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AGENTS AFFECTING LIPID METABOLISM—XV.* BIOCHEMICAL STUDIES WITH THE CHOLESTEROL BIOSYNTHESIS INHIBITOR AY-9944 IN YOUNG AND MATURE RATS

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Abstract—The capacity of young and mature rats to incorporate injected 2-^{14}C-acetate was determined in liver, lung, and brain cholesterol and fatty acids. Rates of incorporation were age dependent, particularly in liver and brain.

A single oral dose of AY-9944 markedly depressed incorporation of the precursor into tissue cholesterol. Appearance of "fast-acting" sterols in brain and liver of treated animals suggests usefulness of AY-9944 in revealing rates of tissue cholesterogenesis.

The importance of age of laboratory animals in studies of cholesterol biosynthesis inhibitors is discussed.

VARIOUS tissues are capable of biosynthesizing cholesterol *in situ*, and the idea has been expressed that this capacity may be a general property of growing cells.[1] It is believed that the enzymatic mechanisms constituting the biogenetic pathway of hepatic and extrahepatic cholesterol synthesis are the same. However, the degree to which local biosynthesis contributes to a given tissue cholesterol pool is usually unknown. With a given dietary cholesterol intake, rates of tissue cholesterol biosynthesis may vary, may depend on age and may, furthermore, differ from species to species. Therefore, the effects of an inhibitor of cholesterol biosynthesis on tissue sterols may, depending on age, also differ from tissue to tissue and reflect species differences.

We have recently reported on *trans*-1,4-*bis*(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride (AY-9944)[2] an inhibitor of hepatic[3–5] and adrenal[6] cholesterogenesis. *In vitro*, the compound blocked the conversion of 7-dehydrocholesterol to cholesterol by liver homogenates;[3,4] *in vivo*, the inhibition caused depressed sterol levels in the serum of laboratory animals.[7]

In the present study we have investigated the acute effect in young and mature rats of AY-9944 on tissue sterol levels and on the incorporation of injected 2-^{14}C-acetate into liver, lung, and brain cholesterol and total fatty acids.

MATERIALS AND METHODS

Experimental design

Two groups of ten normal male hooded rats were used; the first consisted of 9-day-old and the second of 15-month-old animals. Both groups were subdivided into control and treated animals, five each. The rats were fasted for 4 hr and were then given

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by gavage a single dose of 50 μmoles (23·2 mg) AY-9944/kg. Control animals received saline. After 2 hr all rats were injected i.p. with 2-^{14}C acetate (40 μc; 2·6 μc μmole; Merck, Sharp and Dohme of Canada, Ltd.) and were killed 2 hr later by decapitation and exsanguination. The individual organs (liver, lung and brain) were removed, weighed, washed in isotonic saline, cut into small pieces, and saponified with ethanolic KOH (1·0 ml ca. 18 N KOH, 4 ml ethanol, and 5 ml water) at 75° for 1 hr. The nonsaponifiable neutral lipids were extracted repeatedly with hexane. Aliquots were taken for radioactivity and quantitative colorimetric determinations; in the rest, ^{14}C-activity in cholesterol was measured.

Colorimetric assay of sterols

In the liver and brain tissues, cholesterol and "fast-acting"* sterols were determined by the Lieberman-Burchard reagent according to Moore and Baumann.[9] In the serum and lung tissues, total sterol levels were determined by the reagent of Zlatkis *et al.*[12] as modified for the Technicon autoanalyzer (method Np-24). The amount of 7-dehydrochloresterol was calculated from the absorption in the u.v.[13–15] Since 7-dehydrocholesterol produces with the reagent of Zlatkis *et al.* about 40 per cent as much color as cholesterol, the amount of 7-dehydrocholesterol calculated from the u.v. spectrum was multiplied by 0·4 and subtracted from the "total sterol" value; the difference is considered to represent the cholesterol content.

