other hand, a linear rate of removal was observed when ^{51}Cr-labeled platelets, both with and without PGE_1, were stored at 22°C for 24 hours, and when they were frozen with a combination of DMSO and liquid nitrogen. Storage at +4°C for 24 hours produced preservation injury manifested by an exponential loss of platelets from the circulation; PGE_1 did not affect this removal pattern.

The addition of 8 ng of PGE_1 per milliliter of whole blood produced an increase in the number of circulating platelets when transfused from fresh platelet concentrates or from previously frozen platelets (Table 1). This increase reflected only improved recovery of platelets in vitro from whole blood; neither platelet recovery in vivo nor platelet life-span was improved by PGE_1. This treatment of whole blood did not adversely affect the survival of concentrated red cells stored at +4°C for about 1 week. The percent of cells viable after 24 hours was similar to that of concentrated red cells stored for the same period without PGE_1 (*11*). The functional state of the red cells stored with and stored without PGE_1 was similar.

No adverse clinical effects were observed after the administration of either liquid-stored platelet concentrates, previously frozen platelet concentrates, or the small portions of stored, concentrated red cells which had been treated with PGE_1. There were no changes in hematology or chemistry of the recipients during the week after transfusion of these cellular elements.

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References and Notes

1. H. Shio and P. W. Ramwell, *Science* **175**, 536 (1972).
2. PGE_1 and PGE_2 were obtained from Dr. N. Weinshenker.
3. G. Brecher and E. P. Cronkite, *J. Appl. Physiol.* **3**, 365 (1950).
4. R. I. Handin, N. L. Fortier, C. R. Valeri, *Transfusion* **10**, 305 (1970).
5. S. Murphy and F. H. Gardner, *N. Engl. J. Med.* **280**, 1094 (1969).
6. R. I. Handin and C. R. Valeri, *ibid.* **285**, 538 (1971).
7. ———, *Abstr.* American Association of Blood Banks, 24th annual meeting, Chicago, Ill. (1971), p. 91.
8. J. E. Allen and H. Rasmussen, *Science* **174**, 512 (1971).
9. C. R. Valeri, *Clin. Lab. Sci.* **1**, 381 (1970).
10. F. S. Morrison and M. Baldini, *Vox Sang.* **12**, 90 (1967).
11. C. R. Valeri, I. O. Szymanski, C. G. Zaroulis, *ibid.*, in press.
12. Supported by the U.S. Navy.

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Detection of High-Molecular-Weight RNA in Particles from Human Milk

Abstract. *Particles from human milk contain a reverse transcriptase and a high-molecular-weight (60S to 70S) RNA that serves as a template. These particles have two features diagnostic of known RNA tumor viruses.*

Particles morphologically similar to the type-B mouse mammary tumor virus (MMTV), Mason-Pfizer monkey virus, and type-C murine leukemia–sarcoma viruses have been observed in samples of human milk (*1*). Since the causative agents of both murine mammary tumors (*2*) and murine leukemia (*3*) appear in and are transmitted via mothers' milk, the question arose immediately as to the relatedness of the human particles to known RNA tumor viruses.

The oncogenic RNA viruses, or oncornaviruses (*4*), exhibit two biochemical properties unique to them as a group. They possess (*5*) a large single-stranded RNA molecule with a sedimentation coefficient of 60*S* to 70*S*, often referred to as high-molecular-weight RNA. They also contain a reverse transcriptase, an enzyme capable of using the viral RNA as a template to generate a complementary DNA copy (*6*, *7*).

The particles from human milk have a density of 1.16 to 1.19 g/ml and contain a ribonuclease-sensitive DNA polymerase analogous to the reverse transcriptase (*8*). Ribonuclease sensitivity of a DNA polymerase, however, does not per se establish that a reverse

