Chemistry. — *Researches on fat metabolism.* VIII. *Feeding experiments on dogs with sodium salts of normal saturated dicarboxylic acids.* By P. E. VERKADE, J. VAN DER LEE, A. J. S. VAN ALPHEN and M. ELZAS. (From the chem. lab. of the Dutch Commercial University and the State Serum Institute, Rotterdam.)

(Communicated at the meeting of October 30, 1937.)

§ 1. We have shown that excretion of the dicarboxylic acid formed by ω-oxidation of the component acid (*diaciduria*) takes place both in man [1]) and in the dog [2]) to very different degrees after administration of various saturated mono-acid triglycerides under as nearly as possible similar conditions. In both cases this phenomenon has up to the present been observed only after the consumption of a few lower triglycerides.

The conclusion has been drawn by B. FLASCHENTRÄGER and his collaborators [3]) from this fact, as well as from the results of their own feeding experiments on dogs with salts and esters of fatty acids, that the way of degradation of saturated fatty acids discovered by us — consisting of ω-oxidation followed by bilateral β-oxidation of the dicarboxylic acid produced — is restricted to lower members of the series. In their 1936 paper quoted above, B. FLASCHENTRÄGER and K. BERNHARD expressly state that ω-oxidation is restricted to the fatty acids with 8—11 carbon atoms and that one must think of a "spezifische Tendenz zur ω-Oxydation von Seiten der mittleren Fettsäuren". Consequently the error in principle is committed of infering the non-occurrence of ω-oxidation from the absence of diaciduria. In a similar way one might also, or rather might have to, come to the conclusion that there is a question of an appreciable unilateral β-oxidation of fatty acids only in pathological states, namely when ketonuria (ketosis) occurs.

If this standpoint were correct the way of degradation we have found would be physiologically of but little importance since the lower fatty acids occur to an appreciable extent as component acids in only a few of the customary food fats. In our opinion it is, however, untenable. The work to be discussed below leaves indeed little or no reasonable

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1) P. E. VERKADE and J. VAN DER LEE, Biochem. J. **28**, 31 (1934).

2) P. E. VERKADE, J. VAN DER LEE and A. J. S. VAN ALPHEN, Z. physiol. Chem. **247**, 111 (1937).

3) B. FLASCHENTRÄGER, K. BERNHARD, C. LÖWENBERG and M. SCHLÄPFER, Z. physiol. Chem. **225**, 157 (1934); B. FLASCHENTRÄGER and K. BERNHARD, Helv. chim. acta **18**, 962 (1935); Z. physiol. Chem. **238**, 221 (1936); see also K. SCHLUMPF, Diss. Univ. Zürich (1936).

doubt that *all saturated fatty acids* — we are only concerned with these here — *are catabolised in the way we have discovered.*

§ 2. In our opinion it was already very improbable *a priori* that, for example, the terminal methyl group of the fatty acids with 8—11 carbon atoms can indeed be oxidised in the human organism to a carboxyl group and in two cases (capric acid and undecoic acid) recognisably fairly readily, and on the other hand that of the members of the series immediately above or below would be non-oxidisable. One should not lose sight of the fact, already pointed out by us in previous papers [4]) and also by others [5]) that *the oxidation of the methyl group in fatty acids discovered by us and called ω-oxidation for the sake of brevity is certainly not an isolated phenomenon. On the contrary numerous cases of methyl group oxidation in vivo, and indeed in compounds of very divergent constitution, have already been described in the literature* [6]). The following may serve as typical examples: the formation of benzoic acid from toluene, of mandelic acid from methylphenylketone, of HILDEBRANDT's acid from geranic acid, citral, geraniol and dihydromyrcene [7]), of 3-methyl-nonene-(2)-dioic acid-(1,9) from 3-methyl-nonen-(2)-oic acid-(1) [5]), of polyene dicarboxylic acids from monocarboxylic acids of the type (I) and of trans-π-

$$H_3C - (CH = CH)_n - COOH \quad (I)$$

-apocamphor-carboxylic acid-(7) from camphor [9]).

