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" CHOLESTEROL AND FATTY ACID BIOSYNTHESIS IN BRAIN "

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A b s t r a c t

~~The present investigations deal with~~ the problem of cholesterol biosynthesis in brain of suckling rats was studied.

The effect of ~~a number of~~ experimental conditions known to affect liver cholesterol and ~~also~~ brain lipid synthesis was studied.

The administration of Triton WR 1339 results in an increase of cholesterol synthesis in liver and, to a lesser degree, in brain when 1-^{14}C-acetate, but not 2-^{14}C-mevalonolactone, is used as precursor.

Gamma
γ-Irradiation increases the cholesterol synthesis in liver but shows an opposite effect on such a synthesis in brain.

After partial hepatectomy there is an increase of cholesterol synthesis in regenerating liver, using acetate as precursor, but no change occurs in brain.

Exposure to low oxygen tension considerably decreases cholesterol synthesis in liver but shows no effect on brain. → to p. 2

Experiments to elucidate the pathways of cholesterol synthesis in brain show that brain, but not liver slices are more permeable to mevalonic acid (as K salt) than to mevalonolactone . Also mevalonic acid is incorporated into cholesterol better than mevalonolactone by brain homogenates.

Mevalonic kinase is present in brain at a concentration of about 15% compared with that of liver.

An attempt has been made to affect the levels of brain cholesterol by injecting once a day for six days to suckling rats compounds known to lower serum cholesterol.

Thyroxine analogues, biphenylacetic acid derivatives, nicotinic acid, benzmalacene, MER-29 , heparinoid and hexoestrol at various dose levels, do not affect the concentration of brain cholesterol.

A I M S

The investigations carried out in the period lasting from 1st August 1960 to 31st July 1961 were dealing mainly with three problems :

1. difference between liver and brain in cholesterol and fatty acid biosynthesis under various experimental conditions ;
2. biochemical explanation for the different incorporation of 1-^{14}C-Sodium acetate and 2-^{14}C-mevalonolactone in brain cholesterol ;
3. effect of drugs on the accumulation of cholesterol in brain of new born rats.

R E S U L T S

1. Liver and brain cholesterol synthesis in various conditions

Before reporting our results it is useful to briefly summarize the general methods used in this investigation.

ANIMALS - Sprague-Dawley male rats ten day and sixty day old were used . The rats were sacrificed by decapitation and bleeding, and brains and livers were rapidly excised and placed in ice-cold,oxygenated Krebs-Ringer phosphate solution until used.

MATERIALS - The labelled precursors used were : sodium acetate 1-^{14}C (specific activity 10 mC/mM) ; DL mevalono_ lactone 2-^{14}C (1 mC/mM) , obtained from the Radiochemical Center, Amersham, U.K.

2 μCuries of labelled acetate or 0.5 μCuries of mevalonic acid were added to each incubation flask containing 300 ± 5 mg of brain or liver slices suspended in a total volume of 5 ml (Krebs-Ringer phosphate solution).

PREPARATION OF TISSUES AND INCUBATION

300 ± 3 mg of brain or liver slices of 400 μ thickness were incubated for 2 hrs in Warburg flasks in 5 ml of Krebs-Ringer phosphate solution at pH 7.4 and 37.5°C in 100% oxygen for 2 hrs.

ANALYSIS - Incubated tissues were analyzed for substrate oxidation and total cholesterol and fatty acid biosynthesis. Substrate oxidation was determined by the method of Lyon and Geyer (1) counting the radioactivity of CO_2 collected in 20% KOH solution in the central wall of the incubation flask.

Liver and brain slices were collected after incubation and extracted for 2 hrs in chloroform methanol (2: 1) at the boiling point.

The solvent was evaporated under vacuum and the residue taken up in methanolic N KOH was saponified by heating for 2 hrs .

After the evaporation of the solvent, the residue was taken up in distilled water . The unsaponifiable fraction was separated by three extractions in ethyl ether, and the fatty acids, present as soaps in the aqueous phase, were hydrolized with 5 N H_2SO_4 and separated by three extractions with low boiling petroleum ether.

The ethyl ether and the petroleum ether solutions were dehydrated with anhydrous Na_2SO_4.

CHOLESTEROL was precipitated from the unsaponifiable fraction as the digitonide and plated using a special centrifuge (2).

FATTY ACIDS recovered from the petroleum ether solution were plated according to Popjak (3).

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(1) I.Lyon,R.P.Geyer - J.Biol.Chem.,1954,208,529
(2) P.Paoletti,R.Paoletti - Atompraxis ,1957,3,222
(3) G.Popjak - Biochem.J.,1950,46, 460

An end-window G.M. counter was used for all ^{14}C determinations.

The counting time was chosen to give a maximum error of 2% . Counter dead time, self-absorption and background radioactivity were allowed for, in the usual way.

Results are reported as CPM/g of tissue for $^{14}CO_2$, and CPM/mg of cholesterol or fatty acid.

The brain and liver cholesterol synthesis has been studied in the following conditions :

a) after treatment with Triton WR 1339

b) after Y-irradiation

c) during liver regeneration following partial hepatectomy

d) after hypoxia (exposure to low pressure)

A) Effect of Triton WR 1339

A treatment with Triton induces in rats a marked hypercholesteremia (1) which is accompanied by an increase of the cholesterol biosynthesis in liver (2). The effect of this detergent on brain cholesterol and fatty acid synthesis was compared with the effect on liver.

