the sorediate-sterile morph do indeed have different niche characteristics. In every stand where both chemical races were present the norstictic-connorstictic race was encountered more frequently on hardwoods than on conifers, and the stictic-constictic-norstictic race was encountered more frequently on conifers than on hardwoods. At some localities these associations were statistically not significant, but in every instance the trend is in the same direction. However, when the data from stands geographically near each other are pooled (thereby yielding a sample of larger size), the pooled data are significant at the 1 percent or 0.1 percent level. (The only exception is for stands more than 90 km from the coast, where the stictic-constictic-norstictic race becomes rare and the number of encounters in samples was low.) The pooled data and the maps (Fig. 1, C and D) show the overriding effect of gross climatic change from the seaboard to the interior. The complex of environmental parameters that makes up the east-west climatic gradient in the mid-Atlantic states causes an abrupt replacement inland of the coastal stictic-constictic-norstictic race by the norstictic-connorstictic race. At 200 km west of the Atlantic coast the stictic-constictic-norstictic race of the sterile morph is eliminated, but the norstictic-connorstictic race of that morph ranges another 1400 km westward through Missouri (*7*). The dependence of the stictic-constictic-norstictic race of the asexual morph on near-coastal environments is further seen in the disjunctive representation of this race in the Mediterranean region (Fig. 1E) and in southern California and Baja California (Fig. 1F) (*8*).

The norstictic-connorstictic race of the asexual morph must have been derived directly from the corresponding chemical race of its fertile counterpart by the production of soredia and the subsequent suppression of sexuality. The geographic ranges of the races are virtually identical with the exception of the absence of the norstictic-connorstictic race from the Gulf coastal plain. More important, the ecologies of both races are virtually identical—both are highly adapted to life as epiphytes of hardwoods. Although no field studies were made on the norstictic-connorstictic race of the esorediate-fertile morph, 94 percent (254) of the 269 herbarium specimens of this race seen with appropriate substrate data came from hardwoods. Likewise, the ecologically different stictic-constictic-norstictic race of the asexual morph must have evolved from the fertile but chemically identical race now known from only Texas and Louisiana (*9*).

The suggestion that in lichen fungi the chemically variable asexual morphs, which conventional taxonomies treat as species, may be polyphyletic and the product of parallel evolution is new. Poelt (*10*) put forth this theory only this year, by using examples with hypothetical but plausible extinct sexual ancestors to explain certain extant asexual morphs. Many of the commonest lichens are known only as chemically variable asexual morphs, and few asexual-sexual morph pairs coexist today. The research reported here provides the first example of a full set of chemical races of sexual and asexual morphs of which the origin of the latter is better explained by parallelism from the former than by the assumptions of conventional taxonomy.

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7. The essentially allopatric ranges of the chemical races of the sorediate-sterile morph in the eastern United States result from well-differentiated niches, for the organisms grow so widely spaced on tree trunks that competitive exclusion does not (at least now) seem to be a factor.
8. Although long-distance dispersal may be the simplest explanation of this widely disjunct distribution, it is tempting to relate it to the ancient connection of the New World and the Old World.
9. Four of the five known specimens of the latter race came from hardwoods. The ecology of the widely ranging stictic-constictic-norstictic race of the asexual morph is surely more complex than our analysis indicates. For example, this race, associated with conifers in the eastern United States, often grows on the specialized bark of the cork oak (*Quercus suber*) in the Mediterranean. The polyphyletic nature of the asexual morph is underscored by the fact that this and the sexual morph both also have poorly known races that produce alectoronic acid, an orcinol-type depsidone not closely related to the compounds referred to in this report. Organisms with this chemistry are relatively rare, restricted to seashore trees and maritime scrub in the southeastern states, and so ecologically remote from the races discussed above that we did not consider them in this study. But that a race with this very different and unsuspected chemistry evolved independently in both morphs seems far less probable than that the sexual morph of this chemistry gave rise to a sorediate asexual morph.
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DNA Polymerase Activities of Human Milk

