Fatty acid composition of portal fatty liver in lysine- and threonine-deficient rats

R. VIVIANI, A. M. SECHI, and G. LENAZ

Istituto di Chimica Biologica dell'Università di Bologna, Bologna, Italy

SUMMARY The fatty acid composition of total lipids, neutral fat, and phospholipids in liver of rats fed low protein rice diets deficient in and supplemented with lysine and threonine has been studied to extend the knowledge of the chemical composition of portal fatty liver.

More linoleic acid and less 20:4, 20:5, and 22:6 in percentage of fatty acids was found in total liver lipids in lysine- and threonine-deficient rats. An increase in the percentage of linoleic acid in neutral fat, and a decrease of 20:5, 22:5, and 22:6 in phospholipids, was observed in the livers of the rats fed the deficient diets, suggesting a control of lysine and threonine on polyunsaturated fatty acid metabolism.

The absolute amounts of linoleic, palmitic, and oleic acids in fatty livers were increased; slight changes were seen in amounts of palmitoleic, stearic, and arachidonic acids; eicosapentaenoic and docosahexaenoic acids were slightly decreased.

These data seem to indicate some alterations in the metabolism of fatty acids, besides those described by other authors, that accompany fat accumulation in the liver.

A FATTY LIVER of portal type, first described in children suffering from severe protein malnutrition (kwashiorkor) (1) has been reproduced in the growing rat (2–4), pig (5), and monkey (6) fed low protein diets deficient in essential amino acids. Studies of the rats have shown that the lipid increase is due mainly to triglycerides (3, 7). Also both total (8) and esterified cholesterol (3) are increased. Phospholipids do not change (7), or decrease only moderately (3). The iodine number of the lipids in fatty liver is slightly decreased (3); on the other hand, in a preliminary experiment in this laboratory, an increase of linoleic acid and a decrease of arachidonic acid within the polyunsaturated fatty acids of total liver lipids was observed (9).

In order to extend present knowledge of the chemical composition of portal fatty liver, in the present study we assayed the lipids and the fatty acid composition of total lipids, neutral fat, and phospholipids in the liver of rats fed low protein rice diets deficient in and supplemented with lysine (first limiting amino acid) and threonine (second physiologically limiting amino acid). The fatty acid composition of liver lipids of normal rats fed a completely adequate diet was also studied.

METHODS

Twenty-eight weanling male rats of the Wistar strain from our inbred colony, weighing 40 ± 2 g, were divided into two groups and fed low protein rice diets deficient in and supplemented with lysine and threonine. The composition of the diet is reported in Table 1. The diets were isonitrogenous. Supplementation with the limiting amino acids was made according to Rosenberg, Culik, and Eckert (10) to obtain better growth and a physiological level of liver lipids. The fatty acid composition of the diet was determined by means of gas-liquid chromatography after direct saponification of an aliquot of the diet (11) and preparation of methyl esters as described below. As shown in Table 2, the dietary fat contained a high level of linoleic acid and a small quantity of unsaturated fatty acids of the linolenic acid family (20:5, 22:6); arachidonic acid was absent. Food and water were given ad lib. during the 6 weeks of the experimental period. Consumed food was measured for calculation of protein efficiency (Table 3).

At the end of the experimental period, the rats of the two groups were weighed (Table 3) and killed by decapitation. The livers were immediately removed, frozen, and stored at −20°. Total liver lipids were extracted according to Hanahan et al. (12), and phospholipids were precipitated by the addition of acetone (12); the acetone-soluble fraction was designated as the neutral fat fraction. The acetone-insoluble material represented about 92% of the total phospholipids as controlled by lipid phosphorus analysis (13) in total lipids and in the acetone-soluble fraction.

Data for total lipids, neutral fat, and phospholipids, gravimetrically determined, are reported in Table 3, with the content of total and free cholesterol assayed according to Sperry and Webb (14).

In comparison with the two groups of rats cited above, a normal diet (standard diet, Table 6) was fed to rats for the same period of 6 weeks after weaning. Total lipids were extracted from the livers and phospholipid and neutral fat fractions were separated by the methods described above.

