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

IV. THE PHOSPHORYLATION OF THYMIDINE

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SUMMARY

Kinases responsible for the formation of thymine-deoxyribose-5'-monophosphate, diphosphate and triphosphate from thymine-deoxyribose have been purified from extracts of Ehrlich ascites carcinoma.

These enzymes are also found in rabbit tissues, highest levels of activity being observed in thymus and bone marrow.

In rat liver regenerating after partial hepatectomy thymine-deoxyribose, thymine-deoxyribose-5'-monophosphate and diphosphate kinases appear in that order, maximum activities being observed between 30 and 48 h after the operation.

In cultures of L. strain fibroblasts there is again a sequential appearance of thymine-deoxyribose, thymine-deoxyribose-5'-monophosphate and diphosphate kinases during the early part of the growth phase and the activities of all three enzymes decline before the completion of growth. Growth of L. strain fibroblasts in a medium containing thymine-deoxyribose lead to increases in kinase activities which were compatible with enzyme induction.

INTRODUCTION

In previous papers[1-3] we have described an enzyme system in cell free extracts of Ehrlich ascites carcinoma cells which promotes the incorporation of [^{3}H]TdR into carrier DNA and have shown that this process involves the phosphorylation of TdR to the corresponding triphosphate. The present paper describes the kinase systems responsible for such phosphorylation in extracts of ascites cells and of other mammalian tissues such as regenerating rat liver and strain L fibroblasts. Similar enzymes have been described in regenerating rat liver by POTTER *et al.*[4,5] by MANTSAVINOS AND CANELLAKIS[6] and by HIATT AND BOJARSKI[7].

Abbreviations: DNA, deoxyribonucleic acid; TdR, thymidine (thymine-deoxyribose); TMP, TDP and TTP, thymidine 5'-mono-, di-, and tri-phosphates respectively; ATP, adenosine triphosphate; DNase, deoxyribonuclease; Tris, 2-amino-2-hydroxymethyl propane-1, 3-diol. TdR kinase, TMP kinase and TDP kinase denote respectively the enzymes systems responsible for the formation of TMP, TDP and TTP.

MATERIALS AND METHODS

Enzyme assay

5.5 μmoles $MgCl_2$, 5.5 μmoles ATP, 110 μmoles Tris buffer pH 7.9 and 0.5 μC [^{3}H]TdR were incubated in 25-ml flasks for 90 min at 37° with an amount of enzyme corresponding in activity to 0.5 ml whole ascites extract. The final volume was 1.1 ml. The contents of the flasks were then transferred to chilled centrifuge tubes, 0.15 ml 4 *N* $HClO_4$ was added and the precipitated protein centrifuged down. All manipulations were carried out in the cold. The supernatant fluid was brought to pH 7.8 with KOH and the precipitated $KClO_4$ removed by centrifugation. 1 ml of the supernatant fluid was applied to an ECTEOLA cellulose column[8] (0.030 mequiv./ml) 8 cm × 1 cm. The column was eluted successively with 50-ml portions of water, 0.01 *N* HCl, 0.05 *N* HCl and 0.5 *N* HCl to remove TdR, TMP, TDP and TTP respectively. The columns were regenerated between runs by washing with 125 ml water and then resuspending the material in 25 ml water and allowing the column to drain.

0.2-ml portions of each 50-ml eluate were plated on stainless steel planchets and the time required for 500 counts determined in a Nuclear Chicago windowless gas flow counter. The total counts/min in each of the column eluates are expressed as a percentage of the total counts/min recovered from the column. The incorporation of [^{3}H]TdR into DNA was followed in some experiments by the method previously described[1].

Biological material

Cell free extracts were obtained from osmotically disrupted Ehrlich ascites tumour cells as previously described[1].

Rat and rabbit tissues were quickly removed from the exsanguinated animals, blotted with filter paper, finely minced with scissors in an ice-cooled beaker and disintegrated in a Potter type homogenizer, usually in 20 ml water for each liver or in 10 ml water for other organs. The homogenates were centrifuged at 105,000 × *g* for 1 h and the cell and particle-free extracts collected. The protein concentrations were adjusted to 3–6 mg/ml except in the case of liver extracts which usually had a protein concentration of 15–20 mg/ml.

