Thymidine Kinase: Evidence for its Absence from *Neurospora crassa* and Some Other Micro-organisms, and the Relevance of This to the Specific Labelling of Deoxyribonucleic Acid

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(Accepted for publication 12 July 1968)

SUMMARY

Thymidine kinase (EC 2.7.1.21), an enzyme that catalyses the phosphorylation of thymidine to thymidine-5'-phosphate in the presence of adenosine triphosphate and Mg^{2+} , was not detected in cell-free extracts of *Neurospora crassa*, *Aspergillus nidulans*, *Saccharomyces cerevisiae* or *Euglena gracilis*. Deoxyribonucleic acid was not specifically labelled in *Neurospora crassa* grown in the presence of [^{14}C]thymidine, [^{14}C]thymidine monophosphate or [^{14}C]thymidine triphosphate. Instead, the isotope was incorporated into both types of nucleic acid in a ratio which approximated the mole ratio of ribonucleic acid to deoxyribonucleic acid. It is considered that the absence of thymidine kinase prevents the specific incorporation of thymidine into deoxyribonucleic acid in intact cells of *Neurospora crassa*.

INTRODUCTION

The incorporation by micro-organisms of [3H]- and [^{14}C]-labelled thymidine has often been used as a simple and reliable technique for the specific labelling of newly synthesized deoxyribonucleic acid (DNA) (Evans, 1966). This procedure has not been successfully applied to the ascomycete *Neurospora crassa*. Neither thymine- nor thymidine-requiring mutants of *N. crassa* have been reported (*Neurospora* Stock List, Fungal Genetics Stock Centre, Dartmouth College, U.S.A.) and thymidine will not support the growth of pyrimidine-requiring *N. crassa* mutants (Fink & Fink, 1961) which otherwise grow in minimal medium when supplemented with uracil, uridine or cytidine (Loring & Pierce, 1944; Loring, Hammell, Levy & Bortner, 1952; Chakraborty & Loring, 1960). Although the tracer from labelled thymidine is incorporated by *N. crassa* into nucleic acids, the label is not confined to DNA (Chakraborty & Loring, 1960; Fink & Fink, 1961, 1962 *a, b*). The fact that *Neurospora crassa* does not specifically incorporate exogenous thymidine led Chakraborty & Loring (1960) and Fink & Fink (1962 *a*) to postulate that thymidine kinase is absent from this fungus. However, the phenomenon might also be explained by active degradation of added thymidine (Adelstein & Lyman, 1968) or in terms of membrane permeability effects (see Lieberman, Kornberg & Simms, 1955). Radioautographic evidence for other fungi and some algae suggests that other micro-organisms may also be deficient in thymidine kinase (Cleaver, 1967). In labelling experiments, [3H]thymidine was not

detected in the nuclei of two pathogenic fungi, *Puccinia graminis* var. *tritici* (Bhattacharya & Shaw, 1967) and *Uromyces phaseoli* (Staples & Ledbetter, 1960), and the label was found in both ribonucleic acid (RNA) and DNA of *Euglena gracilis* (Sagan, 1965), *Spirogyra grevilleana* (Meyer, 1966) and *Schizosaccharomyces pombe* (Mitchison, 1963).

In the present work, cell-free extracts of several micro-organisms were studied for the presence of the enzyme thymidine kinase (ATP: thymidine 5'-phosphotransferase; EC 2.7.1.21) (Okazaki & Kornberg, 1964). Also, thymidine and thymidine nucleotides were supplied to cultures of *Neurospora crassa* in an attempt to label specifically the DNA.

METHODS

Organisms. The micro-organisms used were *Escherichia coli* strains B and B-185, *Aspergillus nidulans* BI-1, *Saccharomyces cerevisiae* (DELFT 1171), and *Neurospora crassa* strains STA 4 (wild-type) and *pyr-3* (1298). Strain 1298 of *N. crassa* requires a pyrimidine supplement, and is able to grow in a medium containing uracil, uridine or cytidine (Loring *et al.* 1952).

A frozen preparation of broken cells of *Euglena gracilis* was obtained from Dr N. S. Scott (CSIRO Division of Food Preservation, University of Sydney, Australia).