7-Dehydrocholesterol was recrystallized three times from methanol–ethyl acetate before use, $E^{1\%}_{1\,cm}$ 281·5 mμ was 318. According to thin layer chromatography (ethyl acetate:carbon tetrachloride, 4 : 1) the purified compound was free from its usual "fast-acting" contaminant, viz. its transannular 5,8-peroxide;[3] this can also be detected by its absorption in 30 N H_2SO_4: after 15 min at room temperature its $E^{1\%}_{1\,cm}$ 503 mμ was 432·5.

Isolation and assay of ^{14}C-labeled cholesterol

AY-9944 blocks the conversion of 7-dehydrocholesterol, its normal precursor,[3,16] to cholesterol and hence causes accumulation of 7-dehydrocholesterol.[5] In order to measure only the biosynthesized cholesterol, the ^{14}C-labeled cholesterol was isolated by the addition of carrier cholesterol (150 mg), brominated[11,17,18] and the resulting 5,6-dibromocholestan-3β-ol purified; three crystallizations from methanol–ethyl acetate sufficed to produce constant radioactivity. Samples were counted in a D-47 gas-flow counter mounted with a Mylar Micromill window (Nuclear Chicago Corp.). In order to ascertain the degree of possible contamination of the dibromo-derivative with ^{14}C-labeled 7-dehydrocholesterol produced in rats treated with AY-9944, in a separate experiment, 4-^{14}C -7-dehydrocholesterol† was added to 200 mg cholesterol. The initial specific activity of the mixture was 657 dpm/mg. After bromination and three crystallizations as described above, the specific activity of the dibromo-derivative was 2·1 dpm/mg. A liquid scintillation system (Nuclear Chicago Corp., model 720) was used (counting efficiency, 76 per cent).

* The rates of reaction and the intensities of colors developed by different sterols in the Schoenheimer-Sperry-Liebermann-Burchard test[8] depend on the structure of the sterol. Thus, at room temperature, Δ^5-stenols reach maximal intensity only after 30–35 min. In contrast, Δ^7-stenols, $\Delta^{5,7}$-stenadienols, and compounds which are readily convertible into the latter produce a maximum after 1½ min and were therefore termed "fast-acting" sterols.[9–11]

† Prepared from 4-^{14}C-cholesterol according to Kulkarni *et al.*[19] and had a specific activity of 0·57 μc/μmole. We wish to thank Dr. J. F. Bagli for the gift of 4-^{14}C-7- dehydrocholesterol.

Isolation and assay of ^{14}C-labeled fatty acids.

The residual aqueous layer after the extraction of the neutral nonsaponifiable lipids from the saponified mixture was acidified and the total fatty acids extracted,[20] titrated,[21] and their radioactivity measured in a gas-flow counter.

RESULTS

Rats used in our studies were 9 days or 15 months old. In the brain of young animals the cholesterogenic activity, i.e. the capacity to incorporate 2-^{14}C-acetate into cholesterol, was measured in a period of active myelination in the developing rat brain.[22,23] We have chosen 9-day-old rats because of the report[24] that the desmosterol/cholesterol ratio in rat brain is unchanged from the fourth to ninth day but begins to rise rapidly after the tenth day of age. Accordingly, we had expected the radioactivity found in 5,6-dibromocholestan-3β-ol derived from brain cholesterol of 9-day-old rats to account for about 90 per cent of the sterologenic activity of the brain. Actually, the detected radioactivity represented one seventh of that found in the nonsaponifiable neutral lipid fraction (Table 6). If it is assumed that the nonsaponifiable material isolated from the brain contained predominantly sterols, our results suggest that, under the experimental conditions, in the brain of 9-day-old rats the rate of formation of sterolic precursors of cholesterol was considerably greater than the rate of their conversion to cholesterol.

