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Metabolic studies on scorbutic guinea pigs

IV. Fatty acid metabolism in vitro

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With 2 figures and 5 tables

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Fatty acid metabolism in scurvy

The premise that scurvy affects fat metabolism originated with the work of QUASTEL and WHEATLEY (1). Reports in literature have revealed that both fatty acids (2–5) and cholesterol (6–9) metabolisms are disturbed in scurvy. Both anabolism (6, 10, 11) and catabolism (1–3) of fatty acids were reported to be altered in this disease. The oxidation of fatty acids by scorbutic guinea pig liver slices was found to yield lower quantity of acetoacetate compared to the normal animal (1). Addition of ascorbic acid increased the rate of acetoacetate production from which these authors concluded that this vitamin might have some role on the respiration of and on the power to oxidise fatty acids. ABRAMSON (2) has also observed a decreased oxidation of unsaturated fatty acids by different tissues from scorbutic guinea pig and ascorbic acid could correct this subnormal oxidation. DONNAN (3) reported that ascorbic acid plays as an excellant catalyst in the oxidation of unsaturated fatty acids. An increased lipid oxidation by liver slices with ascorbic acid was also shown by OTTOLENGHI et al. (4). The observation by DEBOUS et al. (5) have also indicated that scorbutic guinea pig liver had a lesser capacity for the production of ketone bodies in comparison with the control animal.

The recent report by GUCHHAIT, GUHA and GANGULI (9) has shown that cholesterol-4-^{14}C catabolism to bile acids is depressed in scorbutic guinea pig both *in vivo* and *in vitro* and ascorbic acid could stimulate the catabolism of cholesterol *in vitro*.

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Due to an alteration in carbohydrate metabolism in scurvy (12–15), one might as well expect its reflection on fat metabolism as these metabolic processes are very much interrelated, acetate being the pivot for such interconversions. In addition, GUCHHAIT and GANGULI (8) have put forward evidence which showed that acetate metabolism is also impaired in scurvy. Accumulation of body fat in scorbutic guinea pigs (11) cannot therefore be attributed to an enhanced synthesis of fatty acids from acetate, but more probably due to a defect in fatty acid catabolism. The purpose of this investigation was to examine these contentions.

This paper presents data on fatty acid synthesis and oxidation using ^{14}C-labeled substrates by tissues from scorbutic guinea pigs. The results delineate that ascorbic acid deficiency does cause a depressed fatty acid metabolism in guinea pigs.

Experimental Procedure

Treatment of animals

Scurvy was developed in male guinea pigs in the following manner: Animals weighing from 250 to 300 g were maintained on a diet consisting of green grass, soaked gram, and a scorbutogenic diet (16) for 1 weak. Those which grew well were selected and separated into groups, each group consisting of one pair-fed and one scorbutic guinea pig. The pair-feeding procedure was then followed as described earlier (14) till the animals in the scorbutic group developed acute scurvy. The pair-fed control in each group was also fed 5 mg ascorbic acid daily. All the animals were supplied with 2 drops of a concentrate of vitamins A and D twice a week. Acute symptoms of scurvy developed in the scorbutic group usually during the 4th week was, therefore, the period when the animals were killed.

Methods

Tissue Preparation – (a) *Mitochondria.* The guinea pigs were killed by a blow on the head. The livers were immediately excised, weighed, and immersed in 0.25 M sucrose solution at 0°. Mitochondria were then prepared essentially following the method of HOGEBOOM (17) as described previously by GUCHHAIT and GANGULI (18). The final suspension used in the experiments contained mitochondria derived from 2 g of guinea pig liver suspended in 4 ml of 0.25 M sucrose.

(b) *Slices.* Tissue slices were prepared using a Stadie-Riggs tissue slicer at 4° from the hepatic lobes. Approximately one gram of such tissue slices was used in each incubation flask. The adrenals were sliced with a new fine razor blade freehand to give altogether eight slices from two adrenals per animal

(c) *Homogenate.* Tissue homogenates were prepared in an equal volume of ice-cold 0.1 M phosphate buffer (p_H 7.0) using an ice-cold all-glass homogenizer of POTTER-ELVEHJEM type as indicated earlier (19). The different tissue preparations from scorbutic and pair-fed animals were usually made on two consecutive days.