Culture media and growth conditions. *Escherichia coli* was grown in Hershey's nutrient broth (Chase & Doermann, 1958) with extra glucose 0.5 (g./l.). *Neurospora crassa* was grown in the minimal N medium of Vogel (1956). The carbon source in solid agar media (2%, w/v, Bactoagar) was 1% (w/v) sucrose and 1% (v/v) glycerol; in liquid cultures it was 2% (w/v) sucrose. The media for strain 1298 were supplemented with uracil (Nutritional Biochemicals Corp., U.S.A.) (0.4 mg./ml.), except in labelling experiments when 0.05 mg./ml. was used. In one experiment, the minimal medium was supplemented with 0.2% yeast extract. *Aspergillus nidulans* was grown in minimal medium (Cove, 1966) containing 0.1% yeast extract. *Saccharomyces cerevisiae* was grown in YEPD medium which contains yeast extract (Manney, 1964).

The following Difco products (Difco Laboratories, Michigan, U.S.A.) were used in the preparation of media: nutrient broth, Bacto-peptone, yeast extract and Bacto-agar. All media were autoclaved at 120° for 10 to 15 min. except in the case of labelling experiments to be described later. The micro-organisms were grown in 100 ml. medium in 500 ml. conical flasks. Inocula of *Escherichia coli* and *Saccharomyces cerevisiae* were actively growing liquid cultures. Suspensions of conidia in water, prepared from 5- to 10-day cultures of *Neurospora crassa* and *Aspergillus nidulans*, were used as inocula. Liquid cultures were aerated for 14 to 20 hr at 28° to 30°; at 37° for *E. coli*.

Preparation of thymidine kinase from *Escherichia coli*. A partially purified enzyme was prepared from *Escherichia coli* B-185 according to the method of Okazaki & Kornberg (1964). The enzyme fraction used was fraction 5 of their procedure ('fraction 5 thymidine kinase').

Reagents. The labelled materials used were obtained from the following sources: [2-¹⁴C]thymidine (35.9 mc./m-mole) and [2-¹⁴C]uridine (36.7 mc./m-mole) from the Radiochemical Centre, Amersham, Buckinghamshire, U.K., and [2-¹⁴C]thymidine triphosphate (50 mc./m-mole) from Schwartz BioResearch Inc., Orangeburg, New York, U.S.A.

[2-¹⁴C]Thymidine monophosphate was prepared enzymically from [2-¹⁴C]thymidine with the fraction 5 thymidine kinase as indicated by Okazaki & Kornberg (1964). The product was isolated by low-voltage paper electrophoresis on Whatman 3 MM paper with sodium citrate buffer (0.05 M; pH 3.4), and eluted from the paper with water (Dent, 1947).

[¹⁴C]DNA was extracted by the method of Marmur (1961) from the chemoautotrophic bacterium *Nitrosomonas europaea* grown in medium containing [¹⁴C]carbonate (36.4 mc./m-mole; Radiochemical Centre, Amersham, U.K.).

Unlabelled di- and tri-phosphates of adenosine (ADP, ATP) and mono- and tri-phosphates of thymidine (TMP, TTP) were bought from Calbiochem (California, U.S.A.). Thymine and thymidine (dT) were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). All other chemicals were of analytical reagent grade.