TABLE 1. EFFECT OF AGE ON THE DISTRIBUTION OF ^{14}C-RADIOACTIVITY IN TISSUE CHOLESTEROL AND TOTAL FATTY ACIDS TWO HOURS AFTER INTRAPERITONEAL ADMINISTRATION OF 2-^{14}C-ACETATE TO NORMAL MALE HOODED RATS

Age[a]	Tissue	Total radioactivity	
		Cholesterol[b]	Total fatty acids
		(cpm/organ)	
Days, 9	Liver	1,859 ± 375[c] (100)	3,553 ± 180 (100)
	Lung	513 ± 47 (28)	9,338 ± 790 (263)
	Brain	6,404 ± 260 (344)	68,504 ± 4,000 (1928)
Months, 15	Liver	28,624[d] (100)	100,544 ± 20,000 (100)
	Lung	1,072[d] (3·7)	10,417 ± 2,400 (10)
	Brain	107[d] (0·4)	2,095 ± 115 (2)

[a] Five animals in each age group.
[b] Isolated as 5,6-dibromocholestan-3β-ol.
[c] Standard error.
[d] Pooled nonsaponifiable lipid (sterol) fractions.

The fact that we have isolated radioactive cholesterol (in the form of its dibromo-derivative) from the brain of 15-month-old animals (Table 1) indicates active cholesterol synthesis in the brain of mature rats. Kabara and Okita[25] have demonstrated active cholesterol biosynthesis in mice 9–16 weeks old.

The effect of age in the distribution of radioactivity in tissue cholesterol and total fatty acids after i.p. injection of 2-^{14}C-acetate is illustrated in Tables 1 and 2. The data obtained from our studies are in agreement with those of Pritchard[26] and of Kleine

and Schreier[23]. As expected[25,27] in mature rats the total radioactivity in brain cholesterol was 1/60 of that found in young animals (Tables 1 and 2) but only 1/639 if expressed in terms of specific activities (Table 4.)

The effects of the inhibitor AY-9944 are presented in Table 3 (sterol levels), Table 4 (^{14}C-labeling of cholesterol), Table 5 (^{14}C-labeling of total fatty acids), and Table 6 (summary) respectively.

TABLE 2. RATIOS OF ^{14}C-RADIOACTIVITY FOUND IN TISSUE CHOLESTEROL AND TOTAL FATTY ACIDS OF YOUNG VS. MATURE RATS TWO HOURS AFTER INTRAPERITONEAL ADMINISTRATION OF 2-^{14}C-ACETATE

Tissue	Young (9 days)[a] / Old (15 months)	
	Cholesterol[b]	Total fatty acids
Liver	0·065	0·035
Lung	0·5	0·9
Brain	60	33

[a] Ratios of total ^{14}C-radioactivities, cpm/organ.
[b] Isolated, purified, and counted as 5,6-dibromocholestan-3β-ol.

TABLE 3. EFFECT OF A SINGLE ORAL DOSE OF 50 μMOLES AY-9944 PER KG ON STEROL LEVELS IN RATS 9 DAYS OR 15 MONTHS OLD
(Expressed in mg/100 g tissue or mg/100 ml serum).

Age		Liver		Serum		Lung	Brain	
		Δ^{5a}	"Fast-acting"[b]	Δ^{5}	$\Delta^{5,7c}$	Δ^{5d}	Δ^{5}	"Fast acting"
Days, 9	Control[e]	269 ± 7[f]	0	172 ± 13	0	196 ± 9	447 ± 22	0
	Treated[e]	255 ± 34	3·1 ± 1·5 (1·2%)[g]	172 ± 9	0·6 ± 0·4	157 ± 7	430 ± 9	10·8 ± 4·9 (2·5%)
Months, 15	Control[e]	244 ± 14	0	84 ± 5	0	239 ± 15	1,791 ± 59	0
	Treated[e]	253 ± 18	9·1 ± 4·2 (3·5%)	80 ± 4	0·7 ± 0·4 (0·9%)	230 ± 9	1,804 ± 140	7·5 ± 4·2 (0·4%)

[a] Cholesterol.
[b] Colour development in the Lieberman–Burchard reaction after 1½ min.
[c] 7-Dehydrocholesterol.
[d] No 7-dehydrocholesterol detected.
[e] Five animals per group.
[f] Standard error.
[g] Per cent of total sterols.