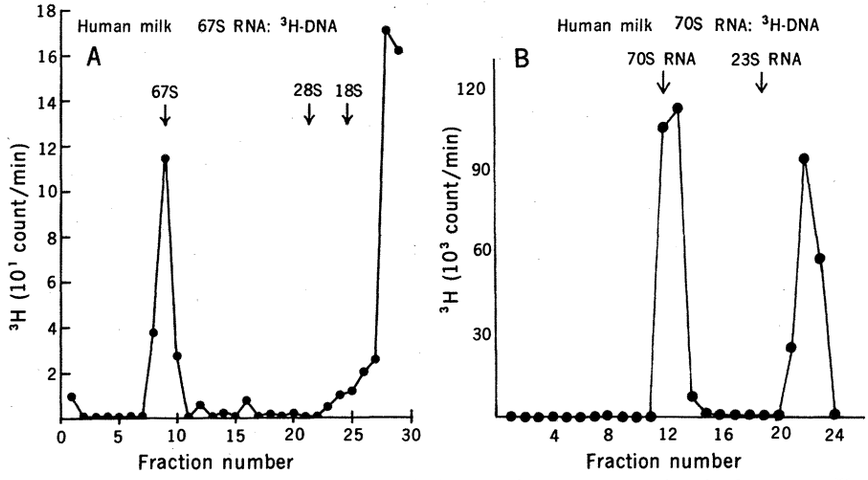

Fig. 1. Detection of high-molecular-weight-RNA : [^{3}H]DNA complex in human milk. (A) Seventy-five milliliters of human milk and 75 ml of 0.15*M* EDTA (*p*H 7.5) were mixed and centrifuged at 3000*g* for 10 minutes. The clear zone between the lipid and precipitated casein layers was removed and incubated at 37°C for 30 minutes in the presence of trypsin (Worthington) at a final concentration of 1 mg/ml. Lima-bean-trypsin inhibitor (0.5 mg/ml) was then added, and the sample was layered over an 8-ml column of 20 percent glycerol in a SW-27 (Spinco) centrifuge tube, and centrifuged at 98,000*g* for 60 minutes at 4°C; the resulting pellet was resuspended in 45 μl of 0.01*M* tris(hydroxymethyl)aminomethane (*p*H 8.3) containing 0.33 percent NP-40 detergent and 0.1*M* dithiothreitol and kept at 4°C for 10 minutes. This suspension was then added to a standard reverse transcriptase reaction mixture (125 μl final volume) containing 6.25 μmole of tris-HCl (*p*H 8.3), 1 μmole of $MgCl_2$, 1.25 μmole of NaCl (instead of KCl to avoid precipitation when sodium dodecyl sulfate is added in a subsequent step), 0.2 μmole each of unlabeled deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and [^{3}H]deoxythymidine triphosphate to a final specific activity of 8900 count min^{-1} $pmole^{-1}$. After a 60-minute incubation at 37°C, the reaction was terminated by the addition of NaCl and sodium dodecyl sulfate to final concentrations of 0.2*M* and 1 percent, respectively. After addition of an equal volume of a phenol-cresol (7 : 1) mixture containing 8-hydroxyquinoline (3.7 g per 100 ml of mixture), the final mixture was shaken at 25°C for 5 minutes and centrifuged at 5000*g* for 5 minutes at 25°C. The aqueous phase was then layered over a linear glycerol gradient (10 to 30 percent) and centrifuged at 40,000 rev/min for 3 hours at 4°C (Spinco SW-41 rotor). External markers were 28*S* and 18*S* [^{3}H]RNA from NC-37 cells. Fractions were collected from below, and portions were assayed for acid-precipitable radioactivity (*7*). (B) Nineteen milliliters of milk was processed as described in (A). Avian myeloblastosis virus 70*S* [^{3}H]RNA and *Escherichia coli* 23*S* [^{3}H]RNA were used as external markers.

transcriptase has been identified because a DNA-directed DNA polymerase primed by short ribo-oligomers might also exhibit this property (*9*). More convincing evidence that the reaction being observed is mediated by the reverse transcriptase of an oncornavirus would be provided by showing that RNA is serving as the guiding template and that this RNA is of high molecular weight (60*S* to 70*S*). This sort of evidence is not easily obtained with the particles from human milk, since they are in short supply and difficult to purify. To obviate these technical difficulties (*10*) an assay was developed to permit the simultaneous detection of reverse transcriptase and the 60*S* to 70*S* RNA unique to the oncogenic RNA viruses.

The test involves sedimentation of the DNA synthesized in the endogenous reverse transcriptase reaction. A positive outcome is signaled by the appearance of newly synthesized DNA complexed to the 60*S* to 70*S* RNA template. The method permits the detection of virus at low concentrations in biological fluids contaminated with enzymatically active cell fragments. It has been successfully applied to find oncornaviruses in mouse milk, mouse plasma, and tissue culture supernatants of cells infected with viruses of avian, murine, feline, and primate origin (*10*). The fact that two features diagnostic of the oncornaviruses are used increases the certainty with which a positive outcome can be accepted.