In experiments with regenerating rat liver partial hepatectomy was kindly performed by Dr. R. Y. THOMSON by the method of HIGGINS AND ANDERSON[9].

Cell cultures

Adult mouse subcutaneous fibroblasts NCTC strain L clone 929 (see ref. 10) were grown in accordance with the methods described by PAUL[11].

The cells (1–20 million) were harvested by incubating for 5 min at 37° with 10 ml trypsin solution and where necessary by scraping the flask with a rubber-tipped glass rod. The cells were washed twice with 0.9 % NaCl solution and suspended in 0.25–1.0 ml water. A portion of the cell suspension was diluted with 0.9 % NaCl and counted in an automatic particle counter. The remainder was disrupted by exposure for 90 sec to the vibrations of a Mullard ultrasonic drill (20 kc./sec). A portion of the disruptate containing 0.25–1.0 mg protein was used for each assay. The same amount of protein was used in each flask.

Protein determinations in all cases were carried out by the method of LOWRY *et al.*[12].

[^{3}H]TMP was prepared by exposure of 250 mg calcium thymidylate to 7 C tritium gas in an 83 % mixture with hydrogen for 17 days. This was carried out at the Atomic Energy Research Establishment, Harwell, England. Subsequently the TMP was purified by lyophilizing several times from a large volume of water to remove labile tritium. It was then fractionated on a Dowex-1-Cl column and further purified by paper chromatography in ammonium isobutyrate[2], butanol–water[13] and ethanol–ammonium acetate[14] until all the radioactivity was found to be associated with the area corresponding to TMP. The final product was converted to the sodium salt by passage through a Dowex-50-Na column and had a specific activity of 10^7 counts/min/μmole as determined in a counter of approx. 30 % efficiency.

RESULTS

Enzyme purification

All manipulations were carried out in the cold.

Precipitation with acid: The supernatant fluid obtained by high speed centrifugation of the ascites tumour cell extract (containing 3–4 mg protein/ml) was brought to pH 4.5 with 1 *N* acetic acid. The precipitate was centrifuged down and the supernatant fluid brought to pH 7 with 1 *N* NaOH. This fluid (fraction AS 4.5) contained all the TMP kinase and TDP kinase and generally more than 66 % of the TdR kinase, and since the total protein concentration was 0.9–1.1 mg/ml, an approximately three-fold purification of TMP kinase and TDP kinase was achieved by this step (Table I).

Ammonium sulphate fractionation: To each 100 ml of AS 4.5, 21.5 g ammonium sulphate were added (final pH 6.4) to give approx. 35 % saturation. The solution was allowed to stand for 30–60 min, and the precipitate centrifuged down and redissolved in two-thirds of the original volume of water or of 0.01 *M* Tris buffer pH 7.8–7.9 giving fraction ASP 35 of protein concentration 0.15–0.30 mg/ml. It retained more than two-thirds of the TdR kinase activity of the AS 4.5.

Precipitates obtained by adding 19–21.5 g ammonium sulphate to the AS 4.5 showed variable retention of TMP kinase activity and could promote the formation of some TDP but not TTP. Similar results were obtained with extracts of rabbit bone marrow and thymus and in some instances with liver. Occasional preparations retained two-thirds or more of the original TMP kinase activity and showed a 15–20 fold

TABLE I

THYMIDINE KINASE PURIFICATION FROM ASCITES CELL EXTRACT

	*Total units**	*Specific activity***
Extract	387	70
AS 4.5	410	171
ASP 35	160	960
ASP 35 ECTEOLA fraction	< 20–25	> 2500

* Per cent TdR phosphorylated for appropriate dilution.
** Per cent TdR phosphorylated/mg protein/ml.

purification. This enzyme was very unstable at this stage and storage overnight led to marked loss of activity. TDP kinase was not detectable either in the precipitate or in the dialysed supernatant fluid. In certain experiments the supernatant fluid obtained on adjusting the pH of the whole ascites extract stepwise to 4.25–4.0 showed ability to form TTP when added to preparations of TdR kinase plus TMP kinase which in themselves had no TDP kinase activity.

