Reports and Letters

Factors in Human Milk Interfering with Influenza-Virus Activities

The recognition of mucoproteins and the isolation of N-containing saccharides in human milk stimulated the study of human milk as a source for factors with antiviral activity (*1*). Mucoproteins interfering with hemagglutination by influenza virus or exhibiting other antiviral activity have been previously found in various biological materials (*2*). In a recent report (*1*) from our laboratory, fractions obtained from human milk were described as showing activity against the multiplication of influenza viruses in the chorioallantoic cavity of the chick embryo.

Further investigations of the nature of these antiviral substances in human milk were undertaken. For these investigations the technique of Fulton and Armitage (*3*), utilizing pieces of chicken chorioallantoic membrane for infectivity titration of influenza viruses, was modified and adapted as a screening procedure for one form of antiviral activity—that is, inhibition of viral multiplication. Influenza-A strain PR8 was used.

It was noted that, in those fractions that showed antiviral activity, there was definite toxicity to the membranes at concentrations slightly higher than those showing antiviral activity. This was evidenced by a loss of elasticity and by a stretched and pale appearance. When these fractions were passed over a cation-exchange column, no antiviral and toxic effects were demonstrable in the filtrate. The material that was eluted from the column, however, showed marked toxicity and antiviral activity. This material was high in copper content, probably owing mainly to contamination during processing of the milk. It was decided, therefore, to test the activity of copper ions, and it was found that copper chloride showed antiviral activity at a level of 10 μg/ml with evident toxicity at 30 μg/ml. It was observed that the antiviral activity of the copper salt varied inversely with the concentration of the virus.

The effect of copper chloride on multiplication of influenza-A strain PR8 in the allantoic cavity of the embryonated chicken egg (10-day-old) was then studied. At a level of 1.0 mg of copper chloride per egg, the results paralleled those reported for the human milk factor (*1*). When the inoculum size of PR8 was 100 ID_{50}, growth of the virus was delayed at 24 hours but reached the same titer as that of the controls in 48 hours. When, however, the inoculum size was 10,000 ID_{50}, the viral titer that was reached after 24 hours in the presence of 1.0 mg of copper chloride per egg was the same as that of the controls. Further studies, with earlier and more frequent sampling, indicated, however, that temporary inhibition with copper chloride was also demonstrable with this level or even higher levels of virus. If the copper chloride was injected 24 hours before the virus, a delayed effect was still evident, although it was slightly less pronounced. At this concentration of copper chloride (1.0 mg per egg), there was no macroscopic evidence of toxicity to the embryo or the chorioallantoic membrane. At 3.0 mg of copper chloride per egg, death of the embryo resulted within 18 hours.

In conclusion, it appears that the copper ion interferes, even in extremely low concentration, with the multiplication of influenza virus in the chorioallantoic membrane. The antiviral activity of fractions obtained from human milk (*1*) was the result of contamination with the copper ion.

For the purification of substances that inhibit hemagglutination by influenza virus, skimmed human milk contained in cellophane casings was dialyzed against 10 vol of distilled water at +6°C for three periods of 2 days each. The dialysis residue was then adjusted to *p*H 6.0 to remove the casein and then was carefully concentrated in a vacuum to a volume suitable for lyophilization. The lyophilized products were then deproteinized by trichloroacetic acid at +6°C or by the method of Sevag. The deproteinized substances were subjected to an alcohol fractionation, as is indicated in Table 1. Hemagglutination inhibitor activity (HAI) was determined on the various fractions, using influenza-A strain PR8 and influenza-B strain Lee as indicator viruses. The virus was passed in 10-day-old chick embryos, and the infected allantoic fluid was harvested after 48 hours of incubation at 36°C and heated at 56°C for an additional 30 minutes. To 0.2 ml of twofold dilutions of the fractions in round-bottom test tubes 55 by 17 mm was added 0.2 ml of indicator virus containing 4 hemagglutinating (HA) units. Phosphate-buffered saline, *p*H 7.2, was used throughout the test. The tubes were shaken and allowed to stand at room temperature for 1 hour, after which 0.2 ml of 1 percent washed chicken red-blood cells was added. The tubes were again shaken and allowed to stand at room temperature for 1 hour, after which they were checked for hemagglutination. The highest dilution of the fraction tested that completely inhibited hemagglutination was taken as the end-point. The fractions were also analyzed for Bial positive material that has been reported to be present in many natural substances showing HAI activity (*4*). The Bial-positive material was reported to be gynaminic acid (*5*). The bifidus activity of these fractions was likewise determined. The results are given in Table 1.

HAI activity against indicator Lee virus was found only in the 50 to 60 percent ethanol fraction. No activity against indicator PR8 was demonstrated. Variation in the level of activity was observed with different preparations. Three preparations of the 50 to 60 percent ethanol fraction showed activity at 5 μg/HA unit of indicater Lee; other preparations showed activity as low as 250 μg/HA unit of indicator Lee. The gynaminic acid content, however, remained close to 6 percent in all these preparations. The 60 to 87 percent

Table 1. Results showing HAI activity with indicator Lee virus, growth-promoting activity for *L. bifidus* var. *Pennsylvanicus* and percentage of gynaminic acid in the human-milk fractions listed.

