XXVII. A QUANTITATIVE STUDY OF THE OXIDATION OF PHENYL-FATTY ACIDS IN THE ANIMAL ORGANISM.

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THE theory that fatty acids undergo in the body a series of successive oxidations at the β -carbon atom rests upon several pieces of evidence. Knoop [1904] first came to this conclusion as the result of experiments which depend on the fact that benzoic and phenylacetic acids in the body are resistant to oxidation. The administration of β -phenylpropionic acid or of δ -phenylvaleric acid to a dog led to the appearance of benzoic acid (conjugated with glycine) in the urine, whereas γ -phenylbutyric acid under similar circumstances yielded phenylacetic acid in its conjugated form. It thus appears that two carbon atoms at a time are removed until one of the resistant acids is produced and excreted. The theory received further support from the observations of Embden and his co-workers [1906, 1908], on the perfusion of the surviving liver with blood containing salts of fatty acids. It was shown that only those acids containing an even number of carbon atoms show an increase in acetone formation. Any objection on the grounds of the absence of an *in vitro* analogy was removed by the discovery of Dakin [1908, 1, 2, 3], that the action of hydrogen peroxide on the ammonium salts of fatty acids leads to the formation of β -oxidation products.

More recently, however, it has been shown that with this reagent types of oxidation other than β -oxidation may occur. The action of hydrogen peroxide on the ammonium salts of a number of straight chain acids of the fatty series was found by Clutterbuck and Raper [1925] to yield products proving the existence of both γ - and δ - as well as of the more usual β -oxidation. It was suggested that γ - and δ -oxidation might occur in the body and be followed by rupture of the chain between the γ - and δ -carbon atoms thus splitting off succinic acid which is known to be readily oxidised *in vivo*. This scheme would result in the splitting off of four carbon atoms at a time and hence lead to the same end product as the β -theory. In this way a theory of γ - or δ -oxidation would explain Embden's results already mentioned and the physiological oxidation of δ -phenylvaleric acid as well as the accepted β -oxidation theory. It thus seemed possible that in the case of this acid β -, γ - and δ -oxidation all occurred and gave rise to the same product. With γ -phenylbutyric acid, on

the other hand, γ -oxidation might possibly give rise to some benzoic acid which would be excreted along with the phenylacetic acid obtained by β -oxidation. In view of this possibility, it seemed worth while to repeat the experiments of Knoop [1904] and Dakin [1908, 5] on this acid to see whether after its administration any benzoic acid above that normally present could be detected in the urine.

The series of phenyl-fatty acids administered by Knoop [1904] and Dakin [1908, 4, 5; 1909, 1] to dogs give as their end products either benzoic or phenylacetic acid. Now benzoic acid when fed to man can be recovered almost quantitatively (96 %) from the urine [Dakin, 1909, 2]. When fed to or injected into dogs, Delprat and Whipple [1921] found that from 70 to 100 %, according to the particular dog used, was excreted in the urine in the form of hippuric acid. If these acids are the sole end products of the oxidation of the higher acids of the same series, then for each molecule of the higher acid, one molecule of benzoic or phenylacetic acid, as the case may be, should be produced. When Dakin's figures are converted so as to represent the quantity of hippuric or phenaceturic acid recovered compared with that theoretically possible if simple quantitative β -oxidation and complete excretion occurred, we get recoveries of 41 to 53 % (in different experiments) for β -phenylpropionic acid and 28 to 33 % for γ -phenylbutyric acid. Dakin gives no figure for δ -phenylvaleric acid but Knoop who does not mention the yield with the above two substances obtained a 33 % recovery in this case. It must be remembered, when comparing these results with those obtained by us, that, on the one hand, the urine was not worked up in such a way as to obtain the whole of the excreted acids and, on the other, the doses given were relatively large, from 0.5 to 1.0 g. per kg. However, it seemed that the 40 to 70 % of these acids which remained unaccounted for might easily have been converted in part into a γ -oxidation product or suffered some other change in the organism and been lost during the working up. It was therefore decided to administer to a dog as many normal phenyl-fatty acids as could conveniently be synthesised in order to see to what extent the products could be recovered when definitely quantitative methods were employed.

