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In a pilot experiment we found striking indication that the inclusion in the diet of a dehydrated egg and milk powder, which is produced for human consumption, induced a high incidence of liver tumors in mice. We postulated that the carcinogen(s) might be oxidation products of cholesterol. Subsequent pilot experiments revealed an increase in the incidence of liver tumors after brief inclusion in the diet of 1.0% 5,6-epoxide of cholesterol, but not after prolonged inclusion of 0.1% 3,5,6-trihydroxycholesterol.

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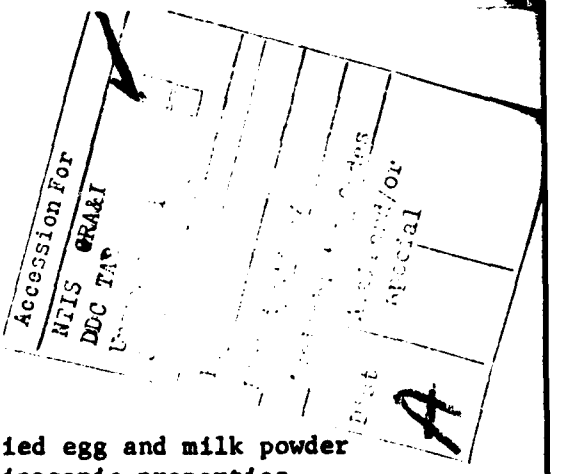

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Section 1. Objectives

The objectives of this study were as follows:

1. To determine whether a component of a dried egg and milk powder sold for human consumption may have carcinogenic properties.

2. To determine whether the specific dried egg and milk powder prepared for the Armed Forces has similar properties.

3. To determine whether the nature of the total diet influences the liver tumor incidence in experimental mice on a regimen containing 25% dried egg and milk powder.

4. To establish whether it is the oxidation products of cholesterol in dried eggs and milk which are responsible for liver tumor development.

Section 2. Dietary Experiments

The induction of liver tumors in mice was selected as the endpoint in determining the potential carcinogenicity of dried eggs and milk because they are employed at the National Cancer Institute, Bethesda, Maryland and at the National Center for Toxicological Research, Jefferson, Arkansas, and the PI has experience in this field.

In all experiments we use B6C3F_1 mice (first generation hybrids of C57BL/6 x C3H/f) as recommended by NCI. According to Chu the percent of spontaneous primary liver tumors in control untreated mice at NCI was first reported as 19.7% of 2034 males and 3.6% of 1985 females. A more recent report indicates liver tumor incidences of 21.9% of 2355 males and 4.0% of 2365 females. We purchased the mice from the Charles River Laboratories, Inc., Wilmington, Mass. They were placed on the experimental diets shortly after weaning when they had attained 18-22 grams body weight.

Section 3. First Experiment with Dried Egg and Milk Diets

A preparation of dried egg yolk and milk available as a powdered custard at local supermarkets was used. It was fed at several concentrations, mixed with a control basal diet which is a modification of a semisynthetic diet employed in this laboratory for other purposes. Fresh mixtures were prepared and fed ad libitum daily. Five diets were employed, the control diet alone, and 25%, 50%, 75%, and 100% custard diets.

The test mice were breeding females that were allowed to raise their litters to 22 days of age and then were returned to the breeding pens (one male and four females per pen). This protocol was followed because females have a lower incidence of spontaneous liver tumors and, also, because we assumed that if

steroid metabolism were significantly altered by the custard-containing diets, it would be expressed by such effects as delayed or reduced frequencies of pregnancy and/or smaller numbers of sizes of offspring born or weaned. It is of interest that breeding females were found by Gellatly (In: Mouse Hepatic Neoplasia, Butler, W.H. & Newberne, P.W., eds., Amsterdam: Elsevier, pp. 77-109, 1975) to have a lower incidence of spontaneous liver nodules than virgin females.

The mice were maintained on the diets for 72-80 weeks before sacrifice and examination. This is the time span used for carcinogenicity studies in a number of laboratories. At necropsy one sample of each of the following organs from all animals was fixed in Bouin's fluid for light microscopy: liver tumors, liver, duodenum, colon, aorta, lung, reproductive organs. To date, slides of the tumors, livers, aortas, and lungs have been examined. In addition, randomly selected liver tumors and livers from mice on each of the diets were preserved for transmission electron microscopy. These are in preparation for examination.

