STUDIES ON THE BIOSYNTHESIS OF DEOXYRIBONUCLEIC ACID IN EXTRACTS OF MAMMALIAN CELLS

II. THE ENZYMIC FORMATION OF DEOXYRIBONUCLEOSIDE TRIPHOSPHATES

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SUMMARY

Extracts of the Ehrlich ascites carcinoma of the mouse prepared by centrifuging the disrupted cells at 105,000 × *g*, have been shown to contain enzymes capable of phosphorylating the monophosphates of deoxyadenosine, deoxycytidine, deoxyguanosine and thymidine to the corresponding triphosphates.

The mobilities of these deoxyribonucleotides in a number of solvent systems are described.

INTRODUCTION

As part of a programme designed to investigate the synthesis of DNA* in disrupted preparations of Ehrlich ascites tumour cells[1,2], the ability of such preparations to produce deoxyribonucleoside triphosphates from the corresponding monophosphates was studied.

Current theories on the mechanism of DNA biosynthesis[3-8] suggest that deoxyribonucleoside triphosphates are the direct precursors of DNA and that they are condensed enzymically to form deoxyribopolynucleotides with the concomitant release of inorganic pyrophosphate. The system described by DAVIDSON *et al.*[1,2] readily incorporates [^{3}H]TDR into DNA, and it might therefore be expected that the TDR together with other deoxyribonucleoside and deoxyribonucleotide precursors present in the system are converted to the deoxyribonucleoside triphosphates prior to incorporation into the DNA.

This paper demonstrates that particle-free extracts of Ehrlich ascites cells contain enzymes which are capable of phosphorylating dAMP, dCMP, dGMP and TMP to the triphosphate level, and describes the methods which have been used for the isolation and characterisation of these compounds.

* The following abbreviations are used: DNA for deoxyribonucleic acid; dAMP, dADP, dATP for deoxyadenosine-5′-mono-, di- and triphosphates; dCMP, dCDP, dCTP for deoxycytidine 5′ mono-, di- and triphosphates; dGMP, dGDP, dGTP for deoxyguanosine-5 -mono, di- and triphosphates; TMP, TDP, TTP for thymidine 5′-mono-, di- and triphosphates; AMP, ADP, ATP for adenosine-5′-mono-, di- and triphosphates; TDR for thymidine; Tris for 2-amino-2-hydroxymethylpropane-1 :3-diol.

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EXPERIMENTAL

Preparation of the enzyme extract

Extracts were prepared from sonically-disrupted preparations of Ehrlich ascites tumour cells as described in the preceding paper[2] and the resulting supernatant fluid was dialysed for 18 h at 4° against 4 × 1 l changes of 0.1 *M* phosphate buffer pH 8.1, or against 4 × 1 l changes of 0.05 *M* tris buffer pH 8.0. The preparation (S) contained 18–25 mg protein/ml and was used as the enzyme source in the ensuing incubation.

Incubation procedure

The components of the incubation mixture in a typical expt. were: deoxyribonucleoside-5'-monophosphate, 5 μmoles; ATP, 15 μmoles; $MgCl_2$, 10 μmoles; phosphate buffer at pH 8.1, 200 μmoles, containing 19 mg protein from S, or tris buffer at pH 8.0, 100 μmoles containing 19 mg protein from S; total volume, 2.25 ml. Incubation was conducted at 37° for 2 h with gentle shaking in stoppered conical flasks. At the end of the incubation period, the flasks were cooled to 0° 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° 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 expts. the reaction was stopped by heating at 100° for 2 min followed by rapid cooling to 0° but it was generally found that the small amounts of protein remaining in solution interfered with subsequent paper chromatography.

Paper chromatography

The deproteinised incubation mixture was applied to sheets of Whatman No. 1 chromatography paper, 33 cm × 46 cm, 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 deoxyribonucleoside monophosphate were also applied. The spots and bands were dried in cold air, and the paper was developed for 18 h as a descending chromatogram in the solvent system of KREBS AND HEMS[9] 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* ethylenediamine-tetra-acetic acid, 1.6 ml. After development, the papers were dried in cold air and scanned in u.v. light to detect the positions of absorbing spots and bands. The channel bearing the spots from the 0.05–0.1 ml sample portion was cut off as a strip and deoxyribose derivatives were detected by spraying with the cysteine–H_2SO_4 reagent of BUCHANAN[10]. The corresponding deoxyribose-containing bands on the remaining paper strips were cut out and eluted in water by capillary flow. The paper eluates were concentrated by lyophilisation and were applied as 2-cm bands to papers, the lower ends of which were serrated. One-dimensional chromatography in the ammonium isobutyrate solvent was then repeated for periods of 24–36 h in order to effect a satisfactory separation of deoxyribose derivatives from the trace amounts of ribose compounds. In some cases, the concentrated paper eluates were applied as spots to papers for two-dimensional chromatography in the ammonium isobutyrate system, followed by the ethanol-ammonium acetate solvent of PALADINI AND LELOIR[11] containing 10^{-2} *M* ethylenediaminetetra-acetic acid, as an ascending chromatogram. The cysteine–H_2SO_4

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test was used on duplicates of such two-dimensional chromatograms. It was sometimes found useful to use the ethanol-ammonium acetate solvent in descending chromatography for periods up to 65 h.