The formation of succinec acid in cultures of *Aspergillus niger* with calcium butyrate as the sole source of carbon reported by H. B. STENT, V. SUBRAMANIAM and TH. K. WALKER [10]) is also interesting in this connection; the workers just quoted however rightly observe that a different way of formation of this succinic acid than by methyl group oxidation is also quite conceivable.

Certain processes observed *in vivo* receive a very satisfactory explanation on the assumption of a primarily occurring methyl group oxidation. Reference may be made to the well known observation of H. THIERFELDER and F. KLENK [11]) that n-propyl- and n-amylbenzene are oxidised to

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[4]) P. E. VERKADE and J. VAN DER LEE, Chem. Weekblad **33**, 163 (1936); P. E. VERKADE, Bull. soc. chim. biol. **18**, 989 (1936).

[5]) R. KUHN, F. KÖHLER and L. KÖHLER, Z. physiol. Chem. **242**, 171 (1936).

[6]) See the reviews of K. FROMHERZ in Handb. norm. path. Physiol. **5**, 1001 (1928), of H. GESENIUS in E. ABDERHALDEN's Handb. d. biol. Arbeitsmeth. IV, **5** (1st half), 863 (1931) and of K. SCHLUMPF, loc. cit.

[7]) H. HILDEBRANDT, Arch. exptl. Path. Pharmacol. **45**, 110 (1901); R. KUHN and K. LIVADA, Z. physiol. Chem. **220**, 235 (1933); R. KUHN, F. KÖHLER and L. KÖHLER, loc. cit.

[8]) R. KUHN, F. KÖHLER and L. KÖHLER, Z. physiol. Chem. **247**, 197 (1937).

[9]) Y. ASAHINA and M. ISHIDATE, Ber. **68**, 947 (1935).

[10]) J. Chem. Soc. 1929, 1987.

[11]) Z. physiol. Chem. **141**, 13 (1924).

benzoic acid and n-butyl- and n-hexylbenzene to phenylacetic acid; the fate of the ω-phenylsubstituted fatty acids produced from these hydrocarbons by ω-oxidation had in fact already been cleared up by the classical researches of F. KNOOP [12]). Other examples are the transformation of 2-methyl(benzenesulphonyl)amino-dodecoic acid-(1) into 2-methyl-(benzenesulphonyl)amino-hexanedioic acid-(1,6) in the dog found by B. FLASCHENTRÄGER, K. BERNHARD, C. LÖWENBERG and M. SCHLÄPFER [3]) and the formation of 3-methyl-hexene-(2)-dioic acid-(1,6) from 3-methyl-dodecen-(2)-oic acid-(1) observed recently in the rabbit by R. KUHN, F. KÖHLER and L. KÖHLER [5]); in both these cases the ω-oxidation would then be followed by a triple β-oxidation.

According to A. CH. CHIBNALL and S. H. PIPER [13]) ω-oxidation may perhaps play a part in the formation of sabinic acid (12-hydroxy-dodecoic acid), thapsic acid (hexadecanedioic acid), juniperic acid (16-hydroxy-hexadecoic acid) and similar products in the plant. Likewise it has already been postulated that civetone is produced from oleic acid and indeed by ring closure of the ω-oxidation product of the latter. It is also surmised that methyl group oxidation plays a part in the transitions between sterols, bile acids, heart poisons, sex hormones, etc. These examples show that the notion of a methyl group oxidation as a general and important biological principle is everywhere a pregnant one.

Our standpoint, that all normal saturated fatty acids are capable of undergoing ω-oxidation in the animal organism, taken up directly after the discovery of the first case of diaciduria [14]) is the result of consideration of the facts brought up in this § and is indeed the only logical one.