Extraction of Fatty acids from Tissues – A weighed portion of the tissue was saponified with 3 g of KOH amd 10 ml of methyl alcohol per g of tissue in a 100 ml standard joint conical flask fitted with an air condenser over a boiling water bath. The total content was refluxed for one hour for complete saponification. The non-saponifiable material was then extracted with petroleum ether as described previously by GUCHHAIT, GUHA, and GANGULI (9). The alcohol-water phase left after such extraction was heated on a steam bath to remove the trace of alcohol. The aqueous layer was then acidified with 6 N HCl to p_H 1.0. The liberated fatty acids after acidification was extracted with petroleum ether (40–60°) and three such extractions were found to be sufficient for the purpose. The petroleum ether

extract was ultimately evaporated and finally dried on a previously weighed container to determine the tissue content of the fatty acids gravimetrically.

Incubation Procedure for Studying Fatty Acid Synthesis from Acetate-^{14}C – Experiments on fatty acid synthesis were carried out in a specially designed flask (50 ml) provided with a central well and rubber stopper (20) as used elsewhere (8). The main compartment of each flask contained the following components: 25 μmoles of a total of inactive and active sodium acetate, acetate-1-^{14}C, 20 μc and 6 μc in case of homogenate and slices respectively, 1 mmole of phosphate buffer, p_H 7.0 and 1 ml of homogenate or 1 g of tissue slices in a total volume of 10.5 ml. The central well contained 0.5 ml of 20% KOH to absorb the respired carbon dioxide by the tissue. The flasks were stoppered and placed in the water bath at 37° with constant shaking and having air as the gas phase for a 3 hours period. The reaction was stopped by injecting 2 ml of 12 N H_2SO_4 through the rubber stopper.

The synthesized labeled fatty acids were then isolated by following a procedure of saponification as described above. Fatty acids thus recovered were dissolved in ether and an aliquot was plated uniformly in stainless steel planchet under an infra red lamp. Plating of the same fatty acid sample was done in triplicate and the average count of such plates was recorded for the sample.

For studying the rate of fatty acid synthesis from acetate-1-^{14}C, experiments were carried out in a similar way as described above where several incubations were started with the same tissue slices only and reactions were terminated at desired intervals. The rest of the procedure was exactly identical.

Experiments on oxidation of labeled fatty acids – The oxidation of labeled fatty acids was carried out by incubating the fatty acid-^{14}C in WARBURG's vessel at 37° where the side arm contained 0.4 ml of 21% trichloroacetic acid in order to stop the reaction after the desired interval (18, 19). The central well of the vessel had 0.2 ml of 20% KOH for absorbing the evolved $^{14}CO_2$. After stopping the the reaction, the flasks were shaken in the bath for an additional period of 10 minutes to ensure total absorption of respired $^{14}CO_2$.

The complete assay system for such oxidation study had : fatty acid, concentration and radioactivity as indicated in respective tables, Tris buffer, p_H 7.2, 200 μmoles, ATP, 3 μmoles, $MgCl_2$, 15 μmoles, and 0.5 ml of mitochondrial suspension in a final volume of 3 ml.

Production of $^{14}CO_2$ from acetate-1-^{14}C was followed in a similar manner (19) with tissue slices only and the rate of formation of carbon dioxide was recorded as done in the case of fatty acid synthesis.

Chemical Analysis – Nitrogen content of mitochondria was determined by the micro-kjeldahl method (21).

Radioactivity Measurements – The evolved $^{14}CO_2$ in acetate and fatty acid oxidation studies was extracted from the central well of incubation flask, precipitated and plated as barium carbonate, and radioactivity measurements were made as described by GANGULI and BANERJEE (14). Measurement of radioactivity in fatty acids was performed according to procedure used by GUCHHAIT and GANGULI (18). A windowless gas flow counter (Nuclear-Chicago Corporation) was used for counting, and corrections were made for self-absorption and background.

Materials

Fatty Acids – All fatty acids used in this study were kindly supplied by Mr. D. GANGULY of the department. Fatty acids were brought to solution as K^+ salts by neutralising warm stock suspensions of fatty acids just prior to use according to LEHNINGER (22). The stock solutions were stored in frozen condition. The labeled acetate and other fatty acids were purchased from the Radiochemical Centre, Amersham, England as their sodium salts. Fatty acids used were all carboxyl labeled with ^{14}C. Solutions were prepared in distilled water and stored at −20° while not in use.

Other Chemicals – ATP was purchased from Schwartz Laboratories, U.S.A. Tris was from Sigma Chemical Company, U.S.A.