Paper ionophoresis

Eluates from the preliminary large-scale paper separations in the ammonium isobutyrate solvent were submitted also to ionophoresis on Whatman No. 1 paper strips in 0.02 *M* citrate buffer at pH 3.75 [12] for 4 h or 8 h. U.v.-absorbing bands were eluted with water by capillary flow.

Analytical methods

Protein in the dialysed tissue extracts was estimated by the procedure of GORNALL, BARDAWILL AND DAVID[13].

The spectra of nucleotides in the paper eluates were determined in acid, neutral and alkaline solution in the Cary Model 11 recording spectrophotometer, and the total phosphorus in the same nucleotide solutions was estimated by the procedure of ALLEN[14]. It was not possible to conduct satisfactory phosphorus estimations in expts. in which phosphate buffer was used in the incubation mixture, as inorganic phosphate was found to have unfavourable mobilities in the chromatographic and ionophoretic systems described above (see Tables I and II). In some cases, the nucleotide solutions were divided into 3 equal parts for estimation of base, deoxyribose and phosphorus, the deoxyribose being estimated by the method of BRODY[15]. Calibration curves for these estimations were constructed using pure samples of deoxyribonucleoside monophosphates, the most satisfactory results in this estimation being obtained using 87.86 % (w/v) H_2SO_4. To 0.2 ml of the deoxyribonucleotide solution plus 0.05 ml 1 % cysteine hydrochloride solution (maintained at a low temperature in a test-tube surrounded by a solid CO_2–alcohol mixture), 5 ml of cold 87.86 % (w/v) H_2SO_4 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° for exactly 25 min, quickly cooled to 0° and the optical densities read at 474 mμ. When the amount of deoxyribose in a nucleotide solution was not thus determined, the evidence that the nucleotide was of the deoxyribose variety was based on the positive result of the cysteine–H_2SO_4 spray technique[10] carried out on a portion of the purified nucleotide run on a descending paper chromatogram in one of the two solvents mentioned above. The method used for the estimation of nucleotide bases was that described by SMELLIE, THOMSON AND DAVIDSON[16].

In some expts. the final nature of the reaction products was confirmed by relating the phosphorus content of the deoxyribonucleotides to the deoxyribonucleoside content as determined from the u.v. absorption. The molar extinction coefficients at pH 7.0 used in these calculations were deoxycytidine, 8,900[17,18]; thymidine, 9,600[17,18]; deoxyadenosine, 15,800[19] and deoxyguanosine, 13,600[18].

RESULTS

Paper chromatography of extracts from control incubation mixtures from which the enzyme source had been omitted, showed that the ATP and the deoxyribonucleoside

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monophosphates did not undergo alteration in the course of incubation and chromatography. Preliminary control expts. in which ATP and the deoxyribonucleoside monophosphates were omitted from the incubation mixtures clearly demonstrated that undialysed preparations of the soluble extract contained fairly large amounts of nucleotides. Removal of these intrinsic nucleotides by dialysis greatly facilitated the chromatographic isolation and purification of deoxyribonucleoside polyphosphates.

After incubation of ATP and a deoxyribonucleoside monophosphate with the dialysed enzyme preparation, chromatography of small portions of the extract in the ammonium isobutyrate solvent showed that there were usually 3 u.v.-absorbing spots which contained deoxyribose as indicated by the cysteine–H_2SO_4 spray test. The spot with the highest R_F value corresponded in chromatographic location to the deoxyribonucleoside monophosphate.

By analogy with the behaviour of ribonucleoside polyphosphates in this solvent, the two slower-moving spots were assumed to be the di- and triphosphates of the deoxyribonucleoside. The bands corresponding to these spots on the adjacent channel of each paper, were cut out, eluted in water and re-applied to paper for longer periods in order to effect a satisfactory separation from the adenosine compounds which in most cases contaminated the bands eluted. During the ensuing chromatographic operations the cysteine–H_2SO_4 test was frequently used to confirm the presence of deoxyribose in the material being purified.

TABLE I

PAPER CHROMATOGRAPHY OF NUCLEOTIDES IN THE AMMONIUM ISOBUTYRATE SOLVENT AT pH 4.6

(A), in the ethanol–ammonium acetate solvent, ascending (B), and in ethanol–ammonium acetate, descending (C), at 22°.

