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

I. INCORPORATION OF [^{3}H]THYMIDINE

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SUMMARY

A particle-free extract has been prepared from Ehrlich ascites tumour cells disrupted sonically or osmotically which promotes the incorporation of [^{3}H]thymidine into DNA in the presence of ATP, Mg^{++} ions and carrier DNA. With sonic extracts glucose and DPN in high concentrations are required for good incorporation; with osmotic extracts a low concentration of DPN suffices.

Incorporation is diminished by dialysis and is promoted by low concentrations of deoxyribonucleoside monophosphates and to a greater extent by deoxyribonucleoside triphosphates. Evidence is presented that incorporation is preceded by conversion of [^{3}H]TDR to [^{3}H]TTP.

Degradation of the [^{3}H]DNA yields among other products [^{3}H]thymidine diphosphate.

Investigation of extracts from a series of different tissues shows that ability to incorporate [^{3}H]TDR into DNA is greater in tissues of high mitotic activity.

INTRODUCTION

In earlier papers[1,2] we have reported studies on the incorporation of [^{14}C]formate, [^{14}C]adenine and [^{32}P]phosphate into the DNA of Ehrlich mouse ascites tumour cells *in vitro* which led us to believe that under *in vitro* conditions these cells are capable of synthesizing DNA to an appreciable extent. The final aim of these experiments was to obtain a cell-free system capable of DNA synthesis and it seemed likely that the ascites tumour cells would provide good starting material. None of the precursors mentioned above, however, is specific for DNA and all require the participation of several enzyme systems before they are likely to serve as immediate precursors of DNA. Our attention was therefore turned to isotopically-labelled thymidine which,

The following abbreviations are used: DNA for deoxyribonucleic acid, RNA for ribonucleic acid, dAMP, dGMP, dCMP and TMP for the 5'-monophosphates of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine respectively, dATP, dGTP, dCTP and TTP for the 5'-triphosphates of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine respectively, TDR for thymidine, ATP for adenosine triphosphate, DPN for diphosphopyridine nucleotide, and DNase for deoxyribonuclease.

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in the form of [^{15}N]TDR[3] or [^{14}C]TDR[4], has been used with success as a precursor of DNA in the tissues of the rat[3], in chick embryo pulp *in vitro*[4], in suspensions of bone marrow cells[5], in slices of various rabbit tissues[5], in isolated thymus nuclei[5], in onion root tips[6] and in tissue cultures[7].

Thymidine labelled with ^{15}N or ^{14}C is not readily available but TDR can easily be labelled with ^{3}H[8] and the resulting product has been used in the study of DNA metabolism both at the cytological level[8–11] and at the chemical level[12–15]. While the use of ^{3}H precursors introduces difficulties in counting procedures, these are outweighed by the advantages of having a specific and close precursor of DNA. In this paper the results of studies on the incorporation of [^{3}H]TDR into DNA by soluble extracts of Ehrlich ascites tumour cells and other mammalian cells are reported. Brief preliminary reports of this work have already appeared[16, 17].

METHODS

Biological

The Ehrlich ascites carcinoma, kindly supplied by Dr. G. POPJAK of Hammersmith Hospital, London, was maintained by serial transplantation in mice of the departmental colony. Chick embryos were obtained from eggs supplied by the Poultry Research Centre, Edinburgh. For the expts. on rabbit tissues, young rabbits weighing from 1500 g to 2000 g were selected from the departmental colony.

Preparation of tissue extracts

Ehrlich ascites tumour extracts: Ascitic fluid was withdrawn from several mice under aseptic conditions and pooled. The suspension of cells was centrifuged at low speed (200–300 × *g*) for 5 min to separate the cells from the plasma. The sediment was resuspended in 5–10 vol. of either 0.25 *M* sucrose in 0.1 *M* phosphate buffer pH 8.1 or 0.1 *M* phosphate buffer pH 8.1 and again centrifuged at 200–300 × *g* for 5 min to separate the tumour cells from erythrocytes. This washing procedure was repeated several times until the sediment of tumour cells was freed from contamination with erythrocytes.

Two procedures were adopted for disrupting the tumour cells: (a) sonic disruption; (b) osmotic disruption. For the sonic disruption, the packed washed cells were resuspended in an equal volume of either 0.25 *M* sucrose in 0.1 *M* phosphate buffer pH 8.1 or 0.1 *M* phosphate buffer pH 8.1 and then exposed in batches of about 15 ml to vibration by a Mullard ultrasonic drill (50 W; 20 kc) for periods of 3 to 5 min[18]. The length of exposure was determined by following cellular breakdown in wet smears stained with crystal violet. The disintegrated cell suspension was centrifuged in a Spinco preparative ultracentrifuge at 105,000 × *g* for 1 h to yield a clear extract.

For osmotic disruption[19] the washed cells were resuspended and centrifuged at 600 × *g* to pack them tightly in the centrifuge tube. 10–12 vol. ice-cold distilled water were then added and the suspension gently homogenised in a Potter-type homogeniser. 3 or 4 passes of the material were sufficient for adequate dispersion. Microscopic 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 the Spinco centrifuge at 105,000 × *g* to yield a clear extract.

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The protein content of the extracts was determined by the method of GORNALL, BARDAWILL AND DAVID[20].

Extracts of chick embryo and rabbit tissues: 17-day chick embryos were collected and homogenised gently in a Potter-type homogeniser in 0.1 *M* phosphate buffer pH 8.0. In some cases these suspensions were exposed to sonic vibrations before centrifuging, while in others they were centrifuged directly at 105,000 × *g* to yield clear extracts. Bone marrow, appendix, thymus, liver and kidney from young rabbits were homogenised in 2 vol. 0.1 *M* phosphate buffer using a Potter-type homogeniser so as to rupture most of the cells without destroying many nuclei. The resulting suspensions were either centrifuged directly at 105,000 × *g* or were first exposed to sonic vibration and then centrifuged so as to yield clear extracts.