We now report the application of this method to particles from human milk. The results show that the human particles contain 60*S* to 70*S* RNA and that this RNA is used as a template in a reverse transcriptase reaction.

For assay, the milk preparations were first clarified by the addition of an equal volume of 0.15*M* (EDTA) followed by centrifugation at 3000*g* for 15 minutes. The intermediate milk-plasma layer was then placed on a column of 20 percent glycerol and centrifuged at 98,000*g* for 60 minutes. The sediment was resuspended in 0.33 percent Nonidet P-40 (NP-40) detergent and 0.1*M* dithiothreitol, and a standard 30- to 60-minute endogenous reverse transcriptase reaction was carried out employing [^{3}H]TTP incorporation into DNA. Nucleic acids were then extracted and subjected to sedimentation analysis (Fig. 1). The fractions were collected, and portions were tested for acid-precipitable radioactive material. A distinct peak of acid-precipitable [^{3}H]TTP, observed in the 70*S* region of the gradient, constitutes a positive. This peak is not observed, however, if the preparation in question is subjected to ribonuclease treatment (40 μg/ml) after disruption of virions with detergent or if any one of the deoxyribonucleoside triphosphates is omitted from the reverse transcriptase reaction mixture. Furthermore, when the 70*S* RNA : [^{3}H]DNA complex is subjected to Cs_2SO_4 equilibrium gradient centrifugation, it bands at the density of RNA (1.650 to 1.680 g/ml).

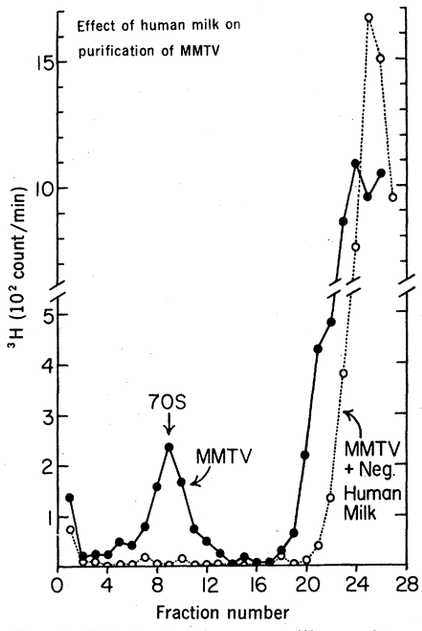

Fig. 2. Effect of a human milk on detection of added mouse mammary tumor virus (MMTV). Five-tenths milliliter of RIII mouse milk (containing MMTV) and 0.5 ml of RIII mouse milk mixed with 25 ml of a "negative" human milk were processed in a manner identical to that described for Fig. 1.

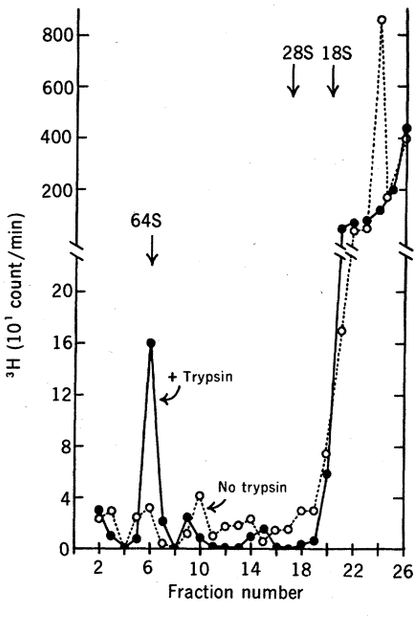

Fig. 3. Effect of trypsin on detection of the 70*S* RNA : [^{3}H]DNA complex in human milk. Human milk (25 ml) was processed as described (Fig. 1). One half of the sample, however, was not treated with trypsin as described (Fig. 1). The external markers used were 28*S* and 18*S* [^{3}H]RNA from NC-37 cells.

Numerous preparations of human milks have been assayed. As is seen in Fig. 1A, an RNA : [^{3}H]DNA complex is observed sedimenting at 67*S*. In one milk, from a woman with a family history of breast cancer, as many as 200,000 count/min were observed in the 60*S* to 70*S* region (Fig. 1B).