§ 3. Experiments of J. BAER and L. BLUM [15]), B. FLASCHENTRÄGER [16]), H. G. SMITH [17]) and E. ANDERSEN [18]) with one or more normal saturated dicarboxylic acids with 6—10 carbon atoms have shown that, after administration of the sodium salts either by subcutaneous injection or *per os* to dogs, a considerable portion of the acid is excreted in the urine. Y. MORI [19]) observed the same thing for adipic acid in the rabbit and E. ANDERSEN [18]) in man; the latter, for example, administered 25 g of adipic acid as sodium salt in daily amounts of 5 g *per os* to two subjects and found not less than 55 % and 72 % respectively of the acid in the

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12) Hofmeisters Beitr. **6**, 150 (1905).

13) Biochem. J. **28**, 2209 (1934).

14) P. E. VERKADE, M. ELZAS, J. VAN DER LEE, Miss. H. H. DE WOLFF, Mrs. A. VERKADE—SANDBERGEN and D. VAN DER SANDE, Proc. Royal Acad. Amsterdam **35**, 251 (1932).

15) Hofmeisters Beitr. **11**, 101 (1908).

16) Z. physiol. Chem. **159**, 297 (1927); B. FLASCHENTRÄGER and K. BERNHARD, ibid. **238**, 221 (1936).

17) J. Biol. Chem. **103**, 531 (1933).

18) See B. FLASCHENTRÄGER, loc. cit., p. 299.

19) J. Biol. Chem. **35**, 341 (1918).

urine. These acids are thus combustible with difficulty in the organism. It has as yet been tacitly assumed that such would also be the case with the higher members of this series. The standpoint of B. FLASCHENTRÄGER *et al.* as regards the way of degradation of the saturated fatty acids discovered by us in obviously based on this assumption.

In the feeding experiments on dogs with the sodium salts of sebacic acid and undecanedioic acid, described in another connection in part VII of this series [20], we were struck by the fact that the latter acid (C_{11}) was excreted to a noticeably smaller extent in the urine than the former (C_{10}). This observation suggested the incorrectness of the above-mentioned assumption and was in fact the motive for comparative feeding experiments with the sodium salts of various normal saturated dicarboxylic acids, which are discussed below.

§ 4. The adult, healthy dogs Tommy (about 12 kg, ♂) and Fox (about 13 kg, ♂) served for the experiments in question. They received twice a day for three successive days, always at the same times (11 h and 17 h), 2.5 g of the neutral sodium salt of the dicarboxylic acid to be investigated, as an addition to a meal, consisting of 20—30 g dog biscuit and 10—20 g lean horse meat. Water was supplied as desired. The urine passed during the test-days and several (at least 5) succeeding days was collected. In the experiments with sebacic acid and undecanedioic acid the course of the excretion of these acids in the urine could be readily followed, namely by testing the successive portions of urine with the aid of concentrated phosphoric acid. A total duration of 8 days for the experiment was always found to be amply sufficient with these acids. There was absolutely no reasonable ground for the supposition that such would not be the case with the other dicarboxylic acids investigated by us. Nevertheless for sake of safety the test-period was extended by some days especially in the investigation of the higher acids. Failures of the experiments due to vomiting, diarrhoea, etc. did not occur.

An adequate space of time always intervened between the various experiments made with one and the same dog, during which the animal resumed its normal mode of life.

The dicarboxylic acids employed (C_4, C_6, C_8, C_{10}, C_{11}, C_{13}, C_{16}) were prepared or purified in our laboratory, with the exception of hexadecanedioic acid. A considerable quantity of this acid (eq. wt. 142,8; m.p. 123—124.5°) was very kindly put at our disposal by the S. A. M. Naef & Cie (Firmenich & Cie, Succrs) of Geneva; to them we offer our hearty thanks.

Two experiments at least were always made with each of the acids on one and the same dog. The urines obtained during these experiments

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[20] P. E. VERKADE, J. VAN DER LEE, A. J. S. VAN ALPHEN and M. ELZAS, Proc. Royal Acad. Amsterdam **38**, 945 (1935).

were worked up separately until it had been adequately proved that they furnished concurrent results; then for the purpose of determining the amount of dicarboxylic acid excreted unchanged they were combined for the sake of simplicity. Indications regarding the manner of working up the urine and the isolation of the dicarboxylic acids present have already been given sufficiently elsewhere [21]).

