that used in the previous experiment, but in the results obtained the control values were again about five times higher than the tests.

As all the previous attempts to demonstrate the occurrence of pyrophosphorolysis had been unsuccessful it was decided to try to demonstrate its occurrence in a crude extract which had been shown to be capable of pyrophosphorolysis when partially purified. Calf thymus homogenate was chosen.

Experiment (11) Calf thymus homogenate, prepared as described in section 8.2c, was assayed as in experiment (10). No evidence of pyrophosphorolysis was obtained

the controls being six or more times as high as the test values.

This experiment was repeated with deoxyribonucleoside triphosphates added to the incubation mixture at the concentration used by Bollum i.e. 40 mmoles/ml. but the results obtained were the same as before. It was also repeated using the lower inorganic pyrophosphate concentration used by Bollum i.e. 4.5 µmoles/ml. and added deoxyribonucleoside triphosphates but the results were unchanged.

From the results of these experiments it seemed clear that it would not be possible to detect the pyrophosphorolysis of DNA in unpurified tissue extracts and so this work was terminated.

Summary

Part I.

1. The ability of extracts of Ehrlich ascites carcinoma to phosphorylate deoxyribonucleoside monophosphates was studied with a view to using this system as a means of preparing the deoxyribonucleoside triphosphates on a large scale.

2. The extracts were found to be able to convert dAMP to dATP in 60% - 80% yield, dGMP to dGTP in 50% - 60% yield, dCMP to dCTP in about 15% yield and TMP to TTP in about 1% yield.

3. A method for the preparation of dATP and dGTP on a large scale, using extracts of Ehrlich ascites carcinoma, is described.

Part II.

1. TTP was prepared from TMP in 18% yield by the method described by Smith and Khorana for the preparation of ATP from AMP.

2. An investigation of the purification led to several improvements.

3. This improved method (Method I) was used in the isolation of TTP-H^3 prepared from TMP-H^3 by the method

of Smith and Khorana. An overall yield of 36% was obtained

4. The effect on the yield of deoxyribonucleoside triphosphate of decreasing the ratio of the deoxyribonucleoside monophosphate to the other constituents of the reaction mixture, was investigated. By using reaction mixtures, involving twice and three times the amounts of the reactants used by Smith and Khorana for the same amount of deoxyribonucleoside monophosphate, greatly improved yields of the deoxyribonucleoside triphosphates were obtained.

5. The effect on the yield of deoxyribonucleoside triphosphate of reacting the deoxyribonucleoside monophosphate in the form of the tri-n-butylammonium salt instead of as the free acid was also studied. Still further improvements in the yields of the deoxyribonucleoside triphosphates were obtained. TMP was converted to TTP in 92% yield, dAMP to dATP in 93% yield, dGMP to dGTP in 92% yield and dCMP to dCTP in 73% yield.

6. A charcoal column procedure for the simultaneous removal of lithium chloride and inorganic polyphosphates from the deoxyribonucleoside triphosphate solution obtained after anion exchange chromatography was developed and incorporated in Method I giving Method II.

7. TMP^{32} was prepared from thymidine by the methods of Hurwitz and Tener.

8. The TMP^{32} thus obtained was converted, via the tri-n-butylammonium salt, to TTP in 90% yield and the TTP thus formed isolated by Method II in 70% yield.

The specific activity of the TTP thus prepared was $1.0 \times 1.5 \times 10^6$ cpm/μmole. A slight modification of the Tener procedure gave TMP^{32} of higher specific activity from which TTP with a specific activity of 3.5×10^6 cpm/μmole was obtained.

Part III.

1. Attempts were made to demonstrate the pyrophosphorolysis of DNA in extracts of Ehrlich ascites carcinoma using ^{32}P-labelled inorganic pyrophosphate and an analytical procedure involving the separation of inorganic pyrophosphate from deoxyribonucleoside triphosphate on charcoal columns. The results obtained were inconclusive because of the variability of the analytical procedure.

2. Attempts to demonstrate the pyrophosphorolysis of DNA in crude and partially purified extracts of Ehrlich ascites carcinoma using DNA labelled with tritiated thymidine and analysis of the deproteinised incubation mixtures by paper chromatography were unsuccessful.

3. A new method of separating inorganic pyrophosphate from deoxyribonucleoside triphosphates was developed.

4. This method was used in conjunction with ^{32}P-labelled inorganic pyrophosphate in attempts to demonstrate the pyrophosphorolysis of DNA in extracts of Ehrlich ascites carcinoma, rabbit thymus glands and calf thymus glands. No evidence for the occurrence of pyrophosphorolysis was obtained.

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