Column fractionation: 5 ml of fraction ASP 35 dissolved in water were applied to a column of ECTEOLA cellulose 3 cm × 1 cm. When the fluid had reached the surface of the column, 5 ml 0.01 *M* Tris buffer pH 7.8 were passed through the column followed by a 5-ml portion of 0.1 *M* Tris buffer pH 7.7. The total activity recovered was about 10–40 % of that applied in less than 20 μg protein. The final purification of TdR kinase was approx. 20–40 fold. Similar results were obtained with DEAE cellulose columns eluted with 0.1 *M* and 0.2 *M* Tris buffer.

Attempts at acetone fractionation or treatment with calcium phosphate gel resulted in marked loss of activity.

Properties of the kinases

Confirmation that the nature of the product of the action of TdR kinase was in fact TMP-5′ was obtained by paper chromatography and by treatment with snake venom 5′-phosphomonoesterase.

All activity disappeared on heating to 65° for 5 min or on altering the pH to above 8.25 or below 4.25. TDP kinase was slightly more acid resistant than TdR kinase or TMP kinase. Under storage at —10° activity declined over a period of weeks.

TdR kinase was stable to dialysis but TMP kinase and TDP kinase lost activity on contact with unwashed dialysis tubing. Consequently dialysis tubing was always washed several times with water, filled with distilled water and soaked for several hours in distilled water before use. With these precautions TMP kinase was entirely stable, and TDP kinase partially stable, in dialysis for several hours against 0.1 *M* Tris buffer pH 7.8–7.9 containing 1 μmole 2-mercaptoethanol/ml and 1 mμmole TMP/ml.

TdR kinase showed optimum activity at pH 7.9 but TMP kinase activity and especially TDP kinase activity tended to decline above pH 7.7.

Mg ions were necessary for activity and kinase activities were maximal in the presence of 5 μmoles/ml of both Mg^{++} and ATP. When the concentration of Mg^{++} and ATP was reduced from 5 μmoles/ml to 1 μmole/ml, the formation of TDP and TTP was diminished without affecting TMP formation. In the presence of optimal concentrations of Mg^{++} and ATP, increasing concentrations of one of several ions (including Tris) tended to decrease yields of TDP and TTP (Table II). Phosphate ions tended to decrease the total yield of thymidine nucleotides.

Studies on the course of formation of phosphorylated derivatives indicated that the formation of TMP and TDP preceded that of TTP. The absolute amounts of substrate phosphorylated by whole ascites extract increased with increasing thymidine concentration between 1 and 50 mμmole/ml. Formation of [^{3}H]TTP from [^{3}H]TMP over this concentration was a nearly constant proportion of the substrate. While the absolute amount of phosphorylation increased slightly with increasing concentrations of TdR, the proportion of TdR phosphorylated decreased markedly. Preincubation of

the enzyme at 37° for 30 min without substrate caused marked loss of activity.

The curve in Fig. 1 shows an approximately linear relationship between enzyme concentration and the extent of phosphorylation over the range 25 to 65 % conversion to phosphorylated derivatives.

Distribution

Kinase activities were found in varying degrees in several tissues (Table III). TMP kinase and TDP kinase in particular tended to be associated, so that no tissue showed a preponderant production of TDP.

Kinases in extracts of regenerating rat liver

As has been mentioned by other workers[4-7] a marked rise in kinase activity in liver tissue occurs during regeneration after partial hepatectomy. The livers of rats

TABLE II

EFFECTS OF VARIOUS IONS ON KINASES

Test substance	*μmoles/ml*	*Per cent formed*		
		TMP	*TDP*	*TTP*
$MgCl_2$ control	5	10	10	69
$MnCl_2$	10	9	17	61
KCl	10	6	15	65
KCl	100	22	17	44
$CaCl_2$	10	16	19	39
$PO_4^{\equiv}$ (Na buffer pH 7.9)	10	4	20	52
$PO_4^{\equiv}$ (Na buffer pH 7.9)	50	3	29	25
NaCl	10	6	25	44
NaCl	100	23	26	31
$(NH_4)_2SO_4$	10	17	15	40
$(NH_4)_2SO_4$	100	44	10	32
NH_4Cl	10	12	29	16
NH_4Cl	50	48	23	4
$FeCl_3$	2	6	51	19
K_2SO_4	10	5	11	64
K_2SO_4	50	6	48	22

