SYMPOSIUM: BIOLOGICAL SIGNIFICANCE OF AUTOXIDIZED AND POLYMERIZED OILS

Presented at the JOCS-AOCS Joint Meeting, Los Angeles

I. HARA, Chairman

H. KAUNITZ, Cochairman

Exacerbation of Heart and Liver Lesions in Rats by Feeding of Various Mildly Oxidized Fats[1]

HANS KAUNITZ and **RUTH E. JOHNSON**, Department of Pathology, Columbia University, New York, New York 10032

ABSTRACT

Groups of 40 male Charles River rats were fed diets containing cottonseed oil, olive oil, corn oil, soybean oil, coconut oil, chicken fat, beef fat, butter oil, lard and saturated medium chain triglycerides. The fats were fed fresh and after 40 hr aeration at 60 C, which hardly changed peroxide values. In addition, fresh and aerated soybean oil and lard were fed to W/Fu rats. Body weights and life span were significantly influenced by the kind of fat fed, but not by aeration. Many hearts exhibited unspecific focal myocarditis and focal fibrosis. The latter was graded in a blind test, which revealed highly significant differences in the incidence of severe lesions; those fed corn oil had the most, followed by cottonseed oil, soybean oil, olive oil, beef fat, saturated medium chain triglycerides, butter, chicken fat and lard, in that order. Feeding of aerated fat resulted in an increased incidence with six of the eight fats. The W/Fu rats had lower incidences, but those fed soybean oil had more than those fed lard, and aeration led to a higher incidence. Some heart sections stained with Light Green SF Yellowish revealed areas of muscle fibrils that did not accept the stain, probably as a conse-

[1]One of seven papers presented in the symposium "Biological Significance of Autoxidized and Polymerized Oils," JOCS-AOCS Joint Meeting, Los Angeles, April 1972.

INDEX

TABLE I

Analyses of One Batch of Fats Fed to Rats from Weaning

Oil	Peroxide value,[a] Meq/kg	Epoxide value,[b] Meq oxirane oxygen/kg	Carbonyl value,[c] Meq/kg		
			Unsaturated	Saturated	Total
MCT[d]	0.1	0	0.4	1.7	2.1
Olive oil					
Fresh	15.2	4	3.2	6.1	9.3
Aerated	18.5	6	3.2	7.4	10.1
Corn oil					
Fresh	1.9	3	7.3	4.1	11.4
Aerated	4.0	3	7.1	3.6	10.7
Cottonseed oil					
Fresh	7.8	5	6.0	0.8	6.8
Aerated	6.7	9	7.1	3.4	10.5
Soybean oil					
Fresh	1.0	0	3.0	2.6	5.6
Aerated	2.3	0	2.9	3.0	5.9
Irradiated	2.3	0	2.1	7.2	9.3
Beef fat					
Fresh	4.9	7	2.8	8.1	10.9
Aerated	3.5	3	2.2	4.9	7.1
Butter fat					
Fresh	1.0	0	1.7	8.4	10.9
Aerated	2.5	0	1.7	6.7	8.4
Chicken fat					
Fresh	1.9	1	0.6	2.9	3.5
Aerated	4.3	1	0.6	3.5	4.1
Lard					
Fresh	2.0	1	1.7	4.7	6.4
Aerated	2.3	10	1.9	5.0	6.9
Irradiated	1.7	0	1.3	8.8	10.1

[a]AOCS Official Method Cd 8-53.

[b]Fioriti et al., JAOCS 43:487 (1966).

[c]Fioriti, JAOCS 42:743 (1965).

[d]Saturated medium chain triglycerides.

quence of cellular damage. Higher incidences of this lesion were associated with the same fats as was severe fibrosis, and feeding of aerated fats led to higher incidences. Many livers revealed marked proliferation of bile ducts. The groups fed cottonseed, soybean, olive and corn oils had higher incidences of severe lesions, and feeding of the oxidized oils led to still higher incidences. None of the results appeared to be associated with the fatty acid composition of the fats, which suggested that these long term effects may have been due to minor constituents in the individual fats.

