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Formation of Lipid-Nucleotide Complex by RNA Tumor Virus Preparations

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RNA tumor virus preparations utilize α-^{32}P-deoxyribonucleoside triphosphates as substrates to form lipid-nucleotide products which are tightly associated with protein.

The adjoining paper (2) described the effect of disruption conditions and other variables on the RNA-DNA covalent linkages formed by the endogenous reaction with three different RNA tumor viruses. During the course of this work, it was recognized that a portion of the labeled deoxyribonucleoside triphosphate substrates were being converted into a nucleotide-lipid which was tightly associated with protein. This was a potential pitfall in these types of studies because misleading results could be obtained if the complex compound was not removed prior to analysis for the RNA-DNA bonds.

Formation of the complex product was initially observed in control radiochromatograms which were routinely run prior to alkaline hydrolysis of the polynucleotide product. When the high levels of isotope that were necessary for sufficient sensitivity to check for complete removal radioactive substrates were examined by this chromatography step, it was found that a fast-moving ^{32}P product (retardation factor [R_f] = 0.7 to 0.9) was present (Fig. 1). Formation of this product was detected only in system B; in the other systems (2) it remained at the origin along with DNA. The R_f of this product was appreciably greater than any of the known mono-, di-, or triphosphates or inorganic orthophosphate (P_i).

This complex product was formed by either NP-40- or ether-disrupted preparations of all RNA tumor viruses studied, namely, avian myeloblastosis virus, B77 virus, and murine leukemia virus. It was formed to the extent of only 0.5 to 3% of the amount of DNA formed (based on ^{32}P incorporation). However, this extent was important, because this was roughly the extent of formation of RNA-DNA linkages (2). It was formed in largest amount when α-^{32}P-dCTP and α-^{32}P-dGTP were the labeled substrates. A lesser amount, or none, was formed when the label was in dATP or dTTP.

We have partially characterized the product as containing phospholipid, protein, and dNMP. Treatment of the compound with alkaline phosphatase (Table 1) provided no change, suggesting that the ^{32}P was bonded through the 5'-phosphate group to another residue. However, the compound was sensitive to alkaline hydrolysis to give dCMP as the product (Table 1). Also, the dCMP was sensitive to alkaline phosphatase to give $^{32}P_i$ as the product. The complex product was completely adsorbable to Norite (data not shown), also indicating that it must contain a nucleotide. Because the complex contained isotope which could be converted into dNMP on alkaline hydrolysis, it was necessary to remove this contamination prior to the assay for the RNA-DNA bond (2). If it were not removed, dNMP would be formed which has chromatographic mobilities similar to some ribonucleotides.

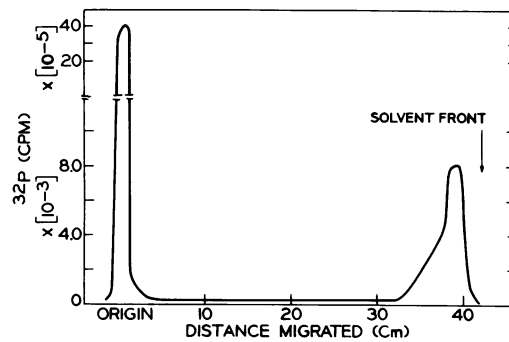

FIG. 1. *Radiochromatogram of reaction products formed by ether-disrupted avian myeloblastosis virus. Reaction conditions and product work-up were as described (2). The labeled substrate was α-^{32}P-dCTP. The polymer product after Sephadex G-75 chromatography (2) was chromatographed in system B (2). Other details were as described (2).*

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TABLE 1. *Summary of characterization of lipid-containing product*

Treatment	Result
Alkaline phosphatase[a]	No apparent change
Alkaline hydrolysis[b]	Product was dCMP
Alkaline phosphatase digest of dCMP product[c]	Product was P_i
Phospholipase C[d]	Partial degradation
Dialysis versus 0.5 M or 0.01 M NaCl solution[e]	Product was nondialyzable
Trypsin[f]	Partial degradation

[a] The source of the complex product was the fast-moving peak (R_f = 0.7 to 0.9) from a chromatogram as shown in Fig. 1; this product was eluted quantitatively from the paper by $CHCl_3$-methanol (1). The organic solvents were evaporated, and the product was dissolved in 0.01 M Tris-hydrochloride (pH 7.5). This was used for all later determinations unless noted otherwise. A reaction mixture (0.10 ml) containing 0.1 M Tris-hydrochloride (pH 8.0), 0.05 M $MgCl_2$, the complex product (1,600 counts/min), and 20 μg of alkaline phosphatase per ml (Worthington preparation which was further purified by DEAE-cellulose chromatography) was kept at 37 C for 2 h. The mixture was chromatographed in solvent A (1) where the product remains at the origin and P_i moves with R_f = 0.9. All radioactivity remained at the origin.

[b] The complex product (1.85×10^3 counts/min) was treated with 0.3 M NaOH at 37 C for 17 h. The solution was neutralized with Tris-hydrochloride and half of the solution was chromatographed in system A and half in system B (2). The radioactivity migrated quantitatively (>90%) with dCMP marker in both systems.

[c] Another sample of the complex product (1.85×10^3 counts/min) was treated with alkali and neutralized as described in footnote *b*.

It was then treated with alkaline phosphatase as described in footnote *a*. Samples were removed at time intervals and chromatographed in system A (2). Radioactivity was converted from the dCMP area to the P_i area (R_f = 0.9); 24,600 and 1,670 counts/min (corrected to total reaction volume), respectively, were observed at R_f = 0.9 after 0, 60, and 120 min of incubation.

