i^{32}P (10 mc), 86 mg of radioactive ATP from 80 mg of ADP was obtained. The specific activity of each phosphorus was

Adenine – Ribose	–	P	–	P	–	P	
		490		1,480		22,010	counts/min/μatom P

Thus, remarkable differences in radioactivity between each phosphorus atom in ATP were obtained by this method, but it was difficult to obtain [γ-^{32}P] ATP of high specific activity because the fractional distillation of $^{32}POCl_3$ could not be carried out on a small scale.

*Method of analysis**

γ-Phosphorus was split from the ATP with ATPase, and the quantity and the radioactivity were evaluated after addition of molybdate and extraction by isobutanol[11,12]. The β- and γ-phosphorus atoms were split off by the combined action of ATPase and adenylate kinase, and the specific activity of the β-phosphorus was obtained by subtracting A from 2C. The specific activity of the α-phosphorus was calculated as 3D–(A + B). Total phosphorus was determined by KING's method[13] chemically, or, if necessary, enzymically. The latter method consisted of successive splitting with ATPase, adenylate kinase and 5'-nucleotidase, and this method is recommended in case of the presence of other phosphorus compounds. The conditions for the enzymic reactions were based on the method of BOWEN AND KERWIN[14], except that the enzyme source employed was rat skeletal muscle, and that the 5'-nucleotidase used was prepared by the simplified method of HEPPEL AND HILMOE[15]. The determination of iP in the reaction mixture was carried out by TAKAHASHI's modification[16] of the method of BERENBLUM AND CHAIN[17]. With this method well-reproducible and satisfactory results are easily obtained.

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* A, B, C and D represent the specific activities of phosphorus at the following positions: A, γ-position; B, β-position; C, β- and γ-positions; D, α-, β-, and γ-positions.

The conversion of ^{14}C-deoxynucleoside-5'-monophosphates to the corresponding di- and triphosphates by soluble mammalian enzymes

Experimental results obtained in this laboratory have indicated that the dialyzed soluble supernatant fraction prepared by centrifugation of rat-liver homogenates at 106,000 × *g* for 90 min is capable of converting uracil, uridine and uridine-5'-monophosphate to uridine di- and triphosphates[1]. In the present communication it will be shown that the deoxy-5'-mononucleotides of adenine, guanine, cytosine and thymine are converted to the corresponding di- and triphos-

phates by a similar soluble enzyme fraction obtained from regenerating rat liver. In addition, a modified method for the separation and isolation of the deoxymononucleotides of deoxyribonucleic acid (DNA) in good yields will be presented.

The ^{14}C-deoxynucleoside-5'-monophosphates which were used as substrates in this investigation were obtained by enzymic hydrolysis of the DNA of *E. coli* grown in the presence of $^{14}CO_2$, according to the method of DOWNING AND SCHWEIGERT[2]. The fractionation and isolation of these nucleotides was facilitated by the use of the ion-exchange chromatography technique of HURLBERT *et al.*[3], modified in that the 1.0 *M* ammonium formate used in the reservoir for elution was adjusted from pH 4.2 to 4.3. This modification permits excellent resolution of the deoxymononucleoside-5'-monophosphates in small effluent volumes, in contrast to the acetate method[4] which requires relatively large volumes; it also avoids the use of solvents of low pH[5,6] which degrade the deoxymononucleotides and therefore decrease the yield. As can be seen from Fig. 1, the best separation of the deoxymononucleotides was obtained when the pH of the ammonium formate was 4.25. Concentration and quantitative isolation of the ^{14}C-deoxymononucleotides obtained by this procedure was accomplished by diluting the isolated fractions 10-fold with water, adjusting the pH to 9–10 with ammonia, and passing them through a 1.0 cm Dowex-1 chloride column from which they were then eluted with 1.0 *M* NaCl. The yield of ^{14}C-deoxymononucleotides from *E. coli* grown in the presence of $Na_2{}^{14}CO_3$ (10 mc) was about 5–7 μmoles of each with a specific activity of $4.5 \cdot 10^6$ to $8.5 \cdot 10^6$ counts/min/μmole. The totally labeled deoxymononucleotides thus isolated were used in the following experiments.

The supernatant fractions (106,000 × *g*) of homogenates of regenerating rat liver 36 h after hepatectomy, before and after a 30-h dialysis against two 4-l changes of 0.05 *M* tris(hydroxymethyl)aminomethane buffer, pH 7.4, were assayed for their capacity to phosphorylate the deoxymononucleotides to the di- and triphosphate stage. These results are summarized in Table I, which shows that the dialyzed enzyme regularly converted 80 to 90 % of the deoxymononucleotides to the triphosphates; without dialysis, conversion was less. Our interpretation of this is that dialysis eliminates the naturally occurring nucleotide mono- and diphosphates which occur in the soluble supernatant fraction of rat liver[3] and may compete with the added radioactive substrates for the phosphorylating system.