For the last 25 years, various studies have been undertaken to determine the extent to which changes induced in fats by thermal or oxidative treatment would produce toxic effects in animals fed these fats. In the early 1950's, feeding experiments were carried out with fats that had undergone rather severe damage by heat or oxidation (1,2). Interest centered on separation of broad classes of compounds produced in such fats. Some of these groups of compounds were so toxic that feeding studies were necessarily of short duration. Rats fed these materials exhibited diarrhea, edema of the gut, and enlarged livers and kidneys (3). All of this accumulating knowledge had social consequences: processors of such foods as potato chips, French fried potatoes and other deep-fried foods became much more careful with regard to how long they used any one batch of oil, and, at the same time, manufacturers of various fats and oils and of foods containing these oils routinely included antioxidant in their products.

Further investigations dealt with possible effects produced by fats after ordinary cooking procedures or after storage. In general, it was found that growth and life span of animals fed such fats were not adversely affected (4-8). However it was reported that rats fed mildly oxidized beef fat appeared to have less linoleate in their depots than those fed fresh beef fat (9). Furthermore it was observed that feeding of

TABLE II

Composition of Purified Diet

A.

Component	%
Vitamin-free test casein[a]	30
Dextrose[b]	44
Salt mixture (Bernhart & Tomarelli)[a]	4
Cellulose (Alphacel)[a]	2
Fat	20

B.

Component	mg/kg	Component	mg/kg
Choline dihydrogen citrate[a]	1000	Vitamin D_2[a]	0.5
Inositol[a]	1000	Thiamin-HCl	4
p-Aminobenzoic acid[a]	300	Pyridoxine-HCl	8
Nicotinamide[a]	100	Riboflavin	8
Vitamin K (Synkayvite)	10	Ca pantothenate	20
α-Tocopherol acetate	100	Folic acid	5
Free α-tocopherol	10	Biotin	0.05
β-Carotene	5	Ascorbic acid	50
		Vitamin B_{12} (0.1% trituration in mannose)	10

[a]Nutritional Biochemicals Corp., Cleveland, Ohio. All other vitamins contributed by Hoffmann-La Roche Inc., Nutley, N.J.

[b]Corn Products Co., Englewood Cliffs, N.J.

mildly oxidized fats led to increased vitamin requirements (10), as had been shown previously for more highly oxidized fats (11).

In an earlier long term feeding study carried out in our laboratory, with eight fresh and mildly oxidized vegetable and animal fats fed to rats in a low iodine diet, the average life spans of the groups fed fresh vegetable oils were shorter than those of the groups fed fresh animal fats, and feeding of the oxidized forms of three of the oils (olive, corn and soybean) resulted in longer life spans (7). Furthermore there was some suggestion that the various fats exerted other effects which were unrelated to their fatty acid composition, and the hypothesis was put forth that fats, particularly vegetable oils, even after refining, may contain biologically active substances in their nontriglyceride fractions which exert long term effects.

Because our early experiment was complicated by a low iodine diet and periodic sacrifices for lipid chemistry, a more comprehensive feeding study was undertaken with larger groups of rats, all of which could be observed until they died of natural causes.

EXPERIMENTAL PROCEDURES

Fats and Oils

The fats studied were cottonseed oil, olive oil, corn oil, soybean oil, coconut oil, chicken fat, beef fat, butter oil, lard and a randomized mixture of saturated medium chain triglycerides (MCT), (mainly C_8 and C_{10}). All of these fats had been prepared for human consumption, and three different batches of each were used in the course of the experiment. Half of each batch (one-third in the case of soybean oil and lard) was aerated at a rate of 1 liter of air per gallon per minute for 40 hr at 60 C. In addition, part of the lard and soybean oil was canned according to FDA specifications for irradiation of foods and was given 4.5 Mrad of gamma irradiation. These treatments brought about only very slight changes in peroxide, epoxide and carbonyl values (Table I). The fats were incorporated in a highly purified diet, the composition of which is given in Table II.

Animals

The main study was carried out on Charles River (CD) male rats. All fresh, aerated and irradiated fats were fed to groups of 40 rats each from the age of 28 days. In addition, four groups of 40 male rats of the smaller W/Fu strain were fed fresh and aerated lard and soybean oil. The rats were assigned to the individual groups on the basis of stratified body weights; all groups had the same weight range and the same average weights.