Abstract. *DNA polymerases have been partially purified from human milk. A DNA polymerase detected by phosphocellulose chromatography is similar to the enzymes of RNA tumor viruses in that a hybrid of polyriboadenylate and oligodeoxythymidylate is a better template than is DNA. However, this polymerase differed from that of the RNA tumor viruses in its chromatographic behavior. Three different methods of detecting "reverse transcriptase" activity failed to correlate with the donor's family history of cancer.*

The reported morphologic and biochemical properties of particles found in human milk appear similar to the properties of known RNA tumor viruses. (i) "Viruslike" particles of several types have been detected by electron microscopy (*1*). (ii) RNA-directed DNA polymerase activity has been detected by two methods: ribonuclease-sensitive incorporation of radioactive thymidine triphosphate (dTTP) into trichloroacetic acid–precipitable material (*2*); and incorporation of dTTP into molecules with sedimentation coefficients of 60 to 70*S* (*3*, *4*). The latter method simultaneously detects the presence of a high molecular weight RNA of a size characteristic of the RNA of RNA tumor viruses and of an enzyme synthesizing DNA that cosediments with this RNA. (iii) RNA extracted from human breast cancers has been found to hybridize specifically with DNA produced in vitro from the mouse mammary tumor virus (*5*), and DNA synthesized by a human milk sample has been reported to hybridize with the polysomal RNA of a human breast carcinoma (*6*). Thus, DNA polymerases and nucleic acids of hu-

man milk have been claimed to have properties characteristic of the DNA polymerases and nucleic acids of RNA tumor viruses.

In this study, DNA polymerases from human milk have been partially purified in order to determine whether these enzymes, when separated from other components of milk, have properties similar to those of the DNA polymerases of RNA tumor viruses. In addition, the presence of "reverse transcriptase" activity as measured by various methods has been correlated with the donor's family history of cancer.

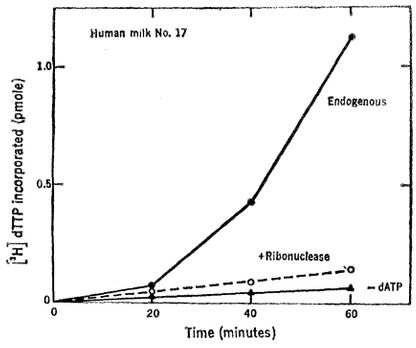

Fig. 1. Kinetics of a positive endogenous reaction in a human milk sample. Milk samples were collected in the hospitals of the Detroit metropolitan area and divided into 15- to 30-ml portions with antibiotic added to prevent bacterial growth. Early samples were stored less than 1 week and shipped to Bethesda at 4°C. Samples were frozen slowly in Dry Ice on arrival, stored in liquid nitrogen, and thawed only once. Later samples were shipped frozen after collection and were stored in liquid nitrogen. Family histories of the milk donors were not decoded before experimentation was completed. Samples of frozen milk (60 ml) were processed essentially by the method of Schlom *et al.* (*2*). After initial purification steps, the material was placed on a sucrose gradient (10 to 60 percent) containing a density gradient marker bead that sediments to a density of 1.16 g/ml. After centrifugation for 16 hours at 90,000*g*, fractions were collected and the absorbance at 260 nm was determined. Peaks of absorbance or the fractions of the gradient within the density region of 1.16 to 1.19 g/ml were pooled, centrifuged at 90,000*g* for 30 minutes, and resuspended in TNE [0.01*M* tris(hydroxymethyl)aminomethane (tris), *p*H 8.3; 0.15*M* NaCl; and 0.002*M* ethylenediaminetetraacetic acid (EDTA)] containing 20 percent glycerol and 0.08 percent Nonidet P-40 (NP-40). Half of the sample was then analyzed by phosphocellulose chromatography, and a portion of the remaining sample was added to the standard reaction mixtures described by Schlom *et al.* (*3*) except that the unlabeled dTTP was omitted; [^{3}H]dTTP (17 c/mmole) was added to a final concentration of $2.4 \times 10^{-6}M$. Samples were incubated at 37°C and 25-μl portions were removed at the indicated times. Samples were considered positive if the endogenous activity after 1 hour of incubation was twice the reagent blank (400 count/min) or greater, showed ribonuclease sensitivity (12 μg/ml present throughout incubation), and demonstrated a requirement for the four deoxyribonucleoside triphosphates; dATP, deoxyadenosine triphosphate.