"Fast-acting" sterols were detected in the liver and brain of both young and mature rats treated with AY-9944 (Table 3). In view of the site of inhibition of AY-9944[3,4] we assume that the "fast-acting" sterols consisted mainly of 7-dehydrocholesterol.[5] Our assumption is based on findings in a group of ten 2-month-old male hooded rats which were given a daily oral dose of 10 μmoles AY-9944/kg; after seven days of treatment, levels of 7-dehydrocholesterol and of "fast-acting" sterols were determined. The results indicated that under these conditions, the "fast-acting" sterols in the

TABLE 4. EFFECT OF A SINGLE ORAL DOSE OF 50 μMOLES OF AY-9944 PER KG ON THE INCORPORATION OF 2-^{14}C-ACETATE INTO TISSUE CHOLESTEROL (Δ^5) IN RATS 9 DAYS OR 15 MONTHS OLD

Age		(cpm/g)[a]	Δ^5 (cpm/mg)
Days, 9	Control[b]		
	Liver	3,690 ± 790[c]	1,371
	Lung	1,571 ± 210	802
	Brain	8,002 ± 380	1,790
	Treated[b]		
	Liver	588 ± 41	231
	Lung	86 ± 32	55
	Brain	240 ± 26	56
Months, 15	Control[b]		
	Liver	1,619[d]	663
	Lung	713	298
	Brain	50	2·8
	Treated[b]		
	Liver	113	45
	Lung	122	53
	Brain	20	1·1

[a] Counts/min in 5,6-dibromocholestan-3β-ol per gram of wet tissue.
[b] Five animals per group.
[c] Standard error.
[d] Pooled samples.

TABLE 5. EFFECT OF A SINGLE ORAL DOSE OF 50 μMOLES OF AY-9944 PER KG ON THE INCORPORATION OF 2-^{14}C-ACETATE INTO TISSUE TOTAL FATTY ACIDS IN RATS 9 DAYS OR 15 MONTHS OLD

Age		(μEq/g)[a]	(cpm/g)[a]	(cpm/μEq)
Days, 9	Control[b]			
	Liver	66 ± 5	7,009 ± 440[c]	107
	Lung	68 ± 2	28,472 ± 3,400	417
	Brain	30 ± 2	85,294 ± 6,000	2,820
	Treated[b]			
	Liver	82 ± 7	11,795 ± 1,600	144[d]
	Lung	60 ± 4	27,701 ± 4,900	450
	Brain	31 ± 2	89,741 ± 1,700	2,920
Months, 15	Control[b]			
	Liver	102 ± 6	5,797[e]	58
	Lung	51 ± 3	6,951	139
	Brain	59 ± 6	979	17
	Treated[b]			
	Liver	102 ± 8	9,877	87
	Lung	42 ± 2	7,843	185
	Brain	54 ± 12	995	20

[a] Microequivalents or counts/min per gram of wet weight of tissue.
[b] Five animals per group.
[c] Standard error.
[d] Statistically significant difference from control ($P < 0.05$).
[e] Pooled samples.

TABLE 6. EFFECT OF A SINGLE ORAL DOSE OF 50 μMOLES OF AY-9944 PER KG ON THE INCORPORATION AND DISTRIBUTION OF 2-^{14}C-ACETATE INTO TISSUE LIPIDS OF RATS 9 DAYS OR 15 MONTHS OLD