Aliquots of total lipids, neutral fat, and phospholipids were saponified in ethanolic KOH. Methyl esters of fatty acids were prepared by refluxing in dry methanolic HCl (15). The methyl esters were determined by gas-liquid chromatography on a Pye apparatus equipped with an argon ionization detector. The temperature was 175° for the polar column (acid-washed Celite coated with polyethylene glycol adipate) and 190° for the nonpolar column (Celite coated with Apiezon L). The flow rate was 50–75 ml/min. The areas of the peaks on the chromatogram were obtained by triangulation.

Peaks of fatty acids were identified by comparing the retention times relative to palmitate with those of standard compounds and with those of known compounds as cited in the literature. Identification of some peaks was made by hydrogenation and bromination of samples (15, 16), and by plotting the logarithm of retention time according to the method of James (15) or determining the carbon number as described by Woodford and Van Gent (17).

Fatty acid composition (in percentage of total fatty acids and in milligrams per gram of liver) is reported in Tables 4, 5, and 6. Statistical analyses were carried out by the "t" test; values of $p < 0.05$ were considered to be significant.

TABLE 1 COMPOSITION OF DIETS

Ingredient	Diet A (Lysine- and Threonine-Deficient)	Diet B (Lysine- and Threonine-Supplemented)
	%	%
Ground white polished rice	89	89.215
Diammonium citrate	1	—
L-Lysine HCl	—	0.425
DL-Threonine	—	0.360
Corn oil	3	3
Cod liver oil	1.5	1.5
Salt mixture*	4	4
Vitamin mixture†	1.5	1.5

* K_2HPO_4, 322 g; $CaCO_3$, 300 g; NaCl, 167 g; $MgSO_4 \cdot 7H_2O$ 102 g; $CaHPO_4 \cdot 2H_2O$, 75 g; $FeC_6H_5O_7 \cdot 6H_2O$, 27.5 g; $MnSO_4 \cdot H_2O$, 5.1 g; KI, 0.8 g; $CuSO_4 \cdot 5H_2O$, 0.3 g; $ZnCl_2$, 0.25 g; $CoCl \cdot 6H_2O$, 0.05 g.

† At the 1.5% level, the vitamin mixture supplies per 100 g diet: choline chloride, 150 mg; inositol, 75 mg; p-aminobenzoic acid, 75 mg; α-tocopherol acetate, 7.5 mg; nicotinic acid, 3 mg; riboflavin, 1.5 mg; calcium pantothenate, 3 mg; thiamine, 0.75 mg; pyridoxine hydrochloride, 0.75 mg; menadione, 0.375 mg; folic acid, 0.075 mg; biotin, 18.75 μg; vitamin B_{12} (0.1% triturate), 1500 mg.

TABLE 2 FATTY ACID COMPOSITION OF DIETARY FATS

Fatty Acid*	Diets A and B
	%†
14:0	1.27
16:0	10.08
16:1	4.42
18:0	2.47
18:1	33.70
18:2	37.92
18:3	0.76
20:1	3.65
20:3	1.26
20:5	2.97
22:6	1.49

* Chain length: number of double bonds.
† Expressed as percentage of total fatty acids.

RESULTS

As shown in Table 3, the rice diet deficient in lysine and threonine induces a fatty liver in growing rats, in addition to a lower growth rate and protein efficiency. In fatty liver of the portal type (confirmed by histological examination), the content of neutral fat and total and esterified cholesterol increases; there is no difference in

TABLE 3 EFFECT OF DIETS DEFICIENT IN AND SUPPLEMENTED WITH LYSINE AND THREONINE ON GROWTH, PROTEIN EFFICIENCY, AND LIVER LIPIDS

	Deficient	Supplemented
Total weight gain, g	74.5 ± 12.3*	144.83 ± 27.9
Protein efficiency†	1.41	2.42
Liver		
Total lipids, mg/g	91.7 ± 13.8‡	58.8 ± 4.2‡
Neutral fat, mg/g	57.4 ± 13.3§	24.7 ± 3.8§
Phospholipids, mg/g	34.3 ± 5.9	34.1 ± 2.7
Total cholesterol, mg/g	4.7 ± 0.59‡	2.4 ± 0.34‡
Free cholesterol, mg/g	1.6 ± 0.29	1.4 ± 0.31
Esterified cholesterol, mg/g	3.1	1.0
Cholesterol esters,‖ mg/g	5.4	1.7
Triglycerides,¶ mg/g	50.4	21.5
Fatty acids**		
In phospholipids, mg/g	24.35	24.21
In neutral fat, mg/g	47.66	20.14
In total lipids, mg/g	72.01	44.35

* Standard deviation.
† Protein efficiency, g gain/g protein consumed.
‡ $p < 0.001$.
§ $p < 0.01$.
‖ The value for cholesterol esters is calculated from that of esterified cholesterol, assuming 43% of fatty acids in the cholesterol esters (24).
¶ Calculated by difference.
** Calculated using the following percentages (24) of fatty acids in the different lipid fractions: phospholipids, 71; triglycerides, 90; cholesterol esters, 43.