TABLE III

DISTRIBUTION OF KINASES IN RABBIT TISSUES

	Per cent formed		
	TMP	*TDP*	*TTP*
Plasma	4	1	0
Erythrocytes	7	1	1
Leucocytes (buffy coat)	9	12	8
Muscle	8	1	1
Brain	17	4	2
Intestine	27	15	6
Liver	17	7	1
Kidney	18	5	3
Spleen	27	12	14
Thymus	8	18	55
Bone marrow	10	16	49

killed 6 and 18 h post-operatively show no higher enzyme levels than did those of control animals but a distinct elevation in TdR kinase and TMP kinase activity was found at 24 h (Fig. 2). A few animals showed elevation of TdR kinase activity only. The peak in TDP kinase activity occurred between 30 and 48 h post-operatively. This correlated well with the results of experiments on the rate of incorporation of [^{3}H]TdR into DNA.

The precipitate obtained from extracts of normal liver by saturation with ammonium sulphate to 25 % represented 8 % of the protein of the extract and contained most of the TdR kinase. Since such a precipitate from extracts of 48 h regenerating liver was at least 10 times more active, there is good evidence for an increase in the amount of enzyme.

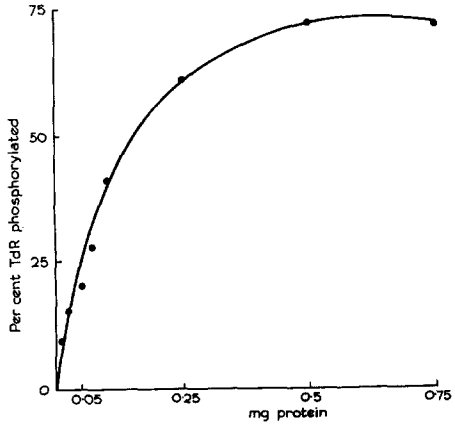

Fig. 1. The effect of enzyme concentration on the phosphorylation of [^{3}H]TdR by the precipitate obtained from fraction AS 4.5 on the addition of $(NH_4)_2SO_4$ to 50 % saturation. The results are expressed as per cent total counts/min recovered in TMP + TDP + TTP.

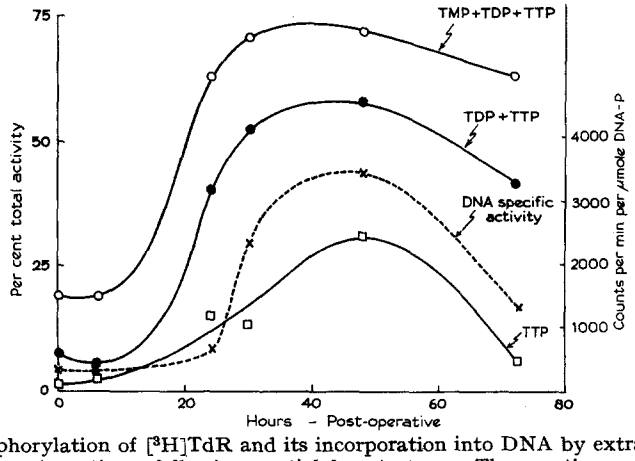

Fig. 2. The phosphorylation of [^{3}H]TdR and its incorporation into DNA by extracts of rat liver regenerating for various times following partial hepatectomy. The reaction vessels contained 0.6 ml of extract of regenerating liver. The results are expressed as per cent total counts/min recovered in TTP, TDP + TTP and TMP + TDP + TTP and represent TDP, TMP and TdR kinase activities respectively.

An attempt was made to induce TdR kinase in rat liver according to the method of HIATT AND BOJARSKI[7] by hourly intraperitoneal injections of 100 mg TdR. Animals were killed 2, 4 and 6 h after the first injection and liver extracts assayed both before and after dialysis. No detectable change in liver enzyme levels was found.

Kinases in cell cultures

L. strain mouse fibroblasts in the resting phase after exhaustion of the medium showed either no kinase activity whatever or only TdR kinase activity. On inoculation into a fresh medium, a period of rapid growth occurred after a lag phase (Fig. 3). TMP kinase activity rose slightly before the onset of rapid growth and was followed by TDP kinase. TDP kinase activity also declined earlier, its peak corresponding to the early portion of the rapid growth phase. Similarly in experiments in which growth began and ended sooner after inoculation, there was an earlier rise and decline in kinase activities.