Preparation of cell-free extracts. All operations following the collection of organisms were done at 0° to 4°. With the exceptions described in the Results section, the buffer used in these experiments was tris-HCl (2-amino-2-hydroxymethylpropane-1,3-diol chloride; 0.05 M, pH 7.5). Organisms were collected by centrifugation at 7000 to 15,000 g for 2 to 10 min., washed in buffer once or twice and resuspended in 2 to 7 ml. buffer/g. wet weight organism. The organisms were extracted by ultrasonic disruption for 2 to 5 min. (MSE 20 kc. Ultrasonic Disintegrator fitted with a 0.9 cm. titanium probe). Each crude homogenate was centrifuged at 5000 g for 10 min. to remove cell debris. Supernatant fractions were used for all enzyme assays. After precipitation with 10% (w/v) trichloroacetic acid, protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Measurement of thymidine kinase activity. The basic reaction mixture for thymidine kinase determinations was essentially that described by Okazaki & Kornberg (1964); the main difference was the inclusion of an ATP regenerating system. The reaction volume (0.07 ml.) contained buffer (tris-HCl, pH 7.5; 5.0 μmoles), bovine serum albumin (20 μg.; Commonwealth Serum Laboratories, Australia), ATP (0.15 μmole), MgCl₂ (0.50 μmole), creatine phosphate (0.7 μmole), creatine phosphokinase (4 μg.; Calbiochem), thymidine (0.012 or 0.017 μmole; specific activity 5.6 × 10⁶ or 1.8 × 10⁷ counts/min./μmole) and cell-free extract (0.01 ml. containing 0.5 μmole buffer and 0.02 to 0.40 mg. protein). Variations from this mixture are recorded in the relevant sections below. Extracts heated in boiling water for 2 min., as well as fraction 5 thymidine kinase, were used in separate control incubations. After 30 min. incubation at 37° each reaction was stopped by immersing the tubes in boiling water for 1.5 min. The precipitate was removed after centrifugation at 2000 g, and duplicate 0.025 ml. samples were analysed by low-voltage paper electrophoresis, together with added marker compounds thymidine, TMP and TTP. Whatman 3 MM paper and 0.10 M-sodium citrate buffer (pH 3.4) were used. Marker areas, located with short wavelength ultraviolet radiation (Mineralight model SL 2537), were cut out and the quantity of isotope in each measured in a liquid scintillation spectrometer (a model 3002 Tricarb Liquid Scintillation Spectrometer, Packard Instrument Co. Inc., Illinois, U.S.A.; or a model 6851 Unilux liquid scintillation system, Nuclear Chicago Corp., Illinois, U.S.A.). The scintillation fluid contained 0.20 g. POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene)/l. and 3.0 g. PPO (2,5-diphenyloxazole)/l. in toluene. Most samples were counted for 20 min. The quantity of [2-¹⁴C]thymine in the reaction mixture after incubation was determined by liquid scintillation counting. The thymine and thymidine

in 0.01 ml. samples of the reaction mixtures were separated by ascending paper chromatography in 0.25 N-formic acid on Whatman 1 paper (Shapiro, Eigner & Greenberg, 1965).

Thymidine kinase activity is expressed in the unit $\mu\text{m-mole}$ TMP produced in 30 min. at 37° per assay. All tabulated data have been corrected by subtraction of the background quantity of label corresponding to TMP in heated-extract control incubations.

Labelling of intact Neurospora crassa. Samples (2.5 ml.) of Vogel's minimal N medium, supplemented with 0.15 mg. uracil and one of the [^{14}C]-labelled compounds, were individually sterilized by passing through a membrane filter (13 mm., 0.22 μ ; Millipore Corp., Mass., U.S.A.). This procedure was adopted because autoclaving was associated with dephosphorylation of labelled nucleotides. Conidia from 6-day cultures of *Neurospora crassa*, STA 4 and 1298, were suspended in minimal medium. Hyphal fragments were removed from this inoculum by filtration through fine nylon mesh. Approximately 2.5×10^8 conidia in 0.5 ml. were added to each incubation medium in an 18 $\times$ 150 mm. sterile test tube. The cultures were vigorously agitated by a wrist-action shaker for 16–18 hr at 28° to 30°. No evidence for bacterial contamination after incubation was detected by the naked eye, by phase-contrast microscopy, or after small samples of some cultures had been streaked onto solid nutrient medium (Hershey's nutrient broth; Chase & Doermann, 1958).