Method 1: Endogenous reaction. Milk samples from 94 donors were tested for ribonuclease-sensitive incorporation of dTTP into acid-precipitable form by the method of Schlom *et al.* (*2*). Figure 1 shows a positive reaction that is sensitive to bovine pancreatic ribonuclease A and requires all four deoxyribonucleotide triphosphates. Such reactions were observed in 15 percent (14 out of 94) of the samples tested.

Method 2: Simultaneous detection. An additional 40 milk samples were tested by the simultaneous detection method of Schlom *et al.* (*3, 4*) in the presence of actinomycin D. Figure 2 shows the glycerol gradient profile of a typical positive sample. This test was scored as positive when the region of the density gradient corresponding to the position of a molecule with a sedimentation coefficient of 60*S* to 70*S* showed radioactivity at least 200 count/min above the background level of 20 count/min. By this technique, 77 percent of the samples tested were positive. This strikingly high percentage of positive donors suggests that this technique measures the presence of a commonly found constituent of human milk. Alternatively, some of these samples may not represent DNA copies of RNA. These data would suggest that for this technique, as for method 1, the incorporation of dTTP should be shown to be ribonuclease-sensitive or the material in the high molecular weight peak should be examined by cesium sulfate centrifugation. Such additional criteria could give assurance that a positive result implies the presence of an RNA-directed DNA polymerase activity.

Method 3: Partial purification of DNA polymerases from human milk. Phosphocellulose chromatography has been extensively used for purification of DNA polymerases from many sources. For this study, columns were packed in capillary pipettes, and samples representing 15 ml of human milk were applied. The milk samples used for chromatography represented half of the material processed by isopycnic banding for method 1 or by pelleting through 20 percent glycerol for method 2. Column effluents were simultaneously assayed with poly(rA)·oligo(dT) (*7*) and activated DNA templates as described (*8*). Column chromatography was used to further characterize enzymes responsible for dTTP incorporation as measured by methods 1 and 2. There was no single phosphocellulose elution pattern which could be correlated with a positive assay by either of these methods. In general, the elution profile of DNA polymerases

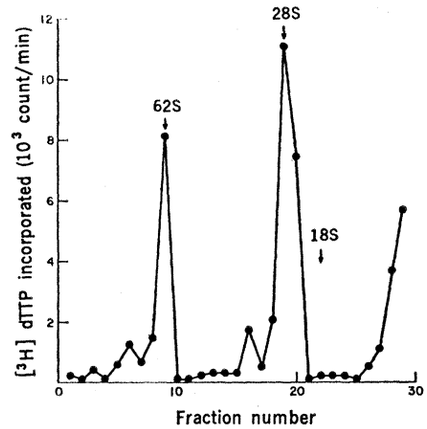

Fig. 2. Gradient profile of a positive simultaneous detection reaction from human milk. Samples of thawed milk (30 ml) were treated essentially as described by Schlom *et al.* (*3*) with the following changes. The pellet resulting from the purification and concentration steps was resuspended in 100 μl of a solution containing 0.01*M* tris-HCl (*p*H 8.3), 3.3 mg of NP-40 per milliliter, and 0.1*M* dithiothreitol. A 45-μl portion of the sample was added to a reaction mixture (final volume, 125 μl) with the components and concentrations described (*3*) plus 9.4 μg of actinomycin D (*11*). After a 30-minute incubation at 37°C, the reactions were terminated by addition of tris-HCl (*p*H 8.3), NaCl, and sodium dodecyl sulfate to final concentrations of 0.1*M*, 0.2*M*, and 10 mg/ml, respectively (final volume, 0.5 ml). An equal volume of phenol-cresol containing 8-hydroxyquinoline (equilibrated with 1*M* tris-HCl at *p*H 8.3) was added and the mixture was shaken and centrifuged as described (*3*). The aqueous phase was layered over a linear glycerol gradient (10 to 30 percent, by volume) in 0.1*M* tris-HCl (*p*H 8.3), 0.15*M* NaCl, and $10^{-3}M$ EDTA, and centrifuged at 40,000 rev/min for 3 hours at 4°C (Spinco SW-40 rotor). External markers were 18*S* and 28*S* ribosomal subunits.

from human milk (Fig. 3) corresponded to either pattern A (DNA-directed DNA polymerases only) or pattern B [poly(rA)·oligo(dT)-directed poly(dT) synthetase present either with or without DNA-directed enzymes], or was completely negative for DNA polymerase activity. A total of 33 individual milk samples were examined by phosphocellulose chromatography. Of these samples, 10 showed pattern A, 12 showed pattern B, and 11 were negative for DNA polymerase.