Incubations

Incubation of the extracts with appropriate additions was carried out in air in stoppered 50-ml Erlenmeyer flasks at 37° with shaking at 80 oscillations/min. The incubation was terminated by immersing the flasks in a mixture of solid CO_2 and ethanol and the frozen material was either processed immediately or stored at —10° until required. The volume per flask was 5 ml.

Analytical procedures

The frozen incubation mixtures were allowed to thaw at 0° and were transferred to 15 ml centrifuge tubes in which subsequent procedures were carried out. The material was first treated with 0.5 vol. ice-cold 2.1 *N* $HClO_4$ to precipitate protein and nucleic acid, and was centrifuged. The supernatant fluid was decanted and the sediment extracted twice more with ice-cold 0.7 *N* $HClO_4$ to remove all acid-soluble products. These acid extracts were combined, neutralised with 5 *N* KOH, centrifuged in the cold to remove $KClO_4$ and were termed the acid-soluble fractions. The sediment after acid extraction was treated successively with acetone, ethanol, ethanol–$CHCl_3$ (3:1), ethanol–ether (3:1) and ether to remove lipid material. The residual material consisting of protein and nucleic acids was then suspended in 0.3 *N* KOH and incubated for 18 h at 37° to hydrolyse the RNA to mononucleotides. After incubation, the material was acidified in the cold with 10 *N* $HClO_4$ and centrifuged to precipitate protein and DNA. The supernatant fluid was removed and discarded and the precipitate washed twice with ice-cold 0.5 *N* $HClO_4$ to remove traces of RNA nucleotides.

The remaining precipitate was extracted twice with 0.5 ml 0.5 *N* $HClO_4$ at 70° for 20 min, the extracts, containing all the DNA in degraded form, being pooled. This material was cooled to 0°, neutralised carefully with 5 *N* KOH and was then centrifuged at low speed at 0° for 1 h to remove as much of the $KClO_4$ as possible. The final extract was diluted with water to 10 ml. One portion of this was used for counting while a second portion was used for measurement of the extinction at 268 mμ in a 1 cm light path. The DNA content of the material was calculated on the assumption that a solution of DNA containing 1 μg DNA-P/ml would give an E_{268} of 0.230 [21].

In some expts. portions of the acid-soluble fraction from the incubations were chromatographed on paper along with added non-radioactive TDR, TMP and TTP. The solvents employed were: (1) in the first dimension, isobutyric acid–NH_4OH–H_2O–versene (ethylenediaminetetra-acetic acid)[22]; (2) in the second dimension,

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ethanol–ammonium acetate[23] with versene present in a concentration of 10^{-2} *M*; (3) followed by butanol–water 86:14[24] in the same direction as the second solvent. Spots were located by scanning the papers in u.v. light. Those corresponding to TDR, TMP, TDP and TTP were eluted and samples of the eluate used for identification by measurement of u.v. spectra and phosphorus content and for counting.

Assay of radioactivity

Counting of ^3H was carried out on samples which had been plated on aluminium planchets which were first thoroughly cleaned by rubbing with "Ajax" cleaner. They were assayed in a Tracerlab windowless flow counter operated in the Geiger and not in the proportional region. The major difficulty in counting ^3H by this technique is that of self-absorption. Preliminary expts. indicated that counting of these samples at infinite thickness could give rise to very large errors due to non-uniform distribution of material on the planchets. At the same time, it was almost impossible to achieve infinitely thin ^3H containing samples. However, by serial dilution it proved possible to obtain samples that approached infinite thinness and this technique was finally adopted. As a further safeguard, all planchets counted had closely similar quantities of DNA applied to them and all expts. were carried out in duplicate, repeat dilutions and counts always being performed where any serious disagreement between duplicates was observed. The greatest care had to be taken to remove as much $KClO_4$ as possible from solution before preparing the planchets.

Isolation of DNA

In some expts. DNA was prepared from the incubation mixture by treatment of the precipitate obtained on acidifying the alkaline digest. This precipitate was suspended in 10 % (w/v) NaCl and the pH adjusted to 7 with *N* NaOH. The suspension was then heated in boiling water for 1 h, centrifuged, the supernatant fluid decanted into a clean tube and the sediment reextracted with 10 % (w/v) NaCl. The combined extracts were then treated with 2–4 vol. ethanol and the precipitated DNA plus some protein collected by centrifuging. This precipitate was then dissolved in 0.9 % NaCl and shaken repeatedly with water saturated $CHCl_3$ to remove protein[25]. The final supernatant fluid was then treated with 2–4 vol. ethanol to precipitate the DNA.

In some experiments [^3H]DNA prepared as described above was degraded as follows: 20-mg portions were degraded by the method of BURTON AND PETERSEN[26]. After removal of excess formic acid and diphenylamine by ether extraction the aqueous phase was lyophylised and the dry powder dissolved in a small volume of water. The pH of this solution was adjusted to 3.5 and portions of this were used for ionophoresis in 0.02 *M* citrate buffer pH 3.5 to separate the degradation products[27]. The u.v. absorbing bands were eluted with water and portions of the eluate taken for determination of the absorption spectrum, the radioactivity and the ratio of phosphorus to base.

Materials

[^3H]TDR of varying specific activity from 118 μC/μmole to 1.9 mC/μmole was obtained from Schwarz Laboratories, Inc. Crystalline sodium ATP was purchased from the Sigma Chemical Corporation and DPN was purchased from the Sigma Chemical Corporation and from C. F. Boehringer.