Some difficulty was at first found in devising a suitable method for the estimation of a mixture of benzoic and phenylacetic acids but one was eventually evolved and is described in a separate paper [Wayne, 1928]. It was realised that account would have to be taken of the amount of hippuric acid normally present in dog's urine and this was estimated before and after each experiment and allowed for. The amounts injected were such as to yield, if completely β -oxidised, approximately 1.0 g. of benzoic or phenylacetic acid, *i.e.* with the dogs employed a dose of only 0.10 to 0.20 g. per kg.

A list of the acids administered together with the results obtained is given in Table I. It will be noticed that, in the case of benzoic and phenylacetic acids, recoveries of approximately 80 % were obtained in the case of dogs "A" and "C" and 85-90 % in the case of dog "B."

Table I.

Dog	Acid administered	% recovery	Excreted (conjugated with glycine) as
A	Benzoic acid	78	Benzoic acid
A	Phenylacetic acid (i)	78	Phenylacetic acid
	(ii)	79	
A	β -Phenylpropionic acid	77	Benzoic acid
A	γ -Phenylbutyric acid	80	Phenylacetic acid
A	δ -Phenylvaleric acid (i)	74	Benzoic acid
	(ii)	75	
B	Benzoic acid	85	Benzoic acid
B	Phenylacetic acid	90	Phenylacetic acid
	ϵ -Phenylcaproic acid	88	"
	ι -Phenyldecoic acid (i)	64	
	(ii)	60	"
C	Benzoic acid	79	Benzoic acid
C	θ -Phenylnonoic acid	64	"

Delprat and Whipple [1921] were unable to correlate a similarly varying incomplete recovery of benzoic acid with age, sex, dose or any other factor. The findings of Quick [1926] agree in this. For the present purpose, however, it is only necessary to compare the figures for these two acids with those for the remainder where oxidation of the side chain has occurred in the organism. It will be seen that, in the case of the acids containing 3, 4, 5 and 6 carbon atoms in the side chain the figures are identical within experimental error with the benzoic or phenylacetic acid figures. Moreover by titration it was possible to show that the final acid obtained was always that demanded by the β -oxidation theory nor were any higher homologues detected. This is of especial interest in the case of γ -phenylbutyric acid where γ -oxidation might have been expected to give rise to some benzoic acid along with the phenylacetic acid produced by β -oxidation. It has therefore been demonstrated that complete quantitative β -oxidation occurs with the four simplest phenyl-fatty acids capable of undergoing this process.

With the two longer chain acids containing 9 and 10 carbon atoms in the side chain definitely lower figures were obtained than would be expected if such quantitative β -oxidation had occurred. The fate of the equivalent of the 25 to 30 % of phenylacetic acid unaccounted for in the case of ι -phenyldecoic acid as of the equivalent of the 15 % of benzoic acid in the case of θ -phenylnonoic acid is not easily explained. There are some phenyl derivatives which are completely oxidised to carbon dioxide and water in the animal organism. These are notably the α -amino- and α -keto-phenylpropionic acids and some of their nuclear substitution derivatives, and it may be that a proportion of these higher acids is converted to some such completely oxidisable product and so escapes detection. It is also conceivable that the higher acids take longer to oxidise than the lower acids because the greater length of the side chain would necessitate a greater number of successive β -oxidations. It may happen therefore, owing to this greater length of time during which the acids with the longer side chains are undergoing oxidation, that a greater opportunity for oxidation and disruption of the benzene nucleus will occur and this would result in a smaller yield of benzoic or phenylacetic acid as the

case may be. Whatever the explanation, quantitative β -oxidation still remains unproved in the case of these long chain phenyl-fatty acids and it must be emphasised that they correspond more closely to the fatty acids normally undergoing metabolism in the body than do the lower members of the series.