The poly(dT) synthetase activity shown in pattern B eluted at $0.6 \pm 0.05M$ KCl in all 12 samples where it was found. An elution position (Fig. 3, arrow) of $0.3 \pm 0.02M$ KCl has been observed in this laboratory for the viral enzymes of murine mammary tumor virus purified from C_3H and RIII mouse milk, Rauscher murine leukemia virus, Gross murine leukemia virus, Rous sarcoma virus (RAV-1), and feline leukemia virus (Rickard). Thus the poly(dT) synthetase activity observed in milk elutes at a higher salt concentration than the DNA polymerase of these tumor viruses.

Since unknown factors in human milk might influence the elution position of viral polymerase, 10^9 particles from a sample of the Rauscher virus, purified twice in sucrose density gradients, were added to 50 ml of untreated human milk. The milk sample was then processed as usual. It was tested for an endogenous reaction by method 1 and found negative, yet it showed a typical viral enzyme peak eluting at $0.3M$ KCl from phosphocellulose. A 50-ml sample of this milk without added virus showed no polymerase activity in the $0.3M$ KCl region. The results of this experiment suggest that column chromatography is as sensitive as the endogenous method, but they do not preclude the possibility that other milk samples might alter the chromatographic behavior of other viral enzymes.

Table 1. "Reverse transcriptase" in milk classified according to cancer history of donor. Method 1 is endogenous reaction, method 2 is simultaneous detection, and pattern B is that shown in Fig. 3B. Methods used to detect "reverse transcriptase" are detailed in the legends to Figs. 1 to 3. Familial cancer histories were scored as positive when at least one sibling, parent, sibling of parents, or grandparent had a documented cancer. Most donors did not have families with multiple occurrences of cancer.

History	Number positive/total number		
	Method 1	Method 2	Pattern B
Breast	1/26	5/7	3/7
Other	7/42	12/16	4/12
Negative	6/26	14/17	5/14