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DNA was prepared from calf thymus and from Ehrlich ascites tumour cells by the procedure of KAY, SIMMONS AND DOUNCE[28]. A preparation of salmon sperm DNA from the California Corporation was also used.

dAMP, dGMP, dCMP and TMP were purchased from the California Corporation. A sample of TTP was kindly provided by Sir ALEXANDER TODD. dATP, dGTP and dCTP were prepared as described by KEIR AND SMELLIE[29].

Crystalline deoxyribonuclease was supplied by Nutritional Biochemicals, Inc. and crude lyophylised Russells viper venom was generously presented by Messrs. BURROUGHS, Wellcome & Company.

RESULTS

Table I illustrates the incorporation of [^{3}H]TDR into the DNA of intact ascites cells *in vitro*. Disruption of the cells, including the nuclei, in the ultrasonic generator produces a preparation which shows diminished incorporation. The supernatant

TABLE I

INCORPORATION OF [^{3}H]TDR INTO THE DNA OF INTACT AND SONICALLY DISRUPTED PREPARATIONS OF EHRLICH ASCITES TUMOUR CELLS

The medium contained 0.25 *M* sucrose, 0.1 *M* phosphate buffer pH 7.4, 2.5 μmoles ATP/ml, 2.5 μmoles DPN/ml, 50 μmoles glucose/ml, 2 μC [^{3}H]TDR/ml, 4 μmoles $MgCl_2$/ml and 20 μmoles NaCl/ml. Incubation time: 2 h.

Fraction	*DNA specific activity (Counts/min/μmole DNA-P)*
Intact ascites cells	9,700
Sonically disrupted cells	600
Supernatant fraction (1000 × *g*) from disrupted cells	1,225
Sediment (1000 × *g*)	322
Supernatant fraction after high speed centrifugation (105,000 × *g*)	1,661

fraction prepared by centrifuging this material shows higher activity than is to be found in the sediment and this high activity persists after the removal of particulate material by centrifugation at 105,000 × *g*. A similarly active particle-free extract is obtained by osmotic disruption of the ascites cells. It was employed in most of the later expts.

While our early results were obtained by sonic disruption of cells in a buffered solution containing 0.25 *M* sucrose and 0.1 *M* phosphate, other buffers were also found to be effective, *e.g.*, 0.1 *M* phosphate or 2-amino-2-hydroxymethyl propane-1:3-diol (tris), which were used in all the later expts. A curve showing activity at different pH values shows a sharp optimum in the neighbourhood of pH 7.8 (Fig. 1).

It was observed at an early stage that addition of a boiled ascites extract increased incorporation to a marked extent (Table II) but that boiled extract which had previously been incubated with deoxyribonuclease was inactive. Accordingly the effect of adding carrier DNA was examined. DNA from several different sources was found to cause a sharp increase in incorporation of [^{3}H]TDR (Table III), the increase in each case being abolished by prior incubation of the DNA with deoxyribonuclease. In all subsequent experiments ascites DNA was added as carrier. The effect

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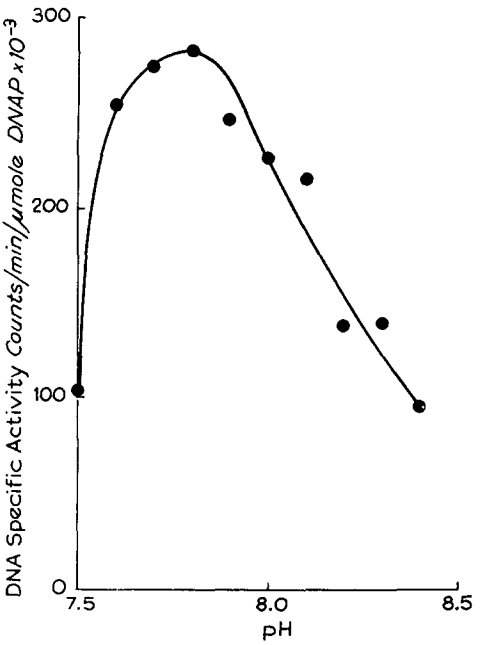

Fig. 1. The effect of variation in pH on the incorporation of [^{3}H]TDR into DNA by osmotic extracts of Ehrlich ascites tumour cells. The medium was 0.1 *M* with respect to tris buffer and contained 5.0 μmoles ATP/ml, 0.25 μmole DPN/ml, 5.0 μg DNA/ml, 5.0 μmoles $MgCl_2$/ml, 0.5 μC [^{3}H]TDR/ml and 3.6 mg protein/ml.

TABLE II

THE EFFECT OF ADDING BOILED EXTRACT OF SONICALLY DISRUPTED ASCITES TUMOUR CELLS AND DEOXYRIBONUCLEASE TREATED BOILED EXTRACT ON THE INCORPORATION OF [^{3}H]TDR INTO DNA BY PARTICLE-FREE EXTRACTS OF SONICALLY DISRUPTED ASCITES TUMOUR CELLS

The medium contained 0.1 *M* phosphate buffer pH 8.1 50 μmoles glucose/ml, 2.5 μmoles ATP/ml, 2.5 μmoles DPN/ml, 4 μmoles $MgCl_2$/ml, 20 μmoles NaCl/ml and 2 μC[^{3}H]TDR/ml. 1 ml of boiled extract or of boiled extract first treated for 18 h with deoxyribonuclease and re-heated to 100° for 5 min, was added in place of a portion of the buffer in the tests. Incubation time: 2 h.