In certain experiments with various dicarboxylic acids — among them all those with brassylic acid and hexadecanedioic acid, in which this was particularly important — also the faeces were tested for the presence of these acids, however, always with a negative result; all dicarboxylic acids were completely resorbed.

§ 5. The accompanying table gives a survey of the results obtained.

	TOMMY		FOX	
	administered	excreted unchanged	administered	excreted unchanged
C_4 succinic acid	21.9 g	none	—	—
C_6 adipic acid	23.1	about 58%	—	—
C_8 suberic acid	24.0	„ 45%	—	—
C_{10} sebacic acid	36.9	„ 33%	24.6 g	about 28%
C_{11} undecanedioic acid	24.9	„ 17%	24.9	„ 7%
C_{13} brassylic acid.	25.4	very little?	25.4	very little?
C_{16} hexadecanedioic acid . . .	26.0	none	39.0	none

In what follows we shall occupy ourselves exclusively with the dicarboxylic acids with six or more carbon atoms and first of all attempt to answer the question why these acids are excreted in the urine to such a typically decreasing degree on proceeding up the series. The possibility had certainly to be kept in mind *a priori* that the cause of this phenomenon might lie in a rapid decrease of the resorbability of these acids (perhaps as a consequence of their rapidly decreasing solubility in water) or of their sodium salts by the intestinal wall on mounting the homologous series. The assumption that *the "combustibility" of these acids in the organism rapidly rises on passing up the series,* was however much more attractive. Obviously then also the possibility had to be borne in mind that both these factors are operative together. The latter assumption can

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[21]) See P. E. VERKADE and J. VAN DER LEE. Z. physiol. Chem. **227**, 215 (1934); P. E. VERKADE, J. VAN DER LEE, A. J. S. VAN ALPHEN and M. ELZAS, Proc. Royal Acad. Amsterdam **38**, 945 (1935).

best be tested by means of comparative experiments in which the neutral sodium salts of the dicarboxylic acids are supplied by injection. Experiments of this nature are now in progress.

In the meantime it has however been shown in the following way that the just mentioned assumption is indeed correct:

It has already been shown in part VII of this series [20] that in feeding experiments on dogs with sebacic acid (C_{10}) and with undecanedioic acid (C_{11}), dicarboxylic acids with 8 and 6 or 9 and 7 carbon atoms respectively were also present in the urine. Naturally we have also examined the urine obtained in feeding experiments with the other dicarboxylic acids from this point of view. In the experiments with suberic acid (C_8) the dicarboxylic acid with 6 carbon atoms was also found to be present. In those with brassylic acid (C_{13}), which itself practically failed to be excreted, we nevertheless found lower dicarboxylic acids, namely those with 11, 9 and 7 carbon atoms, in appreciable amounts in the urine. Finally also in the experiments with hexadecanedioic acid (C_{16}), which was itself never encountered in the urine, lower dicarboxylic acids were however found to be excreted and indeed those with 10, 8 and 6 carbon atoms. These facts are summarised in the accompanying table. They lead to the unavoidable conclusion that *the dicarboxylic acids with 6—11 carbon atoms are more difficultly catabolised by the organism, at least by that of the dog, than the higher members of the series.*

Administered	*Present in the urine*
C_4 succinic acid	
C_6 adipic acid	C_6
C_8 suberic acid	C_8 C_6
C_{10} sebacic acid	C_{10} C_8 C_6
C_{11} undecanedioic acid	C_{11} C_9 C_7
C_{13} brassylic acid	C_{11} C_9 C_7
C_{16} hexadecanedioic acid	C_{10} C_8 C_6

These experiments on the "combustibility" of dicarboxylic acids *in vivo* did not furnish any indication of a contrast in this respect between the even and the odd members of the series. That does not however mean to say that in our opinion such a contrast is to be considered as definitely excluded [22].