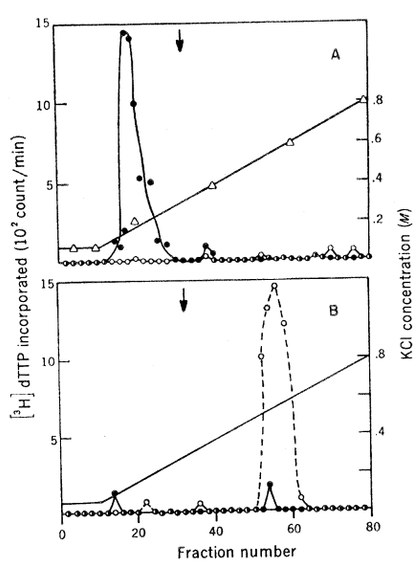

Fig. 3. Phosphocellulose elution profiles of DNA polymerases from different human milk samples. The arrow marks the gradient elution position of DNA polymerases of known RNA tumor viruses (murine mammary tumor virus, Rauscher and Gross murine leukemia viruses, Rous sarcoma virus RAV-1, and feline leukemia virus). Samples of 50 to 100 μl were received after processing as described for Figs. 1 or 2. These samples were diluted with an equal volume of buffer containing $0.1M$ tris-HCl (*p*H 7.8), $1.0M$ KCl, 2 mM dithiothreitol, 2 percent Triton X-100, and 40 percent (by volume) glycerol and incubated for 30 minutes at 37°C. The disrupted sample was centrifuged at 100,000*g* for 1 hour at 4°C, and the pellet was discarded. All subsequent steps were performed at 4°C. The basic buffer for phosphocellulose chromatography contained $0.05M$ imidazole (*p*H 6.5), 1 mM dithiothreitol, 0.1 percent (by volume) Triton X-100, $10^{-4}M$ EDTA (disodium salt), and 20 percent (by volume) glycerol. Phosphocellulose (Whatman, P-11 powder) was prepared as described (*8*) and packed with 0.1 atm of N_2 pressure to just below the constriction in short-form Pasteur capillary pipettes with glass wool plugs (column volume, 2.0 ml). Columns were equilibrated in the basic buffer containing $0.1M$ KCl. Samples were dialyzed against the equilibration buffer for two 1-hour periods and applied to the columns which were then washed with at least 10 ml of the same buffer. Flow rates of 8 ml/hour were maintained with peristaltic pumps. DNA polymerases were eluted with a 40-ml linear gradient from 0.2 to $0.8M$ KCl in the basic buffer. Portions (0.01 ml) of the 0.75-ml fractions were assayed in 0.05-ml reaction mixtures as described (*8*). Symbols: ●, DNA-dependent DNA polymerase activity assayed with "activated" calf thymus DNA and Mg^{2+}; ○, hybrid template–directed DNA polymerase activity assayed with poly(rA)·oligo(dT) and Mn^{2+}; △, KCl concentration.

Phosphocellulose purification indicates that some human milk samples contain a DNA polymerase that prefers the hybrid template poly(rA)·oligo(dT) to "activated" DNA. This enzyme differs from viral enzymes described previously in its elution position after phosphocellulose chromatography. In addition, this enzyme, in contrast to viral enzymes, has proved to be very labile. Purified viral DNA polymerases have been stored for months in this laboratory without loss of activity at −70°C in solutions containing 20 percent glycerol and 0.1 percent Triton. However, the purified enzyme from milk was completely inactivated in 24 hours under these conditions at 0°, −20°, or −70°C. Others have described cellular enzymes that synthesize poly(dT) with poly(rA)·oligo(dT) primers (*9*). The relation of the milk enzyme described here to cellular or viral enzymes remains to be investigated.

The various methods for detecting the presence of "reverse transcriptase" can be correlated with each other by means of the data reported here. When samples were tested by both the endogenous reaction and simultaneous detection, all milk samples that were positive by the first method were also positive by the second. These results can be explained by a greater sensitivity of the simultaneous detection method. In several instances, different portions of the same sample were tested by all three methods, and results were reproducible. There was no correlation between the detection of a poly(dT) synthetase on phosphocellulose chromatography and the result of either method 1 (endogenous reaction) or method 2 (simultaneous detection).

Some samples that showed only DNA-dependent DNA polymerase activity (Fig. 3A) after phosphocellulose chromatography were positive by the criteria of the endogenous reaction or simultaneous detection. In addition, some samples that were negative by these criteria did show a poly(dT) synthetase peak at $0.6M$ KCl. These data could be explained if a viral enzyme of insufficient quantity or stability to be found by phosphocellulose chromatography is detected by the other methods. Alternatively, enzymes other than "reverse transcriptases" may pro-

duce positive results by methods 1 or 2. Other studies (*10*) demonstrate no correlation between the detection of "viruslike" particles by electron microscopy (*1*) and detection of "reverse transcriptase" by methods 1 or 2.

The data of Table 1 show that the occurrence of "reverse transcriptase" was not significantly higher in milk donors with a positive family history of breast cancer or any other cancer than in donors with normal histories. Donors in this study were sampled only once, and few donors with positive family histories had more than one relative with breast cancer. However, the lack of correlation is not due to small numbers of positive determinations but to an equal distribution of positives among the three classes.

Thus, there is no correlation of the "viruslike" enzyme activity observed in human milk with a positive family cancer history of the donor. The enzyme activity being measured may still be virus-associated. If so, then the presence of a viral agent containing this enzyme might be a necessary cause but could not be sufficient cause for the development of breast cancer.