Nature of experiment	*DNA specific activity (Counts/min/μmole DNA-P)*
Control	4,800
Boiled extract added	12,800
Deoxyribonuclease treated boiled extract added	1,180

of varying concentrations is shown in Fig. 2, which makes it clear that the optimum effect is obtained at 25–50 μg DNA/ml and that at lower concentrations the amount of [^{3}H]TDR incorporated increases linearly with increasing amounts of DNA.

While early expts. were carried out with sonic extracts, most of the later work involved osmotic extracts which have the advantage of a much lower DNA content. From the results in Table IV it is clear that at equivalent levels of protein content, osmotic extracts are more active. Since osmotic extracts contain virtually no nuclear

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TABLE III

THE EFFECT OF ADDING DNA AND DEOXYRIBONUCLEASE TREATED DNAS FROM DIFFERENT SOURCES ON THE INCORPORATION OF [^{3}H]TDR INTO DNA BY EXTRACTS OF SONICALLY DISRUPTED EHRLICH ASCITES CELLS

The composition of the reaction mixtures was as in Table II. 50 μg/ml DNA or degraded DNA were added to the tubes shown. Samples of the intact solutions of DNA were incubated with deoxyribonuclease at a level of 50 μg/mg DNA for 18 h and heated to 100° for 5 min to yield degraded DNA. Incubation time: 2 h.

	DNA specific activity (Counts/min/μmole DNA-P)
Control	8,575
Ascites DNA	99,700
Degraded ascites DNA	2,075
Calf thymus DNA	100,500
Degraded calf thymus DNA	2,375
Salmon sperm DNA	56,500
Degraded salmon sperm DNA	2,440

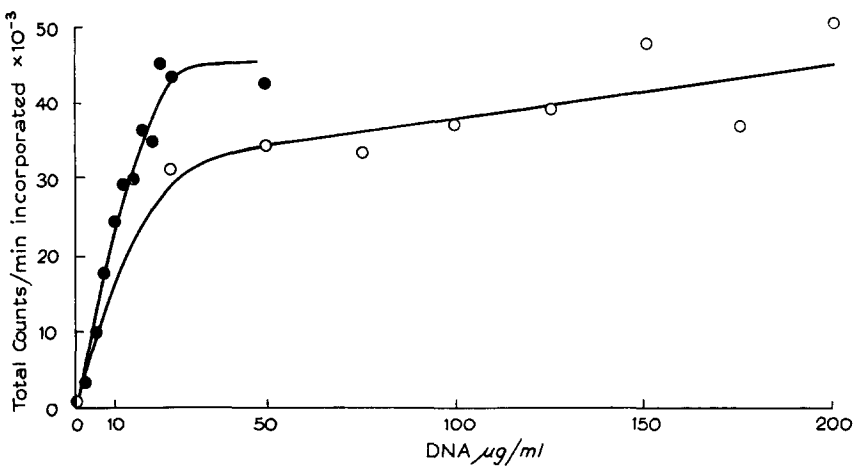

Fig. 2. The effect of adding varying amounts of ascites DNA on the incorporation of [^{3}H]TDR into DNA by two separate batches of soluble extracts of osmotically disrupted Ehrlich ascites tumour cells. The medium was 0.1 *M* with respect to tris buffer pH 7.9 and contained 5.0 μmoles ATP/ml, 0.25 μmoles DPN/ml, 5.0 μmoles $MgCl_2$/ml, 0.5 μC [^{3}H]TDR/ml, and 3.3 mg protein/ml

material it would appear that the enzyme systems involved in the incorporation are cytoplasmic in origin. This has been confirmed by taking the nuclear sediment obtained in the preparation of an osmotic extract and disrupting it sonically. In this way a comparison of a nuclear and a cytoplasmic extract from the same cells was possible. Table IV shows that the activity is more pronounced in the cytoplasmic material.

In an examination of the other factors involved, it became clear that osmotic and sonic extracts differed in their requirements for such substances as ATP, DPN and glucose.

ATP is necessary in both instances at concentrations of from 2 to 5 μmoles/ml. When phosphate buffer is used in the incubation medium, higher concentrations of

TABLE IV

THE INCORPORATION OF [^{3}H]TDR INTO DNA BY (A) SONIC AND OSMOTIC EXTRACTS OF EHRLICH ASCITES TUMOUR CELLS AND (B) CYTOPLASMIC AND NUCLEAR EXTRACTS

The medium contained 0.1 *M* phosphate buffer pH 8.1, 5.0 μmoles ATP/ml, 0.25 μmole DPN/ml, 5.0 μmoles $MgCl_2$/ml 50 μg DNA/ml, 50 μmoles glucose/ml and 0.5 μC. [^{3}H]TDR/ml. In (A) the sonic extract was diluted to the same protein content as the osmotic extract, and in (B) the nuclear extract was diluted to the same protein content as the cytoplasmic extract. The extracts in (A) and (B) were made from separate batches of ascites cells and are therefore not strictly comparable.

		Protein content (mg/ml reaction mixture)	*Total counts/min incorporated*
(A)	Sonic extract	3.3	42,600
	Osmotic extract	3.3	61,100
(B)	Cytoplasmic extract	4.5	93,900
	Nuclear extract	4.5	28,500

ATP lead to a reduction in the amount of [^{3}H]TDR incorporated (Fig. 3). With tris buffer, on the other hand (Fig. 4), the maximum activity is reached at concentrations of 2.5 μmoles ATP/ml and remains almost unchanged between 2.5 and 10 μmoles/ml.