Age		Average wet wt. of organ (g)	Total radioactivity, in cpm/organ		
			Nonsaponifiable neutral lipids	Cholesterol[a]	Total fatty acids
Days, 9	Control[b]				
	Liver	0·512	2,256 ± 280	1,859 ± 375[c]	3,553 ± 180
	Lung	0·332	1,424 ± 76	513 ± 47	9,338 ± 790
	Brain	0·801	45,036 ± 2,342	6,404 ± 260	68,504 ± 4,000
	Treated[b]				
	Liver	0·460	2,508 ± 593	267 ± 10 (−86%)[d]	5,348 ± 610[e]
	Lung	0·351	1,292 ± 120	30 ± 12 (−94%)	9,656 ± 1,500
	Brain	0·835	47,036 ± 2,512	200 ± 18 (−97%)	74,979 ± 2,400
Months, 15	Control[b]				
	Liver	17·680	29,659 ± 6,075	28,624[f]	100,544
	Lung	1·504	1,362 ± 228	1,072	10,417
	Brain	2·146	380 ± 44	107	2,095
	Treated[b]				
	Liver	18·580	21,098 ± 8,490	2,100 (−93%)	196,280[e]
	Lung	1·546	1,456 ± 171	189 (−82%)	11,682
	Brain	1·954	374 ± 54	39 (−64%)	1,925

[a] Isolated, counted, and purified as 5,6-dibromocholestan-3β-ol.
[b] Five animals per group.
[c] Standard error.
[d] Per cent inhibition.
[e] Statistically significant difference from control ($P < 0.05$).
[f] Pooled samples.

TABLE 7. LEVELS OF "FAST-ACTING" STEROLS AND OF 7-DEHYDROCHOLESTEROL IN THE BRAIN AND LUNGS OF RATS TREATED WITH 10 μMOLES OF AY-9944 PER KG PER DAY FOR A PERIOD OF 7 DAYS

	"Fast-acting" sterols (mg/100 g)	7-Dehydrocholesterol (mg/100 g)
Brain	88 ± 2·6[a]	67 ± 5·3
Lung	300 ± 22·3	278 ± 15·4

[a] Standard error.

nonsaponifiable fractions of brains and lungs consisted predominantly of 7-dehydrocholesterol (Table 7).

DISCUSSION

In the first part of this study we have examined the effect of age on the incorporation of intraperitoneally injected 2-^{14}C-acetate into cholesterol and fatty acids in rat liver, lung, and brain. In accordance with earlier conclusions,[23,26] we consider that the labeled cholesterol isolated from the brain was biosynthesized *in situ* and that it

was not due to radioactive cholesterol transported into the brain from other organs[25]. ^{14}-C-Labeled cholesterol isolated from the livers was mainly formed in the liver itself and the rest most likely in the intestinal tract.[1] As far as the lung is concerned, available experimental data[1,25,26] are not sufficient to decide whether the labeled cholesterol isolated from the lungs was biosynthesized locally or whether it was transported from other cholesterogenic tissues, e.g. liver or intestinal tract.

As expected,[23,26] incorporation of 2-^{14}C-acetate into cholesterol and into total fatty acids varied with age. The dependence on age is particularly striking if the brain/liver incorporation ratios are compared. Thus, young rats contained 3·4 times more radioactivity in brain than in liver cholesterol. In contrast, mature rats incorporated 268 times more labeled acetate into the cholesterol in the liver than into cholesterol in the brain. The corresponding ratios for total fatty acids were 19 in young and 50 in mature rats.

Administration of a single oral dose of 50 μmoles (23·2 mg) of AY-9944/kg, 4 hr before death and 2 hr before i.p. injection of 2-^{14}C-acetate, caused interference with cholesterol biosynthesis. This was reflected (1) in the appearance of "fast-acting" sterols in the liver and notably the brain and appearance of 7-dehydrocholesterol in the serum; and (2) in a greatly depressed incorporation of 2-^{14}C-acetate into cholesterol of all the examined tissues, particularly into brain cholesterol of young animals (−97 per cent). Since brain cholesterol is believed to be predominantly biosynthesized locally[28,29] our finding may suggest the capacity of AY-9944 to be transported to the sites of cholesterol biosynthesis in the brain. The lower degree of inhibition of acetate incorporation (−64 per cent) into brain cholesterol of mature rats may be due to decreased permeability of the old brain membranes to the inhibitor.[30,31] In this respect it is of particular interest to note the rapid appearance of some "fast-acting" sterols in the brain of both young and mature rats treated with AY-9944. In view of the primary site of inhibition of AY-9944, it is probable that the "fast-acting" sterols produced by the inhibitory action of the agent consist mainly of 7-dehydrocholesterol. Since it is likely that 7-dehydrocholesterol does not readily penetrate into the brain, its presence may indicate the capacity of AY-9944 to enter the brain* and cause appearance of 7-dehydrocholesterol by preventing *in situ* its enzymatic conversion to cholesterol. Hence, treatment with AY-9944, i.e. an inhibitor of cholesterol biosynthesis, appears to reveal more completely the cholesterogenic activity in brain tissues† than do other methods based on determination of radioactivity in brain lipids after intraperitoneal injection of a labeled precursor,[35] usually acetate.[36] The low incorporation values found with this method in mature brain may reflect minimized penetration of the precursor(s) into the brain rather than decreased cholesterogenic activity.[25]