It was possible to show that the final acid excreted as a result of the oxidation of these two acids also was solely that required on the hypothesis of the removal of two or a multiple of two carbon atoms at a time. There was, moreover, no indication that the oxidation of the acid administered was not completed within the experimental period. The amount of benzoic or phenylacetic acid excreted in the after period was not different from that obtained in other experiments with the simpler acids. Moreover, no acid intermediate between the acid administered and benzoic or phenylacetic acid in the two cases could be detected. The demonstration by Dakin [1909, 1] of derivatives of phenylpropionic acid in the urine after the administration of δ -phenylvaleric acid occurred after large doses (0.75 g. per kg.) of the latter acid had been given.

Flaschenträger and co-workers in a series of papers [see Flaschenträger 1926] have examined the conduct in the body of a series of substituted ω -amino-fatty acids of general formula $C_6H_5SO_2N(CH_3).(CH_2)_n.COOH$. These undergo β -oxidation in a similar fashion to the phenyl-fatty acids and are excreted as the unoxidisable propionic or butyric acid of the series according to the number of carbon atoms in the fatty acid chain. The results of these workers though not strictly quantitative are very similar to those obtained by us. Quantitative β -oxidation seems to occur with the lower members of this series and no intermediate products were detected even with the higher members.

The question of the nature of the intermediate products formed during the oxidation of even so simple a substance as β -phenylpropionic acid is by no means settled. It is unlikely that a single reaction is involved in the shortening of the carbon chain by two carbon atoms in the course of a β -oxidation and it has been suggested that oxidation of a saturated acid gives rise, in the first stage, to the α - β unsaturated acid, *i.e.* cinnamic acid in the above case rather than to a β -keto- or β -hydroxy-acid.

Kay and Raper [1924] found when dogs were injected with β -phenyl- α -methylpropionic acid and α -methylcinnamic acid respectively that whereas the former yielded 77 % of the expected amount of benzoic acid if all had undergone β -oxidation the latter under comparable conditions only gave 30 %. It was concluded, therefore, that the formation of the unsaturated acid was not an obligatory stage in the oxidation of the saturated acid, for otherwise the yields should have been about the same. It seemed possible that conjugated double bonds might in general confer, as in this case, a greater susceptibility to oxidation upon the benzene nucleus. It was hoped that an examination of the extent to which the oxidation products of cinnamic and phenyl-*iso*-crotonic acids could be recovered when compared with the results in the case of β -phenylpropionic and γ -phenylbutyric acids might throw similar light

on the problem of the mode of oxidation of these latter substances. The results are given in Table II. It will be seen that these unsaturated acids yield β -oxidation products to the same extent, allowing for experimental error, as the saturated acids and therefore remain quite possible intermediate substances in the course of oxidation of the latter.

Table II.

Dog	Acid administered	% recovery	Excreted (conjugated with glycine) as
A	β -Phenylpropionic acid	77	Benzoic acid
A	Cinnamic acid	74	"
A	γ -Phenylbutyric acid	80	Phenylacetic acid
A	Phenyl- <i>iso</i> -crotonic acid	77	"

EXPERIMENTAL.

Preparation of materials.

γ -Phenylbutyric acid. The most rapid method of preparing this acid is from γ -phenyl-*n*-propyl alcohol. This was converted to γ -phenyl-*n*-propyl bromide by the method of Rupe and Bürgin [1910]. The compound with magnesium was then formed by the Grignard reaction and this was decomposed by carbon dioxide giving the required acid [Rupe and Proske, 1910]. In this way 8 g. of pure acid were obtained from 27 g. of the alcohol.