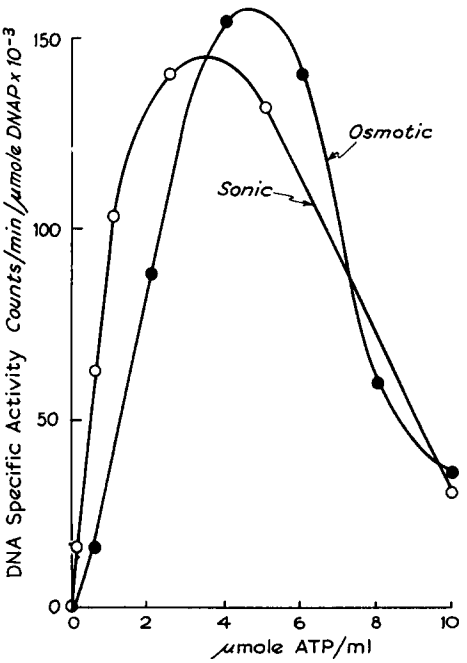

Fig. 3. The effect of varying concentrations of ATP on the incorporation of [^{3}H]TDR into DNA by sonic and osmotic extracts of Ehrlich ascites tumour cells in phosphate buffer. The medium contained 0.1 *M* phosphate buffer, pH 8.0, 0.25 μmole DPN/ml, 10.0 μmoles $MgCl_2$/ml, 50 μmoles glucose/ml, 50 μg DNA/ml, 1.0 μC [^{3}H]TDR/ml and 4.5 mg protein/ml.

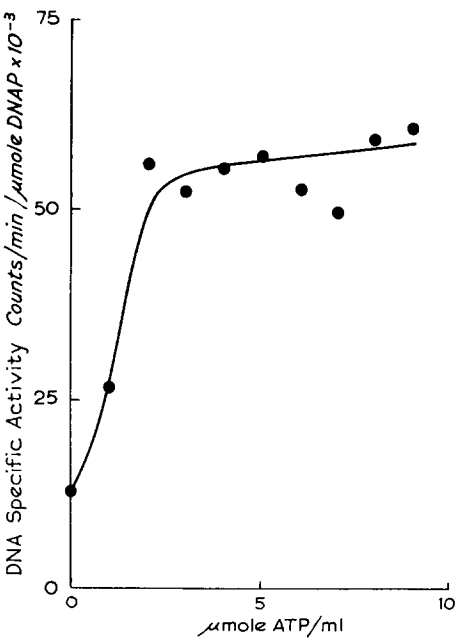

Fig. 4. The effect of varying concentrations of ATP on the incorporation of [^{3}H]TDR into DNA by osmotic extracts of Ehrlich ascites tumour cells in tris buffer. The medium was 0.1 *M* with respect to tris buffer pH 7.9 and contained 0.25 μmole DPN/ml, 5.0 μmoles $MgCl_2$/ml, 50 μg DNA/ml, 0.5 μC [^{3}H]TDR/ml and 3.3 mg protein/ml.

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For incorporation with sonic extracts DPN is necessary at the rather high concentration of 2.5 μmoles/ml[17]. With osmotic extracts, however, a concentration of DPN of 0.25 μmole/ml is adequate (Table V).

TABLE V

THE EFFECT OF DPN AND GLUCOSE ON THE INCORPORATION OF [^{3}H]TDR INTO DNA BY EXTRACTS OF OSMOTICALLY DISRUPTED ASCITES CELLS

The reaction mixture was 0.1 *M* with respect to tris buffer pH 7.9 and contained 50 μg DNA/ml, 5.0 μmoles ATP/ml, 10.0 μmoles $MgCl_2$/ml, 0.5 μC [^{3}H]TDR/ml and 3 mg protein/ml. Incubation time: 1 h.

DPN (μmole/ml)	*Glucose (μmole/ml)*	*DNA specific activity (Counts/min/μmole DNA-P)*
0	50	22,100
0	5	23,000
0.25	0	46,900
2.5	0	57,300
0.25	50	55,800
2.5	50	60,700
0.25	5	60,300
2.5	5	59,400

With sonic extracts glucose is also necessary at the relatively high concentration of 50 μmoles/ml[17]. It may be replaced by other energy sources such as hexose diphosphate but not by pyruvate or glutamate. Osmotic extracts, however, show as good activity in the absence of glucose as in its presence (Table V). With both extracts Mg^{++} ions are essential, the optimum concentration being about 5.0 μmole/ml (Fig. 5).

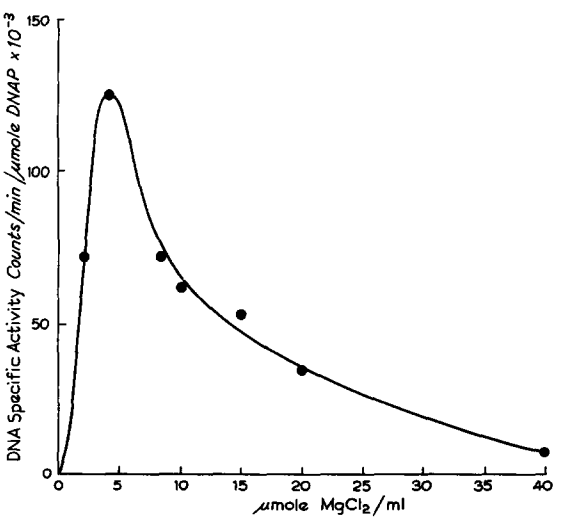

Fig. 5. The effect of varying concentrations of Mg^{++} on the incorporation of [^{3}H]TDR into DNA by osmotic extracts of Ehrlich ascites tumour cells in tris buffer. The medium was 0.1 *M* with respect to tris buffer pH 7.9 and contained 5.0 μmoles ATP/ml, 0.25 μmole DPN/ml, 50 μg DNA/ml, 0.5 μC [^{3}H]TDR/ml and 3.6 mg protein/ml.