Results

Effect of ascorbic acid deficiency on the tissue reserve of fatty acids

Tissues like liver, adrenal, kidney, and spleen from both scorbutic and pair-fed guinea pigs were analysed for the fatty acid content in order to get a preliminary idea on the effect of ascorbic acid deficiency on fat metabolism. These results are borne out in Table 1. It is apparent from the results that the scorbutic guinea pig tissues have increased in both their wet weights and fatty acid concentration. Amongst the tissues studied, maximum weight increase took place in the case of spleen (110.4%) whereas fatty acid content was maximum in the liver tissue (189.3%). This table therefore shows that there is an accumulation of fatty acid in these tissues due to scurvy.

Table 1. Weight and fatty acid content of tissues of guinea pigs under scurvy. The results are expressed as averages of six individual animals

Tissue	Condition of animal	Weight of tissue g	% Increase	Fatty acids %	% Increase
Liver	Scorbutic	8.186	31.0	11.0	189.3
	Pair-fed	6.246		3.8	
Adrenals	Scorbutic	0.275	32.2	12.8	61.0
	Pair-fed	0.207		7.7	
Kidneys	Scorbutic	2.695	27.1	3.3	17.8
	Pair-fed	2.120		2.8	
Spleen	Scorbutic	0.724	110.4	3.2	23.0
	Pair-fed	0.344		2.6	

Incorporation of Acetate-1-^{14}C into Fatty Acids by Tissue Preparations from Scorbutic Guinea Pigs – While studying the incorporation of acetate-1-^{14}C into fatty acids by tissue preparations from scorbutic guinea pigs, we felt it necessary to standardize the incubation system with the different tissues from normal guinea pigs as such systems on fatty acid synthesis with guinea pig tissues are lacking. As pointed out by EMERSON and VAN BRUGGEN (23), it is also essential to find out the effective tracer concentration to work with a new radioactive substrate in a metabolic study and therefore such preliminary experiments were carried out and reported elsewhere (8). It was observed that for such synthetic studies, 4 μc and 20 μc of acetate-1-^{14}C are found to be reasonable tracer concentrations with tissue slices and homogenates respectively.

Having determined the optimal conditions for the incorporation of acetate into fatty acid, we then applied these methods to tissues from scorbutic guinea pigs. Results with scorbutic and corresponding pair-fed animals are shown in Table 2. It is clear from the results that considerable depression in the fatty acid synthesis from acetate-1-^{14}C took place in the case of both scorbutic liver and adrenal tissues. The percentage depression in the incorporation was found to be 60% in case of liver slices or homogenate and adrenal homogenate whereas adrenal slices showed a depression of 32%. Fig. 1 gives a more clear picture about the effect of scurvy on the rate of fatty acid synthesis by the liver tissue

slices. Liver slices from normal animal shows even better synthesis than the pair-fed animal tissue.

Table 2. Fatty acid synthesis from acetate-1-^{14}C by tissue preparations from scorbutic guinea pigs.

The incubation systems used for studying the incorporation of acetate-1-^{14}C into fatty acids by the tissue preparations are as described in the text. The results are expressed as averages of six pairs of experimental and control guinea pigs

Tissue	Condition of animal	Incorporation in fatty acids c.p.m.	Specific activity	% Depression
Slices				
Liver	Scorbutic	320	2.8	59
	Pair-fed	510	11.9	
	Normal*	986	50.2	
Adrenal	Scorbutic	2804	16.1	32
	Pair-fed	6246	23.9	
Homogenate				
Liver	Scorbutic	814	21.1	60
	Pair-fed	962	54.7	
	Normal*	1832	109.1	
Adrenal	Scorbutic	3580	176.4	60
	Pair-fed	5475	692.8	

* These are animals which received 5 mg of ascorbic acid daily and the scorbutogenic diet (16) *ad libitum*.

*Hepatic oxidation of acetate-*1-^{14}C *to* $^{14}CO_2$ *by scorbutic guinea pigs* in vitro – It has been reported earlier (19) that oxidation of acetate is impaired during scurvy. Experiments were performed on the rate of oxidation of acetate to carbon dioxide by hepatic slices from scorbutic guinea pigs. Fig. 2 presents the rate of $^{14}CO_2$ production from acetate-1-^{14}C by hepatic slices from scorbutic, pair-fed and normal guinea pigs *in vitro* indicating a significant lower rate of acetate oxidation to carbon dioxide by the scorbutic tissue compared to either the pair-fed or the normal animal. The difference between the rates of oxidation becomes more prominent after a period of 2 hours incubation.