We have not only observed that milks from different women vary in their enzyme content (60*S* to 70*S* RNA : [^{3}H]DNA and reverse transcriptase), but that milks from an individual woman differ from day to day. This may be due to an actual variation of particles in the milk or to the variable presence of inhibitors in milks that may obscure the results of our assays. An example of the latter situation is observed from the results of a reconstruction experiment in which RIII mouse milk containing MMTV was placed in a known negative human milk. As can be seen in Fig. 2, when the RIII milk is processed alone, a high-molecular-weight RNA : [^{3}H]-DNA complex is observed. When, however, the RIII milk is mixed with the human milk and processed in the identical manner no such complex is observed. Subsequent to such findings, we used trypsin in our milk purification procedure because we have found that trypsin treatment of RIII milk enhances the endogenous reverse transcriptase reaction of MMTV (Fig. 3).

We have also observed [^{3}H]DNA in the 35*S* region of the gradient in the processing of certain human milks, corresponding to similar results obtained in the assay of MMTV (*10*) and of other oncogenic RNA viruses. Milk specimens (30) from 20 individuals have thus far been examined for either the 35*S* or the 70*S* RNA : [^{3}H]DNA complex. Of the 20 women tested, 10 were positive. Of the 30 specimens tested, 8 had only the 70*S*, 5 had only the 35*S*, and 4 had both. Negative results may be due to either the absence of particles or to the presence of inhibitors.

Our data do not establish the viral nature of the particles found in human milks; their propagation in cells will

constitute acceptable proof that they are in fact viruses. However, these results do provide evidence that human milks contain particles that are similar in several respects to the known RNA tumor viruses of animals: a density of 1.16 to 1.19 g/ml and the presence of both the reverse transcriptase and the 60*S* to 70*S* RNA.

Further experiments relating particles to human neoplasia now become possible. Human breast tumors contain RNA that is homologous to the RNA of the MMTV (*11*). Of particular interest will be the outcome of molecular hybridization experiments that examine the extent of homology between the RNA of the particle in human milk and the RNA of human neoplastic tissues as well as the RNA of known animal tumor viruses.

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References and Notes

1. D. H. Moore, N. H. Sarkar, C. E. Kelly, N. Pillsbury, J. Charney, *Texas Rep. Biol. Med.* **27**, 1027 (1969); D. H. Moore, J. Charney, B. Kramarsky, E. Y. Lasfargues, N. H. Sarkar, M. J. Brennan, J. H. Burrows, S. M. Sirsat, J. C. Paymaster, A. B. Vaidya, *Nature* **229**, 611 (1971); W. F. Feller, H. C. Chopra, F. Bepko, *Surgery* **62**, 750 (1967); W. F. Feller and H. C. Chopra, *Cancer* **24**, 1250 (1969); H. C. Chopra and W. F. Feller, *Texas Rep. Biol. Med.* **27**, 945 (1969).
2. J. J. Bittner, *Science* **84**, 162 (1936).
3. L. W. Law, *Proc. Soc. Exp. Biol. Med.* **111**, 615 (1962).
4. R. C. Nowinski, L. J. Old, N. H. Sarkar, D. H. Moore, *Virology* **42**, 1152 (1970).
5. W. S. Robinson and P. H. Duesberg, *Proc. Nat. Acad. Sci. U.S.* **58**, 825 (1967).
6. H. M. Temin and S. Mizutani, *Nature* **226**, 1211 (1970); D. Baltimore, *ibid.*, p. 1209.
7. S. Spiegelman, A. Burny, M. R. Das, J. Keydar, J. Schlom, M. Travnicek, K. Watson, *ibid.* **227**, 563 (1970).
8. J. Schlom, S. Spiegelman, D. Moore, *ibid.* **231**, 97 (1971).
9. N. C. Goodman and S. Spiegelman, *Proc. Nat. Acad. Sci. U.S.* **68**, 2203 (1971).
10. J. Schlom and S. Spiegelman, *Science* **174**, 840 (1971).
11. R. Axel, J. Schlom, S. Spiegelman, *Nature* **235**, 32 (1972).
12. We thank S. Hullett, D. Colcher, S. Mitchell, D. Burton, and M. Mason for assistance. We also thank Drs. W. Feller and Royo-Burman for providing some of the milk samples. This study was conducted under contract 70-2049 within the special virus cancer program of the National Cancer Institute, and grant CA-02332.