The question as to the cause of this increase of the "combustibility" on ascending the series can best be dealt with in connection with the discussion of the injection experiments previously mentioned. Consequently reference may be made to part XI for this. To prevent misunderstanding it may be mentioned here that by the term *"combustibility"* we merely wish to indicate in a very general way the possibility of being catabolised

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22) See F. P. MAZZA, Arch. sci. biol. (Ital.) **22**, nr. 3 (1936).

and that the "velocity of oxidation" of a dicarboxylic acid [23]) — already in itself a very complex concept — need in no wise be *a priori* regarded as the only factor which determines its "combustibility".

Finally these experiments confirm in a very convincing way our idea, based as yet really only on our experiments with sebacic acid and undecanedioic acid, *that all normal saturated dicarboxylic acids are catabolised by bilateral β-oxidation.*

§ 6. The above facts in our opinion leave little or no reasonable doubt *that in fact all normal saturated fatty acids are catabolised by the dog besides by the classical way of unilateral β-oxidation by ω-oxidation and subsequent bilateral β-oxidation of the dicarboxylic acids produced.* Direct evidence is still missing only for the ω-oxidation of the *higher* fatty acids; unfortunately with regard to these we had as yet to be satisfied with pointing out the general character of methyl group oxidation (§ 2) and with a few demonstrations that the higher dicarboxylic acids are very well acceptable as intermediate products (§ 5). Naturally our attention remains fixed on this gap and we hope to arrive at an exact proof in this case also.

In our opinion it is quite beyond dispute that than the way of degradation of the fatty acids discovered by us does play a part also with other species of animals and in man. Further researches on this matter — among others on the rabbit (which on the basis of the work of R. KUHN, F. KÖHLER and L. KÖHLER may be considered as a very suitable experimental animal) and on man — are on our program. It may be expected that the quantitative picture of the phenomena will vary therewith.

We can leave undiscussed here the question first raised in the literature by B. FLASCHENTRÄGER and K. BERNHARD [24]) and which had already occurred to us before that, whether an "anchoring" of the carboxyl group of the fatty acid is favourable or necessary for the occurrence of ω-oxidation.

At the same time the facts mentioned do to some extent explain why only lower simple triglycerides give rise to diaciduria in dogs [2]) and also in man [1]). The variation of the diacidogenic properties of these glycerides on ascending the homologous series is however by no means quite clear as yet. In particular there is no parallelism between the diacidogenic action of these substances in the dog and the "combustibility" observed there of the corresponding ω-oxidation products administered *per os*: tricaprylin (C_8) was here found to be the most strongly diacidogenic, the most difficultly "combustible" on the other hand not the corresponding dicarboxylic acid but very probably adipic acid (C_6). Further research

[23]) See F. P. MAZZA, loc. cit.; N. L. EDSON, Biochem. J. **30**, 1855 (1936).

[24]) Helv. chim. acta **18**, 962 (1935); see also B. FLASCHENTRÄGER, K. BERNHARD, C. LÖWENBERG and M. SCHLÄPFER, Z. physiol. Chem. **225**, 157 (1934) and K. SCHLUMPF, loc. cit.

is necessary here. We shall not anticipate regarding the many questions which arise in this connection.

§ 7. Unfortunately very little can yet be answered with certainty to the important question as to the quantitative relationship in normal cases between ω-oxidation followed by bilateral β-oxidation on the one hand and unilateral β-oxidation (and possibly other ways of degradation of the fatty acids) on the other hand. It must be expected that it is different for each kind of animal, for each individual and also for each fatty acid and furthermore depends on the form in which the fatty acid is administered (sodium salt, triglyceride, etc.). We are convinced that the first mentioned way of degradation can play an important part. The question raised by B. FLASCHENTRÄGER and K. BERNHARD [25]) whether the above mentioned ratio can change in the case of illness must certainly be answered in the affirmative. An argument for this is already to be found in the connection of the two ways of degradation of the fatty acids known up to the present with carbohydrate metabolism which will be discussed in later communications from our laboratory. Moreover, we found that even a "healthy" man does not always show the same tendency to dioic acid-acidosis and diaciduria.