Triton WR 1339 (p.iso-octyl-polyossi-ethylen-phenole) was administered to ten-day old rats by intra-peritoneal route, at the dose of 500 mg/kg , 8 hrs before killing , and to sixty-day old rats intravenously at the dose of 200 mg/kg .

All the animals (treated and controls) were deprived of food after injection of Triton, the adult ones beeing also fasted 18 hrs before the injection . Glucose was added at the concentration of 4 mg/ml in the brain incubation medium.

The results obtained are reported in the following tables.

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(1) M.Friedman, S.O.Byers - J.exp.Med.,1953,97,117

(2) S.Garattini,P.Paoletti,R.Paoletti - "Biochemistry of Lipids",Pergamon Press,London, 1960,184

TABLE N. 1 - Effect of Triton WR 1339 (°) on acetate 1-^{14}C "in vitro" utilization by liver and brain of growing rats

treatment	$^{14}CO_2$ CPM/g tissue ± S.E.	CHOLESTEROL mg/g tissue	CHOLESTEROL CPM/mg ± S.E.	FATTY ACIDS CPM/mg ± S.E.
a) LIVER				
(6) Controls	94,697±13,512	3.10±0.38	706±148	44 ± 7
(6) Triton	81,642±6,939	3.22±0.36	2,015±320	99 ± 9
	p > 0.05		p < 0.01	p < 0.01
b) BRAIN				
(6) Controls	172,660±7,792	6.10±0.61	4,046±305	1,306±185
(6) Triton	155,944±9,215	6.62±1.68	5,262±347	1,693±121
	p > 0.05		p = 0.01	p > 0.05

(°) Triton was injected i.p. at the dose of 500 mg./kg .
In brackets is reported the number of determinations.

TABLE N. 2 - Effect of Triton WR 1339 (°) on acetate 1-^{14}C "in vitro" utilization by liver and brain of adult rats

treatment	$^{14}CO_2$ CPM/g tissue ± S.E.	CHOLESTEROL mg/g tissue ± S.E.	CHOLESTEROL CPM/mg ± S.E.	FATTY ACIDS CPM/mg ± S.E.
a) LIVER				
(5) Controls	81,969±13,602	2.57±0.28	201±35.8	13±0.89
(5) Triton	73,377±1,091	2.63±0.37	504±81.4	28±1.84
	p > 0.05		p = 0.01	p = 0.01
b) BRAIN				
(5) Controls	66,030±8,411	10.98±0.65	58±9.5	26±2.2
(6) Triton	71,680±6,996	11.07±0.63	53±10.3	28±0.6
	p > 0.05		p > 0.05	p > 0.05

(°) Triton was injected i.v. at the dose of 200 mg/kg. Similar results were obtained when Triton was given i.p. at the dose of 500 mg/kg. In brackets is reported the number of determinations.

TABLE N. 3 - Effect of Triton WR 1339 on mevalonic acid 2-^{14}C "in vitro" utilization by liver and brain of growing rats

treatment	$^{14}CO_2$ CPM/g tissue ± S.E.	CHOLESTEROL mg/g tissue ± S.E.	CHOLESTEROL CPM/mg ± S.E.
a) LIVER			
(6) Controls	1,788±305	3.80±0.16	2,857±873
(6) Triton	1,685±266	3.83±0.26	2,429±691
	p > 0.05		p > 0.05
b) BRAIN			
(6) Controls	183±25	6.81±0.49	291±15.5
(6) Triton	248±20	6.27±0.51	367±21.6
	p > 0.05		p > 0.05

For legends see table 1 .

TABLE N. 4 - Effect of Triton WR 1339 on mevalonic acid 2-^{14}C "in vitro" utilization by liver and brain of adult rats

treatment	$^{14}CO_2$ CPM/g tissue ± S.E.	CHOLESTEROL mg/g tissue ± S.E.	CHOLESTEROL CPM/mg ± S.E.
a) LIVER			
(3) Controls	1,600±108	2.77±0.0	2,832±128
(3) Triton	1,240±188	2.74±0.03	5,640±80
b) BRAIN			
(3) Controls	60±0	10.93±0.04	16±2
(3) Triton	187±18	11.17±0.09	25±3

For legends see table 2 .

Triton WR 1339 increases cholesterol and fatty acid biosynthesis from 1-^{14}C acetate in liver of growing and adult rats 8 hrs after its administration. An increase is also present in the brain cholesterol synthesis of growing , but not of adult rats . On the contrary fatty acid synthesis remains unchanged (see tables n. 1 and n. 2).

When mevalonic acid-2-^{14}C was used as precursor, the increased cholesterol synthesis occurred only in the liver of adult rats (see tables n. 3 and n. 4).

In conclusion, Triton WR 1339 appears more active in enhancing the cholesterol and fatty acid synthesis in liver than in brain.

B) Effect of γ-irradiation

Ten-day old Long-Evans rats kept in plastic cages were submitted to total body γ-irradiation in a single dose of 3750 r , at the rate of 150 r/sec. using a ^{60}Co GammaCell 220 from Atomic Energy Canada Ltd.,Ottawa, Canada . Controls and treated rats were killed 24 hrs after irradiation.