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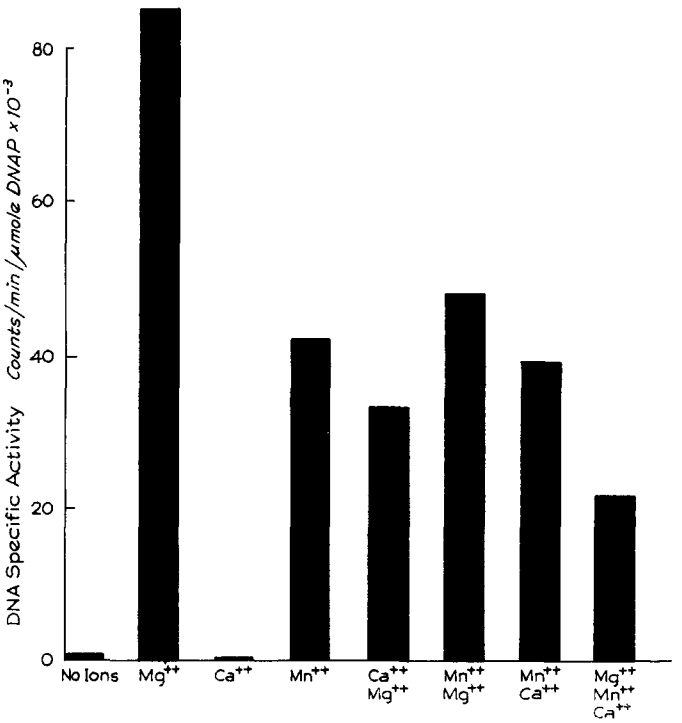

Fig. 6. The effect of different ions on the incorporation of [^{3}H]TDR into DNA by osmotic extracts of Ehrlich ascites tumour cells. The medium was 0.1 *M* with respect to tris buffer pH 7.9 and contained 5.0 μmoles ATP/ml, 0.25 μmole DPN/ml, 50 μg DNA/ml, 0.5 μC [^{3}H]TDR/ml and 3.2 mg protein/ml. $MgCl_2$ when added was at a concentration of 5.0 μmoles/ml, $MnCl_2$ at 2.5 μmoles/ml and $CaCl_2$ at 2.5 μmoles/ml.

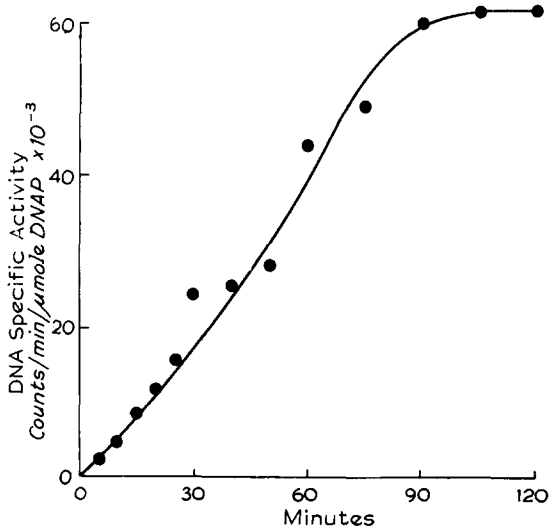

Fig. 7. The time course of [^{3}H]TDR incorporation into DNA by osmotic extracts of Ehrlich ascites tumour cells. The medium was 0.1 *M* with respect to tris buffer pH 7.9 and contained 5.0 μmoles ATP/ml, 0.25 μmole DPN/ml, 50 μg DNA/ml, 5.0 μmoles $MgCl_2$/ml, 0.5 μC [^{3}H]TDR/ml. and 3.3 mg protein/ml.

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The effect of other ions is shown in Fig. 6. Mg^{++} can be replaced to some extent by Mn^{++} but not by Ca^{++}. Combinations of ions are not so effective as Mg^{++} alone.

The time course of the reaction varies with the conditions and with the type of extract. With osmotic extract under optimum conditions incorporation is complete after 90-min incubation (Fig. 7).

If the incorporation of [^{3}H]TDR represents DNA synthesis it must be assumed that the other 3 bases are provided in the extract probably as the corresponding deoxyribonucleosides or nucleotides. As might be expected, therefore, supplementation with low concentrations of a mixture of deoxyribonucleotides was found to increase incorporation (Table VI). Higher concentrations have an inhibitory effect. On the other hand, dialysis diminishes incorporation (Table VII) and activity is not restored by the addition of the deoxyribonucleotides of adenine, guanine and cytosine (Table VII). The activity of the dialysed extract is not restored by addition of dialysate or of boiled extract.

TABLE VI

THE EFFECT OF ADDING MONO- AND TRI-PHOSPHATES OF THE DEOXYNUCLEOSIDES OF ADENINE, GUANINE AND CYTOSINE, ON THE INCORPORATION OF [^{3}H]TDR INTO DNA BY EXTRACTS OF OSMOTICALLY DISRUPTED ASCITES CELLS

The reaction mixture was 0.1 *M* with respect to tris buffer pH 7.9, and contained 50 μg DNA/ml, 5.0 μmoles ATP/ml, 0.25 μmole DPN/ml, 5.0 μmoles $MgCl_2$/ml, 0.5 μC [^{3}H]TDR/ml and 3 mg protein/ml. Incubation time: 1 h.

dAMP dGMP dCMP (mμmole each/ml)	*dATP (mμmole/ml)*	*dGTP (mμmole/ml)*	*dCTP (mμmole/ml)*	*DNA specific activity (Counts/min/μmole DNAP)*
0	0	0	0	54,900
2.5	0	0	0	99,500
5.0	0	0	0	63,900
10.0	0	0	0	37,800
0	5	0	0	82,000
0	0	5	0	84,400
0	0	0	5	107,300
0	2.5	2.5	2.5	138,000
0	5.0	5.0	5.0	149,000
0	10.0	10.0	10.0	170,500