The rats were housed, two per cage, on racks holding 20 cages (10 with rats fed a fresh fat and 10 with rats fed the corresponding aerated

TABLE III

Relation of Average Body Weight at Early Age to Eventual Average Life Span in Groups of Charles River Rats Fed Various Fats

Oil	Fresh		Aerated	
	Average wt at 238 days, g	Average life span, days	Average wt at 238 days, g	Average life span, days
Coconut oil	738±15.8[a]	592±28.4[a]	735±13.2	549±29.5
Chicken fat	723±17.2	613±19.7	712±18.1	655±23.5
Butter	703±19.2	626±21.2	694±12.5	640±18.7
Cottonseed oil	701±12.9	641±24.2	725±23.2	650±25.0
Corn oil	691±15.5	652±21.2	687±14.0	664±22.2
Lard	688±16.8	639±20.3	697±26.6	665±20.7
Soybean oil	678±12.5	670±22.1	683±12.2	635±21.9
Olive oil	646±10.7	665±17.9	662±12.5	642±29.3
Beef fat	626±14.1	703±19.2	650±16.0	690±17.1
MCT[b]	614±10.7	703±24.5		
	P < .001	P = .005	P = .01	P < .01

[a]Standard error.

[b]Saturated medium chain triglycerides.

fat). The other halves of the same groups were housed on a second rack on the opposite side of the room, and the racks were rotated frequently. All rats of one strain were kept in the same room.

The groups fed coconut oil were placed in the experiment one year after the rest. The coconut oil diets initially contained a supplement of 0.5% of linoleic acid, but early mortality among these animals suggested that this was too low. When the rats were 9 months of age, the linoleic acid supplement was raised to 1.5% and the high death rate subsided.

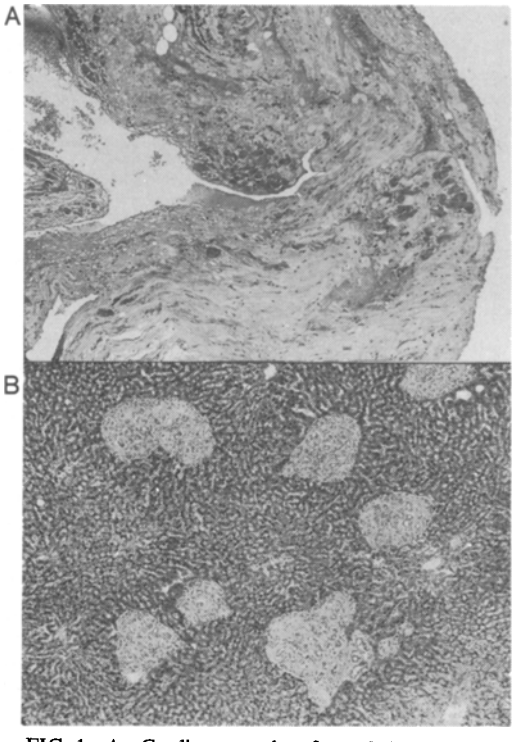

FIG. 1. A: Cardiac muscle of rat fed cottonseed oil. Muscle fibers are largely replaced by fibrous tissue (H & E; x 90). B: Proliferation of bile ducts in liver of rat fed corn oil (H & E; x 70).

The animals were inspected daily and any dead or moribund were autopsied as soon as possible. At least 10 tissues were fixed in Bouin's solution for histology, together with any tissues suspected of being abnormal. Sections of all tissues were stained with hematoxylin and eosin, and sections of hearts were also stained with Carr's chloranilic acid stain for calcium with Light Green SF Yellowish as counterstain.

The data for each animal were coded and key punched into an 80 column IBM card, which was used as input to an IBM 360/91 computer at the Columbia University Computer Center.

RESULTS AND DISCUSSION

When the rats had been on their diets for three months, there was a spread of 60 g in the median weights of the Charles River groups fed fresh fats, and this difference increased to ca. 200 g at the age of 605 days. In addition to intergroup differences, there was a considerable spread in the body weights of an individual group (over 300 g at 133 days of age in the group fed fresh corn oil). At 238 days of age, there was a spread of over 100 g in the average weights of the groups fed fresh fats (Table III), and an analysis of variance showed that the intergroup differences were significant.

Because of the frequently cited inverse relationship between early overweight and even-

TABLE IV

Observed and Expected Incidences of Severe (3+ and 4+) Cardiac Fibrosis and of Marked Discoloration after Heart Sections Were Stained with Light Green SF Yellowish