Liver and brain slices were incubated with 1-^{14}C acetate and 2-^{14}C mevalonic acid (lactone form) in the conditions previously reported . The results obtained are summarized in table 5 and 6 .

TABLE N. 5 - Effect of γ-irradiation on acetate-1-^{14}C "in vitro" utilization by liver and brain of growing rats.

treatment	$^{14}CO_2$	CHOLESTEROL		FATTY ACIDS
	CPM/g tissue ± S.E.	mg/g tissue	CPM/mg ± S.E.	CPM/mg ± S.E.
a) LIVER				
(5) Controls	57,718±8,432	3.6±0.2	96±9	14±0.6
(6) γ-irradiation	58,444±1,551	3.2±0.2	295±25	34±2.3
	$p > 0.05$	$p > 0.05$	$p < 0.01$	$p < 0.01$
b) BRAIN				
(5) Controls	117,438±4,771	8.3±0.3	776±35	1,013±35
(6) γ-irradiation	103,164±4,491	8.6±0.2	339±12	765±12
	$p > 0.05$	$p > 0.05$	$p < 0.01$	$p < 0.01$

TABLE N. 6 - Effect of γ-irradiation on mevalonic acid-2-^{14}C "in vitro" utilization by liver and brain of growing rats.

treatment	$^{14}CO_2$	CHOLESTEROL
	CPM/g tissue	CPM/mg ± S.E.
a) LIVER		
(6) Controls	1,179±134	2,588±263
(6) γ-irradiation	1,379±209	3,549±183
	$p > 0.05$	$p < 0.01$
b) BRAIN		
(6) Controls	136±31	191±14
(6) γ-irradiation	133±32	180±8
	$p > 0.05$	$p > 0.05$

The results described are comparable with those previously obtained submitting young animals to a X-irradiation treatment (1)(2). γ-Irradiation increases cholesterol biosynthesis from acetate or mevalonic acid in liver, but depresses (acetate) or has no effect (mevalonic acid) on brain cholesterol synthesis.

These data also show a different pattern of lipid biosynthesis in liver and brain.

C) Effect of liver regeneration

In previous work it was observed that lipid biosynthesis in liver is increased during liver regeneration after partial hepatectomy (2).

Ten-day old Long Evans rats were submitted to partial hepatectomy under light ether anesthesia, according to the method described by Higgins and Anderson (3). Animals were killed 48, 72 or 144 hr after the operation and their livers and brains were sliced and incubated with 1-^{14}C acetic acid in the usual conditions.

The results obtained are summarized in table 7 .

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(1) E.Grossi,P.Paoletti,R.Paoletti - Arch.Int.Physiol. Bioch.,1959,67,651
(2) S.Garattini,P.Paoletti,R.Paoletti - "Biochemistry of lipid", Pergamon Press, London, 1960,184
(3) G.M.Higgins, D.L.Anderson - Arch.Pathol.,1931,12,186

TABLE N. 7 - Lipid biosynthesis after partial hepatectomy in growing rats.
a) from 1-^{14}C acetic acid

treatment	time after operation hr	tissue	% liver regeneration	$^{14}CO_2$ CPM/g	CHOLESTEROL mg/g	CHOLESTEROL CPM/g	FATTY ACIDS CPM/g
Controls(6)	-	liver	-	87,003 ±5,254	2.8 ±0.5	262 ±42	21 ± 1
partial hepatectomy (6)	48	liver	60	40,722 ±7,112	3.0 ±0.5	1210 ±193	37 ± 2
				p<0.01	p>0.05	p<0.01	p < 0.01
controls(6)	-	brain	-	175,571 ±18,627	7.0 ±0.9	3315 ±115	1325 ±124
partial hepatectomy (6)	48	brain	-	163,460 ±11,256	7.3 ±1.7	3135 ±290	1358 ±27
				p>0.05	p>0.05	p>0.05	p>0.05
controls(6)	-	liver	-	58,394 ±7,350	3.5 ±0.2	92 ± 7	34 ± 1
partial hepatectomy (6)	72	liver	122	39,188 ±11,543	3.8 ±0.3	236 ±30	46 ±3
				p>0.05	p>0.05	p<0.01	p<0.01
controls(6)	-	brain	-	97,754 ±5,949	9.8 ±0.4	2707 ±147	962 ±26

partial hepatectomy (6)	72	brain	-	125,867 ±5,206	9.6 ±0.5	2242 ±151	889 ±65
				p = 0.01	p>0.05	p>0.05	p>0.05
controls(3)	-	liver	-	92,714	3.1	276	29
partial hepatectomy (3)	144	liver	135	91,114	3.7	564	57
controls(3)	-	brain	-	164,462	10.2	1308	567
partial hepatectomy (3)	144	brain	-	161,025	9.3	1198	526

b) from 2-^{14}C mevalonic acid

treatment	time after operation hr	tissue	$^{14}CO_2$ CPM/g	CHOLESTEROL mg/g	CHOLESTEROL CPM/g
controls(6)	-	liver	2,298±277	2.4±0.2	7,881±695
partial hepatectomy (4)	48	liver	1,363±90	3.8±0.2	7,204±542
controls(6)	-	brain	87±21	6.3±0.7	223±15
partial hepatectomy (5)	48	brain	76±31	8.5±0.9	89±12

In brackets is reported the number of determinations.