TABLE 4 FATTY ACID SPECTRUM IN LIVER LIPIDS OF RATS FED DIETS DEFICIENT IN AND SUPPLEMENTED WITH LYSINE AND THREONINE*

Fatty Acid	Total Lipids		Neutral Fat		Phospholipids	
	Deficient (7)	Supplemented (6)	Deficient (4)	Supplemented (4)	Deficient (4)	Supplemented (4)
14:0	1.04† ± 0.45‡	0.71 ± 0.19	1.61 ± 0.70	1.27 ± 0.17	0.42 ± 0.29	0.33 ± 0.20
16:0	31.94 ± 4.25	29.95 ± 1.76	32.63 ± 2.83	32.91 ± 3.46	29.41 ± 2.30	26.07 ± 3.32
16:1	3.74 ± 1.05	4.33 ± 1.33	5.96 ± 2.24	5.89 ± 1.15	1.83 ± 0.40	2.12 ± 0.49
18:0	9.04 ± 2.62	11.97 ± 1.29	4.98 ± 1.34	5.23 ± 1.29	19.92 ± 2.45	20.12 ± 1.15
18:1	20.39 ± 4.26	18.22 ± 1.73	22.95 ± 4.54	25.44 ± 3.26	7.81 ± 1.09	9.38 ± 2.38
18:2	20.73 ± 2.65§	15.69 ± 1.84§	23.99 ± 2.57§	16.84 ± 2.57§	16.30 ± 2.16	13.94 ± 0.81
18:3	0.65 ± 0.55	Tr.	Tr.	Tr.	0.61 ± 0.41	Tr.
20:1	0.22 ± 0.17	Tr.	Tr.	Tr.		
20:3	1.79 ± 1.53	1.22 ± 0.98	0.50 ± 0.15	0.69 ± 0.90	2.70 ± 1.94	1.87 ± 0.30
20:4	4.22 ± 0.93§	5.77 ± 1.01§	1.56 ± 0.31	2.39 ± 1.59	12.32 ± 1.30	10.99 ± 0.10
20:5	0.71 ± 0.47‖	1.55 ± 0.84‖	1.15 ± 1.39	1.63 ± 0.74	Tr.	1.84 ± 0.42
22:5	1.24 ± 0.84	1.96 ± 1.00	1.33 ± 0.98	1.92 ± 1.37	0.67 ± 0.41‖	1.99 ± 0.66‖
22:6	4.79 ± 1.28‖	9.00 ± 4.18‖	3.28 ± 1.80	5.46 ± 2.71	7.81 ± 1.46§	11.32 ± 2.24§
Ratios:						
20:3/20:4	0.42	0.21				
20:3/20:5	2.52	0.79				
20:4/18:2	0.20	0.37				

* The numbers in parentheses are the numbers of determinations (livers of two animals pooled per determination).
† Percentage of total area of gas-liquid chromatographic elution diagram.
‡ Standard deviation.
§ Significant difference, $p < 0.01$.
‖ Significant difference, $p < 0.05$.

TABLE 5 FATTY ACID LEVELS IN LIVER LIPIDS OF RATS FED DIETS DEFICIENT IN AND SUPPLEMENTED WITH LYSINE AND THREONINE*

Fatty Acid	Total Lipids		Neutral Fat		Phospholipids	
	Deficient	Supplemented	Deficient	Supplemented	Deficient	Supplemented
	mg/g liver		*mg/g liver*		*mg/g liver*	
14:0	0.75	0.31	0.77	0.25	0.10	0.08
16:0	23.00	13.28	15.55	6.63	7.16	6.31
16:1	2.69	1.92	2.84	1.19	0.44	0.51
18:0	6.51	5.31	2.37	1.05	4.85	4.87
18:1	14.68	8.08	19.94	5.12	1.90	2.27
18:2	14.93	6.96	11.43	3.39	3.97	3.37
20:3	1.29	0.54	0.24	0.14	0.66	0.45
20:4	3.04	2.56	0.74	0.48	3.00	2.66
20:5	0.51	0.69	0.55	0.33	Tr.	0.44
22:5	0.89	0.87	0.63	0.39	0.16	0.48
22:6	3.45	3.99	1.56	1.10	1.90	2.74