TABLE VII

THE EFFECTS OF DIALYSIS AND OF ADDING DEOXYNUCLEOSIDE MONOPHOSPHATES ON THE INCORPORATION OF [^{3}H]TDR INTO DNA BY SOLUBLE EXTRACTS OF OSMOTICALLY DISRUPTED EHRLICH CELLS

The reaction mixture contained 0.1 *M* phosphate buffer pH 8.1, 50 μg DNA/ml, 2.5 μmoles ATP/ml, 50 μmoles glucose/ml, 0.5 μmole DPN/ml, 5 μmoles $MgCl_2$/ml, and 1 μc [^{3}H]TDR/ml. Incubation time: 1 h. The control extract was stored at 4° during the period of dialysis.

Nature of experiment	*DNA specific activity (Counts/min/μmole DNA-P)*
Control	224,000
Extract dialysed for 18 h against 2 × 2 l changes of distilled water at 4°	38,300
As above + 5 mμmoles/ml each of dAMP, dGMP and dCMP	29,200

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With undialysed extract activity is greatly increased by addition of the triphosphates of the deoxynucleotides of adenine, guanine and cytosine (Table VI). Each triphosphate alone is effective but the greatest response is produced by a mixture of all three, the effect being greater with higher concentrations of the added triphosphates.

These results indicate the participation of deoxynucleoside triphosphates in the incorporation process. Accordingly an examination was made of the acid-soluble nucleotide fraction, after the addition of inactive carriers, TDR, TMP and TTP before chromatography. The corresponding fractions recovered from the chromatogram showed the activities given in Table VIII. It is clear from these results that an appreciable amount of the [^{3}H]TDR is converted to TTP by the ascites extract.

TABLE VIII

THE DISTRIBUTION OF RADIOACTIVITY IN THE THYMIDINE DERIVATIVES OF THE ACID-SOLUBLE FRACTION AFTER THE INCORPORATION OF [^{3}H]TDR INTO DNA BY EXTRACTS OF OSMOTICALLY DISRUPTED ASCITES CELLS

The reaction mixture was 0.1 *M* with respect to tris buffer pH 8.3 and contained 50 μg DNA/ml, 5.0 μmoles ATP/ml, 0.25 μmole DPN/ml, 10 μmoles $MgCl_2$/ml, 0.5 μC [^{3}H]TDR/ml and 3.3 mg protein/ml. Incubation time: 1 h.

	Percentage of total acid-soluble activity
TTP	51
TDP	34
TMP	7
TDR	8

This evidence that TTP is produced during incorporation is confirmed by the observation that the process is inhibited by pyrophosphate to an extent of 40 % at a concentration of 2.5 μmoles/ml and 95 % at 25 μmoles/ml.

Early control expts. had confirmed that when the bases, prepared by the hydrolysis of [^{3}H]DNA, were separated by chromatography[1], the activity was confined to the thymine and that when the DNA was treated with deoxyribonuclease acid-soluble radioactive breakdown products were released. In the degradation by the

TABLE IX

THE INCORPORATION OF [^{3}H]TDR INTO DNA BY SOLUBLE EXTRACTS PREPARED FROM SONICALLY DISRUPTED RABBIT AND CHICKEN TISSUES

The reaction mixture contained 0.1 *M* phosphate buffer pH 8.1, 50 μmoles glucose/ml, 2.5 μmoles ATP/ml, 2.5 μmoles DPN/ml, 4 μmoles $MgCl_2$/ml, 500 μg/DNA/ml, 20 μmoles NaCl/ml, and 2 μC [^{3}H]TDR/ml. Incubation time: 2 h.

Tissue	*DNA specific activity (Counts/min/μmole DNAP)*
Rabbit bone marrow	24,750
Rabbit thymus	9,275
Rabbit appendix	1,485
Rabbit liver	131
Rabbit kidney	71
Chick embryo	12,025

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BURTON[26] method of DNA isolated after incorporation, products were obtained which were separated by ionophoresis. The fastest moving band contained material with the u.v. spectral properties of a thymidine derivative (E_{250}/E_{260}, 0.66 and E_{260}/E_{280}, 0.72: the corresponding figures for thymidine are 0.64 and 0.73 respectively). On the assumption that it was a thymidine derivative its content of thymine was determined and also its content of P. The molar ratio was 1:2, and the material was therefore identified as thymidine diphosphate. Activity measurements showed that approximately 10 % of the counts applied to the paper could be recovered in this material.

The slower moving bands obtained on electrophoresis also showed radioactivity. From their ionophoretic mobilities and their u.v. spectra it was concluded that they were oligonucleotides containing varying proportions of cytosine and thymine.

The results so far described have all been obtained with extracts of ascites tumour cells. Extracts prepared in a similar manner from a series of rabbit tissues and from chick embryo showed activities varying over a wide range (Table IX). The differences between tissues was less evident when carrier DNA was omitted.

DISCUSSION

It is clear that the material into which the [^{3}H]TDR is incorporated is DNA, since it is insoluble in acid, is precipitated by acid after alkaline incubation, can be isolated as DNA which on hydrolysis yields the expected 4 bases of which thymine alone is radioactive, and is broken down by deoxyribonuclease and diesterase to yield acid-soluble radioactive fragments. It has, however, not yet been found possible to demonstrate net synthesis. This is scarcely surprising, since from the activity and amount of the isolated DNA it can be calculated that although some 2.5–5 % of the counts present in the administered [^{3}H]TDR are incorporated into DNA, this represents quantities of the order of 0.2 – 0.5 m μmole TDR. Since the amount of DNA-P added to the reaction mixture is of the order of 0.2–0.5 μmole, an increase of 0.2–0.5 m μmole would be outside the limits of chemical estimation by the usual analytical methods.