The increase of cholesterol and fatty acid biosynthesis by 1-^{14}C acetate in regenerating liver is confirmed by these experiments and in growing rats as well. This increase is still present 144 hr after the operation, when the liver regeneration is completed . 48 and 72 hr after partial hepatectomy the increase of lipid biosynthesis is accompanied by a depression of the acetate oxidation . On the contrary no changes in lipid bio - synthesis have been observed in brain. During liver regeneration cholesterol biosynthesis was not modified in liver or brain when mevalonolactone 2-^{14}C was used as precursor . The decrease of the cholesterol biosynthesis in brain with the passing of time is related to the increase of the cholesterol contents in brain and to the fact that the animals are drawing near the age of achieved mielynization. These results show also that a stimulus increasing lipid biosynthesis in liver remains without effect on brain lipid synthesis.

D) Effect of hypoxia

The animals (suckling and mothers) were kept in a thermostat Vaccum Oven (Thomson and Mercier, Croydon U.K.), with an internal pressure of 0.5 atm. at 22 ± 0.5°C.

After 4 or 8 days of hypoxia the animals were killed and brain and liver slices (both from controls and treated) incubated with 1-^{14}C acetate in the conditions previously described.

The results are summarized in table n. 8 and n.9.

The increase of cholesterol and fatty acid biosynthesis by 1-^{14}C acetate in regenerating liver is confirmed by these experiments and in growing rats as well. This increase is still present 144 hr after the operation, when the liver regeneration is completed . 48 and 72 hr after partial hepatectomy the increase of lipid biosynthesis is accompanied by a depression of the acetate oxidation . On the contrary no changes in lipid bio - synthesis have been observed in brain. During liver regeneration cholesterol biosynthesis was not modified in liver or brain when mevalonolactone 2-^{14}C was used as precursor . The decrease of the cholesterol biosynthesis in brain with the passing of time is related to the increase of the cholesterol contents in brain and to the fact that the animals are drawing near the age of achieved mielynization. These results show also that a stimulus increasing lipid biosynthesis in liver remains without effect on brain lipid synthesis.

D) Effect of hypoxia

The animals (suckling and mothers) were kept in a thermostat Vaccum Oven (Thomson and Mercier, Croydon U.K.), with an internal pressure of 0.5 atm. at 22 ± 0.5°C.

After 4 or 8 days of hypoxia the animals were killed and brain and liver slices (both from controls and treated) incubated with 1-^{14}C acetate in the conditions previously described.

The results are summarized in table n. 8 and n.9.

TABLE N. 8 - Effect of 4-day hypoxia on acetate-1-^{14}C "in vitro" utilization by liver and brain of suckling rats and their mothers.

treatment	CO_2 CPM/g tissue ± S.E.	CHOLESTEROL CPM/mg ± S.E.	CHOLESTEROL mg/g tissue ± S.E.	FATTY ACIDS CPM/mg ± S.E.
a) LIVER				
6 controls (12 day old)	78,431±12,225	1576 ± 624	2.94±0.5	143 ± 33
6 Hypoxia (12 day old)	78,838 ± 13,131	160 ± 35	3.71 ± 0.3	23 ± 3
	p > 0.05	p < 0.01	p > 0.05	p < 0.01
1 control mother	204,755	3557	5.64	756
1 hypoxia	210,689	2231	3.54	300
b) BRAIN				
6 controls (12 day old)	139,506±32.711	3929 ± 246	4.86 ± 1.05	1426 ± 278
6 Hypoxia (12 day old)	116.276±27.696	2836 ± 343	5.95 ± 0.71	1138 ± 175
	p > 0.05	0.05, p, 0.02	p > 0.05	p = 0.4
1 control mother	198.980	19	15.72	55
1 hypoxia	156.503	31	14.00	34

TABLE N. 9 - Effect of 8 day hypoxia on acetate-1-^{14}C "in vitro" utilization by liver and brain of suckling rats and their mothers.

treatment	CO_2	CHOLESTEROL		FATTY ACIDS
	CPM/g tissue ± S.E.	CPM/mg ± S.E.	mg/g tissue ± S.E.	CPM/mg ± S.E.
a) LIVER				
6 Controls (12 day old)	77,462±4,414	4,319±534	3.26±0.17	143±1.83
6 Hypoxia (12 day old)	65,282±2,094	151±49.8	5.57±0.18	21±4.03
	$p > 0.05$	$p < 0.01$	$p < 0.01$	$p < 0.01$
2 Controls (mothers)	136,413	5,678	3.14	220
2 Hypoxia (")	104,685	2,139	3.22	63
b) BRAIN				
6 Controls (12 day old)	127,019±6,516	2,491±167,6	7.84±0.15	753±93.5
6 Hypoxia (12 day old)	111,318±4,976	2,019±231,3	9.13±0.99	684±97.2
	$0.05 > p > 0.02$	$p > 0.05$	$p > 0.05$	$p > 0.05$
2 Controls (mothers)	80,703	21	15.47	26
2 Hypoxia (")	75,045	20	17.19	24

Keeping the suckling rats for periods of 4 or 8 days at a low pressure and consequently at a low oxygen tension, causes a considerable decrease of cholesterol and fatty acid biosynthesis from 1-^{14}C acetate in liver . The total cholesterol content is, on the contrary, significantly increased after 8 days. No marked reduction could be seen in the $C^{14}O_2$ resulting from the oxidation of 1-^{14}C acetate.

