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The Effect of Surface-active Agents on Bacterial Glutamic Decarboxylase and Glutaminase

By D. E. HUGHES, *Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield* 10

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In previous papers (Krebs, 1948; Hughes, 1949) it was shown that *n*-hexadecyltrimethylammonium bromide (Cetavlon) accelerates the decarboxylation of glutamate and the deamidation of glutamine by intact cells and extracts of *Clostridium welchii*, *Proteus morgani* and *Escherichia coli*. Kinetic studies (Hughes, 1949) suggested that the effect of the detergent might be due to the removal of a competitive inhibitor. In the present work the effect of a variety of surface-active agents on the bacterial glutamic acid decarboxylase and glutaminase has been studied. Acceleration was given only by those cationic surface-active agents which can form micelles, and the concentration of the agents required to form micelles was about the same as that producing maximum acceleration.

MATERIAL AND METHODS

Organisms and extracts. Intact washed cells and extracts of *Cl. welchii*, *Pr. morganii* and *Esch. coli* were prepared as previously described (Hughes, 1949).

Glutamine. Crude L-glutamine (66% pure as judged by amide/N estimation) was prepared from mangel-wurzels by the procedure described by Vickery, Pucher & Clark (1935). It contained, among other impurities, 2% arginine and traces of Zn (see Hughes, 1948), and was freed from these by the following method (see Archibald, 1945). A solution of crude glutamine (35 g.) in 400 ml. water at 60° was cooled to room temperature, saturated with H_2S, and left for 2 hr. After addition of norite (4 g.) the suspension was filtered through paper on to a column (1·5 cm. diameter and 30 cm. long) of 60–80 mesh permutite (Permutite Co. Ltd., Gunnersbury Avenue, London, W. 4), which had been previously washed with 0·5% NaCl. The glutamine solution was allowed to run through by gravity, and the column was washed twice with 20 ml. water. The combined washings and filtrate were evaporated under reduced pressure at 40° until crystals appeared. After addition of warm ethanol (2 vol., at 40°), the solution was left at room temperature and then at 2° until crystallization had finished. The glutamine crystals were collected, recrystallized from water-ethanol as before, washed with 60% ethanol, 40% water (v/v) mixture and dried *in vacuo* over P_2O_5. Yield 22 g. (Found: amide N, 9·4; total N, 18·9; CO_2 evolved by the method of Gale (1947), 28·6; arginine, 0·01. Calc. for $C_5H_{10}O_3N_2$: amide N, 9·6; total N, 19·2; CO_2, 29·6%.)

n-Alkyltrimethylammonium bromides. The Cetavlon (*n*-hexadecyltrimethylammonium bromide) was a specially pure sample supplied by Mr S. Ellingworth, Imperial Chemical Industries (Pharmaceuticals) Ltd. Other homologues in this series were synthesized from the *n*-alkyl bromides by treatment with trimethylamine. The alkyl bromides were prepared from the corresponding alcohols which were obtained as follows: commercial samples of *n*-heptyl, *n*-decyl and *n*-dodecyl alcohols were supplied by L. Light and Co. Ltd., Wraysbury, Bucks, and *n*-octadecyl alcohol by British Drug Houses Ltd. These were purified by fractional distillation. Tetradecyl alcohol was prepared by the method of Levene & Taylor (1924) from recrystallized

and redistilled myristic acid (British Drug Houses Ltd.). Docosyl alcohol ($C_{22}H_{49}O$) was obtained from erucic acid, prepared from rape-seed oil. The acid was converted into the ethyl ester (m.p. 48–49°) (Levene & Taylor, 1924), which was recrystallized from ethanol and converted into docosyl alcohol according to Adam (1925). The yield of the latter (m.p. 83°) was 15% of the erucic acid used; the greatest loss occurred in the last step of preparation.

The alcohols were converted to the corresponding *n*-alkyl bromides by the method of Kamm & Marvel (1920). Refluxing the *n*-alkyl bromides in an excess of alcoholic trimethylamine (Scott & Tartar, 1943) resulted in the formation of the corresponding *n*-alkyltrimethylammonium bromides, which were recrystallized twice from acetone or acetone-benzene according to solubility. The yield of the conversion of *n*-alkyl bromides to the alkyltrimethylammonium bromides was 60–80%. The halogen content was estimated by Volhard's method and was correct within ±2%.

Measurement of enzyme activity. The rate of decarboxylation of glutamate and glutamine was determined as described previously (Hughes, 1949). The rates are expressed as Q_{CO_2} (μl./mg. dry wt. cells/hr.). When extracts were used, Q_{CO_2} referred to the dry wt. of cells from which the extracts had been prepared.

Chemical estimations. Amide N was determined by hydrolysis at pH 6·0 at 100° (Vickery, Pucher, Clark, Chibnall & Westall, 1935) or enzymically with suspensions of *Cl. welchii* (Krebs, 1948). Arginine was determined by estimation of the urea formed on the addition of arginase. Zn was estimated by the dithiozone method of Vallee & Gibson (1948).