δ -Phenylvaleric acid. Sodium (3.3 g.) was dissolved in alcohol (50 cc.) and to the well-cooled solution diethyl malonate (23 g.) was added followed by γ -phenyl-*n*-propyl bromide (28 g.). After standing for half an hour the mixture was heated on the water-bath for 3 hours, cooled, water added and the oil extracted with ether. The ether was distilled off after drying and the oil fractionated *in vacuo*. The diethyl δ -phenyl-*n*-propylmalonate (24 g.) so obtained was collected at 190–200°/15 mm. and hydrolysed by boiling with alcoholic potash for 2 hours. The alcohol was evaporated and the residue dissolved in water was treated with hydrochloric acid precipitating δ -phenyl-*n*-propylmalonic acid. This agreed in its properties with the acid differently prepared by Borsche [1912]. The acid was heated *in vacuo* till no further gas was evolved and then distilled *in vacuo* when δ -phenylvaleric acid (6 g.) passed over at 190–193°/30 mm. and was recrystallised from hot water, when it had M.P. 58.5–59.5°.

The higher acids were prepared by the same general method. The appropriate dibasic acid of the succinic series was converted to its acid ethyl ester and thence to the carbethoxy-acyl chloride. By the Friedel-Crafts reaction with benzene this gave an ω -benzoyl-fatty acid ester which was reduced to the required phenyl-fatty acid by Clemmensen's method [1914].

ϵ -Phenylcaproic acid. Adipic acid was converted to ethyl hydrogen adipate by esterification with insufficient alcohol as described by Blaise and Koehler [1910]. The normal ester also produced in this reaction was then semi-hydrolysed by the method of Grün and Wirth [1922] described for the semi-hydrolysis of ethyl sebacate. Ethyl hydrogen adipate was converted to the

acid chloride by means of thionyl chloride. δ -Carbethoxyvaleryl chloride (25 g.) in dry benzene (200 g.) was treated with anhydrous aluminium chloride (33 g.). After the vigorous initial reaction had subsided the mixture was heated on the water-bath for an hour when it was cooled and poured on to ice with the addition of hydrochloric acid. From the benzene layer, washed with water and sodium bicarbonate solution and dried, the benzene was distilled off and the residue vacuum-distilled. In this way ethyl δ -benzoylvalerate (16 g.) was obtained and collected at 200–210°/30 mm. A little on hydrolysis gave δ -benzoylvaleric acid agreeing in properties with that described by Bauer [1914]. The ester (15 g.) was placed in a flask with amalgamated zinc [Clemmensen, 1914], alcohol (100 cc.) and 30 % hydrochloric acid (100 cc.) and the whole boiled vigorously for 20 hours during which time an additional 300 cc. of 30 % hydrochloric acid was added. The cooled contents were then extracted with ether, the ether evaporated and any ester remaining was hydrolysed by boiling with caustic soda for half an hour. The acid liberated by treatment with hydrochloric acid was extracted with ether and distilled *in vacuo*. The fraction collected at 190–205°/25 mm. gave a low equivalent. It was dissolved in caustic soda, the solution extracted with ether and after acidification the acid extracted with light petroleum. In this way ϵ -phenylcaproic acid was obtained after evaporation of the light petroleum, equivalent 191.5 (calculated 192). It is a liquid agreeing in its properties with that described by v. Braun [1911].

theta-Phenylnonoic acid. Azelaic acid was converted to ethyl hydrogen azelate by the same methods as were used for adipic acid. The acid ester was converted to η -carbethoxy-*n*-octoyl chloride by means of thionyl chloride [Robinson and Robinson, 1926]. The Friedel-Crafts reaction was conducted precisely as is described above for the lower homologue. On evaporating the benzene at the end of the reaction a mixture of crude acid and crude ester was obtained in this case. This was treated with alcohol saturated with hydrogen chloride so as to convert the whole to ester and the ester distilled *in vacuo*, the fraction boiling between 175–200°/12 mm. being collected and hydrolysed. The η -benzoyloctoic acid so obtained was recrystallised from a large amount of light petroleum to separate it from traces of azelaic acid and finally recrystallised from dilute alcohol. The acid forms fine colourless irregular plates from this solvent; M.P. 74.5–75°. Equivalent 249.5 (calculated 248).

Analysis of η -benzoyloctoic acid: found, C 72.67, H 8.18 %; calculated for $C_{15}H_{20}O_3$, C 72.59, H 8.64 %.