*Oxidation of Octanoate-*1-^{14}C *to* $^{14}CO_2$ *by liver mitochondria from Guinea Pigs under Scurvy* – As no report was available on the cofactor requirement for fatty acid oxidation by liver mitochondria from guinea pigs, a systematic study was, therefore, made earlier (18) with the liver mitochondria from normal animal and such conditions were extended for studying the oxidation of octanoate-1-^{14}C to $^{14}CO_2$ by scorbutic guinea pig tissue. Results in Table 3 indicate that the oxidation of octanoate by liver mitochondria is depressed to the extent of 63% due to the scorbutic condition of the animal. Whether this depression in oxidation is at the enzymatic level or due to shortage of cofactors, an experiment was performed with or without added cofactors in the incubation system. Table 4 shows that essential cofactors like ATP and Mg^{++} have no effect in improving the depressed oxidation of octanoate.

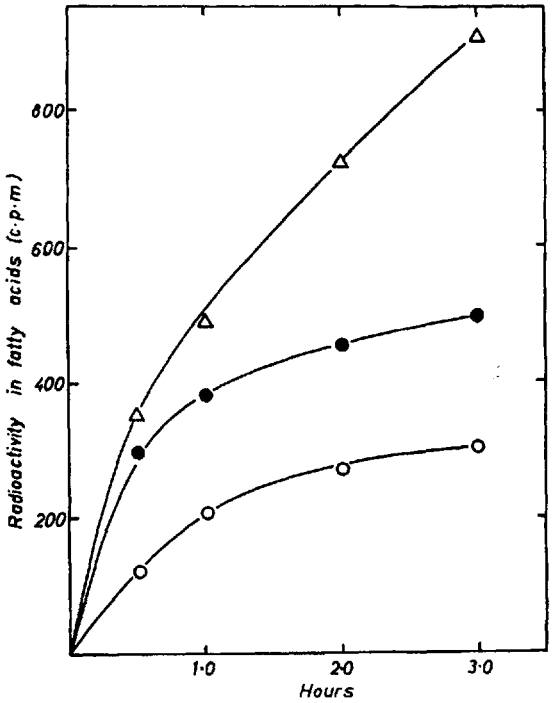

Fig. 1. Rate of fatty acid synthesis from acetate-1-^{14}C by liver tissue slices from guinea pigs under scurvy. Experimental conditions were as described in Table 2. Reactions were terminated at desired intervals as indicated in the figure. –○–○–○– Scorbutic, –●–●–●– Pair-fed and –△–△–△– Normal tissue slices.

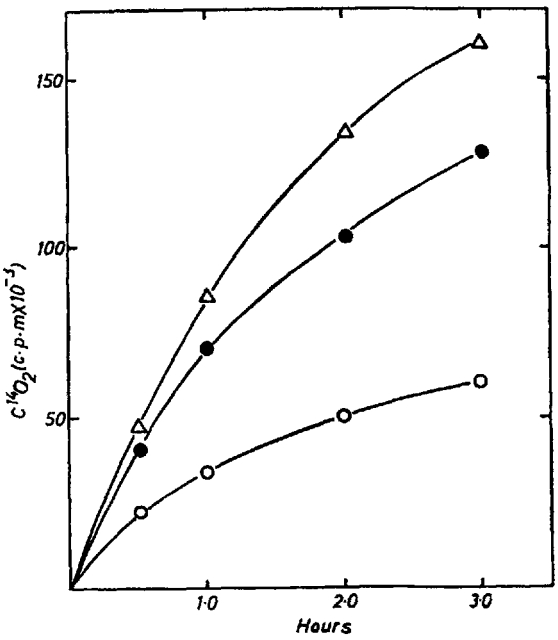

Fig. 2. Effect of scurvy on the oxidation of acetate-1-^{14}C to $^{14}CO_2$ by liver tissue slices from guinea pigs. Conditions of experiments are as described in the text. –○–○–○– Scorbutic, –●–●–●– Pair-fed and –△–△–△– Normal tissue slices.

Table 3. Oxidation of octanoate-1-^{14}C to $^{14}CO_2$ by hepatic mitochondria from guinea pigs under scurvy.

