appreciably influenced by the nature of the dietary fat. The present work suggests that certain marsupials are not unlike ruminants in this respect, inasmuch as these animals also modify the dietary fat in the alimentary tract by hydrogenation of highly unsaturated acids.

SUMMARY

1. An examination of the depot fats of ruminants showed the presence of substantial amounts (3·5–11·2 %) of *trans*-acids.

2. The fats of non-ruminants were found to contain not more than 0·9 % of *trans*-acids, apart from those of certain marsupials, such as the wallaby and the quokka, in which the *trans*-acid contents varied from 18·1 to 21·0 %.

3. From the available evidence it appears that the *trans*-acids arise mainly from hydrogenation of dietary unsaturated acids by bacteria in the rumen, or in the rumen-like stomach in marsupials.

Investigations on the effects of rumen contents on unsaturated fatty acids is being continued, and it is hoped that the results of the investigations now in progress will be published shortly.

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Synthesis of Citrovorum Factor in Rats Given Chloretone

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Numerous clinical and experimental observations have indicated that ascorbic acid may function in certain anaemias (Vilter, 1947). Pernicious anaemia has responded to ascorbic acid therapy, and a deficiency of ascorbic acid favours the development of megaloblastic anaemia in monkeys and in human patients (May, Nelson, Lowe & Salmon, 1950; May, Sundberg & Schaar, 1950). Although pteroylglutamic acid does not minimize the need for ascorbic acid (Silverman & Mackler, 1951), megaloblastic anaemia of scorbutic monkeys is alleviated by pteroylglutamic acid or, in smaller doses, by citrovorum factor (N^5-formyltetrahydropteroylglutamic acid) (May, Sundberg & Schaar, 1950). Correction of scurvy with ascorbic acid abolishes megaloblastic anaemia without any administration of pteroylglutamic acid or citrovorum factor (May, Sundberg & Schaar, 1950). Dietary ascorbic acid is reported to promote growth in chicks and storage of pteroylglutamic acid in their livers

(Dietrich, Nichol, Monson & Elvehjem, 1949). Urinary excretion of citrovorum factor by rats or humans given pteroylglutamic acid increases 2- to 3-fold on ascorbic acid administration (Welch, Nichol, Anker & Boehne, 1951). These findings suggest a function for ascorbic acid in the metabolic alteration of pteroylglutamic acid. More directly, it was demonstrated by Nichol & Welch (1950) that ascorbic acid aids in the conversion *in vitro* of pteroylglutamic acid into citrovorum factor by liver enzymes. Although the effect of ascorbic acid has been attributed to a greater release of bound citrovorum factor (Hill & Scott, 1952), the formation of citrovorum factor in chick-liver homogenates is apparently aided by ascorbic acid (Nichol, 1953). The stimulatory influence of ascorbic acid on citrovorum-factor synthesis from pteroylglutamic acid has also been observed to occur with micro-organisms (Broquist, Kohler, Hutchison & Burchenal, 1953; Mitbander & Sreenivasan, 1954).

Chloretone (1:1:1-trichloro-2-methylpropan-2-ol) and other narcotics are known to increase the biosynthesis of ascorbic acid in the rat (Longenecker, Musulin, Tully & King, 1939; Longenecker, Fricke & King, 1940; Smythe & King, 1942); the role of chloretone is stimulatory and not due to any direct participation in biosynthesis (Jackel, Mosbach, Burns & King, 1950). A concomitant increase in glucuronic acid synthesis (Smythe & King, 1942) suggested that ascorbic acid elaboration in the chloretonized rat is incidental to a step-up in detoxication mechanisms, but it is now known that glucuronic acid is an intermediate in ascorbic acid biosynthesis (Horowitz & King, 1953; Isherwood, Chen & Mapson, 1954). Since chloretone stimulates ascorbic acid excretion in the albino rat as well as the *in vitro* synthesis of ascorbic acid by liver tissues (Smythe & King, 1942), it was of interest to ascertain whether treatment with the narcotic promotes synthesis of citrovorum factor.

The observations presented here provide further evidence of the participation of ascorbic acid in citrovorum-factor synthesis *in vivo* in rats.

