References and Notes

1. I. P. Pavlov, *Conditioned Reflexes,* translated by G. V. Anrep (Oxford Univ. Press, London, 1927), p. 385.
2. ———, *Complete Works* (Akad. Nauk S.S.S.R., Moscow, ed. 1, 1949), vol. 3, p. 58.
3. G. Razran, *Science* **126**, 1107 (1957).
4. I. P. Pavlov, *Pavlov's Wednesdays* (Akad. Nauk S.S.S.R., Moscow, 1949), vol. 3, p. 252.
5. ———, *ibid.*, vol. 2, p. 444.
6. ———, *ibid.*
7. In one place (*ibid.*) Pavlov states: "I simply assume that he [Sherrington] is ill, although he is only seventy years old, that these [his views on brain and mind] are symptoms of old age, decrepitude" (Pavlov was 84 at the time). And later, discussing religious-philosophical distortions of science, he concedes: "Take my wife—she is a dualist. She is religious yet she shows no distorted attitude towards things" (*ibid.*). (The excerpts are from informal talks which Pavlov did not prepare for publication, and probably he never thought they would be published.)

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Effect of Dietary Fats on Fatty Acid Composition of Human Erythrocytes and Chick Cerebella

Abstract. Through gas-liquid chromatography it can be shown that increasing the ingestion of linoleic acid-containing fats increases the deposition of linoleic acid in erythrocytes and in brain tissue. Such changes are probably causally related to the tocopherol requirement, the incidence of chick encephalomalacia, and the peroxide hemolysis test. Whether similar mechanisms are involved in the ability of unsaturated oils to lower serum cholesterol levels remains to be determined.

Although it has been generally acknowledged that the composition of depot and plasma lipids can vary with the type of fat ingested, it had not been proved that structural lipids, such as are found in brain or in the stroma of erythrocytes, could also be varied by diet. This report shows that such changes do take place and that they may have physiological significance.

The controlled dietary regimen of the patients whose blood was used in this study was described in previous publications (*1*, *2*) in which the effects of fats on the tocopherol requirement of man were reported. Chick brains were obtained from birds which were fed tocopherol-deficient diets containing lipids with different levels of linoleic acid. The production of encephalomalacia in chicks, with characteristic gross and microscopic cerebellar lesions, depended upon the concentration of linoleic acid-containing fats in the diet, as well as on the absence of tocopherol (*3*, *4*).

Five-milliliter samples of centrifuged packed red blood cells were washed three times with 0.16*M* NaCl and hemolyzed with water (after the addition of 0.2 ml of 0.5-percent *dl*-α-tocopherol to minimize peroxidation), and the lipids were extracted with methylal-methanol (4+1). The lipids were dried under nitrogen, extracted with petroleum ether according to the method of Morris *et al.* (*5*), and methanolyzed according to the procedure of Stoffel *et al.* (*6*). Saponification of blood lipids followed by methylation of the fatty acids gave results which were similar to those obtained by direct methanolysis. Individual cerebella from brains of 1-month-old chicks were similarly prepared after homogenization in methylal-methanol solutions. Most of the methyl esters were chromatographed at column temperatures of 208°C in the Lovelock ionization chamber (*7*) on Apiezon M glass columns having efficiencies of 6000 to 10,000 plates, calculated for methyl stearate according to Desty's method (*8*). The columns were 8 ft long, with an internal diameter of 1 mm. The celite used was 150 to 200 mesh; argon gas pressure was 110 lb; gas flow was 8.5 ml per minute; and time required to complete analyses through C_{22} was 9 hours. The absence of significant amounts of products larger than C_{22} was confirmed through the use of columns containing the succinate polyester of diethylene glycol (*9*) and of high-temperature (300°C) silicone columns with thermal conductivity cells as detectors (*10*). Retention volumes of methyl esters of fatty acids obtained on Apiezon M were similar to those reported by Insull and Ahrens (*11*).

Some of the analyses of chick cerebella were conducted on Apiezon L columns, which required lower gas pressures and took less time for complete analyses, but fewer separation plates were achieved with these faster-acting columns. The resolution of all known fatty-acid components was not obtained by the techniques used. For example, the "linoleic acid" peak includes any linolenic acid which may be present, and the "oleic acid" may contain other C_{18} monoenoic isomers.

As to fatty-acid content, erythrocytes from 19 subjects who for 2 years received a controlled diet of 2300 calories containing 60 gm of corn oil were compared with those from eight patients who had received the regular institutional diet ad libitum for the same period (Table 1). An increased ratio of "linoleic acid" to "oleic acid" was observed in subjects given the corn oil diet. Percentages of linoleic acid in chick cerebella were much lower than those in human erythrocytes, but again relatively larger ratios of "linoleic" to "oleic" acids (.078) were observed in samples from chicks on the corn oil diet, as compared with those obtained from birds on the cod liver oil diet (.014). Lower "linoleic"-acid levels were also seen in cerebella from birds which were fed diets containing very little linoleic acid, such as 4 percent coconut oil, 10 percent oleic acid, and fat-free diets, which, incidentally, do not require tocopherol supplementation for normal chick growth. In all cases, both in the series reported and in subsequent experiments, the linoleic-acid content of a given tissue was higher in subjects that had been fed high levels of linoleic acid than in individuals that had received the lower levels of linoleic acid.