The cultures were collected by centrifugation at 5000 g for 5 min. at 4°. Except where otherwise stated, all subsequent operations were done at 0° to 4°. After washing twice with 3 ml. water, the organisms were suspended in a total volume of 2.5 ml. of water containing 0.10 μmole DNA-phosphorus as calf thymus DNA. They were disrupted ultrasonically for 4 to 6 min. and centrifuged at 5000 to 7000 g for 5 min. Each pellet was washed with 0.5 ml. water and the washing was bulked with the respective supernatant fraction. The supernatant fluids were then fractionated according to a variation of the Schmidt–Thannhauser procedure (Munro & Fleck, 1966), to yield the following fractions: perchloric acid-soluble; acid-insoluble but alkali-hydrolysable (RNA); acid-insoluble and alkali-non-hydrolysable (DNA). The lipid extraction step was omitted and the period of hydrolysis of RNA at 37° was from 2 to 4 hr. Samples of each fraction, dried on Whatman 3 MM paper, were counted in a liquid scintillation spectrometer for 40 min. Nucleic acid bases were released by hydrolysis of further samples with 99% formic acid in sealed Pyrex-glass tubes at 175° for 30 min. (Wyatt, 1951). Marker bases were derived from either uracil+calf thymus DNA or thymine+yeast RNA (Schwartz BioResearch Inc.). These were included with the sample being hydrolysed. This also enabled a check to be made that each hydrolysis was complete. The dried hydrolysates, dissolved in 0.1 N-HCl, were separated by ascending paper chromatography on Whatman 1 paper in *iso*-propanol+water+conc. HCl (65+18.4+16.6 by vol.; Wyatt, 1951). After detection with short-wavelength ultraviolet radiation, areas containing nucleic acid bases were cut out and counted in a liquid scintillation system. The possibility that label was present in other areas of the chromatograms was tested by dividing replicate chromatograms into 0.25 in. sections and counting each section in sequence. Moreover, when compared with a photographic print made with ultraviolet radiation (Markham & Smith, 1949) the resulting pattern of label corresponded closely with the ultraviolet radiation-absorbing material.

RESULTS

Thymidine kinase activity in cell-free extracts

Except for extracts of *Escherichia coli* B, significant thymidine kinase activity was not detected in extracts of any of the micro-organisms tested (Table 1). When cell-free extracts of *E. coli* B were mixed in turn with the other extracts, the resultant activity was greatly diminished (Table 1). This suggested the presence of an active inhibitor, a thymidine degradative activity, or a TMP phosphatase activity. Most of the ATP was still present at the completion of each incubation, suggesting that this result was not due entirely to the presence of an ATPase. However, when the mixed incubations were repeated with the fraction 5 thymidine kinase instead of a cell-free extract of *E. coli*, the inhibition of the thymidine kinase activity was markedly less (Table 1).

Table 1. *Thymidine kinase activities in cell-free extracts of various micro-organisms, each assayed separately and when mixed with enzyme fractions of Escherichia coli*

Organism	Protein concn. (mg./assay)	Thymidine kinase activity		
		Extract alone	Combined with cell-free extract of <i>E. coli</i> †	Combined with fraction 5 thymidine kinase
Control	—	0.000	0.565	0.943
<i>N. crassa</i> STA 4	0.11	0.004	0.088	0.634
<i>N. crassa</i> STA 4*	0.10	0.001	0.146	0.598
<i>A. nidulans</i>	0.10	0.001	0.183	0.685
<i>S. cerevisiae</i>	0.40	0.004	0.035	0.748
<i>E. gracilis</i>	0.02	0.001	0.325	0.872

* Organisms grown in minimal medium supplemented with yeast extract. Assays as described in Methods. Values mean of four determinations: μ m-mole TMP produced in 30 min./assay.

† Protein added in *E. coli* crude extract was 0.10 mg./assay.

When the time-course of the reaction was followed (Fig. 1 *a*), the reaction catalysed by the *Escherichia coli* extract was non-linear over a 30 min. period and the enzyme activity was significantly depressed when *E. coli* extract was mixed with that of *Saccharomyces cerevisiae*. In contrast, corresponding reactions with the fraction 5 thymidine kinase yielded curves which closely correspond (Fig. 1 *b*). When the concentration of ATP was increased in reaction mixtures containing extracts of both *E. coli* and *S. cerevisiae* (Table 2), the final yield of TMP was increased. These data (Table 2) also showed that little of the initial thymidine remained after a 30 min. incubation, and that the rate of degradation of thymidine to thymine was decreased in the presence of greater concentrations of ATP. It was also observed that when an *E. coli* extract alone was used with normal amounts of ATP (0.15 μ mole/assay), practically all the initial thymidine was degraded. In incubation mixtures which did not include a crude extract of *E. coli*, the degradation of thymidine never exceeded 10% of the amount originally added. These data have been collectively interpreted to indicate that cell-free extracts of *E. coli* degrade thymidine, and that this effect is decreased by high concentrations of ATP. Addition of other extracts may promote the hydrolysis of ATP, resulting in an effective increase in this activity. For this reason,

fraction 5 thymidine kinase was used instead of *E. coli* cell-free extract in subsequent control incubations.