* These represent the major fatty acids; values were derived from the percentage of the individual fatty acids (Table 4) times the total amount of the fatty acids of total lipids, neutral fat, or phospholipids per gram of liver (Table 3) divided by 100.

the liver phospholipid level between the two groups of animals.

There is significantly more linoleic acid ($p < 0.01$) and significantly less arachidonic ($p < 0.01$), eicosapentaenoic ($p < 0.05$), and docosahexaneoic ($p < 0.05$) acids (in percentage of total fatty acids) in the total liver lipids of the lysine- and threonine-deficient animals (Table 4).

The ratios of 20:3 to 20:4 and of 20:3 to 20:5 (proved to be useful parameters for describing, respectively, linoleate and linolenate metabolism [18]) are increased, and the ratio of 20:4 to 18:2 (index of the transformation of linoleate into arachidonate [11]) is decreased in total liver lipids of deficient rats (Table 4).

Comparison of the fatty acid compositions of neutral fat of livers in the two groups of animals indicates that the only fatty acid showing a significant difference is linoleic acid, which increases in deficient rats ($p < 0.01$).

In the phospholipid fraction, the percentage of linoleic acid is the same in both types of animals, but the deficient animals have significantly less 20:5, 22:5, ($p < 0.05$), and 22:6 ($p < 0.01$) than the ones with diet supplements.

Table 5 shows the levels of the different fatty acids in total lipids, neutral fat, and phospholipids, expressed in milligrams per gram of liver. In total lipids, the most notable differences are in the considerable increase of myristic, palmitic, oleic, linoleic, and eicosatrienoic acids. Slight modifications are seen in palmitoleic, stearic, and arachidonic acids; on the other hand, eicosapentaenoic and docosahexaenoic acids slightly decrease. The change in total lipid fatty acids particularly reflects the increase of neutral fat.

In the phospholipids, whose content is the same in the two groups, the differences in fatty acids, expressed in milligrams per gram of tissue, paralleled the differences expressed in percentage of total fatty acids.

The fatty acid compositions of total lipids, neutral fat, and phospholipids of rats fed the standard diet, together with the fatty acid composition of the diet, are reported in Table 6.

TABLE 6 FATTY ACID COMPOSITION OF DIETARY FAT AND OF LIVER LIPIDS* OF RATS FED THE STANDARD DIET†

Fatty Acid	Dietary Fat	Liver Lipids		
		Total Lipids	Neutral Fat	Phospho-lipids
14:0	2.80‡	0.35§	0.67§	0.21§
16:0	28.05	20.04	18.24	17.13
16:1	Tr.	1.59	3.08	1.25
18:0	4.01	18.56	13.53	20.13
18:1	10.00	9.57	16.35	7.18
18:2	49.95	24.96	31.46	23.90
18:3	5.15	1.38	Tr.	1.73
20:3	—	Tr.	Tr.	Tr.
20:4	—	19.87	13.76	21.71
20:5	—	Tr.	Tr.	Tr.
22:5	—	Tr.	Tr.	Tr.
22:6	—	2.77	1.24	3.38

* Lipids (mg/g liver): total lipids, 49.5; neutral fat, 20.3; phospholipids, 29.2.

† The composition of the standard diet per 100 g of ration is: casein, 16.6 g; alfalfa meal, dehydrated, 12.5 g; powdered skimmed milk, 8.2 g; powdered whole milk, 8.2 g; ground wheat, 33.0 g; wheat germ, 12.5 g; powdered yeast, 5.0 g; $CaCO_3$, 1.0 g; iodized salt, 1.0 g; corn oil, 2.0 g; vitamin A, 4,000 IU; vitamin D, 200 IU.

‡ Percentage of total area of gas-liquid chromatographic elution diagram.

§ Average of two determinations (pool of two livers per determination).