Nucleotide	R_F		R_{ATP}
	A	*B*	*C*
AMP	0.53	0.13	4.44
ADP	0.42	0.07	2.09
ATP	0.34	0.04	1.00
dAMP	0.62	0.18	5.64
dADP	0.53	0.12	2.48
dATP	0.44	0.055	1.28
dCMP	0.53	0.18	6.50
dCDP	0.41	—	3.81
dCTP	0.29	0.055	1.48
dGMP	0.38	0.12	3.50
dGDP	0.30	—	1.57
dGTP	0.19	0.035	0.76
TDR	0.66	0.88	—
TMP	0.44	0.27	10.90
TDP	0.31	0.11	5.24
TTP	0.24	0.08	2.91
IMP	0.31	—	—
Inorganic $PO_4^{\equiv}$	0.38	—	5.75

Table I shows the R_F values of various nucleotides in the solvent systems used. The figures quoted are averages of those found in a large number of expts. with partially isolated deoxyribonucleotides and slight variations were observed from

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time to time. The R_F values tended to be slightly lower when all the components of a reaction mixture were undergoing preliminary separation in the ammonium isobutyrate system. The presence of inosine-5'-monophosphate on chromatograms was frequently observed, arising presumably from deamination of AMP during the incubation.

For analytical purposes it was sometimes convenient to use ionophoresis on paper as a final check on the purity of a compound. The mobilities of some of the nucleotides in the system used are recorded in Table II.

TABLE II

PAPER IONOPHORESIS OF NUCLEOTIDES AND TDR IN CITRATE BUFFER
pH 3.75, for 4 h and 8 h, at 14 V/cm.

Compound	*Distance moved (cm)*	
	4 h	*8 h*
AMP	11.3	15.3
ADP	18.8	23.7
ATP	20.1	27.2
dAMP	11.4	16.2
dADP	19.5	25.1
dATP	22.2	28.1
dCMP	10.6	14.3
dCDP	—	—
dCTP	19.9	—
dGMP	15.2	20.6
dGDP	22.4	—
dGTP	24.8	—
TDR	10.0	12.0
TMP	19.4	25.8
TDP	—	—
TTP	26.2	—
Inorganic $PO_4^{\equiv}$	24.5	—

The conversion of the deoxyribonucleoside monophosphates to the triphosphates could be demonstrated with dAMP, dCMP, dGMP and TMP. The yields of dCTP, dGTP and dATP (based on the initial quantity of deoxyribonucleoside monophosphate) were respectively 15 %, 50–60 % and 60–80 %. However, the extent of conversion of TMP to TTP was much less being of the order of 1 %. The amounts of deoxyribonucleoside diphosphates formed were very low in all cases.

TABLE III

RESULTS OF CHEMICAL ANALYSIS OF PURINE DEOXYRIBONUCLEOTIDES
Values for deoxyribose and total phosphorus are expressed as μmoles/μmole of base.

Deoxynucleotide	*Base*	*Deoxyribose*	*Total P*
dATP	Adenine	0.87	2.82
dGTP	Guanine	0.97	2.87

With the 2 purine deoxyribonucleosides relatively large amounts of the triphosphates were recovered and complete analyses indicating the proportions of base to deoxyribose and phosphorus were carried out as shown in Table III. These

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results clearly demonstrate the identity of the compounds as the deoxyribonucleoside triphosphates. With the two pyrimidine deoxyribonucleotides, yields of the triphosphates were much smaller and the cysteine–H_2SO_4 reaction[15] failed to give reproducible results. In order to economise on material, therefore, analyses were restricted to relating the phosphorus to the deoxyribonucleoside content of the compound. The results of these analyses on both the purine and pyrimidine derivatives are shown in Table IV.

TABLE IV

RESULTS OF CHEMICAL ANALYSIS OF DEOXYRIBONUCLEOTIDES

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

Deoxyribonucleotide	*λ max ($m\mu$)*	*Total P per μmole of deoxyribonucleoside*
dADP	259	2.04
dATP	259	3.00
dGDP	253	2.10
dGTP	252	2.93
dCTP	271	3.07
TTP	267	2.62