The increase in the linoleic-acid content of the erythrocytes in individuals on diets containing corn oil helps explain why it has been so difficult to obtain normal data on the peroxide hemolysis test (*1*) after long-term feeding of tocopherol-low corn oil diets, followed by tocopherol supplementation (*2*), since the rate of oxidation of linoleic acid is about 12 times that of oleic acid. The data on chick-brain fatty acids help to explain the high incidence of cerebellitis in chicks on diets

Table 1. Fatty-acid composition (percentage ± standard deviation) of human erythrocytes and chick cerebella from subjects that had been fed diets high and low, respectively, in linoleic acids. Number of subjects in parentheses.

Fatty acid	Human erythrocytes		Chick cerebella	
	Corn oil diet (19)	Institution diet (8)	4% Corn oil diet (4)	10% Cod liver oil diet (4)
Lauric	0.5 ± 0.5	0.4 ± 0.2	0.2 ± 0.2	0.1 ± 0.1
Myristic	0.8 ± 0.6	0.7 ± 0.3	0.7 ± 0.4	0.6 ± 0.3
Palmitoleic	0.5 ± 0.2	0.6 ± 0.4	1.2 ± 0.2	1.4 ± 0.6
Palmitic	27.2 ± 2.6	30.2 ± 3.1	36.8 ± 3.8	39.5 ± 6.6
"Linoleic"	15.3 ± 1.6	8.3 ± 1.5	2.0 ± 0.9	0.5 ± 0.2
"Oleic"	17.1 ± 1.5	20.4 ± 3.3	26.5 ± 4.1	33.6 ± 2.2
Stearic	19.2 ± 1.4	20.4 ± 1.8	19.4 ± 0.2	17.3 ± 2.4
C_{20} Penta+tetraene	10.0 ± 3.2	10.4 ± 3.6	4.9 ± 3.2	1.3 ± 1.0
C_{20} Triene	2.0 ± 0.8	1.9 ± 0.7	0.8 ± 0.4	0.3 ± 0.1
C_{22} Hexaene	1.1 ± 2.3	1.2 ± 0.7	2.4 ± 2.4	2.3 ± 2.2
C_{22} Pentaene	1.6 ± 1.0	2.1 ± 1.0	2.0 ± 1.9	0.7 ± 0.5
Others	4.7 ± 2.3	3.4 ± 2.2	3.1 ± 1.7	2.4 ± 0.7
Linoleic:oleic ratio	0.90 ± 0.13	0.42 ± 0.10	0.078 ± 0.048	0.014 ± 0.005
Plasma cholesterol	167 ± 36	184 ± 40 (mg %)		

high in linoleic acid-containing fats (*4*) without tocopherol supplementation and also fortify the concept that tocopherol functions as a biological antioxidant, which is apparently required only where strongly peroxidizable lipids are present. Since such lipids are normal constituents of tissue, one would expect to find that tocopherol is required in amounts in some way related to the amounts of such lipids present.

Of some interest is the observation in a current experiment in which an attempt to reduce the high linoleic-acid content of erythrocytes in patients who had been on a corn oil diet is being made by substituting coconut oil for the corn oil. After 1 month on coconut oil (60 gm per day), there has been little change in the relative amounts of linoleic acid in the erythrocytes of these patients—amounts which remain much higher than those found in nonexperimental subjects. Apparently (although final conclusions must await the lapse of more time to permit a complete turnover of the erythrocytes), the ability of tissue to hold onto linoleic acid tenaciously, if other sources of energy are available, conforms with the observation (*4*) that while 2-percent corn oil diets, from which tocopherol has been removed, seldom produced encephalomalacia in chicks, 2-percent corn oil plus 7-percent coconut oil which had been similarly treated produced the same high incidence of encephalomalacia (over 70 percent) that 4-percent corn oil did. One should also be aware that linoleic acid is a variable component of lard (varying from 2 to 12 percent, depending upon what the hog ate), and studies of tocopherol or cholesterol metabolism in which lard is used (*1*) should take the linoleic-acid content of the lard into account.

It is of interest to note that the same lipid mixtures which increase the tocopherol requirement and promote encephalomalacia in the chick (*4*) also reduce the plasma cholesterol levels in the rat (*12*) and in man (*13*).