With the experimental conditions of the present study, AY-9944 did not affect the incorporation of labeled acetate into lung or brain fatty acids. However, in the fatty

* In this respect AY-9944 appears to differ from triparanol (MER-29), which caused an increase of 24-dehydrocholesterol (desmosterol) in the brain of young animals only, e.g. in mice.[31–33] We have made a similar observation in a pig fed simultaneously AY-9944 and triparanol: whereas the liver contained $\Delta^{5,7,24}$-cholestatrien-3β-ol, the brain contained only $\Delta^{5,7}$-cholestatrien-3β-ol (7-dehydrocholesterol), thus suggesting the inability of triparanol to pass the blood–brain barrier.[16] This conclusion is supported by the recent finding of desmosterol in the brain of adult rats after administration of 20,25-diazacholesterol, an agent with triparanol-like mechanism of inhibition of cholesterol biosynthesis.[34]

† A similar suggestion was recently made by Fumagalli and Niemiro in a study of the effects on rat brain sterols of two other inhibitors of cholesterol biosynthsis, viz. 20,25-diazacholesterol and triparanol.[34]

acids of the livers of both young and mature animals treated with AY-9944 we have detected a statistically significant increase of radioactivity.

The findings in the present investigation illustrate the importance of age of laboratory animals used in experiments designed to study the effects of an inhibitor of cholesterol biosynthesis. The effects will largely depend on two factors: (1) the rate of cholesterol biosynthesis by a given cholesterogenic tissue, and (2) the capacity of the inhibitor to penetrate to the site of cholesterogenesis in a given tissue. Both the rate of topical cholesterogenesis and, possibly, the transportability of the inhibitor are age dependent. Hence, in young, vigorously growing animals the consequences of suppressed cholesterol formation in a number of growing organs may differ considerably from those in mature, fully grown animals. It is obvious that mature laboratory animals, whose rate of cholesterol biosynthesis is highest in the liver, are preferred in preclinical studies of inhibitors of cholesterol biosynthesis, particularly in studies of the effect on serum and tissue sterol levels. On the other hand, administration of an inhibitor of cholesterol biosynthesis, such as triparanol* or AY-9944, which produces a readily determinable precursor, may be useful in determining the rate of biosynthesis in normal or abnormal, e.g. malignant, growth.