Oil	Fibrosis		Resistance to green stain	
	Fresh	Oxidized	Fresh	Oxidized
Corn oil	26/14.5=1.80	25/16.0=1.57	17/7.9=2.16	22/8.9=2.47
Cottonseed oil	20/16.3=1.23	24/15.7=1.53	12/8.0=1.50	14/8.2=1.71
Soybean oil	16/15.6=1.03	22/15.1=1.46	9/8.3=1.09	15/8.3=1.80
	(Irrad.)	18/15.3=1.17[a]	(Irrad.)	4/8.3= .48[a]
Olive oil	16/15.9=1.01	18/15.7=1.15	13/9.1=1.42	16/7.9=2.02
Beef fat	16/16.9= .94	20/17.0=1.17	6/9.6= .62	10/9.9=1.01
MCT[b]	16/17.6= .91[a]		2/8.1= .25[a]	
Butter	11/15.3= .72	10/15.3= .65	3/6.9= .43	5/8.5= .59
Chicken fat	10/14.5= .69	13/14.5= .84	5/7.2= .69	8/9.1= .88
Lard	9/14.7= .61	19/16.5=1.15	3/8.4= .36	4/9.6= .41
	(Irrad.)	12/16.3= .74[a]	(Irrad.)	2/8.4= .24[a]
Chi square =	23.89	26.48	27.89	53.40
P (7 df)	$\ll .01$	$< .001$	$\ll .001$	$\ll .001$

[a]Not in chi square calculations.

[b]Saturated medium chain triglycerides.

tual life span, we selected the body weights at 238 days as indicative of early overweight. Table III shows highly significant differences among the average life spans of the groups fed fresh fats and an inverse relationship between weight and life span. The comparatively short average life span of the group fed coconut oil is due partly to the early deaths in the initial period of linoleate deficiency. The groups fed beef fat and MCT consistently had lower body weights, and only 6 fed beef fat and 10 fed MCT had died by the age of 600 days.

Feeding of oxidized fats did not result in any significant alteration in body weight or life span. There were highly significant intergroup differences with regard to weight and life span, but no apparent inverse relationship between the two.

The W/Fu rats had longer average life spans, ranging from 695 to 745 days for the four groups. The differences were not significant.

Degenerative Lesions

The heart was the most frequent site of degenerative changes. The latter included focal, unspecific myocarditis, fibrosis and, occasionally, calcium deposits. The myocarditis was characterized by invasion of focal areas of the heart by modified histiocytes and destruction of muscle fibrils. Fibrous tissue replaced some of the muscle fibers, as shown in Figure 1A. The severity of these fibrotic changes was evaluated in a blind test on a scale of 0 to 4+ for statistical treatment. It was apparent that the incidence of severe focal fibrosis was age-dependent, increasing from 26% of those dying between 400 and 500 days to 80% of those dying over 900 days of age; therefore any comparison of groups would have to take into account their different mortality patterns. We adapted the age-specific analysis used by Ross and Bras (12), in comparing tumor incidences in different dietary groups with different average life spans. We pooled the data from all groups fed fresh fats and established incidence factors for successive 100 day periods (number of cases of severe fibrosis per number of rats dying during that period); these age-specific incidence factors were then applied to an individual dietary group, to arrive at an estimate of the number of severe cases to be expected in each 100 day period if the group conformed to the average for the whole population. These "expected" incidences were added, to arrive at the expected incidence for the whole experimental period, and this could be compared with the incidence actually observed. The ratio of observed to expected incidence was a value that lent itself to intergroup comparisons.

The observed and expected incidences of severe (3+ and 4+) cardiac fibrosis are shown in Table IV. Among the groups fed fresh fats, the observed incidence ranged from 180 to 61% of that expected on the basis of the population as a whole, and the intergroup differences were highly significant (Chi square = 23.9; with 7 df, $P < .01$). The groups fed coconut oil have been omitted because of the higher early incidence of cardiac damage associated with the initial transitory linoleate deficiency. The groups fed vegetable oils, particularly corn and cottonseed oil, had more focal fibrosis than did those fed animal fats, particularly chicken fat and lard.

In order to bring out any possible effect of oxidation and strain, the age-specific incidence

TABLE V

Observed and Expected Incidences of Severe (3+ and 4+) Bile Duct Proliferation

Oil	Fresh	Aerated	Irradiated
Cottonseed oil	15/ 9.2=1.62	21/ 9.0=2.34	
Soybean oil	14/ 9.7=1.44	16/ 8.0=2.00	15/8.3=1.81
Olive oil	11/ 9.1=1.21	9/ 8.3=1.08	
Corn oil	12/10.0=1.20	17/10.0=1.70	
Butter	10/ 8.5=1.17	3/ 9.5= .32	
Beef fat	9/10.2= .89	10/ 9.9=1.01	
Chicken fat	7/ 8.0= .88	4/ 8.2= .49	
MCT[a]	8/ 9.9= .81		
Lard	5/ 9.6= .52	5/ 9.1= .55	3/8.3= .36
Coconut oil	2/ 8.9= .22	3/ 7.7= .39	
Chi square = (8 df)	18.22	54.555	
	P = .02	P ≪ .001	