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Procollagen: Conversion of the Precursor to Collagen by a Neutral Protease

Abstract. *An enzymatic activity (procollagen peptidase), capable of converting the biosynthetic precursor procollagen to collagen at neutral* p*H, has been identified in rat and chick calvarial bone. Limited proteolysis of procollagen with chymotrypsin resulted in a similar transformation. The activity in bone can be demonstrated in vitro despite inhibition of new collagen synthesis by cycloheximide. Preservation of the collagen precursor in preparations extracted with acetic acid results from inhibition of the enzymatic activity at low* p*H.*

Recent studies have provided evidence for the existence in rat calvaria of a biosynthetic precursor of collagen, procollagen (*1*). Procollagen was shown to contain the pro-α1 chain (*2*) which differed from α1 of extracellular fibrous collagen in its chromatographic properties, molecular weight, and relative proline and hydroxyproline contents. We have evidence that the procollagen molecule also contains an analogous pro-α2 chain.

A time-dependent conversion of molecules containing pro-α1 to those containing α1 was shown by short-term labeling experiments (*1*). Such conversion could be simulated in vitro by limited proteolysis with pepsin. A collagen fraction with some of the properties of procollagen was also identified in the medium of cultured human fibroblasts (*3*) and chromatographic evidence for the pro-α1 chain was obtained in studies of collagen synthesis in which a cell-free system was used (*4*). In chick calvaria, inhibition of proline hydroxylation with the chelating agent α,α'-dipyridyl resulted in the synthesis of underhydroxylated precursors to the pro-α1 chain (*5*).

It was proposed that the additional sequences in procollagen serve to initiate helix formation and to inhibit intracellular fibrogenesis (*1*). The conversion of procollagen to collagen would therefore be required before a collagen molecule could be incorporated into a functional extracellular fiber. The limited proteolysis necessary for this conversion might occur intracellularly during the secretion of collagen, on the cell membrane, or extracellularly. We now report that a soluble proteolytic activity obtained from chick and rat calvaria, for which we propose the trivial name procollagen peptidase, is capable of converting procollagen to collagen. Some characteristics of this activity and a similar limited proteolysis of procollagen by chymotrypsin are also described.

Calvaria (frontal and parietal bones) from newborn rats or 17-day-old chick embryos were incubated in Dulbecco's modification of Eagle's medium supplemented with β-aminopropionitrile fumarate (64 μg/ml), sodium ascorbate (100 μg/ml), penicillin (8 unit/ml), and streptomycin (8 μg/ml). After an initial 60-minute incubation at 37°C, bones were transferred to medium containing L-[2,3-^{3}H]proline (20 μc/ml) and incubated for 18 minutes. A labeled procollagen fraction was extracted from homogenized bones with cold 0.5*M* acetic acid and dialyzed extensively against this solution. In other experiments labeled bones were extracted with 1*M* NaCl containing 0.05*M* tris-HCl, *p*H 7.5, or with the NaCl-tris buffer with 0.1 percent Triton X-100.

In order to prepare procollagen peptidase, chick or rat calvaria were homogenized and extracted at 4°C for 18 hours with a buffer containing 0.15*M* NaCl, 0.05*M* tris-HCl, *p*H 7.5, and $5 \times 10^{-3}M$ $CaCl_2$. The extract was centrifuged at 100,000*g* for 60 minutes, and the supernatant was concentrated by pressure filtration. Incubation of the enzymatic activity with labeled procollagen substrate was performed at 20°C for 24 hours, and the reaction was terminated by dialysis against *p*H 4.8 acetate buffer at 4°C.

The ability of a neutral salt extract of chick calvaria to convert chick calvarial procollagen to collagen is demonstrated in Fig. 1. In these and subsequent chromatograms the procedure for the fractionation of collagen chains on carboxymethylcellulose (*6*) was modified by addition of 4*M* urea to all buffers (*1*). The content of procollagen and collagen in the starting material, as judged by the relative sizes of the pro-α1 and α1 peaks in Fig. 1A, was very similar to that in preparations from rat calvaria (*1*). Since the recovery of counts was the same in the two chromatograms, selective degradation or precipitation of procollagen by the tissue extract is excluded. Incuba-