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REFERENCES

1. E. SCHWENK, G. J. ALEXANDER and C. A. FISH, *Arch. Biochem.* **58**, 37 (1955).
2. L. HUMBER, Belg. Pat. 627,610, July 15 (1963).
3. D. DVORNIK, M. KRAML, J. DUBUC, M. GIVNER and R. GAUDRY, *J. Amer. Chem. Soc.* **85**, 3309 (1963).
4. M. KRAML, J. BAGLI and D. DVORNIK, *Biochem. biophys. Res. Commun.* **15**, 455 (1964).
5. C. CHAPPEL, D. DVORNIK, P. HILL, M. KRAML and R. GAUDRY, *Circulation* **28**, 651 (1963).
6. M. L. GIVNER and D. DVORNIK, *Proc. Soc. exp. Biol. (N.Y.)* **117**, 3 (1964).
7. C. CHAPPEL, J. DUBUC, D. DVORNIK, M. GIVNER, L. HUMBER, M. KRAML, K. VOITH and R. GAUDRY, *Nature (Lond.)* **201**, 497 (1964).
8. R. SCHOENHEIMER and W. M. SPERRY, *J. biol. Chem.* **106**, 745 (1934).
9. P. R. MOORE and C. A. BAUMANN, *J. biol. Chem.* **195**, 615 (1952).
10. D. R. IDLER and C. A. BAUMANN, *J. biol. Chem.* **203**, 389 (1953).
11. R. P. COOK, *Analyst* **86**, 373 (1961).
12. A. ZLATKIS, B. ZAK and A. J. BOYLE, *J. Lab. clin. Med.* **41**, 486 (1953).
13. R. DORFMAN, *Chem. Rev.* **53**, 1 (1953).
14. M. GLOVER, J. GLOVER and R. A. MORTON, *Biochem. J.* **51**, 1 (1952).
15. E. I. MERCER and J. GLOVER, *Biochem. J.* **10**, 552 (1961).
16. D. DVORNIK, M. KRAML and J. F. BAGLI, *J. Amer. Chem. Soc.* **86**, 2739 (1964).
17. E. SCHWENK and N. T. WERTHESSEN, *Arch. Biochem.* **40**, 334 (1952).
18. L. FIESER, *J. Amer. Chem. Soc.* **75**, 5421 (1953).
19. B. D. KULKARNI, G. BLONDIN and W. R. NES, *Steroids* **1**, 21 (1963).
20. A. PERRIN and R. COMOLLI, *J. Gerontol.* **17**, 260 (1962).
21. J. DRYSDALE and J. D. BILLIMORIA, *Clin. chim. Acta* **5**, 828 (1960).
22. J. FOLCH-PI, in *Biochemistry of the Developing Nervous System*, H. WAELSCH, Ed., p. 121, Academic Press, New York, (1955).
23. T. O. KLEINE and K. SCHREIER, *Klin. Wschr.* **15**, 785 (1963) and references cited therein.
24. D. KRITCHEVSKY and W. L. HOLMES, *Biochem. biophys. Res. Commun.* **7**, 128 (1962).
25. J. J. KABARA and G. T. OKITA, *J. Neurochem.* **7**, 298 (1961).

26. E. T. PRITCHARD, *J. Neurochem.* **10**, 495 (1963) and references cited therein.
27. H. W. MOSER and M. L. KARNOVSKY, *J. biol. Chem.* **234**, 1990 (1959).
28. M. D. MORRIS and I. L. CHAIKOFF, *J. Neurochem.* **8**, 226 (1961).
29. R. CLARENBURG, I. L. CHAIKOFF and M. D. MORRIS, *J. Neurochem.* **10**, 135 (1963) and references cited therein.
30. W. SCHNELL, K. SCHREIER, I. WIEDERHOLT and W. HARTMANN, *Clin. chim. Acta* **6**, 229 (1961) and references cited therein.
31. T. G. SCOTT and V. C. BARBER, *J. Neurochem.* **11**, 423 (1964).
32. T. R. BLOHM, T. KARIYA and M. W. LAUGHLIN, *Arch. Biochem.* **85**, 250 (1959).
33. T. J. SCALLEN, R. M. CONDIE and G. J. SCHROEPFER, JR., *J. Neurochem.* **9**, 99 (1961).
34. R. FUMAGALLI and R. NIEMIRO, *Life Sci.* **3**, 555 (1964) and references cited therein.
35. H. WAELSH, W. M. SPERRY and V. A. STOYANOFF, *J. biol. Chem.* **135**, 297 (1940).
36. P. A. SRERE, I. L. CHAIKOFF, S. S. TREITMAN and L. S. BURSTEIN, *J. biol. Chem.* **182**, 629 (1950).
37. R. FUMAGALLI, E. GROSSI, P. PAOLETTI and R. PAOLETTI, *J. Neurochem.* **11**, 561 (1964).