Incubation mixture contained: octanoate-1-^{14}C with 579×10^3 c.p.m. and a total of 2 μmoles of radioactive and carrier octanoate; 200 μmoles tris buffer, p_H 7.2; 3 μmoles ATP; 15 μmoles $MgCl_2$ and 0.5 ml of mitochondrial suspension. The central well of each flask contained 0.2 ml of 20% KOH. Incubation was carried out at 37° with constant shaking in a WARBURG bath for one hour. Reaction was stopped by tipping 0.4 ml of 30% trichloroacetic acid present in the side arm of the flask. Mean values are the averages of separate determinations on six pairs of experimental and control guinea pigs and are expresses per mg tissue nitrogen.

Condition of animal	$^{14}CO_2$ from octanoate-1-^{14}C		Per cent depression in scurvy
	Range	Mean	
	%		
Scorbutic	2.4 – 2.9	2.60	63
Pair-fed	7.0 – 7.5	7.22	

Table 4. Effect of cofactors on octanoate-1-^{14}C oxidation by hepatic mitochondria from scorbutic guinea pigs

The incubation medium for the oxidation study was as described in Table 3. ATP (3 μmoles) and $MgCl_2$ (15 μmoles) were used as cofactors where indicated. The results are expressed as averages of six such experiments with each tissue mitochondria and per mg tissue nitrogen

Condition of animal	System used	$^{14}CO_2$ from octanoate-1-^{14}C %	Per cent depression in scurvy			
			Complete	-ATP	-Mg^{++}	-ATP, Mg^{++}
Scorbutic	Complete	2.45	66	55	59	52
	-ATP	1.59				
	-$MgCl_2$	1.89				
	-(ATP + $MgCl_2$)	0.81				
Pair-fed	Complete	7.25				
	-ATP	3.56				
	-$MgCl_2$	4.62				
	-(ATP + $MgCl_2$)	1.71				

Hepatic Mitochondrial Oxidation of Higher Fatty Acids under Scurvy – Experiments were performed on the oxidation of higher fatty acids labeled with ^{14}C in the carboxyl group to $^{14}CO_2$ by hepatic mitochondrial preparation from guinea pigs suffering from scurvy. Such results as described in Table 5 indicate that the oxidation of higher fatty acids like lauric, myristic and stearic acids are impaired due to this disease. The scorbutic tissue preparations showed a significant amount of depressed oxidation as revealed by the $^{14}CO_2$ values produced from the terminally labeled substrates. Maximum depression was observed with myristic (36%), next comes lauric (26%) and least with stearic acid (18%). The extent of oxidation without cofactors (ATP and Mg^{++}) was found to be much less and depression in oxidation due to scurvy was proportionately more compared to systems with cofactors. Cofactors, therefore, seem to participate in correcting the depressed oxidation of higher fatty acids caused due to scurvy.

Table 5. Hepatic mitochondrial oxidation of different fatty acid-1-^{14}C to $^{14}CO_2$ by scorbutic guinea pigs

Conditions used in this experiment were as described in Table 4. Radioactive substrates used were either laurate-1-^{14}C, myristate-1-^{14}C or stearate-1-^{14}C with 576.2×10^3, 869.8×10^3 and 138.8×10^3 c.p.m. respectively. Rest of the experiments were as described in the text. Results are averages of six individual experimental groups of animals and per mg tissue nitrogen

Fatty acid used	Condition of animal	System used	$^{14}CO_2$ %	Per cent depression in scurvy	
				Complete	-Cofactors
Laurate-1-^{14}C	Scorbutic	Complete	5.86	26	47
		-Cofactors	0.65		
	Pair-fed	Complete	7.85		
		-Cofactors	1.22		
Myristate-1-^{14}C	Scorbutic	Complete	3.05		
		-Cofactors	0.93	36	51
	Pair-fed	Complete	4.75		
		-Cofactors	1.89		
Stearate-1-^{14}C	Scorbutic	Complete	3.65		
		-Cofactors	2.68	18	27
	Pair-fed	Complete	4.48		
		-Cofactors	3.66		

Discussion

It is well known that both anabolism and catabolism of fatty acids have a direct relation with acetate metabolism. Acetate being the key substrate, a defect in its metabolism in scurvy (8, 10, 19) is expected to disturb the metabolic pattern of fatty acids. The observation that in scurvy, the tissue content of fatty acids has increased (Table 1) immediately indicates an alteration in fatty acid metabolism in this disease. It therefore seems that this apparent increase in fatty acids in tissues will either be due to an increased synthesis or decreased oxidation of fatty acids by the scorbutic guinea pigs.