While it is known that the dietary fatty acids (18, 19), and probably other dietary constituents, may affect the pattern of fatty acids found in the liver lipids, a tentative comparison between the normal rats fed the standard diet and the two groups receiving the rice diets was made (Table 7).

The liver lipids of the rats fed the rice diet with lysine and threonine supplementation and of the normal rats show a fatty acid spectrum that appears to be dependent on the fatty acid composition of the two diets: the higher level of 22:6 in liver lipids of the animals with supplemented diets might be due to the presence of cod liver oil in the diet (19). The total percentage of polyunsaturated acids of the C_{20}–C_{22} series in total lipids and in phospholipids, however, is in both cases higher than in fatty livers of rats with deficient diets. The total percentage of polyenoic acids that we found in the livers of rats fed supplemented diets is also in accordance with the findings of Clement et al. on phospholipids (8), and of Getz and co-workers on total lipids of normal rats (11, 20). This observation, together with the biological data (growth, absence of steatosis), can support the view that the animals with supplemented diets represent a more normal metabolic state than those with deficient diets.

DISCUSSION

Our results regarding neutral fat and phospholipids in portal fatty liver are in agreement with those found by Yoshida and Harper (21) and Clement et al. (8). In regard to the results of Singal et al. (3), who used a pure amino acid ration, our data are in agreement with their findings except for phospholipids. This might be ascribed to dietary differences.

Experiments have been carried out on metabolic defects that cause fatty livers in rats, either in threonine imbalance (21) or in lysine (3) and in threonine (3, 22) deficiency. While the metabolic lesions in these fatty livers (which are in any case of portal type) could be different from those observed by us, we think it may be worthwhile to report these studies in this discussion.

Yoshida and Harper (21), in studies on rats fed a low protein diet (9% of casein supplemented with cystine) deficient in threonine, observed that a stimulation of fat synthesis accompanied the fat accumulation in the livers of the rats.

The amounts of C^{14} from injected acetate-1-C^{14}, incorporated not only in liver neutral fat but also into body fat, were significantly greater in deficient rats than in those fed the threonine-supplemented diet. No evidence was obtained of impaired ability to oxidize acetate or palmitate or to transfer fatty acids from the liver to the body, nor of increased transfer of fatty acids from the body to the liver.

The increase of neutral fat that we have observed in the liver of lysine- and threonine-deficient rats could be attributed to a general stimulation of neutral fat synthesis or to other metabolic lesions; the change in

TABLE 7 COMPARISON OF THE PERCENTAGE OF FATTY ACID SERIES IN LIVER LIPIDS OF RATS FED RICE DIETS DEFICIENT IN AND SUPPLEMENTED WITH LYSINE AND THREONINE, AND OF RATS FED THE STANDARD DIET*

Fatty Acids	Total Lipids			Neutral Fat			Phospholipids		
	Deficient	Supplemented	Standard	Deficient	Supplemented	Standard	Deficient	Supplemented	Standard
	% total fatty acids			*% total fatty acids*			*% total fatty acids*		
Saturated	42.02	42.63	38.95	39.22	39.41	32.44	49.75	46.52	37.47
Monoenoic	24.35	22.55	11.16	28.91	31.33	19.43	9.64	11.50	8.43
Dienoic	20.73	15.69	24.96	23.99	16.84	31.46	16.30	13.94	23.90
Trienoic	2.44	1.22	1.38	0.50	0.69	—	3.31	1.87	1.73
Tetra-, penta-, hexaenoic	10.96	18.28	22.64	7.32	11.40	15.00	20.80	26.14	25.09

* Values obtained by summation of the percentages of individual fatty acids (Tables 4 and 6).

percentage of linoleic acid in neutral fat, however, indicates that the amino acid deficiency does not influence the metabolism of all fatty acids to the same extent. But since linoleic acid is not synthesized by the rat, the percentage increase of this fatty acid in the neutral fat fraction of portal fatty liver could be attributed to (*a*) increased transfer from the diet to the liver or from the fat stores to the liver, (*b*) decreased transfer from the liver to the body, or (*c*) decreased oxidation or conversion to the polyunsaturated higher fatty acids.