In the liver of the mothers the same effect is evident except for the amount of total cholesterol that is not increased.

In brain of both young rats and mothers the decrease of lipid biosynthesis is not statistically significant . A small increase of cholesterol content seems to be present.

Table 10 summarizes the results obtained and shows the differences between brain and liver lipid synthesis in growing rats under various experimental conditions.

TABLE N. 10 -

Experimental condition	CHOLESTEROL BIOSYNTHESIS IN			
	LIVER		BRAIN	
	from acetate	from mevalono lactone	from acetate	from mevalono lactone
Triton WR 1339	increase	no effect	increase	no effect
γ-Irradiation	increase	increase	decrease	no effect
Liver regeneration	increase	no effect	no effect	decrease
Hypoxia	decrease	-	no effect	-

It is clear that the biosynthesis of cholesterol in brain is not influenced in the same way by the factors that enhance or inhibit the synthesis in liver.

2. Incorporation of acetic acid and mevalonolactone in brain cholesterol

Previous experiments have shown that 2-^{14}C mevalonolactone is but poorly incorporated into cholesterol in the brain of growing rats , while it is incorporated at a high degree into liver cholesterol of the same animals. On the other hand, 1-^{14}C acetate is better incorporated into cholesterol of brain than of liver.

These data may be explained at least on the basis of the following hypothesis :

a) mevalonic-kinase is present in brain only in small concentrations ;

b) the brain structures where cholesterol synthesis occurs, are not permeable to mevalonolactone ;

c) cholesterol synthesis in brain occurs through two pathways, one of these does not involve the synthesis of mevalonolactone ;

d) mevalonolactone is utilized in brain for others metabolic processes than those of cholesterol synthesis.

Points a) and b) only have been considered up to now.

a) Mevalonic-kinase

The enzyme has been assayed by various methods reported in the Quarterly Technical Status Reports.

The more satisfactory results have been obtained when extracting the enzyme according to Levy and Popjack (1) and measuring the activity according to Markley and Smallman (2).

Liver from 150 g. Long Evans rats and brain from 10-day old Long Evans rats were homogenized and extracted with a water solution containing sucrose , $KHCO_3$, EDTA and KCl . The filtrate was precipitated with protamine sulfate and then with $(NH_4)_2SO_4$ at the concentration of 85% . The precipitate was dissolved with buffer phosphate, reprecipitated with 30% $(NH_4)_2SO_4$ and redissolved with buffer phosphate.

The enzymatic activity was measured by incubation with 2-^{14}C mevalonic acid . Each 0.5 ml sample contains : 0.3 ml of the enzymatic preparation ; 1 µmole ATP ; 5 µmoles $MgCl_2$; 10 µmoles cysteine ; 25 µmoles K phosphate buffer pH 7.3 ; 0,1 ml 2-^{14}C mevalonic acid (K salt)corresponding to 65.000 CPM . Incubation was carried out for 1 hour at 36°C.

After stopping the reaction, 0.05 ml of the centrifuged medium was chromatographed on paper using n.butanol, acetic acid and water (77, 10, 13). The radioactivity of the spots corresponding to mevalonic acid and phosphomevalonic acid was measured.

The concentration of the mevalonic-kinase present in the brain of growing rats is about 1/6 of the concentration of mevalonic-kinase present in liver of adult rats.

(1) H.R.Levy, G.Popjack - J.Biol.Chem., 1960, 75, 417
(2) R.Markley, E.Smallman - Bioch.Biophys.Acta, 1961, 47, 327

Before drawing any conclusion, further investigations are necessary to establish if the activity of mevalonic-kinase in brain is adequate to account for the incorporation of mevalonic acid into cholesterol.

b) Permeability

Among the various experiments performed here is reported a comparison between the incorporation of 1-^{14}C acetate, 2-^{14}C acetate , 2-^{14}C mevalono_ lactone (MVA-L) and 2-^{14}C-K-mevalonate (MVA-K) in slices or homogenates of brains from suckling rats.

The reason for utilizing mevalonolactone and K-mevalonate was in connection with the possibility that the slices and particles of the homogenates to be more permeable to the acid than to the lacton form . The technical details of the experiments are the following:

Brain slices - One gram of liver or brain slices was incubated in a glucose-Krebs Ringer phosphate solution with 1 μC (= 1 μM) of radioactive substrate (2.220.000 DPM) for 2 hours in the same conditions as those previously reported . The substrates used were 1-^{14}C acetate, 2-^{14}C acetate, 2-^{14}C mevalono_ lactone (MVA-L) or 2-^{14}C K-mevalonate (MVA-K), the latter prepared according to Levy and Popjack (1), preincubating mevalonolactone with a small excess of KOH for 30 minutes,at 37°C .

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(1) H.R.Levy, G.Popjack - Biochem.J.,1960,75,417

After the incubation the medium was removed by centrifugation and the slices were washed in saline.