[a]Saturated medium chain triglycerides, omitted from chi square calculation.

factors derived from the whole population of CR rats fed fresh fats (except coconut oil) were used to calculate the expected incidences in the groups fed oxidized and irradiated fats and in the groups of W/Fu rats. The table shows that with six of the eight fats, intake of the oxidized fat was associated with an increase in the incidence of severe fibrosis, whereas irradiated fats were associated with only a slight increase.

The observed incidence of severe fibrosis among the W/Fu rats was much lower, although they lived somewhat longer and thus had higher expected incidences. Nevertheless, in this strain of rats, feeding of soybean oil was associated with a higher incidence than was lard, and oxidation led to an increase in the incidence in both groups.

Many of the heart sections stained with hematoxylin and eosin had deep blue areas suggestive of calcification. Therefore an additional section from each heart was stained with chloranilic acid (13) and counterstained with Light Green SF Yellowish stain. We were unable to find any of the brownish microcrystals formed between chloranilic acid and calcium, probably because the tissues had been fixed in Bouin's fluid, an acid fixative. However we did observe that the centers of many muscle fibrils were not green, but appeared light brown. Duplicate sections stained only with Light Green SF Yellowish revealed that these areas were evidently resistant to this stain (appearing only light yellow). Lesions of the myocardial fibrils, possibly similar to the one noted here, have been described by others. Wartman and Hill (14) described a change in older myocardial infarcts, in which dead muscle is not replaced by scar tissue and there is no obvious loss of architecture.

Since the discoloration noted by us seemed to vary in amount with age and with dietary fat, we graded the extent of this material on a scale of 0 to 4+ in a blind test; the data were given the same kind of age-specific analysis used in evaluating fibrosis, because the incidence of extensive involvement varied from 4% among rats dying between 400 and 500 days to 32% among those dying after 800 days.

Table IV shows the observed and expected incidences of marked discoloration. The observed incidences among the rats fed fresh fats varied from 216 to 36% of that expected on the basis of the population as a whole; the differences between groups were highly significant. It is apparent from the table that cardiac fibrosis and "muscle fibril damage" vary in nearly the same order from group to group.

The age-specific incidence factors derived from the population fed fresh fats were used to calculate the expected incidences in the groups fed oxidized and irradiated fats and in the W/Fu groups. The table shows that, in all groups, feeding of the oxidized fat increased the incidence over that seen in the group fed the corresponding fresh fat; irradiation, on the other hand, did not appear to have this effect (if anything, there was less involvement). In agreement with our observations on fibrosis, the W/Fu rats showed less fibril involvement. Among the W/Fu groups, those fed fresh soybean oil also had a higher incidence; and feeding of the oxidized lard, at least, led to an increase in incidence over that in the group fed fresh lard.

Renal lesions were present, to some extent, in most of our older rats. They were characterized by atrophy or dilatation of tubules and hyaline casts ("thyroidization") and by tubular calcification. Similar changes have been described by others (15,16). The glomerular

changes observed by Bras and Ross (17) were mild in our rats. Severe renal changes were nearly always associated with hypertrophy of the parathyroids. The severity of the renal lesions was graded on a scale of 0 to 4+ in a blind test, and the results were given an age-specific analysis. There were no statistically significant intergroup differences; if anything, there were somewhat fewer cases of severe kidney damage among those fed vegetable oils. Furthermore feeding of oxidized fats did not increase the incidence of this disease. There was no correspondence between the distribution of severe cardiac damage and severe kidney thyroidization among the various dietary groups.

Many livers of the Charles River rats had a characteristic lesion of the bile ducts. This is shown in Figure 1B. There were proliferation and distension of the bile ducts and accumulation of considerable amounts of connective tissue usually without inflammatory cells. We graded the liver sections as to the extent of this bile duct proliferation and analyzed the results by means of an age-specific analysis because this disease also increased with age. The observed and expected incidences of the severe form of this lesion are shown in Table V. There were significant differences among the groups, with the groups fed unsaturated vegetable oils having more lesions. Feeding of mildly oxidized vegetable oils induced a higher incidence, which was not the case with the animal fats. Irradiation appeared to have the same effect as oxidation. In view of the fact that there did not appear to be any biliary obstruction or inflammatory cells, it seems likely that the proliferation was caused by some circulating toxic material.