Concerning the phospholipids, Yoshida and Harper (21) observed in fatty liver of threonine-deficient rats an increased incorporation of acetate-1-C^{14}, but not of palmitate-1-C^{14}. The amount of phospholipids was unchanged. On the other hand, Singal et al. (3, 22) found, in steatosis obtained with suboptimal levels of lysine or threonine in low protein and amino acid rations, a decrease in the phospholipid content and a lower biosynthesis of the same ones measured with P^{32}.

With the lysine- and threonine-deficient diet, we have observed no change in the liver phospholipid content, but lowered levels of 20:5, 22:5, and 22:6 in this fraction; this decrease could be attributed to a decreased biosynthesis. Moreover, the increase in total lipids of the fatty livers of the ratios of 20:3 to 20:4 and of 20:3 to 20:5 (suggesting a lowered biosynthesis of polyenoic acids of both the linoleate and linolenate family [18]), and the decrease of the ratio of 20:4 to 18:2 (indicating a decreased transformation of linoleate into arachidonate [11]), also provide evidence for an alteration in the metabolic processes of polyunsaturated fatty acids.

So, in addition to vitamin B_6 (23), lysine and threonine also seem able to control the metabolism of polyunsaturated fatty acids.

This work was performed with the aid of a grant from the Consiglio Nazionale delle Ricerche, Rome, Italy.
Manuscript received March 20, 1963; accepted August 21, 1963.

References

1. Davies, J. N. P. *Lancet* **254:** 317, 1948.
2. Shils, M. E., R. De Giovanni, and W. B. Steward. *J. Nutr.* **56:** 95, 1955.
3. Singal, S. A., S. J. Hazan, V. P. Sydenstricker, and J. M. Littlejohn. *J. Biol. Chem.* **200:** 867, 1953.
4. Sidransky, H., and T. Baba. *J. Nutr.* **70:** 463, 1960.
5. Barnes, R. H., E. Kwong, W. Pond, R. Lowry, and J. K. Loosli. *J. Nutr.* **69:** 269, 1959.
6. Deo, M. G., and V. Ramalingswawi. *Lab. Invest.* **9:** 319, 1960.
7. Viviani, R., A. M. Sechi, and F. Sanguinetti. *Quaderni Nutr.* **19:** 165, 1959.
8. Clement, G., J. Clement, and E. Le Breton. In *Biochemical Problems of Lipids*, edited by G. Popjak, Butterworths Scientific Publ., London, 1956, p. 385.
9. Viviani, R., and A. M. Sechi. *Boll. Soc. Ital. Biol. Sper.* **37:** 1762, 1961.
10. Rosenberg, H. R., R. Culik, and R. E. Eckert. *J. Nutr.* **69:** 217, 1959.
11. Getz, G. S., and W. Bartley. *Biochem. J.* **78:** 307, 1961.
12. Hanahan, D. J., J. C. Dittmer, and E. Warashina. *J. Biol. Chem.* **228:** 685, 1957.
13. Bartlett, G. R. *J. Biol. Chem.* **234:** 466, 1959.
14. Sperry, W. M., and M. Webb. *J. Biol. Chem.* **187:** 97, 1950.
15. James, A. T. *Methods Biochem. Analy.* **8:** 1, 1960.
16. Farquhar, J. W., W. Insull, Jr., P. Rosen, W. Stoffel, and E. H. Ahrens, Jr. *Nutr. Rev. (Suppl.)* **17:** 29, 1959.
17. Woodford, F. P., and C. M. Van Gent. *J. Lipid Res.* **1:** 188, 1960.
18. Mohrhauer, H., and R. T. Holman. *J. Lipid Res.* **4:** 151, 1963.
19. Witting, L. A., C. C. Harvey, B. Century, and M. K. Horwitt. *J. Lipid Res.* **2:** 412, 1961.
20. Getz, G. S., W. Bartley, F. Stirpe, B. M. Notton, and A. Renshaw. *Biochem. J.* **80:** 176, 1961.
21. Yoshida, A., and A. E. Harper. *J. Biol. Chem.* **235:** 2586, 1960.
22. Singal, S. A., S. J. Hazan, V. P. Sydenstricker, and J. M. Littlejohn. *J. Biol. Chem.* **200:** 875, 1953.
23. *Nutr. Rev.* **20:** 109, 1962.
24. Swell, L., M. D. Law, P. E. Schools, Jr., and C. R. Treadwell. *J. Nutr.* **74:** 148, 1961.