On comparison of the relative increase in tissue fatty acids, liver was found to have the maximum value (Table 1). The increase in tissue weights can probably be attributed more to the accumulation of fatty acids in the tissue. Our observation concur with that of SHEPPARD and MCHENRY (11) who have also observed a considerable increase in body fat in guinea pigs under ascorbic acid deficiency.

The results on the incorporation of acetate-1-^{14}C to fatty acids (Fig. 1, Table 2) clearly indicate that the observed increase in tissue concentration in fatty acids cannot be due to an increase in the synthetic rate. Synthesis of fatty acids from acetate, both by liver slices (Fig. 1) and by tissue preparations (Table 2) from scorbutic guinea pigs is found to be depressed in this condition. The diminution in the incorporation rate is more prominent from the specific activities of the synthesized fatty acids by the scorbutic tissues. There exists also a remarkable difference in the incorporation rates between the pair-fed and 'ad libitum' fed normal guinea pigs, the tissue from the later showing much higher synthesis of fatty acids than the former. This is probably due to partial

fasting condition of the pair-fed animal as fasting is reported to cause a decrease in fatty acid synthesis from acetate (24, 25).

As the hepatic oxidation of acetate-1-^{14}C to $^{14}CO_2$ by scorbutic guinea pigs *in vitro* (Fig. 2) was found to be depressed compared to the pair-fed animals, it is not unlikely to presume that the oxidation of fatty acids will also be hampered as the later are believed to be oxidized via a common route. Results on octanoate-1-^{14}C (Table 3 and 4) and other labeled fatty acids (Table 5) have revealed that undoubtedly there is a defective oxidation by the scorbutic tissue mitochondria and therefore this fact may explain the rise in tissue fatty acids level. The oxidation of higher fatty acids (lauric, myristic stearic) seems to be affected not only at enzymatic level, but also due to cofactor deficiency as added cofactor stimulated the depressed oxidation rate. The observation by GANGULI and ROY (26) on the diminished tissue level of ATP in scurvy clearly explains that ATP will be a limiting factor for fatty acid oxidation in this disease. Disturbed tricarboxylic acid cycle operation in scurvy (13, 19, 27, 28) will also be an additional cause for the incomplete oxidation of fatty acids.

Several reports (29, 30) have also indicated that insulin has an important role in the regulation of lipid metabolism and a diminished production of insulin in scurvy (13, 31) might be a reason for depressed oxidation of fatty acids. It is therefore tempting to remark that ascorbic acid deficiency resulted in an impaired oxidation of fatty acids through hypoinsulinism.

From the above findings, the important outcome is, therefore, that less oxidation of fatty acids resulted to an accumulation of such metabolite in the tissue in scurvy. The present report together with previous observation from this laboratory (8–10, 14, 15, 19, 26) indicate that metabolisms of carbohydrate (14–15), cholesterol (8–10) and fatty acids are dependent on ascorbic acid, directly or indirectly.

Summary

1. The tissue weight and fatty acid concentration were observed to be higher in the case of scorbutic guinea pigs compared to the pair-fed control animals. Amongst the tissues analysed, maximum increase in fatty acid level was found with the liver tissue.

2. Fatty acid synthesis by the scorbutic tissue *in vitro* was diminished. Both tissue homogenates and slices had less capacity to convert acetate-1-^{14}C to labeled fatty acids. The specific activities of the synthesised fatty acids by tissues of scorbutic guinea pigs were appreciably less than that synthesised by the corresponding tissue preparations from the pair-fed animals. Incorporation by tissue from normal animal fed '*ad libitum*' was more compared to the tissue from pair-fed one.

3. Hepatic oxidation of acetate-1-^{14}C to $^{14}CO_2$ by slices was depressed in scurvy.

4. Mitochondria prepared from scorbutic liver tissue exhibited a lower oxidation of octanoate-1-^{14}C to $^{14}CO_2$. Addition of essential cofactors like ATP and Mg^{++} in the system did not improve the depressed oxidation.

5. The *in vitro* oxidation of terminally labeled higher fatty acids like lauric, myristic and stearic to $^{14}CO_2$ by the mitochondrial tissue preparation from scorbutic guinea pigs was appreciably depressed as a result of this disease. ATP and Mg^{++} could fortify considerably this defect.

6. The significance of these findings in relation to lipid metabolism in scurvy is discussed.

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