[d] A reaction mixture (0.10 ml) containing 0.1 M Tris-hydrochloride (pH 7.3), 0.05 M $CaCl_2$, 2,630 counts/min of the product (as in footnote *a*) and 100 μg of phospholipase C per ml (Worthington) was incubated at 37 C for 2 h. The mixture was chromatographed in system B (2). Approximately half (1,200 counts/min) of the isotope was converted to a heterogenous product with R_f = 0.3 to 0.6; the remaining 1,400 counts/min migrated at R_f = 0.8.

[e] The partially purified reaction mixture (15 to 20,000 counts/min in 1.0 ml), as described in Fig. 2, was dialyzed at 5 C for 19 h versus 6 liters of either 0.5 M or 0.01 M NaCl solutions containing 1×10^{-4} M EDTA (pH 7.5). The solutions were chromatographed in solvent B (2). The radiochromatogram was as shown in Fig. 1.

TABLE 1—*Continued*

[f] A reaction mixture (0.10 ml) containing 50 mM Tris-hydrochloride (pH 8.0), 10 mM $CaCl_2$, the complex product (as in footnote *a*, 1,380 counts/min) and 200 μg of highly purified trypsin per ml (gift of W. H. Orme-Johnson, University of Wisconsin) was kept at 37 C for 3 h. The mixture was chromatographed in system B (2). The 450 counts/min were decreased to a streak from R_f = 0.3 to 0.6, and 860 counts/min remained at the position of the unreacted complex (R_f = 0.8 to 0.9).

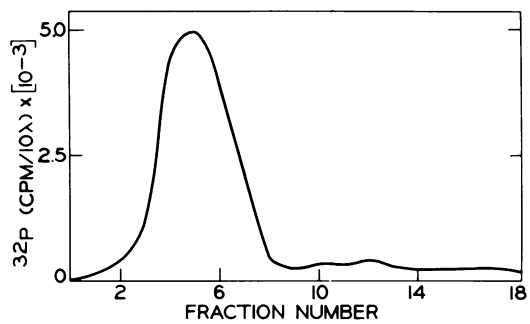

FIG. 2. *Sephadex G-75 chromatography of reaction products formed with α-^{32}P-dCTP substrate by ether-disrupted avian myeloblastosis virus. A reaction mixture as described (2) was purified by chromatography on a column (0.3 × 75 cm) of Sephadex G-75. Elution was with 0.01 M NaCl-0.001 M Tris-hydrochloride solution (pH 7.2). Samples from the fractions were spotted on paper disks and washed with acid (2) before counting. The void volume was at fractions 5 to 6. Trace amounts of unreacted triphosphates eluted at fractions 11 to 15, as judged by radioactivity determination before acid wash. A total of 6.26 × 10^5 counts/min were applied to the column; recovery was >90% (including dNTP peak).*

That it contained lipid-like moieties was demonstrated by its high R_f in system B and by degradation with phospholipase C (Table 1). In addition, the complex product is completely extractable into acidified chloroform-methanol (1) as indicated above. Extraction of an impure DNA polymerase reaction mixture (2) with acidified $CHCl_3$-methanol quantitatively removed this complex product into the organic phase (data not shown). In addition the compound had an identical chromatographic mobility (R_f = 0.8 to 0.9) in system B with an authentic sample (3) of chemically synthesized CDP-dipalmitate (di-Tris) (generous gift of C. Raetz and E. P. Kennedy, Harvard University).

The product was polymeric for it was excluded by Sephadex G-75 chromatography (Fig. 2). The bulk of the polymer material (fractions 2 to 7) was pooled, and radioactivity was quantitatively precipitated with cold 1.0 N perchloric acid; the product was dissolved in water and

chromatographed in system B (2). The developed chromatogram was as shown in Fig. 1. Hence, the product was excluded by Sephadex G-75 chromatography and was acid precipitable. Also, it was nondialyzable (Table 1).

The product also contained a proteinaceous portion for it was sensitive to digestion with trypsin (Table 1). Similar results were found with Pronase treatment (data not shown). That this change in mobility was not due to a general adhesion of the product to added proteins was suggested by its lack of change in mobility on incubation with phosphatase.

Hence, we believe this product to be a lipid-nucleotide which is tightly associated with (or bonded to) protein. Thus, a phospholipid biosynthetic activity can be added to the growing list of biosynthetic activities associated with RNA tumor viruses. Recent work (3, 4) acknowledges the synthesis and utilization of dCDP-diglyceride in other systems.

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LITERATURE CITED

1. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. **37**:911-917.
2. Flügel, R. M., U. Rapp, and R. D. Wells. 1973. RNA-DNA covalent bonds between the RNA primers and the DNA products formed by RNA tumor virus DNA polymerases. J. Virol. **12**:1491-1502.
3. Raetz, C. R. H., and E. P. Kennedy. 1973. Function of cytidine diphosphate-diglyceride and deoxycytidine diphosphate-diglyceride in the biogenesis of membrane phospholipids in *E. coli*. J. Biol. Chem. **248**:1098-1105.
4. Schegget, J. T., H. van den Bosch, M. A. van Baak, K. Y. Hostetler, and P. Borst. 1971. The synthesis and utilization of dCDP-diglyceride by a mitochondrial fraction from rat liver. Biochim. Biophys. Acta **239**:234-242.