Reproductive activity on all diets was only minimally affected. There was a delay in the onset of the first pregnancy in many of the mice, and an occasional offspring or entire litter was smaller than usual. Usually, fresh males previously maintained on a routine lab chow diet were added to the breeding pens from time to time to assure fertility. Three, however, remained in the experimental pens on the 75% custard diets throughout the experiment, and these remained fertile. The reproductive organs of these three males and all females are being prepared for histological study.

Twelve breeding females were placed on each of four custard diets, i.e. those containing 25%, 50%, 75%, and 100% custard powder, and the fifth group was fed the control basal diet alone. At postmortem 72-80 weeks later the only grossly visible abnormality was the presence in some of the mice of one to three liver tumors, usually quite large, as follows:

Liver Tumors

Diet	# Breeding ♀ mice w/large liver tumors	Size of tumors in mm
Control	0/19	
25% custard	6/12	1. 8x7x7 2. 15x10x7 3. 30x25x30 and 25x20x10 4. 5x4x4 5. 15x10x10 and 5x4x4 6. 23x18x10 and 9x9x9
50% custard	4/12	1. 12x12x12* and 8x6x2 2. 4x4x4 and 1x1x1 3. 22x20x5 and 20x20x5 4. 9x8x2
75% custard	1/12	1. 18x10x6
100% custard	0/12	

*A portion of this tumor was transplanted into the spleens of 10 young adult hosts of the same genetic background.

In addition, 1/3 males that remained on 75% custard for 80 weeks developed a liver tumor 3x3x2 mm in size.

Light microscopy of the liver tumors has revealed that all of them were of hepatocyte origin. They were mostly well circumscribed nodules, forming pressure lines in adjacent normal liver tissue. They consisted of compact masses of well-differentiated cells which varied from nodule to nodule in size and degree of cytoplasmic vacuolization. The larger numors frequently contained more than one size class of modified hepatocytes. Narrow sinusoid-like vascular channels were present often, but the lobular pattern of normal liver tissue was absent. According to the descriptions in the Mouse Hepatic Neoplasia Workshop, we would classify most of our hepatic tumors as benign liver cell adenomas, or Type A nodules, or Type 2 nodules. We do not consider them to be hyperplastic nodules because of their large size. Two of the liver tumors, however, or at least a portion of them, could be classified as malignant hepatocellular carcinomas or Type B or Type 3 nodules, because one contained broad cords or trabeculae of cells separated by wide sinuses and the other exhibited a classical papillary organization. Although no counts have been made, mitotic figures seemed to be especially abundant in the latter.

The biological activity of two or our liver tumors demonstrates their neoplastic and, in one case, metastatic properties. First, one tumor, 12x12x12mm in size from a host fed the 50% custard diet, was chosen at random and a portion transplanted intrasplenically into 10 young adult females of the same genetic background to ascertain its neoplastic properties. One recipient that appeared unusually large was sacrificed recently, 7 months after transplantation, and three nodules, each approximately 3 mm in diameter, containing hepatocyte-like tumor cells were found in the spleen. Other recipients of the transplant will be sacrificed soon. That portion of the donor tumor that was fixed for light microscopy is of the benign adenoma type histologically. Second, although no macroscopically visible metastatic nodules were found in the lungs at necropsy, microscopic examination revealed that one lung contained several small hepato-cellular nodules. The tumor in the liver of that mouse proved to be the one containing abundant mitoses and the papillary type of growth characteristic of malignant hepatocellular carcinomas.

One sample of each liver near the tip of the left lateral lobe was preserved for microscopic observation. Several nodules of microscopic size were found in custard-fed mice which, in one case, differed cytologically but, usually, just formed pressure lines in the surrounding parenchyma. None were hyperbasophilic. Some exhibited extensive glycogen vacuolization, which is thought to precede hyperbasophilia, which, in turn, may precede neoplastic change. Thus, these microscopic nodules may represent either preneoplastic or neoplastic foci, and they are not included in our enumeration of tumors.