The acid (0.4 g.) heated with semicarbazide hydrochloride (0.26 g.) and sodium acetate (0.5 g.) in 90 % acetic acid for half an hour on the water-bath gave the semicarbazone of η -benzoylnonoic acid. This separates from alcohol in fine needles; M.P. 151.5–152°.

Analysis: found, C 62.5, H 7.3 %; calculated for $C_{16}H_{23}O_3N_3$, C 62.9, H 7.5 %.

The acid was esterified and reduced by Clemmensen's method in the same way as is described above for δ -benzoylvaleric acid. θ -Phenylnonoic acid so produced distills at $215^{\circ}/12$ mm. and solidifies in ice to a waxy solid; m.p. $29-30^{\circ}$. Equivalent 233 (calc. 234).

Analysis: found, C 77.03, H 9.08 %; calculated for $C_{15}H_{22}O_2$, C 76.93, H 9.39 %.

ι -Phenyldecoic acid. θ -Carbethoxy-*n*-nonoyl chloride was prepared from sebacic acid by the method described above for the valeryl and octoyl compounds. By the Friedel-Crafts reaction conducted as has been described for these latter compounds this was converted into θ -benzoylnonoic acid, m.p. 84° , identical in properties with that described by Borsche and Wollemann [1911] by whose method it was also made but with less satisfactory results. The ethyl ester of θ -benzoylnonoic acid was reduced by Clemmensen's method to *ι -phenyldecoic acid* and purified as described in the case of ϵ -phenylcaproic acid. *ι -Phenyldecoic acid*, distilling at $196^{\circ}/0.4$ mm., is a white waxy solid; m.p. 42° . Equivalent 249 (calc. 248). It has been described by Borsche [1919] and the specimen agreed in its properties with the description of this observer.

GENERAL METHOD OF CONDUCTING THE EXPERIMENTS.

The subjects of the experiments, fox terrier bitches of age 1-2 years and about 10 kg. weight, were fed on a standard diet consisting of Spratt's ovals, potatoes and lard. The amounts were varied until the animal maintained a constant weight. During an experiment the animal was kept in a metabolism cage. Each morning it was transferred to a fresh cage and the urine passed during the preceding 24 hours was collected, the cage being well washed down with about a litre of water. The urine and washings were filtered, made slightly alkaline and boiled down to a small bulk on the water-bath.

For two days before an injection the urine was collected and the benzoic acid present during this time determined to give the *initial blank*. The acid to be injected was weighed out, carefully neutralised with standard sodium hydroxide and made up to about 25 cc., the solution being weighed. In the case of cinnamic acid the ammonium salt was employed, as sodium cinnamate is rather insoluble in water. About 10 cc. of solution were taken up from a known weight into a hypodermic syringe and injected into the dog, the site of injection being the loose subcutaneous tissues of the posterior cervical region. The weight of solution injected was determined by difference. The appropriate amount of solution in the case of phenylnonoic and phenyldecoic acids was divided into two and injected on two consecutive days, as these acids tended to produce toxic symptoms in the animal. The urine was collected for 3 days following the last injection, the specimens added together, boiled down to a small bulk and made up to 250 cc. Duplicate determinations were then made on measured volumes of the solution giving the *experimental figure*.

Table III.