No activity was detected after extraction of *Neurospora crassa* in a ground-glass homogeniser. In contrast, TMP kinase activity was readily measured in extracts prepared by homogenization and ultrasonic disintegration. Thymidine kinase activity was not detected even when two strains of *N. crassa* (1298, STA 4) were extracted

Fig. 1. The effect of cell-free extracts of *Saccharomyces cerevisiae* on *Escherichia coli* thymidine kinase. Assays as described in Methods. Incubations terminated at specified times after adding the enzyme fractions. *a*, Extract of *S. cerevisiae* assayed alone and when mixed with cell-free extract of *E. coli*. *b*, Extract of *S. cerevisiae* assayed alone and when mixed with fraction 5 thymidine kinase: 1, Cell-free extract of *S. cerevisiae*; 2, cell-free extract of *E. coli* B; 3, mixture of 1 and 2; 4, fraction 5 thymidine kinase; 5, mixture of 1 and 4.

Table 2. Influence of initial ATP concentration on the amount of TMP synthesized, and on thymidine degradation to thymine by cell-free extract of *Escherichia coli* mixed with that of *Saccharomyces cerevisiae*

Assay conditions as described in Methods (initial concentration of thymidine constant at 11.7μ m-mole/assay), except that the initial quantity of ATP per assay was increased to the value listed in the table. The listed quantities of TMP, thymidine and thymine are in units of μ m-mole of compound present per assay after a 30 min. incubation.

Cell-free extract	Initial quantity of ATP (μ mole)	TMP produced	Thymidine remaining	Thymine released
<i>E. coli</i> B	1.35	0.95	—	—
<i>E. coli</i> + <i>S. cerevisiae</i>	0.15	0.10	1.8	9.8
<i>E. coli</i> + <i>S. cerevisiae</i>	0.75	0.56	3.1	8.1
<i>E. coli</i> + <i>S. cerevisiae</i>	1.35	0.65	4.2	6.8

and assayed in the presence of each of three buffers (tris-HCl, potassium phosphate, potassium phosphate + 10^{-4} M-EDTA; all 0.05 M, pH 7.5). It was calculated from these data (Student's *t* test) that at the 95% confidence level this set of assays would have detected as significant a mean activity in excess of that for the control assays equal to 0.010μ m-mole TMP produced in 30 min. per reaction mixture. Allowing for the range in the quantity of protein in the individual assay reactions, this rate corre-

sponds to a minimal detectable specific activity of 0.24 to 0.44 $\mu\text{m-mole}$ TMP synthesized/mg. protein in 30 min.

When mixtures of fraction 5 thymidine kinase and cell-free extracts of *Neurospora crassa* STA4 were tested for activity, with and without an ATP regenerating system (creatine phosphate + creatine phosphokinase), the results showed that the apparent inhibition of the thymidine kinase activity by the crude extract was completely reversed in the presence of an adequate amount of ATP (Table 3). It was therefore concluded that the non-detection of a thymidine kinase activity in extracts of *N. crassa* did not result from the presence of an inhibitor, a TMP phosphatase activity, an ATPase or a thymidine degradative enzyme.

Table 3. *Activity of fraction 5 thymidine kinase with and without a cell-free extract of Neurospora crassa and an ATP-regenerating system*

Assays as described in Methods. Buffer was potassium phosphate (0.05 M; pH 7.5); 0.05 mg. protein in *N. crassa* extract per assay. The ATP regenerating system was creatine phosphokinase + creatine phosphate (see Methods). Units are $\mu\text{m-mole}$ TMP produced in 30 min./assay.