Experiments pertinent to this problem have been published by SABLE *et al.*[10] and by HECHT

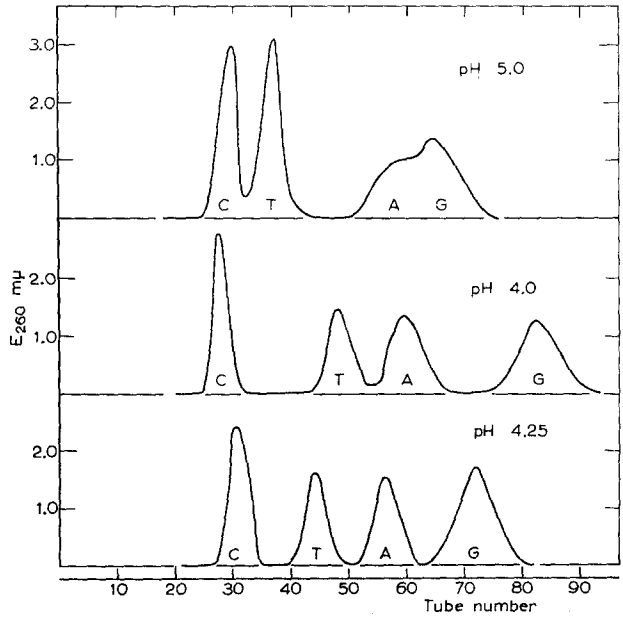

Fig. 1. Elution patterns of deoxymononucleotides at different pH values. A mixture of 2.0 mg each of: deoxycytidylic acid (C), thymidylic acid (T), deoxyadenylic acid (A) and deoxyguanylic acid (G) were added to a 20 × 1 cm Dowex-1 formate column and eluted by gradient elution according to the method of HURLBERT *et al.*[3]. The mixing flask contained 500 ml water and the reservoir contained 1.0 *M* formate which had been adjusted to the indicated pH values by the addition of ammonium hydroxide. Effluent volume per tube, 5–6 ml.

et al.[11] who have described the phosphorylation of some of the deoxymononucleotides by muscle and by particulate rat-liver fractions. In addition KLENOW and his collaborators[12] have shown that deoxyadenylic acid can be phosphorylated in the presence of purified enzymes.

TABLE I

CONVERSION OF DEOXYNUCLEOSIDE-5'-MONOPHOSPHATES TO THE CORRESPONDING DI- AND TRIPHOSPHATES BY ENZYMES FROM REGENERATING RAT LIVER

The incubation mixture consisted of $6 \cdot 10^{-4}$ to $1 \cdot 10^{-3}$ μmole of the deoxyribonucleotide containing 30,000 c.p.m. of ^{14}C, 3.2 μmoles adenosine triphosphate, 15.3 μmoles creatine phosphate, 200 μg creatine kinase (prepared according to the method of KUBY *et al.*[7] up to but not including the crystallization step) 33 μmoles $MgCl_2$, 62 μmoles tris(hydroxymethyl)aminomethane buffer, pH 7.4, and 0.5 ml of enzyme (20 mg protein/ml), prepared from the 106,000 $\times$ *g* supernatant fraction of a rat-liver homogenate 36 h after hepatectomy, before and after dialysis against 0.05 *M* tris buffer for 30 h: final volume, 1.0 ml; incubation time, 20 min. At the end of the incubation the samples were cooled in ice, the proteins were precipitated with 8 ml 0.4 *N* $HClO_4$ and the acid-soluble fraction was neutralized with KOH. After removal of the $KClO_4$, the solution was chromatographed on a 10 $\times$ 1 cm Dowex-1 formate column, as described in Fig. 1.

Substrate	*Treatment of the enzyme*	*Distribution of deoxyribonucleotides (%)**		
		Mono-P	*Di-P***	*Tri-P***
Deoxyadenylic acid	Dialyzed	10	10	80
	Non-dialyzed	60	20	20
Deoxyguanylic acid	Dialyzed	5	10	85
	Non-dialyzed	15	30	55
Deoxycytidylic acid	Dialyzed	10	10	80
	Non-dialyzed	30	30	40
Thymidylic acid	Dialyzed	5	5	90
	Non-dialyzed	15	30	55

* The radioactivity present in each nucleotide form is expressed as % of the total in the three forms.

** The di- and triphosphates of the deoxymononucleotides were identified by comparison with the corresponding synthetic di- and triphosphates prepared chemically from the deoxynucleotide-monophosphates by the methods of KHORANA[8,9] and of POTTER *et al.*[6].

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