EXPERIMENTAL AND METHODS

Male rats (inbred Wistar strain), 80–100 g. in weight, were maintained on a complete stock diet of the following composition (g./100 g. diet): wheat flour 65, casein (vitamin-free) 10, wheat bran 4, food yeast 4, arachis oil 8, shark-liver oil 2, fish meal 3 and salt mixture (U.S.P. No. 2) 4. Groups of four animals, each receiving chloretone with or without pteroylglutamic acid, and appropriate controls, were used. Chloretone was given orally as a 10% (w/v) solution in coconut oil, the dosage being 20 mg./100 g. body wt./day/rat for 4 days. Pteroylglutamic acid (4 μg./day/rat) was injected intraperitoneally for the four following days. Some groups received chloretone or pteroylglutamic acid only, and a control group was maintained on the basal stock ration. Urine collections were made with the usual precautions during the 24 hr. period preceding death. The animals were killed by decapitation on the ninth day after grouping, bled and the livers excized and chilled in cracked ice. Livers were then homogenized with water, made to volume (1:5) and a portion was autolysed at pH 7·6 in an equal volume of 0·2 M phosphate buffer under toluene for 20 hr. The autolysed samples were steamed for 15 min., the pH was adjusted to 6·8 and the solution made to volume. Pteroylglutamic acid and citrovorum-factor activities were estimated (Mitbander & Sreenivasan, 1954) in the clear supernatants after centrifuging, *Streptococcus faecalis* R and *Leuconostoc citrovorum* 8081 respectively being used as assay organisms; citrovorum-factor activity was determined in terms of Leucovorin (Lederle). Turbidity readings were taken after 20 hr. in a Klett–Summerson photocolorimeter with a 660 mμ. filter for both assays.

Liver ascorbic acid was determined in aliquots of the liver homogenates after deproteinizing with trichloroacetic acid and according to the procedure of Williams (1951). For ascorbic acid estimation in urine, the method based on 2:6-dichlorophenol-indophenol titration was followed (Evelyn, Malloy & Rosen, 1938).

Aliquots of the homogenates were used for assays of certain oxidase systems known to be influenced by pteroylglutamic acid and ascorbic acid. Xanthine-oxidase activity was determined by the method of Dhungat & Sreenivasan (1954). Liver-choline-oxidase and tyrosine-oxidase activities were determined by the procedures of Dinning, Keith & Day (1949) and Sealock & Goodland (1949) respectively. For the determination of xanthine-oxidase and tyrosine-oxidase activities, supernatants after centrifuging for $\frac{1}{2}$ hr. at 0° against 25 000 *g* were used; for choline-oxidase activity, the particulate fraction so obtained was resuspended in an appropriate volume of buffer.

Values reported are the averages for all four animals in each group.

RESULTS AND DISCUSSION

Chloretone administration results in the expected increase in urinary excretion of ascorbic acid as well as in a much smaller increase in liver storage. Supplementation with pteroylglutamic acid does not affect the stimulation of ascorbic acid metabolism by chloretone. Liver stores of pteroylglutamic acid in the vitamin-supplemented groups are nearly twice those in the control groups, chloretone exerting no effect in this respect. However, in the chloretonized animal, there is increased citrovorum-factor activity in the liver, both in the unsupplemented and pteroylglutamic acid supplemented groups (Table 1).

The administration of chloretone to the animals on the basal diet results in a large fall in liver-xanthine-oxidase activity (Table 2). Such an effect of the narcotic is not observable in the group receiving pteroylglutamic acid, which, when given alone (group 3), depresses enzyme activity. There are conflicting reports relating to the effect of pteroylglutamic acid on liver-xanthine-oxidase

activity *in vitro* and *in vivo* (cf. Fatterpaker & Sreenivasan, 1951). An inhibition of xanthine oxidase by ascorbic acid has been reported by Feigelson (1952).

The changes in tyrosine-oxidase activity follow the same trend as those for xanthine-oxidase activity. Pteroylglutamic acid and ascorbic acid have both been implicated in tyrosine oxidation, and it has recently been shown that, although the influence of pteroylglutamic acid on this system is indirect, ascorbic acid functions as a co-factor in (Swendseid, Bethell & Ackerman, 1951). The present findings are not in accord with this observation. The activities for pteroylglutamic acid were assayed by using *Strep. faecalis* R. Since leucovorin is only half as active as pteroylglutamic acid for this organism (Weiland, Hutchings & Williams, 1952), and since invariably *Strep. faecalis* R activity assayed for rat-liver preparations was higher (Table 1) than citrovorum-factor activity, it would follow that liver contains folic acid as both pteroylglutamic acid and citrovorum factor.

Table 1. In vivo *synthesis of citrovorum factor by chloretone-fed rats*

Animals on the laboratory stock diet (see text) were each fed on 20 mg. of chloretone/100 g. body wt./day for 4 days. For the next 4 days pteroylglutamic acid (4 μg./day) was given intraperitoneally. Animals were killed on the ninth day after grouping; urine collections were made during the 24 hr. period preceding death. The results are averages for four animals in each group $\pm$S.E.M. Pteroylglutamic acid, citrovorum factor and ascorbic acid were assayed as described in the Experimental section.