Liver histology varied with the diet. The livers of mice on the control diet were normal. Vacuolization of the cytoplasm was somewhat greater in mice fed 25% custard and it was pronounced in those fed higher levels of custard. The latter mice exhibited spherical vacuoles characteristic of lipid, some so large that they resembled those of adipocytes. An unusual phenomenon (to the PI at least) was the greater concentration of the lipid in the periportal zone of the hepatic lobule rather than the centrilobular zone. Vacuolization characteristic of glycogen storage also was present. The actual contents of the vacuoles can be verified in the samples fixed for transmission electron microscopy.

Section 4. Second Experiment with Dried Egg and Milk Diets

To corroborate and to decrease the statistical probability of error of our observations on a small number of mice in the first experiment, we initiated another set of experiments with the dried egg and milk powder.

In March, 1979 we placed 40 mice, 20 of each sex, on each of two control diets and three experimental diets, as follows:

	Diet 135 Control	Diet 136 75% Control Diet 25% Custard	Diet 137 25% Custard + Modified Basal Constituents
Vitamin test casein (Teklad*)	38	28.5	37
Cornstarch (Argo)	37	27.75	14
Salt Mix #2 (Teklad)	9	6.75	9
Brewer's Yeast (Teklad)	8	6.0	8
Vitamin Mix (Teklad)	2	1.5	2
Peanut Oil (Planters)	6	4.5	5
Custard	0	25.0	25
	100%	100%	100%
Protein content	46	35.5	46
Carbohydrate content	37	50.75	37
Fat content	6	5.5	6

*This casein is vacuum dried and temperatures do not exceed 160°F. Prior to extraction, it contains 1.0% fat.

	Diet 138 Chow Control	Diet 139 Chow Custard
Chow	100	75
Custard	0	25
	100%	100%
Protein content	22.3	17.7
Carbohydrate	?	? + 23 from custard
Fat	5.3	4.4
Fiber	4.2	3.15

The experiment was scheduled to terminate in November, 1980, after the standard 80 weeks of feeding the regimen.

Section 5. Regimen with 5,6-epoxide of Cholesterol

We used 5,6-epoxide of cholesterol at 1.0% by weight level in Charles River's mouse diet. The animals consumed the diet readily and at the same level as the controls. Unfortunately, the high cost of the epoxide forced us to cease feeding it after 6 weeks rather than the standard 80 weeks. The mice, however, were kept on the diet for an additional 80 weeks before sacrifice on the basis that small foci of neoplastic cells might have time and opportunity to grow to detectable size.

Among 13 males 5/13 appeared normal, 5/13 had liver tumors (3 of them very large), 2/13 had abnormal spleens (1 weighing 2½ g in a mouse with a liver weighing 13 g; 1 with enlarged nodules), 2/13 had nodules on one kidney. Among 12 females 8/12 appeared normal macroscopically, 1/12 had a large liver tumor, and 2/12 had abnormal spleens (1 very large; 1 with enlarged nodules). Histoloigcal preparations of the organs are being completed at this time. Among those completed and examined to date, all the liver nodules are hepatomas. One animal exhibits massive invasion of the kidney by mitotically active lymphoid cells, and another has similar proliferating lymphoid cells in its spleen and liver. As compared with controls on the laboratory chow during the same period, the incidences of liver tumors were as follows:

Sex	Control diet	1.0% Epoxide
♂	1/13	5/13
♀	1/12	1/12

It is relevant to point out that Dr. Lee-Shin Tsai of the USDA Western Regional Research Center, Berkeley, California, recently analyzed two types of dried egg powders for the 5,6-epoxide of cholesterol and found it to be present in one of them but not the other. It appears possible that the procedure for drying the eggs may be the critical factor.

Section 6. Regimen with 3,5,5-trihydroxycholesterol

In another experiment, 3,5,6-trihydroxycholesterol was administered in commercial mouse laboratory chow as above. The oxygenated cholesterol product was first administered at the 0.1% and the 1.0% levels, but the latter had to be dropped because the mice limited their food intake drastically when 1.0% trihydroxycholesterol was present. On the other hand, the 0.1% level was consumed as readily as the control chow. After 80 weeks on this regimen the incidence of liver tumors was as follows:

Sex	Control Diet	0.1% Trihydroxycholesterol
♂	3/13	2/12
♀	1/12	1/12

All of the liver tumors were of hepatocellular origin. Histologically two had morphological characteristics of malignant hepatocellular carcinomas and the rest were benign. It would appear that the trihydroxycholesterol at this dose level does not alter hepatic carcinogenesis significantly.