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Milk Carbohydrates of the Echidna and the Platypus

Abstract. *The principal neutral carbohydrate of three samples of echidna milk was identified as a trisaccharide, fucosyllactose. That of a sample of platypus milk was a tetrasaccharide, difucosyllactose. Free lactose was found in small amounts only. The milk carbohydrate of monotremes is distinguished from that of both marsupials and placental mammals by its high fucose content.*

The principal carbohydrate of the milk of placental mammals is lactose (*1*). Marsupial milk contains oligosaccharides that, upon acid hydrolysis, yield mainly galactose (*2*), but the identity of the carbohydrate of the milk of monotremes (egg-laying mammals) has been in doubt (*3*, *4*). We now report on the carbohydrate composition of milk of the echidna (*Tachyglossus aculeatus*) and the platypus (*Ornithorhynchus anatinus*).

Echidna milk sample 1 was from an animal caught in October 1969 on Kangaroo Island, South Australia; its pouch young weighed 300 g. The other two samples were from one animal from Rankin Springs, New South Wales, which was milked in October 1971 (sample 2a) and again in November 1971 (sample 2b), when its pouch young weighed 150 and 312 g, respectively. The platypus milk was from an animal caught in the Bendora Dam, Australian Capital Territory, on 9 February 1972. Milking was done by squeezing the mammary glands immediately after the injection of synthetic oxytocin (*5*).

After the removal of fat and protein (*6*), the samples were analyzed for total carbohydrate (*7*), sialic acid (*8*), and fucose (*9*), and the results were compared with those we obtained with milk from three different species of marsupials (Table 1). Echidna milk was rich in sialic acid, in which respect it resembled marsupial milk; but of the samples examined only those of the monotremes contained detectable amounts of fucose.

For the separation and partial identification of the milk carbohydrates, we used a column of Sephadex G-15 gel that had been calibrated with maltodextrins and other small saccharides. Neutral carbohydrates were eluted in order of decreasing molecular size and could be separated from each other (Fig. 1), but those containing sialic acid appeared earlier than expected, presumably because of the exclusion effect exerted by negative charges in the Sephadex gel (*10*).

Figure 1a shows the elution pattern obtained with echidna milk sample 2a; the patterns obtained with samples 1 and 2b were very similar. The material was resolved into five peaks, of which the first contained some protein and the second contained sialyllactose. The other three peaks contained neutral carbohydrates which were eluted at volumes (V_e/V_0) corresponding to those of tetra-, tri-, and disaccharides, respectively. Peaks 3 and 4 contained fucose. The carbohydrate of platypus milk was also separated into five peaks (Fig. 1b); the major fraction (peak 4) emerged at a volume expected for tetrasaccharides and contained fucose. Peak 5 was a disaccharide.

The column fractions containing the neutral carbohydrates were lyophilized and studied qualitatively by paper chromatography and quantitatively with the aid of glucose oxidase and galactose dehydrogenase (*11*). The milk disaccharides of both species cochromatographed with lactose and were hydrolyzed to glucose and galactose by β-galactosidase. Hydrolysis with 0.5*M* sulfuric acid (2 hours at 100°C) yielded only glucose and galactose in equimolar amounts. The disaccharides were therefore identified as lactose. The trisaccharide from echidna milk (peak 4 in Fig. 1a) cochromatographed with authentic 2′-fucosyllactose; was resistant to β-galactosidase; and yielded glucose, galactose, and fucose in the molar ratio 1 : 1 : 1 during hydrolysis with sulfuric acid. Partial hydrolysis with 1*M* acetic acid (2 hours at 100°C) resulted in the formation of lactose, fucose, and traces

Table 1. Carbohydrate composition of milk from the echidna, the platypus, and three species of marsupials: gray kangaroo, *Macropus giganteus*; brush-tailed possum, *Trichosurus vulpecula*; and short-nosed bandicoot, *Isoodon macrourus*.

Animal	Carbohydrate (grams per 100 g of milk)		
	Total free carbohydrate*	Sialic acid	Fucose
Echidna			
Sample 1	0.90		
Sample 2a	1.1	0.47	0.18
Sample 2b	1.0	0.43	0.17
Platypus	1.7	0.05	0.91
Kangaroo	5.9	0.43	< 0.01
Possum	6.4	0.33	< 0.01
Bandicoot	4.7	0.55	< 0.01

* Lactose used as standard.