Fraction assayed	No ATP regenerating system	+ ATP regenerating system
Extract of <i>N. crassa</i> STA 4	0.00	0.00
Fraction 5 thymidine kinase	0.83	0.87
<i>N. crassa</i> + fraction 5 thymidine kinase	0.53	0.90

Labelling of intact cells of Neurospora crassa

When *Neurospora crassa* strains 1298 and STA4 were grown in the presence of [^{14}C]thymidine, the isotope was recovered in both the RNA and DNA fractions (Table 4). Had a specific thymidine kinase been active, it is reasonable to expect that the greater part of the label would have been in the DNA, instead of the RNA as observed (ratio RNA:DNA, Table 4). Several lines of evidence show that this accumulation of label in the RNA fraction cannot be explained simply by hydrolysis of DNA during the fractionation: (1) In an experiment with non-labelled *N. crassa* to which [^{14}C]DNA had been added before extraction and fractionation, less than 6% of the acid-insoluble labelled material was hydrolysed by the alkaline treatment. (2) In RNA fractions from labelled organisms, the tracer was recovered in uracil but not in thymine (Table 5). (3) The ratio of labelled RNA:labelled DNA in the cultures (5 to 16, Table 4) agreed with the value of 8 determined previously (Chakraborty & Loring, 1960).

Similar distributions of the label between RNA and DNA were observed regardless of whether *Neurospora crassa* was grown in the presence of [$2\text{-}^{14}\text{C}$]thymidine, TMP, TTP or uridine (Table 4). TTP was incorporated with relatively low efficiency compared with the other compounds. The similarity of the labelling patterns in cultures containing thymidine derivatives and in those supplemented with uridine strongly suggested that the four labelled compounds tested were incorporated by way of a common metabolic intermediate. This might be uridine or a derivative (Fink & Fink, 1962 *b*). This hypothesis was supported by the data of Table 5, which show that the tracer was recovered in the three major pyrimidine bases. The amount of label in the purine bases (adenine, guanine) was negligible, never exceeding 5% of the total recovered isotope. Cytosine and thymine derived by hydrolysis of each DNA fraction, were labelled with similar specific activities. This followed from the fact that when the

mole ratio (expressed as mole % guanine+cytosine) was calculated for DNA from the proportions of labelled bases, the figure obtained (52.1%) was in agreement with a previously reported value for *N. crassa* (52 to 55%; Storck, 1966). The estimated value was calculated from the data of Table 5, excluding those for the uridine-labelled material. The quantity of labelled cytosine in DNA was obtained by subtraction of the cytosine estimated to be derived from RNA contaminating the DNA fractions. The RNA-cytosine was estimated from the amount of uracil in the DNA fractions, assuming a value of 1.025 for the ratio cytosine:uracil in *N. crassa* RNA (Storck,

Table 4. *Neurospora crassa*: distribution of [^{14}C] among fractions derived from intact cells grown in medium containing labelled pyrimidine nucleosides and nucleotides

Expt. no.	Strain of <i>N. crassa</i>	Compound	[^{14}C]-substrate		Proportion of label added initially			Ratio of label in RNA: label in DNA
			Quantity added ($\mu\text{m-mole}$)	Proportion incorporated	Acid-soluble	RNA	DNA	
1	1298	dT	3.83	0.64	0.306	0.220	0.032	6.96
		TMP	3.78	0.80	0.311	0.334	0.065	5.14
2	1298	dT	3.92	0.69	0.114	0.422	0.044	9.69
		TMP	3.78	0.78	0.136	0.499	0.049	10.22
		TTP	4.00	0.28	0.084	0.164	0.013	12.88
		Uridine	5.44	0.54	0.056	0.359	0.023	15.92
3	1298	dT	3.92	0.83	0.214	0.444	0.052	8.57
		TMP	3.78	0.89	0.219	0.461	0.059	7.75
		TTP	4.00	0.46	0.103	0.196	0.025	7.92
		Uridine	5.44	0.87	0.166	0.526	0.052	10.14
4	STA 4	dT	3.83	0.72	0.199	0.421	0.045	9.41

Table 5. Distribution of [^{14}C] among the pyrimidine bases within RNA and DNA fractions prepared from labelled *Neurospora crassa*

The data were obtained, as described in Methods, by hydrolysis and fractionation of RNA and DNA obtained from organisms supplied with the specified labelled substrate. The RNA and DNA fractions listed correspond to those of Expts. 2 and 4 of Table 4. Values are expressed as proportion of label recovered from each chromatogram.