The saponification of the slices was carried out in N-KOH (in ethanol 95%) at boiling point . After cooling, unsaponifiable material was extracted according to Knauss et al. (1) with three-10 ml portions of petroleum ether . In the combined extracts cholesterol was purified as digitonide and the unsaponifiable compounds not precipitating with digitonine were considered as squalene and derivatives (1). Dibromide cholesterol was prepared according to Schwenk and Werthessen (2).

Measurements of radioactivity were carried out on aliquots of the incubating medium and on each of the fractions obtained (unsaponifiable, digitonine precipitable material, squalene, cholesterol-dibromide).

Brain homogenates - The homogenates were prepared according to Rabinowtz (3) . Animals were sacrificed in the cold room. Liver and brain were rapidly excised and washed in cold buffer . Tissues were homogenized in 1.5 volumes of the medium in a loose Potter Elvelijem homogenizer of 0.5 mm. Five strokes were used for the homogenization. The preparation was centrifuged at 1000 X g for 6 minutes.

0.1 M phosphate buffer, pH 7.0 , enriched with 0.006 M $MgCl_2$ and 0.03 M nicotinamide was used as medium.

The incubation system contained also ATP 5 µmoles , Glucose-1-phosphate 22.5 µmoles, glutathione 30 µmoles, Co-A-SH 0.2 µmoles, DPN 1,3 µmoles, TPN 1.4 µmoles and homogenate 4 ml (protein content 31.5 mg/ml) , radioactive substrate in 0.4 ml . Final volume 5.5 ml .

The used substrates were : 2-^{14}C acetate (1 µC =

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(1) H.J.Knauss, J.W.Porter,G.Wasson - J.Biol.Chem.,1959, 234,2835

(2) E.Schwenk, N.T.Werthessen - Arch.Biochem.Biophys.,1952, 40,334

(3) J.Rabinowitz - J.Biol.Chem.,1957,229,825

1 µmole), 2-^{14}C mevalonolactone (2 µC = 1 µmole) and 2-^{14}C K-mevalonate prepared as previously described (1 µC = 1 µmole).

The incubation was carried out for 2 hrs . The separation of the fractions was the same as that prescribed for brain slices.

Table 11 summarizes the results obtained with brain and liver slices.

TABLE N. 11 - Utilization of mevalono-lactone, K-mevalonate and acetate from brain and liver slices of suckling rats.

Group	% of the radioactivity added			Unsaponifiable	
	$^{14}CO_2$	Medium	Slices after digestion with KOH (a)	% of the total (b)	% of (a)
BRAIN					
MVA-L 1	(5) 0.32±0.16	(9)78.0±4.52	(9) 6.96±0.64	(9) 3.34±0.45	(9) 50.5±7.74
MVA-K 2	(5) 0.23±0.04	(9)70.6±4.60	(9)14.07±0.98	(9) 7.61±0.40	(9) 56.2±4.94
MVA-L + KCl 3	(3) 0.16±0.03	(3)74.6±3.51	(3) 5.74±1.63	(3) 3.41±0.24	(3) 65.9±11.5
Acetate 1-^{14}C 4	(3)27.87±3.61	(3)21.46±4.20	(3)13.97±0.81	(3) 3.61±0.40	(3) 25.6±1.56
Acetate 2-^{14}C 5	(4)15.84±2.83	(4)39.73±0.84	(4) 9.40±0.92	(4) 4.63±0.59	(4) 50.3±6.69
LIVER					
MVA-L 6	(3) 1.85±0.60	(6)64.7±4.70	(6)18.0±5.91	(6) 11.79±1.4	(6) 67.2±6.28
MVA-K 7	(3) 0.79±0.046	(6)60.16±5.18	(6)12.5±1.27	(6) 8.97±1.33	(6) 70.9±5.02
Acetate 2-^{14}C 8	(4) 7.81±0.83	(4)62.6±2.25	(4) 4.77±1.40	(4) 1.41±0.29	(4) 29.6±9.59
Significance for groups (p)					
1 - 2	> 0.05	= 0.05	< 0.01	< 0.01	> 0.05
6 - 7		> 0.05	> 0.05	> 0.05	> 0.05

In brackets is reported the number of determinations.

	Group	Squalene and others			Digitonine precipitable material		
		% of the total	% of (a)	% of (b)	% of the total	% of (a)	% of (b)
BRAIN							
MVA-L	1	(5) 1.18±0.16	(5)19.7±3.34	(5)38.0±5.8	(9)1.10±0.07	(9)15.7±2.0	(9)35.0±4.34
MVA-K	2	(5) 1.68±0.32	(5)14.2±4.04	(5)21.6±4.01	(9)3.44±0.28	(9)24.6±1.53	(9)45.7±3.10
MVA-L + KCl	3	(3) 0.86±0.12	(3)18.0±5.66	(3)25.7±4.90	(3)1.25±0.07	(3)25.5±6.62	(3)37.2±4.34
Acetate 1-^{14}C	4	(3) 0.45±0.08	(3) 3.20±0.51	(3)12.5±1.91	(3)2.97±0.42	(3)21.1±1.82	(3)82.2±2.38
Acetate 2-^{14}C	5	(4) 0.61±0.24	(4) 6.40±2.49	(4)11.8±3.65	(4)3.11±0.47	(4)33.9±5.20	(3)67.1±3.24
LIVER							
MVA-L	6	(3) 1.68±0.20	(3) 9.90±0.79	(3)17.7±1.56	(6)6.16±0.65	(6)37.3±5.00	(6)54.2±5.68
MVA-K	7	(3) 1.25±0.21	(3)12.6±1.21	(3)18.6±1.00	(6)4.76±1.18	(6)35.6±6.51	(6)50.0±8.55
Acetate 2-^{14}C	8	(4) 0.25±0.07	(4) 5.2±2.73	(4)17.2±2.95	(4)1.04±0.23	(4)19.1±6.16	(4)74.2±4.94
Significance for groups (p)							
1 - 2		> 0.05	> 0.05	=0.05	< 0.01	< 0.01	= 0.05
6 - 7					> 0.05	> 0.05	> 0.05