The basic question is actually always to what extent a fatty acid can be catabolised in the organism via its ω-oxidation product without the threshold concentrations of the "difficultly combustible" dicarboxylic acids in the blood being exceeded and consequently excretion of these acids by the kidneys taking place.

In this connection the following remark of a polemical nature must be made. In § 1 it was already stated that according to B. FLASCHENTRÄGER and K. BERNHARD the way of degradation we have discovered is restricted to the fatty acids with 8—11 carbon atoms. Furthermore these workers [26]) now believe that they can argue that in man and in the dog only a small or even practically insignificant part of these fatty acids undergoes ω-oxidation. Their argumentation consists of a combination of the results of feeding experiments with fatty acid derivatives (sodium salts; esters; triglycerides) and those of feeding or injection experiments with the sodium salts of dicarboxylic acids. Serious objections can be raised to this argumentation, making it worthless. First of all B. FLASCHENTRÄGER and K. BERNHARD, basing themselves on the older and very fragmentary researches on the matter (see § 3), certainly estimate the "combustibility" *in vivo* of the dicarboxylic acids in question too low. Secondly they take no account of the well known fact [27]) that, as a consequence of the

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[25]) Helv. chim. acta **18**, 967 (1935).

[26]) Helv. chim. acta **18**, 966 (1935); Z. physiol. Chem. **238**, 221 (1936).

[27]) See *e.g.* H. J. PRINS, Chem. Weekblad **11**, 483, 784 (1914); **23**, 389 (1926); F. R. GOSS and C. K. INGOLD, J. Chem. Soc. **127**, 2776 (1926).

increased reactivity of newly formed molecules, reactions frequently proceed differently or go further than corresponds to the properties of the ordinary (non-active) forms of the species of molecules taking part therein. The normal saturated dicarboxylic acids in particular offer fine examples of this phenomenon; a number of cases, in which dicarboxylic acids formed by oxidation processes were found to possess a much increased vulnerability to oxidative degradation at the instant of their formation, are enumerated in a paper by P. E. VERKADE [28]). It therefore certainly will not do to apply quantitatively the results of experiments with dicarboxylic acids administered *per os* or subcutaneously, in which the body is flooded from time to time with these substances, to cases in which these acids are formed *in vivo* as intermediate products. A too unfavourable impression of the importance of ω-oxidation and related phenomena is doubtless obtained in such a way.

We are indebted to the VAN 'T HOFF-Fund and the HOOGEWERFF-Fund for financial assistance in the execution of these investigations.

Rotterdam, September 1937.

28) Rec. trav. chim. **46**, 200 (1927).

Chemistry. — *On the analysis of the provitamins A in blood serum.* By A. G. VAN VEEN and J. C. LANZING.

(Communicated at the meeting of October 30, 1937.)

Introduction:

The quantitative determination of vitamin A in blood serum is complicated by the fact that by the side of vitamin A there occur in the serum carotinoids, some of which may serve as provitamin A, since they may be converted into vitamin A, especially in the liver. The qualitative composition of these carotinoids largely depends on the carotinoids in the food consumed.

It is an open question whether one has to reckon only with the concentrations of vitamin A itself or also with the provitamins A present, when wishing to show a relation between the determinations of vitamin A in the blood and the clinical deficiencies (xerophthalmia, hemeralopia) occurring among the population. This relation between clinical deficiencies [1]) and A concentration (both vitamin A itself and provitamin A from the carotinoids) of the blood appeared to us to be much less simple than supposed at first. Perhaps the fact that clinical symptoms develop only some time after the A level of the organism has sunk and that, after a

1) On Java, as also in other tropical countries, ophthalmic deviations as a result of vitamin A deficiency prove to be much more frequent than was formerly supposed.