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References and Notes

1. M. K. Horwitt, C. C. Harvey, G. D. Duncan, W. C. Wilson, *Am. J. Clin. Nutrition* **4**, 408 (1956).
2. M. K. Horwitt, C. C. Harvey, B. J. Meyer, *Federation Proc.* **17**, 245 (1958); M. K. Horwitt, *ibid.* **18**, 530 (1959).
3. B. Century and M. K. Horwitt, *ibid.* **17**, 473 (1958).
4. B. Century, P. Bailey, M. K. Horwitt, *A.M.A. Arch. Neurol. Psychiat.*, in press.
5. S. G. Morris and R. W. Riemenschneider, *Arch. Biochem. Biophys.* **78**, 138 (1958).
6. W. Stoffel, F. Chu, E. H. Ahrens, Jr., *Anal. Chem.* **31**, 307 (1958).
7. J. E. Lovelock, *J. Chromatog.* **1**, 35 (1958).
8. D. H. Desty, *Vapour Phase Chromatography* (Butterworth, London, 1957), p. xiii.
9. S. R. Lipsky, R. A. Landowne, M. R. Godet, *Biochim. et Biophys. Acta* **31**, 336 (1959).
10. S. D. Nogare and L. W. Safranski, *Anal. Chem.* **30**, 894 (1958).
11. W. Insull, Jr., and E. H. Ahrens, Jr., *Biochem. J.* **72**, 27 (1959).
12. D. M. Hegsted, A. Gotsis, F. J. Stare, *J. Nutrition* **63**, 377 (1957).
13. We are indebted to many who were associated with this project, which was sponsored by the Food and Nutrition Board of the National Research Council. The support of the Illinois Mental Health Fund, of the National Vitamin Foundation, Inc., and of the U.S. Public Health Service (grant No. 1126) is gratefully acknowledged.

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Experimental Evidence in Support of the Dormant Tumor Cell

Abstract. When rats were injected intraportally with as few as 50 Walker-256 carcinosarcoma cells and then examined 5 months later for hepatic tumor growth, none was evident. If, however, 3 months after injection the rats were subjected to repeated laparotomy and liver examination at 7-day intervals, 100 percent had a tumor within a few weeks.

When specific numbers of viable tumor cells are injected into animals known to be receptive to the growth of such experimental neoplasms, a certain number of "takes" may be expected to occur within a given time. It has been suggested that animals demonstrating no such tumor growth have been able in some fashion to destroy the cells injected. That such cells might not be destroyed but will continue to exist indefinitely in a dormant state has been suggested by experimental and clinical evidence (*1, 2*). Few factual data, however, are available (*3*). Dormancy implies "malignant cells which, although remaining alive in the tissues for relatively long periods, show no evidence of multiplication during this time, yet retain all their former and vigorous capacity to multiply" (*1*).

Recently, while investigating factors which might influence the take and growth of artificially induced hepatic metastases (*4, 5*), we observed that rats injected intraportally with small numbers of Walker-256 carcinosarcoma tumor cells failed to show evidence of tumor growth for inordinately long periods of time. The rats might well have been considered free of tumor. If, however, they were subsequently subjected to repeated surgical trauma in the form of laparotomy with liver manipulation, growth of dormant tumor cells occurred, so that 100 percent of the rats demonstrated hepatic neoplasms.

All animals were adult female Sprague Dawley rats weighing 150 to 175 gm at the time of injection. They were allowed to eat *ad libitum* on a stock diet of Chow checkers and water. The preparation of tumor cell suspensions and the technique of intraportal injection have previously been described in detail (*4*). An aliquot of a filtered saline plasma tumor brei was used for cell counting in a fashion similar to that used for leucocyte counting. Proper dilutions of tumor cell suspensions were made so that each 0.5 ml of the suspension to be injected contained either 250 (experiment 1) or 50 tumor cells (experiment 2). The animals were lightly anesthetized with ether, celiotomy was performed, and the animals were injected intraportally via a large mesenteric vein with 0.5 ml of the saline plasma solution containing tumor cells. To insure maximum viability of the tumor cells, no suspension was used longer than 1.5 hours after its preparation. Approximately 30 rats could be injected during this time. After varying periods of time, exploration for tumor was carried out by an aseptic technique. Animals were considered to have a tumor only after gross examination and later confirmation by microscope. All rats grossly free of tumor were reexamined at weekly intervals until growth was evident, or until they died.

In experiment 1, 122 rats were injected with 250 tumor cells each. Groups of 30 were first examined at 2, 3, and 4 weeks after injection, and 32 were inspected at 8 weeks. The results of this experiment are shown in Fig. 1. At first inspection there was no remarkable difference in the number of positives, whether the animals were looked at as soon as 2 or as long as 8 weeks, there being 20, 20, 27 and 16 percent with demonstrated neoplasms. But on subsequent examinations an increasing number became positive, so that after four laparotomies the 114 rats that survived the multiple surgical procedures all had tumors in their livers. In this experiment the finding of major significance was that all animals first examined at 2, 3, or 4 weeks and surviving multiple laparotomy were positive by 8 weeks, whereas only 16 percent of those opened for the first time at 8 weeks showed tumors.

When it was evident that animals showing no tumor for as long as 8 weeks after injection (84 percent) could be triggered into tumor growth by laparotomy and liver manipulation, experiment 2 was performed.

In experiment 2, 50 rats were injected intraportally with 50 tumor cells; 17 were explored for the first time at 12 weeks, 16 at 13 weeks and 17 at 21 weeks. No tumor was present in any of the animals at the time of first examination (Fig. 2) but, with repeated laparotomy between the 12th and 20th