Incorporation of radioactive precursors into RNA in cell-free systems from mammalian sources has been demonstrated by a number of authors[30–41] but there is good evidence to show that when labelled ATP is incorporated into RNA by cell-free systems a high proportion of it is to be found attached to a terminal monoesterified cytidylic acid of RNA[31, 33, 36, 38, 41].

The possibility that the TDR in our system is merely attached in a terminal position to DNA chains is rendered unlikely by the observation that radioactive thymidine-3′:5′-diphosphate can be isolated from the DNA, but it cannot be completely excluded on this evidence alone. The greatly increased incorporation of [^{3}H]TDR in the presence of carrier DNA suggests that the mechanism involves addition of nucleotides to existing DNA chains but this addition clearly cannot go on indefinitely since a saturation level is reached as shown in Fig. 2. This effect of DNA is more obvious in extracts from osmotically disrupted cells which contain very little DNA, than in extracts from sonically disrupted nuclei. The need for carrier DNA in biosynthesis was first demonstrated in the elegant work of KORNBERG and his associates on DNA synthesis in extracts of *Esch. coli*[42] and has been shown also by BOLLUM[14] and by MANTSAVINOS AND CANELLAKIS[43] working with regenerating

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liver extracts. In our system DNA from any source would appear to be effective. The fact that prior treatment with DNase abolishes its reactivity indicates a requirement for polynucleotide rather than for the component mononucleotides.

If incorporation of [^{3}H]TDR does in fact represent true DNA synthesis it must be assumed that the source of the other three bases is to be found in the small pool of deoxyribonucleosides and deoxyribonucleotides which is known to exist in mammalian tissues[44–51]. This view is supported by our observation that addition of dAMP, dGMP, and dCMP to our system increases the incorporation of [^{3}H]TDR (Table VI).

It is significant that stimulation of incorporation is found with concentrations of the deoxyribonucleotides which are of the same order of magnitude as the amount of thymidine present. The inhibition found with higher concentrations might be due to competition for phosphorylating mechanisms or might be similar to the effect noted by KLENOW, LANGER AND LICHTLER[52], of inhibition by deoxyadenosine of [^{14}C]formate incorporation into the DNA of intact ascites cells.

The reason for the reduction in activity by dialysis is obscure but it is not due entirely to loss of low molecular weight substances found in the dialysate (Table VII). The possibility of enzyme denaturation cannot be excluded but it should be noted that the phosphorylation of the deoxyribonucleotides to the corresponding triphosphates can be carried out readily by dialysed extracts[29].

While there is excellent evidence from the recent work of OCHOA and his colleagues[53] that RNA synthesis under the influence of bacterial polynucleotide phosphorylase proceeds by way of the ribonucleoside diphosphates, the work of KORNBERG[42, 54, 55] has shown very clearly that the phosphorylation of deoxyribonucleosides to the triphosphate level is an essential preliminary to DNA synthesis in extracts of *Esch. coli*. The same requirement would appear to hold in extracts of regenerating rat liver[14, 43]. It is highly probable that it holds also in extracts of ascites cells, for the results shown in Table VIII make it clear that a considerable proportion of the [^{3}H]TDR is converted to TTP. The inhibition by pyrophosphate which has been described for liver by BOLLUM[14] supports this argument.

The evidence available from several laboratories suggests that the biosynthesis of DNA takes place in 2 steps-phosphorylation of deoxyribonucleosides and deoxyribonucleotides to the triphosphate level and the assembly of the triphosphates into the polynucleotide chain. The ability of mammalian enzymes to phosphorylate deoxyribonucleotides has already been demonstrated with kidney mitochondria[56] rat-liver homogenates[57, 58] and bone-marrow extracts[59] and it has been shown[29] that the extracts of disrupted ascites cells are capable of phosphorylating all 4 deoxyribonucleotides to the triphosphate stage. The main need for ATP in the system is clearly for this phosphorylation process. In osmotic extracts ATP alone is required to promote incorporation of [^{3}H]TDR while in sonic extracts glucose and DPN are also necessary, presumably for the regeneration of ATP by glycolysis. It may be that in the cruder sonic extracts there is more destruction of added ATP, hence the need for its regeneration.

BOLLUM AND POTTER[12–15] and MANTSAVINOS AND CANELLAKIS[43] have recently described cell-free systems prepared from the supernatant fraction of regenerating rat liver homogenates which will promote incorporation of [^{3}H]TDR or [^{14}C]TDR into DNA by way of the deoxyribonucleoside triphosphates. These systems show the same requirement for Mg^{++} as does the ascites extract and presumably involve the

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same DNA synthetase. Of particular interest is the observation that in regenerating rat-liver extracts, the enzyme which is responsible for the synthesis of a substance peculiar to the nucleus should be located in the cytoplasm; our expts. with nuclear and cytoplasmic extracts of ascites cells amply confirm the cytoplasmic location of the synthetase.

Since it would be reasonable to suppose that an enzyme which is involved in DNA synthesis would be particularly abundant in tissues in which cell division is active, the results shown in Table IX for different tissues are not surprising. The highest activity is observed in extracts of proliferating tissues such as bone marrow and chick embryo while non-proliferating tissues such as liver and kidney show only very slight activity. These findings are in agreement with the observations of FRIEDKIN AND WOOD[5] on the incorporation of [^{14}C]TDR into tissue slices and with our previous observations on the incorporation of [^{32}P]phosphate[60], [^{14}C]formate and [^{14}C]adenine in rabbit tissues *in vivo*[61].

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