When alternate flasks of L. cells were treated with TdR on day 0 at a final concentration of 10^{-4} *M* and replenished with the same amount on day 4, no appreciable differences in enzyme activity levels between treated and control flasks were observed for the first 3 days (Fig. 4). Although the growth rates were identical in control and treated flasks throughout, TdR kinase activity did not decline so rapidly in the treated cells as in the controls while TMP and TDP kinases showed a second and prolonged peak of activity. All levels fell at the end of the experiment.

In other experiments inocula of $3 \cdot 10^6$ rather than $5 \cdot 10^6$ cells were used. TdR was added either at time 0 or after 3 days of growth to a final concentration of 10^{-4} *M*. After 7 days the medium was replaced with fresh medium containing TdR where appropriate, so as to permit continuous growth of the cultures for 9 days.

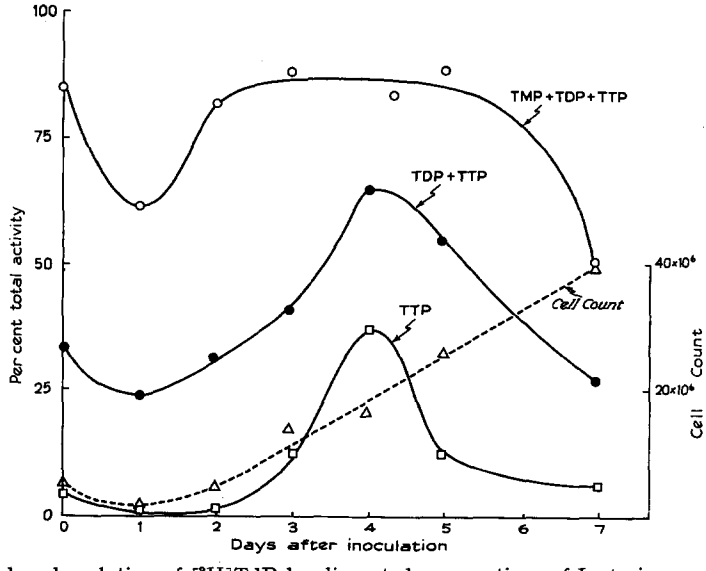

Fig. 3. The phosphorylation of [^{3}H]TdR by disrupted preparations of L strain mouse fibroblasts at various times during growth. Each reaction vessel contained 1.0 mg total protein. Results are expressed as per cent total counts/min recovered in TTP, TDP + TTP and TMP + TDP + TTP representing TDP, TMP and TdR kinases respectively.

In this group of experiments the TdR concentration proved to be mildly toxic so that the treated cells grew more slowly than the controls. The activities of TMP and TDP kinases rose earlier in the cells treated with TdR at time o than in the more rapidly growing controls, remained elevated until shortly before the growth rate declined and then fell sharply.

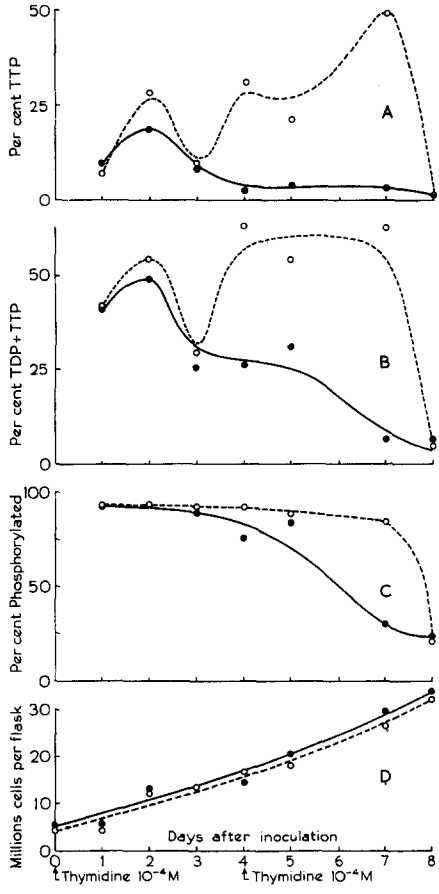

Fig. 4. A, TDP kinase; B, TMP kinase; C, TdR kinase; D, growth curves of L. strain mouse fibroblasts grown in the presence and absence of TdR. Where present, TdR was added at the times shown, to a concentration of 10^{-4} *M*. Solid lines represent control and dotted lines test cultures. The total protein per reaction vessel was between 1.0 and 1.5 mg.