<i>N. crassa</i> strain	Labelled supplement	RNA			DNA		
		Thymine	Uracil	Cytosine	Thymine	Uracil	Cytosine
STA 4	dT	0.03	0.45	0.48	0.29	0.16	0.51
1298	dT	0.03	0.50	0.41	0.36	0.11	0.49
1298	TMP	0.04	0.47	0.42	0.34	0.13	0.50
1298	TTP	0.03	0.53	0.37	0.37	0.11	0.49
1298	Uridine	0.03	0.46	0.46	0.38	0.09	0.50

1965). This agreement between the estimated and reported values of the mole ratio rendered it unlikely that even a small proportion of the thymidine was incorporated via a direct metabolic pathway into DNA (presumably including thymidine kinase), since this would have resulted in a marked decrease in the estimated mole ratio for the

DNA fractions. The data of Tables 4 and 5 agree with those previously obtained from similar labelling experiments with *N. crassa* (Chakraborty & Loring, 1960; Fink & Fink, 1961; 1962 *a, b*).

Although these experiments showed that label from [2-¹⁴C]thymidine, TMP and TTP entered *Neurospora crassa* and was incorporated into nucleic acids, the distribution of the isotope revealed that it was not incorporated directly into DNA. These data are therefore consistent with the postulate that *N. crassa* lacks a specific thymidine kinase.

Fig. 2. Metabolic sequence for the synthesis of thymine residues of DNA in *Escherichia coli* (derived from Okazaki & Kornberg, 1964).

DISCUSSION

The metabolic relationship between the *de novo* and 'salvage' pathways of thymidine incorporation into DNA in *Escherichia coli* is shown in Fig. 2. Indirect evidence suggests that the enzyme thymidine kinase may not be present in some micro-organisms, including *Neurospora crassa* (Chakraborty & Loring, 1960; Fink & Fink, 1961; 1962 *a*; Staples & Ledbetter, 1960; Mitchison, 1963; Sagan, 1965; Meyer, 1966; Bhattacharya & Shaw, 1967). Direct experimental evidence given in the present paper strongly suggests that thymidine kinase is not present in cell-free extracts of these micro-organisms. In the absence of thymidine kinase, the normal salvage route from thymidine to DNA cannot operate. This would explain the lack of efficient or direct incorporation of thymidine into the DNA of such micro-organisms. However, tracer was certainly incorporated into the nucleic acids of *N. crassa* when supplied as [2-¹⁴C]thymidine, [2-¹⁴C]TMP or [2-¹⁴C]TTP, although the molar quantities were very small. In fact, a supplement of thymidine does not support growth of pyrimidine-requiring mutants of *N. crassa* in minimal medium (Fink & Fink, 1961). This implies that the incorporation of label involves a relatively inactive metabolic pathway. The incorporation of thymidine is thought to involve the removal of the 5-methyl group (Fink & Fink, 1962 *a, b*), leaving a uridine derivative which is then further metabolized to yield all the required pyrimidine nucleotides. This may involve the enzyme thymine 7-hydroxylase, as suggested by Abbott, Kadner & Fink (1964) and Abbott *et al.* (1967). Thymidine can be utilized as a substrate by this enzyme (Abbott *et al.* 1964).

The lack of thymidine kinase in *Neurospora crassa* can explain the observed absence of specific labelling of its DNA in the presence of labelled thymidine. TMP and TTP are believed to be intermediates in the synthesis of thymine residues of DNA (Okazaki & Kornberg, 1964). Yet, data from the intact-cell labelling experiments indicate that isotope supplied as TMP and TTP was not incorporated directly into DNA either. Since this may reflect cell-membrane impermeability to these compounds (see Loring *et al.* 1952), a method for increasing cell-membrane permeability might overcome this problem (see Buttin & Kornberg, 1966). This in turn might enable DNA to be labelled with at least some increase in specificity.

The authors thank Professor D. J. D. Nicholas for helpful discussions and Miss Marlene Short and Mr G. J. Megaw for invaluable technical assistance. Financial assistance in the forms of a CSIRO Postgraduate Scholarship to A. R. G. and a U.S. Public Health Service research project grant (No. R05 TW268) to J. F. J. is gratefully acknowledged.

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