DISCUSSION

The various fats under study exerted long term nutritional effects which did not appear to be related to their fatty acid composition or triglyceride structure. Even such mild oxidation as was carried out in this experiment had effects on hearts and livers. It is true that most severe degenerative changes were brought on by the unsaturated fats, including olive oil, and that these changes were increased significantly by mild oxidation. However the effects of the oxidized fats occurred without an appreciable rise in the peroxide, carbonyl and epoxide values, which makes it seem unlikely that the effects were due to changes in the fatty acids. Therefore these results support our original hypothesis that pharmacologically active materials present in the nontriglyceride fraction may be responsible for some of the long term effects of fats and that these substances may be altered by mild oxidation, with reduction or production of toxic materials.

The fact that particularly the nontriglyceride fraction of vegetable oils may contain active materials can be assumed because of the practically limitless number of such materials in plants (18), many of which are fat soluble. Furthermore environmental contaminants were detected in some of our samples. One batch of chicken fat contained traces of chlorinated pesticides, while one batch of soybean oil contained 100 ppb polychlorinated biphenyls. Fats also may contain hormones given to animals in their feed; they frequently have added antioxidants, the biological effects of which are now under discussion. All of this may explain why different long term experiments often give different results and why future work purporting to study the effects of triglycerides should be carried out with carefully purified fats. Because of the uncertainty as to what contaminants may be present in any fat, there is at present little point in condemning one fat more than another. Improved processing of all fats for human consumption seems necessary.

ACKNOWLEDGMENTS

This work was supported by a contract from the National Cancer Institute. J. Furth, Institute of Cancer Research, Columbia University, was consulted regarding histopathology. Computer analysis was done by J. Lopatin, Calculogic Corp., and by A. Varma and L. Turgeon, School of Public Health, Columbia University; analyses for trace substances in fats were performed by H. Enos, Perrine Primate Research Branch, Perrine, Fla., D. Firestone, FDA, R. Sinnhuber, Oregon State University, and J. Fioriti, General Foods Corp.

REFERENCES

1. Crampton, E.W., R.H. Common, F.A. Farmer, A.F. Wells and D. Crawford, J. Nutr. 49:333 (1953).
2. Kaunitz, H., C.A. Slanetz, R.E. Johnson, H.B. Knight, D.H. Saunders and D. Swern, Fed. Proc. 14:408 (1955).
3. Kaunitz, H., C.A. Slanetz, R.E. Johnson, H.B. Knight, D.H. Saunders and D. Swern, JAOCS 33:630 (1956).
4. Alfin-Slater, R.B., S. Auerbach and L. Aftergood, Ibid. 36:638 (1959).
5. Artman, N.R., and J.C. Alexander, Ibid. 45:643 (1968).
6. Poling, C.P., W.D. Warner, P.E. Mone and E.E. Rice, J. Nutr. 72:109 (1960).
7. Kaunitz, H., R.E. Johnson and L. Pegus, Proc. Soc. Exp. Biol. Med. 123:204 (1966).
8. Lang, K., Z. Ernaehrungswiss. 10:234 (1971).
9. Lea, C.H., and L.J. Parr, Brit. J. Nutr. 18:369 (1965).

10. Kummerow, F.A., C. Hemans and E.G. Perkins, Fed. Proc. 31:Abstr. 2674 (1972).
11. Kaunitz, H., R.E. Johnson and C.A. Slanetz, J. Nutr. 46:151 (1952).
12. Ross, M.H., and G. Bras, Ibid. 87:245 (1965).
13. Carr, L.B., O.N. Rambo and T.V. Feichtmeir, J. Histochem. Cytochem. 9:415 (1961).
14. Wartman, B., and W.T. Hill, in "Pathology of the Heart," Second edition, Edited by S.E. Gould, Charles C Thomas Publishers, Springfield, Ill., 1960, p. 497.
15. Wilens, S.L., and E.E. Sproul, Amer. J. Pathol. 14:201 (1938).
16. Saxton, J.A., and G.C. Kimball, Arch. Pathol. 32:951 (1941).
17. Bras, G., and M.H. Ross, Toxicol. Appl. Pharmacol. 6:247 (1964).
18. Leopold, A.C., and R. Ardrey, Science 176:512 (1972).

[Received January 18, 1973]