Group no.	Diet and treatment	Urinary ascorbic acid (mg./100 g. body wt./24 hr.)	Liver concentration of (μg./g. dry wt.) Pteroylglutamic acid	Citrovorum factor	Ascorbic acid
1	Basal	0·20	3·70±0·12	1·42±0·16	303±27
2	Basal + chloretone	2·06	3·69±0·06	2·71±0·10	408±30
3	Basal + pteroylglutamic acid	0·22	6·78±0·18	3·69±0·20	316±30
4	Basal + chloretone + pteroylglutamic acid	2·12	6·88±0·12	4·05±0·08	390±21

Table 2. *Effects of chloretone-feeding on certain liver oxidases*

For details see Table 1 and text. Results are averages for four animals in each group $\pm$S.E.M.

Group no.	Diet and treatment	Xanthine oxidase (μl. O_2/g. dry wt./1½ hr.)	Enzyme activity (μl. O_2/g. dry wt./½ hr.) Choline oxidase	Tyrosine oxidase
1	Basal	561±12	1791±34	180±21
2	Basal + chloretone	261± 9	1526±46	20±18
3	Basal + pteroylglutamic acid	446±18	1964±26	165±24
4	Basal + chloretone + pteroylglutamic acid	461±18	2499±29	150±21

two of the steps of tyrosine oxidation to fumarate and acetoacetate (Williams & Sreenivasan, 1953). Chloretone also depresses choline-oxidase activity, but there is considerable protection by pteroylglutamic acid, which, when given alone, somewhat stimulates choline oxidation (Table 2).

The relationship of pteroylglutamic acid to choline oxidation is apparently more specific than to the other two oxidation systems studied here. Williams, Sreenivasan, Sung & Elvehjem (1953) have shown that there is a close correlation between the choline-oxidase activity and folinic acid content of rat-liver mitochondria. The changes in activity of the oxidase systems when chloretone was fed are therefore probably due to a direct effect of the narcotic on the general metabolism of the animals rather than to increased synthesis of citrovorum factor.

It has been reported that pteroylglutamic acid in rat liver is stored largely as citrovorum factor

Although intraperitoneal administration of pteroylglutamic acid results in increased storage of citrovorum factor in the chloretonized animal (Table 1), this difference is not as pronounced as would be expected from considerations of increased ascorbic acid availability. Swendseid *et al.* (1951) have reported that administered pteroylglutamic acid is not completely converted into citrovorum factor *in vivo*. It seemed possible that, if the samplings for citrovorum-factor assay were done on successive days after a single intraperitoneal dose of pteroylglutamic acid, the effect of chloretone feeding on citrovorum-factor synthesis by the liver enzymes would be more clearly demonstrated.

In the next experiment (Table 3) grouping and chloretone feeding of the animals were exactly as before. The animals were killed at daily intervals for 4 days after the first and only dosage (10 μg./animal) of pteroylglutamic acid, namely between the sixth and the ninth day after grouping. Liver

Table 3. In vivo *synthesis of citrovorum factor in the chloretonized rat during successive days after pteroylglutamic acid administration*

For explanation of Table, see Table 1 and text. Animals were killed at daily intervals for 4 days after the administration of pteroylglutamic acid (10 μg./animal). Values are the averages for four animals in each group. The citrovorum-factor activity of livers from a control group which was kept on the basal stock diet is given in the text.

Diet and treatment	Increase in synthesis of citrovorum-factor activity over the control group (μg./g. of dry liver)			
	1st day	2nd day	3rd day	4th day
Basal + chloretone	1·13	1·04	1·26	0·84
Basal + pteroylglutamic acid	0·90	1·84	1·94	2·04
Basal + chloretone + pteroylglutamic acid	2·23	3·90	4·03	3·98

samples were assayed for citrovorum-factor activity and Table 3 gives the average increase in citrovorum-factor activity. The values for citrovorum-factor activity of the control group on the basal stock diet were $1·08 \pm 0·19$ and $1·02 \pm 0·17$ on the first and fourth days of sampling respectively. The extent of conversion of pteroylglutamic acid into citrovorum factor increases with time after pteroylglutamic acid administration. The fact that in the chloretone-fed groups the values for citrovorum-factor activity decrease after 3 days indicates a reversal of the effect of chloretone on the synthesis of ascorbic acid at this stage. *In vitro* chloretone does not stimulate citrovorum-factor synthesis from pteroylglutamic acid in liver slices, homogenates or fractionated enzyme preparations (unpublished results).

SUMMARY

1. The effect of ascorbic acid on increased citrovorum-factor synthesis is shown *in vivo* with chloretonized rats, which elaborate increased amounts of ascorbic acid. The observations have been extended by a study of the *in vivo* conversion of intraperitoneally administered pteroylglutamic acid into citrovorum factor by normal and chloretonized animals.

2. From a study of the effect of chloretone feeding on liver-choline-oxidase, xanthine-oxidase and tyrosine-oxidase activities, it is concluded that the direct effects of the narcotic on the animal are probably far greater than those attributable to increased conversion of pteroylglutamic acid into citrovorum factor.

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