DISCUSSION

The demonstration that the mammalian tumour used in these expts. is capable of phosphorylating deoxyribonucleoside-5′-monophosphates to the corresponding triphosphates lends further support to the hypothesis that these triphosphates are involved in the biosynthesis of DNA. In the previous paper[2] it has been shown that [^{3}H]TDR can be incorporated readily into DNA under the influence of enzymes in the 105,000 $\times$ g supernatant extract of ascites tumour cells, and that radioactivity from the [^{3}H]TDR also appears in TMP, TDP and TTP. Two enzyme systems are clearly involved in the process (a) the phosphorylating system, and (b) the polymerising system. In the present study, it has been shown that the phosphorylating system of the ascites tumour extracts operates not only with TMP but also with dAMP, dCMP and dGMP as substrates. It is clear therefore that the tumour extract is capable of supplying all 4 deoxyribonucleoside triphosphates to the polymerising system, in accordance with the hypothesis set out by KORNBERG and his colleagues[3–5], for DNA synthesis in preparations from *Esch. coli*. This hypothesis has been substantiated by further work with purified *Esch. coli* enzymes and much information is now available regarding both the phosphorylating and polymerising systems from *Esch. coli*[4,5]. An interesting feature of the work is the finding[4] that there is a distinct kinase for the phosphorylation of each of the nucleotides dAMP, dCMP, dGMP and TMP. This may account in some measure for the observation in the present study that the rates of production of triphosphates from monophosphates differ quite considerably, and may indicate that in the intact cell, the TMP kinase is the regulating factor in the production of triphosphates for consumption by the polymerising system in DNA synthesis. It is interesting to note that CANELLAKIS[20] has observed that the cytoplasmic fraction from regenerating rat liver is capable of

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phosphorylating dAMP, dCMP, dGMP and TMP whereas normal rat liver can phosphorylate only dAMP, dCMP and dGMP to a significant extent.

Other reports of biological sysnthesis of deoxyribonucleoside triphosphates have appeared. SABLE, WILBER, COHEN AND KANE[21] showed that muscle enzymes and mitochondria prepared from rabbit kidney cortex are capable of synthesising dADP and dATP from dAMP in the presence of an energy donor. HECHT, POTTER AND HERBERT[22] demonstrated that rat liver enzymes can phosphorylate dCMP and TMP to dCTP and TTP and KLENOW AND LICHTLER[23] found that aqueous extracts of bone marrow and muscle contain enzymes which catalyse the phosphorylation of dAMP and dGMP. BOLLUM[24] and CANELLAKIS AND MANTSAVINOS[7] have synthesised dATP, dCTP, dGTP and TTP using the high-speed supernatant extract from regenerating rat liver homogenates as a source of enzyme. Similar reactions have also been described for bacterial preparations[25].

The natural occurrence of these deoxyribonucleoside triphosphates has also been reported, thus POTTER[26] and his associates have demonstrated the occurrence of the mono-, di- and triphosphates of TDR and deoxycytidine in acid extracts of calf thymus tissue, and LEPAGE[27] was able to demonstrate the presence of dATP in acid extracts of the Flexner-Jobling carcinoma of the rat.

From all these studies it is apparent that a great variety of tissues and organisms is capable of the phosphorylation of deoxyribonucleoside monophosphates to the corresponding triphosphates and that the kinases responsible for these reactions are widely distributed. These observations together with the reported occurrence of small amounts of deoxyribonucleoside triphosphates in a number of tissues provide further support for the hypothesis that DNA biosynthesis is carried out by a polymerising system utilising as substrates the deoxyribonucleoside triphosphates.

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ON THE EFFECT OF SOME ADENINE DERIVATIVES ON THE INCORPORATION *IN VITRO* OF ISOTOPICALLY LABELLED COMPOUNDS INTO THE NUCLEIC ACIDS OF EHRLICH ASCITES TUMOR CELLS

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SUMMARY

1. The incorporation of [^{14}C]formate into DNA thymidylic acid of Ehrlich ascites tumor cells *in vitro* is slightly stimulated by addition of adenine or adenosine. Deoxyadenosine has, however, a pronounced inhibitory effect on this process. All 3 adenine compounds reduce the incorporation of [^{14}C]formate into DNA purine deoxyribotides to very low levels.

2. The specific radioactivity of pooled acid-soluble thymine compounds is not reduced by the addition of either adenine or deoxyadenosine to cell suspensions. The total radioactivity of acid-soluble thymine compounds is reduced in the presence of adenine, but in the presence of deoxyadenosine this activity is doubled. Addition of adenine, adenosine or deoxyadenosine reduces the total radioactivity of the purines of the acid-soluble fraction.

3. The incorporation *in vitro* of [^{32}P]orthophosphate into DNA of Ehrlich ascites tumor cells is slightly activated by adenine or adenosine but is markedly reduced by deoxyadenosine. A 50 % inhibition of the rate is obtained at about 1 μmole of deoxyadenosine/ml cell suspension. The effect of deoxyadenosine is unchanged after 3 recrystallizations and is abolished by weak acid hydrolysis. At concns. up to about 2 μmoles/ml the 3 adenine compounds have little effect on [^{32}P]labelling of RNA, but all inhibit it at high concns. (6.6 μmoles/ml).

4. It is postulated that deoxyadenosine inhibits the DNA synthesis in Ehrlich ascites tumor cells *in vitro*.

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