	Group	Dibromide			
		% of total	% of (a)	% of (b)	% of digitonide
BRAIN					
MVA-L	1	(3)0.38±0.01	(3) 8.1±3.26	(3)1.15±5.05	(3) 40.8±13.4
MVA-K	2	(6)0.37±0.10	(6)2.95±0.67	(6)4.51±1.20	(6) 24.9±4.00
MVA-L + KCl	3	(3)0.37±0.06	(3)7.0±1.36	(3)10.8±1.24	(3) 30.4±6.24
Acetate 1-^{14}C	4	(1) 0.89	(1) 6.4	(1) 25.4	(1) 32.1
Acetate 2-^{14}C	5	(4)0.95±0.17	(4)10.1±1.43	(4)20.4±2.06	(4) 30.7±3.12
LIVER					
MVA-L	6	(5)1.11±0.01	(5)6.7 ±2.05	(5)11.4±3.82	(5) 23.4±6.96
MVA-K	7	(5)0.66±0.15	(5)6.2±2.26	(5) 9.2±3.69	(5) 27.6±12.38
Acetate 2-^{14}C	8	(4)0.32±0.06	(4)6.9±2.36	(4)23.4±2.17	(4)35.7±2.35
Significance for groups (p)					
6 - 7		> 0.05	> 0.05	> 0.05	> 0.05

2-^{14}C mevalonate (K-salt) enters in brain slices twice more than the lactone form, and therefore the unsaponifiable fraction (including squalene and digitonide), contains more radioactivity.

Referring the percentages of incorporation into squalene and digitonide to the total radioactivity of the slices no difference could be seen for squalene, while digitonide is once more significantly higher with MVA-K in respect to MVA-L.

The addition of KCl , in order to obtain the same concentration of K^+ as in the experiments with MVA-K , does not influence the penetration of the lactone form or its utilization in the slices.

Mevalonolactone and K-mevalonate, on the contrary, enter in liver slices at the same concentrations and no difference could be seen in the incorporation.

Table 12 summarizes the results obtained with brain homogenates.

TABLE N. 12 - Utilization of 2-^{14}C acetate, 2-^{14}C mevalonolactone and 2-^{14}C-K Mevalonate from brain homogenates of suckling rats (values are expressed as % of the added radioactivity)

Precursor	Concentration	$^{14}CO_2$	Unsaponifiable	Squalene and others	Digitonine precipitable material	Dibromide
2-^{14}C acetate	0.125 µC	23.60	12.52	6.86	2.63	0.69
	0.25 "	22.53	14.60	9.41	1.53	0.49
	0.5 "	19.89	10.66	6.48	1.42	0.29
	1.0 "	11.39	5.42	-	0.73	-
	2.0 "	4.82	6.40	-	0.54	-
	4.0 "	5.69	1.62	-	0.19	-
2-^{14}C MVA L	0.125 µC	1.13	6.88	4.89	-	0.38
	0.25 "	0.87	7.89	3.15	0.76	0.32
	0.5 "	0.70	7.90	3.68	0.70	0.11
	1.0 "	1.03	7.39	-	0.70	-
	2.0 "	0.56	7.59	-	0.57	-
	4.0 "	0.59	7.33	-	0.51	-
2-^{14}C MVA-K	0.125 µC	3.01	36.52	20.69	3.63	0.93
	0.25 "	2.94	-	9.04	-	0.57
	0.5 "	2.87	33.53	7.46	4.62	0.68
	1.0 "	2.38	42.24	-	6.04	-
	2.0 "	2.15	33.13	-	3.63	-
	4.0 "	2.34	28.51	16.54	5.23	0.61

The results with the homogenates largely confirm the data obtained with brain slices . Furthermore the radioactivity of the digitonine precipitable material is several times higher when using K-mevalonate rather than acetate.

It is possible that one of the major reasons for explaining the poor utilization of mevalono_ lactone from brain of suckling rats be a problem of permeability .

3. Effect of drugs on brain cholesterol

A number of drugs known as cholesterol lowering agents was used with the purpose to affect brain cholesterol .

Seven-day old Long Evans suckling rats were injected daily i.p. with the compounds under investigation for 6 successive days . 24 hrs after the last treatment animals were killed . Serum cholesterol (on pooled sera) was estimated according to Bloor (1), brain and liver cholesterol according to Grigaut (2).