Preparation	HAI activity/HA unit indicator Lee (μg)	Activity for *L. bifidus* var. *Penn.* (1 unit in mg)	Quantitative Bial (% gynamic acid)	Yield (g)
Sevag deproteinized	0	1		5
0 to 51% C_2H_5OH	0	1.9		0.80
50 to 60% C_2H_5OH	5 to 250	0.50	6	0.13
60 to 87% C_2H_5OH	0	0.54	16	0.66
Residue	0	2.8		2.37

ethanol fraction, which contained 16 percent gynaminic acid, showed no HAI activity. This is in agreement with findings by Odin (*4*), showing that no quantitative relationship was found between the presence of Bial-positive material and the HAI of various substances. Growth-promoting activity for *L. bifidus* var. *pennsylvanicus,* however, was found in both the 50 to 60 percent and 60 to 87 percent fractions where the Bial-positive material was present.

The HAI activity of these preparations was lost on incubation with active Lee virus, active PR8 virus, and Vibrio cholerae filtrate (RDE). HAI activity was not lost on heating in boiling water for 10 minutes. These properties are similar to those of the Francis inhibitor of human serum.

In conclusion, it may be stated that the presence of normal hemagglutinin-inhibitor in human milk has been demonstrated.

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Chlorosis Produced by Fluorine on Citrus in Florida

A chlorotic condition of citrus trees in the Bartow area of Polk County, Fla., was noted in April 1950. This chlorotic pattern was unique in that it differed from known chloroses resulting from nutritional deficiencies or toxicities. Although certain instances of typical iron, zinc, manganese, and other deficiency symptoms could be found in this area, the most prevalent pattern resembled the pattern on citrus that results from boron toxicity. However, there was no gumming on the undersides of the affected leaves, which is always associated with boron toxicity (*1*).

The appearance of this chlorosis seemed to be associated with a triple-superphosphate manufacturing plant that had recently been put into operation. Qualitative tests of citrus leaf ash from affected trees indicated the presence of fluorine. Shortly after this first observation of chlorosis, additional scrubbing facilities were installed at the triple-superphosphate manufacturing plant, and the chlorotic pattern on citrus was alleviated.

Fig. 1. Chlorosis of citrus leaves induced by fluorine.

However, in January 1954, several other instances of chlorosis were observed. This pattern, shown in Fig. 1, occurred in groves on several different types of soil, and under several different systems of management. As the number of new triple-superphosphate manufacturing plants in the vicinity has increased (there are seven at present), the extent of the area affected has also increased.

It is known that Florida pebble rock phosphate contains from 2 to 4 percent fluorine (*2*). In the production of triple-superphosphate, the rock is treated first with sulfuric acid, then with phosphoric acid, and in each treatment fluorine is released.

Analyses of citrus leaves in California (*3*) that had been obtained near an industrial plant indicated that up to 211 ppm of fluorine on a dry weight basis could be found in leaves from trees that were suspected of being damaged. However, no instances of chlorosis were reported. Chlorotic leaves from affected Florida groves ranged in fluorine content from 370 ppm to as low as 48 ppm. Chlorotic citrus leaves were observed up to 6 miles away from the nearest plant, but analyses of these leaves did not prove that the leaves were abnormally high in fluorine content. Thus, chlorosis was not in all cases associated with an unusually high fluorine content. It would appear that fluorine could cause leaf chlorosis, then be dissipated by translocation or otherwise lost, an observation similar to that made by others (*4*). Normal-appearing leaves away from the affected area contained from 12 to 30 ppm of fluorine.

Since the degree of chlorosis was not always related to the fluorine content of affected leaves, samples of an air plant, *Tillandsia usneoides,* commonly called Spanish moss, growing in the affected area, were taken at varing distances from one of the triple-superphosphate manufacturing plants and analyzed. The results of these analyses (Table 1) indicated that fluorine was being released by the plant operation. The deviations from the general trend can be ascribed to the topography of the area sampled.

During the spring of 1955, sprays of aqueous HF, H_2SiF_6, and H_3PO_4 were applied at a concentration of 0.1*N* to 4-year-old Ruby Red grapefruit trees located north of Lake Alfred, 20 miles away from the nearest triple-superphosphate plant. Approximately 1 lit of solution was applied per tree, per spray application. After the application of seven sprays during a 2-month period, a chlorotic leaf pattern appeared that was identical to the pattern observed in the affected area. This pattern occurred with both the HF and H_2SiF_6 sprays and was confined to the growth produced during the period of spraying. It appeared that the growth that was about three-fourths matured was the most susceptible to the fluorine injury. A similar observation was made in the area affected by the triple-superphosphate plants.

A small amount of leaf burn resulted from the H_3PO_4 spray, but no pattern appeared. Sulfuric acid sprays were not used because it had been previously noted that chlorotic leaf symptoms were present near one plant that produced defluorinated phosphate rock but did not operate a sulfuric acid plant.

It has been observed by others (*5*) that gaseous fluorine compounds are more toxic to many species of vegetation than are equivalent concentrations of sulfur dioxide and also that the greatest accumulation usually occurs at the tips or margins of leaves. This results in an inhibition of photosynthesis or degradation of chlorophyll. The reason chlorosis develops in Florida and not in California may be the result of differences in climatic conditions, primarily the higher

Table 1. Fluorine content of Spanish moss.

Distance from manufacturing plant (mi)	F (ppm)
0.20	9400*
1.5	688
2.5	688
3.5	888
4.0	132
5.0	110
6.0	60
7.0	100
20.0	100

* This sample was taken within 400 yards of the plant. It is likely that part of the fluorine present was in the form of rock and triple-superphosphate dust.