Dog	Acid administered	Amount injected g.	Equivalent to benzoic (B) or phenylacetic (P) acid g.	Initial blank benzoic acid g. per diem	Final blank benzoic acid g. per diem	Average blank benzoic acid g. per diem	Amount of benzoic acid normally in urine during experi- mental period (Y) g.	Total of acids excreted during experi- mental period (X) g.	Average of esti- mations of (X) g.	Excess of acid present during experi- mental period X-Y g.	Recovery % 78
10.5 kg. A	Benzoic	1.00	1.00 (B)	0.040	0.058	0.049	0.147	0.910	0.925	0.778	78
A	Phenylacetic	1.02	1.02 (P)	0.045	0.063	0.054	0.162	0.940	0.955	0.793	78
A	"	1.045	1.045 (P)	0.063	0.035	0.049	0.147	0.953	0.973	0.826	79
A	Phenylpropionic	1.28	1.04 (B)	0.031	0.064	0.047	0.141	0.973	0.940	0.799	77
A	Phenylbutyric	1.23	1.025 (P)	0.050	0.040	0.045	0.135	0.945	0.960	0.825	80
A	Phenylvaleric	1.42	0.975 (B)	0.037	0.057	0.047	0.141	0.945	0.875	0.734	75
A	"	1.45	0.990 (B)	0.038	0.071	0.054	0.162	0.855	0.897	0.735	74
A	Cinnamic	1.20	0.990 (B)	0.032	0.062	0.046	0.138	0.895	0.866	0.728	74
A	Phenyl-iso-crotonic	1.17	0.980 (B)	0.055	0.055	0.055	0.165	0.873	0.917	0.752	77
8.7 kg. B	Benzoic	1.03	1.03 (B)	0.069	0.066	0.067	0.201	0.860	1.080	0.879	85
B	Phenylacetic	1.04	1.04 (P)	0.067	0.049	0.058	0.174	1.080	1.112	0.938	90
B	Phenylcaproic	1.37	0.975 (P)	0.057	0.064	0.060	0.180	1.045	1.042	0.862	88
B	Phenyldecoic	1.79	0.985 (P)	0.052	0.053	0.0525	0.157	1.040	0.789	0.632	64
B	"	1.81	0.995 (P)	0.042	0.032	0.037	0.148 (4 days)	0.777	0.740	0.592	60
C	Benzoic	1.01	1.01 (B)	0.034	0.039	0.0365	0.108	0.800	0.905	0.797	79
7.0 kg. C	Phenylnonoic	1.945	1.01 (B)	0.040	0.036	0.038	0.152 (4 days)	0.735	0.797	0.645	64
								0.744			
								0.910			
								0.795			
								0.800			

The amount of benzoic acid present in the urine during 2 days following the experimental period was also estimated giving the *final blank*. This served to ensure that the benzoic acid excretion had fallen back to its normal value. In agreement with the observations of other investigators it was found that 3 days were sufficient for the complete excretion of products of injected acids of this type.

The average of the *initial* and *final blanks* was used to determine the amount of benzoic acid which would normally have been excreted during the experimental period and this was deducted from the *experimental figure*.

The recovery was then expressed as the percentage of that which would have occurred if one molecule of the acid injected had given one molecule of phenylacetic or benzoic acid, as the case may be, and the whole of this had been excreted.

The experimental figures are given in Table III. It will be seen that in the different dogs the blank figures are fairly constant, especially the average blank which is the average over 4 days. Some error is, however, necessarily introduced in calculating the amount of benzoic acid normally present in the urine during the experimental period from this figure. There is a further possible source of error in the estimation of the acid excreted during the experimental period and it is probable that differences of less than 5 % in the figure expressing the recovery are due to experimental error. In those cases where phenylacetic acid was the end product of oxidation the benzoic acid excreted along with it as the result of the normal metabolism of the dog was estimated by titration of the weighed sublimate. This figure for benzoic acid agreed within experimental error with the amount calculated from the average blank.

SUMMARY.

(1) Normal phenylpropionic, phenylbutyric, phenylvaleric and phenylcaproic acids when administered to dogs yield amounts of benzoic and phenylacetic acid, as the case may be, which indicate that the fatty acid side chain is oxidised quantitatively in accordance with the theory of β -oxidation.

(2) Under the same conditions, phenylnonoic and phenyldecoic acids yield smaller amounts of benzoic and phenylacetic acid respectively than would be expected if quantitative β -oxidation of the side chain takes place.

(3) This suggests that some other mode of oxidation, in addition to β -oxidation, takes place with phenyl-fatty acids containing side chains approaching in length those of the higher fatty acids, but evidence as to the nature of this additional mode of oxidation has not been obtained.

(4) Cinnamic and phenyl-*iso*-crotonic acids yield the same amounts of benzoic and phenylacetic acid respectively as the corresponding saturated acids.

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