In the group of cells where TdR was added after 3 days there was a reduction in rate of growth. Four days after addition of TdR there was a fall in TMP and TDP kinase activities and in the rate of growth. This was followed, however, by a second rise in cell count and in TMP and TDP kinase activities.

In both test and control cultures the TDP kinase activity tended to fluctuate more and to parallel the growth rate more closely than did the TMP kinase. TdR kinase remained elevated through most of the growth phase in all cultures.

DISCUSSION

The results of fractionation experiments on extracts of Ehrlich ascites cells have shown that three enzymes are involved in the formation of TTP from TdR. TdR kinase has been purified 20–40 times by acid precipitation followed by ammonium sulphate fractionation and chromatography on columns of ECTEOLA or DEAE cellulose. TMP kinase proved to be extremely labile but 15–20 fold purification was obtained in some samples by acid precipitation followed by ammonium sulphate fractionation. TDP kinase also proved highly labile although 2–3 fold purifications were regularly obtained by removal of inactive protein by acid precipitation.

The TMP kinase appears to be quite distinct from the relatively non-specific nucleoside monophosphate kinase described by STROMINGER *et al.*[15] since it is much more labile, has a much lower reaction velocity and behaves differently on ammonium sulphate fractionation. The bacterial TMP kinase of HURWITZ[16] also differs from the ascites enzyme in its precipitation with ammonium sulphate and in its stability.

It has previously been shown that TTP may be formed from TdR by preparations from regenerating rat liver[4,5,7,17–20] and that regenerating liver differs from normal liver in possessing the necessary kinases[4,7,20]. The results in Fig. 3 show that the appearance of the kinases after partial hepatectomy tends to be sequential, TdR and TMP kinases appearing before TDP kinase. By 24 h the TdR and TMP kinases are increasing sharply reaching a maximum of activity around 30 h. TDP kinase activity develops more slowly and reaches a peak at about 48 h. Measurements of the incorporation of [^{3}H]TdR into DNA show little difference between normal and regenerating liver until about 30 h after partial hepatectomy, and maximum uptake is observed at about 48 h. Subsequently all kinases decline in activity. In the experiments of BOLLUM AND POTTER[4] the greatest uptake of TdR into DNA was observed between 24 and 60 h following partial hepatectomy and this is substantially confirmed by our findings. TMP kinase activity has been studied by HIATT AND BOJARSKI[7] who found that the activity started to rise at 20 h post-operatively, reached a maximum at about 42 h and then declined. These findings are in agreement with our observations in Fig. 3.

A sequential pattern of kinase appearance is also found in cultures of L. cells. Fig. 3 shows that TdR kinase appears early, rises sharply and remains elevated. TMP kinase activity increases later but before TDP kinase and both enzymes show a peak of activity during the early part of the growth phase and decline before growth has stopped. The results of an effort to induce TdR and TMP kinases by growing L. cells in the presence of TdR are shown in Fig. 4. While there is no effect on the net rate of growth or on the kinases during the early period when the control cultures show maximal kinase activity, TdR kinase remains elevated for a much longer period in the test cultures and TMP and TDP kinases rise to a second peak during the period when these activities in the control cultures decline. These differences between control and test culture were observed only during growth.

These results, together with those from other experiments reported in the results section, suggest that such elevations in kinase activities can only be produced during the growth phase and could be due to true enzyme induction. An alternative explanation that the results might be due to *in vivo* protection of the enzymes by their respective substrates seems less likely in view of the observation that enzyme levels fell sharply when cell growth ceased but could rise equally sharply on replenishment of the medium irrespective of the length of exposure to TdR.

Attempts to induce TdR kinase in rat liver by the methods which HIATT AND BOJARSKI[7] employed to induce thymidylate kinase proved unsuccessful. These, however, were short term experiments.

The results presented here are consistent with the possibility of sequential induction of enzymes concerned with the synthesis of TTP and support the view that enzymes converting TdR to TTP are specifically concerned with processes relating to cell division.

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