The compounds and the doses tested are reported in table 13 . For a survey on the drugs used see a recent article review (3).

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(1) W.R.Bloor - J.Biol.Chem.,1916,24,227
(2) A.Grigaut - C.R.Soc.Biol.,1910,68,791
(3) S.Garattini,R.Paoletti,L.Bizzi,E.Grossi,R.Vertua - "Drugs affecting lipid metabolism". Elsevier Publ. Co., Amsterdam,1961,p.144

TABLE 13 - Effect of various cholesterol lowering agents on serum, liver and brain cholesterol of suckling rats.

N. rats	Treatment mg/kg i.p. x 6 days	Weight in g. at the beginning	Weight in g. at the end	Cholesterol (± S.E.) Serum (mg/100 ml)	Liver (mg/g)	Brain (mg/g)
5	controls	17.4	27.7	242±26	3.81±0.23	10.3±0.4
6	L-Thyroxine 1	17.2	24.7	190±7	3.86±0.16	9.7±0.2
5	α-Biphenylbutyric acid 100	17.6	29.5	258±22	3.21±0.14	10.1±0.6
4	Difencsenic acid 100	17.4	27.3(°)	199±13	3.31±0.20	10.0±0.2
6	Nicotinic acid 300	16.5	28.0	306±31	4.37±0.41	10.4±0.3
6	Controls	14.5	24.3	210±18	3.26±0.08	9.5±0.2
5	Heparinoid 100	18.5	22.8	205±6	3.43±0.13	9.1±0.3
5	Triiodothyropropionic acid 1	16.4	23.8	217±14	3.57±0.12	9.9±0.3
6	D-Thyroxine 5	17.8	23.8	146±10	3.51±0.21	10.2±0.2
6	Controls	17.0	30.0	261±29	3.49±0.06	8.33±0.2
6	D-Thyroxine 5	17.0	26.6	179±9	3.37±0.21	10.03±0.2
6	D-Thyroxine 10	16.0	23.6	186±22	3.68±0.39	9.95±0.2
6	Triiodothyropropionic acid 5	16.0	25.6	191±13	3.94±0.60	10.13±0.4
6	Benzmalacene 50	17.0	23.8	214±23	3.19±0.35	9.13±0.4

6	Controls		17.0	30.0	273±18	3.72±0.37	8.28±0.2
6	MER-29	50	16.0	23.8	251± 8	3.39±0.15	7.78±0.2
6	Hexoestrol	0.7	16.0	27.7	268±13	3.51±0.09	9.41±0.4

(°) The dose used was toxic .

None of the drugs used was able to decrease the levels of brain or liver cholesterol in suckling rats, although some of the drugs showed a definite effect in lowering serum cholesterol.

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FUTURE OBJECTIVES

In relation with the above mentioned results it seems worth interesting in the future the following points :

1. Studies on other experimental conditions able to affect the cholesterol synthesis in brain of newborn rats . Hormonal treatments (thyroxine , antithyroxine, growth hormone, oestrogens, testosterone) may be of particular interest.
2. Studies on the incorporation of 2-^{14}C-mevalonolactone in brain cholesterol . Differences between incorporation of 2-^{14}C-mevalonolactone and 2-^{14}C-K mevalonate considering various concentrations and various times of incubation . Studies on the possibility that the low level of mevalonic-kinase in brain may be a limiting factor in the cholesterol synthesis .
3. Effect of various psychotropic drugs (sedatives or stimulants), administered to the mother or

to the newborn rats, on the concentration of brain cholesterol . Possibility to delay or accelerate the mielynization by means of drugs interfering with the lipid metabolism.

S.GARATTINI
L.BIZZI
K.GOPAL
E.GROSSI
P.PAOLETTI
M.POGGI

Personel utilized during the reporting period (1st August, 1960 - 31st July,1961) :

prof.S.Garattini (principal investigator) ; dr.L.Bizzi ; dr. K.Gopal ; dr. E. Grossi ; dr. P. Paoletti ; dr.M.Poggi.

Summary of the expenses

S a l a r i e s

Name	Average hours/week	Salary		Salary	
		Lit/hr	$ /hr	Lit/year	$ /year
L.Bizzi	45	500	0.80	1.080.000	1,739.13
K.Gopal	25	300	0.49	360.000	579.71
E.Grossi	45	420	0.68	900.000	1,449.27
P.Paoletti	25	1200	1.93	1.440.000	2,318.84
M.Poggi	45	390	0.63	840.000	1,352.65

(1 U.S. dollar = 621 Lit.)

	1st Quart.	2nd Quart.	3rd Quart.	Final	Total
Salaries paid in dollars	1,481.14	1,862.90	1,858.29	1,858.29	7,060.62
Laboratory supply and glassware	120.22	438.06	521.37	805.40	1,885.05
Chemicals and radioisotopes	694.26	853.79	841.90	1,462.30	3,852.25
Animals and diets	-	97.46	177.13	312.50	587.09
Miscellaneous (including reports)	-	35.07	38.64	130.20	203.91
Total	2,295.62	3,287.28	3,437.33	4,568.69	13,588.92

No important property was acquired during the contract period at direct contract expense.

Silvio Garattini

S. Garattini