RESULTS

The effect of n-*alkyltrimethylammonium bromides*

Concentration of Cetavlon and decarboxylation of L-*glutamate.* Cetavlon had no appreciable effect on the decarboxylation of glutamate by intact cells and extracts of *Cl. welchii* when the concentration was below 5×10^{-5}M. When the concentration was raised above 5×10^{-5}M the degree of acceleration rose sharply with increasing concentration and was maximal between 2·5 and $5{\cdot}0 \times 10^{-4}$M. The rate of decarboxylation was reduced approximately 10–15% by 10^{-2}M-Cetavlon, and this inhibition increased as Cetavlon concentration was raised above 10^{-2}M. When the rates of decarboxylation (Q_{CO_2}) were plotted against the negative log. of the Cetavlon concentration, sigmoid curves were obtained (see Table 1 and curve 'Hexadecyl', Fig. 1), resembling curves showing the effect of the concentration of surface-active agents on other properties of their solutions. For instance, the change in electrical conductivity shows the same rapid approach to maximal effect with increasing concentration (Scott & Tartar, 1943). A similar curve is found when surface tension is measured (Ralston, 1946). According to McBain (1944), the abrupt change in the properties of surface-active agents is connected with the formation of micelles.

Homologous n-*alkyltrimethylammonium bromides.* The concentration at which surface-active agents form micelles ('critical point') decreases with increasing chain length of the alkyl substituent. The measurements of electrical conductivity of Scott & Tartar (1943) indicate that in the *n*-alkyltrimethylammonium bromide series a minimum chain length of at least eight carbon atoms is necessary before micelle formation can occur. If the ability to form micelles is essential for the action of a quaternary salt on the glutamic decarboxylase, it would be expected that homologues with an alkyl chain of less than eight carbon atoms would have no effect.

Table 1. *Rate of decarboxylation of glutamate at varying concentrations of Cetavlon*

(Each flask contained 2·2 ml. solution (extract of *Cl. welchii* equivalent to 2 mg. dry wt. cells; 0·1M-acetate buffer, pH 4·1; 0·0045M-glutamate; Cetavlon solution 0·5 ml.). All concentrations are final concentrations. After completion of decarboxylation, 2 drops of 0·04% methyl orange were added.)

Concentration of Cetavlon (M × 10^{-5})	Q_{CO_2} (first 10 min.)	Colour of methyl orange*
250	306	Canary yellow
125	306	Canary yellow
50	306	Canary yellow
37·5	306	Yellow orange
18·8	300	Orange yellow
12·5	132	Orange
6·25	132	Orange
3·25	132	Orange
0·14	132	Orange
None	129	Orange

* See text, p. 233.

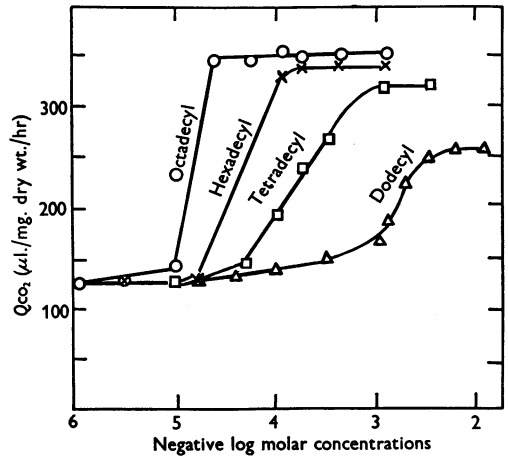

Fig. 1. Homologous *n*-alkyltrimethylammonium bromides and the rate of decarboxylation of glutamate by an extract of *Cl. welchii*. Each flask contained 2·2 ml. solution (extract equivalent to 3·5 mg. dry wt.; 0·1M-acetate buffer, pH 4·1; 0·0045M-glutamate; 0·5 ml. solution of the homologue, indicated in the figure). All concentrations are final concentrations.

Examination of a number of *n*-alkyltrimethylammonium bromides showed that this is the case. The ethyl and *n*-heptyl homologues did not accelerate the decarboxylation of glutamate by intact cells or extracts in concentrations up to 10^{-1}M, and were inhibitory at higher concentrations. The decyl homologue accelerated (20%) in all batches of intact cells, but only in some batches of extracts, at a concentration of 5×10^{-2}M, while homologues with alkyl chains of between twelve and twenty-two carbon atoms accelerated the enzymic reaction in all batches of cells and extracts. Fig. 1 shows the concentration and effect of some members of the series on the rate of decarboxylation of glutamate in an extract. Similar results were obtained with intact cells. It will be seen that as the length of the alkyl chain increased the maximum acceleration was greater and the concentration of the homologue required to give maximum acceleration was lower. Another point of interest is that the slope of the ascending parts of the curves was the steeper the longer the alkyl chain. The maximum effect was obtained by homologues with an alkyl chain of sixteen carbon atoms in the case of cells, and from eighteen to twenty carbon atoms in the case of extracts (see Table 2).

Table 2. *Maximum acceleration of the decarboxylation of glutamate by homologous* n-*alkyltrimethylammonium bromides*

(Each flask contained 2·2 ml. solution (extract or cells of *Cl. welchii* equivalent to 3·15 mg. dry wt.; 0·1M-acetate buffer, pH 4·1; 0·0045M-glutamate; 0·5 ml. solution of homologue). All concentrations are final concentrations.)

Homologue added	Minimum concentration of homologue causing maximum acceleration (M)	Q_{CO_2} (first 10 min.) Intact cells	Extract
None	—	85	65
Ethyl	2×10^{-1}	85	65
Heptyl	5×10^{-2}*	85	65
Decyl	5×10^{-2}	102	65
Dodecyl	3×10^{-2}	146	68
Tetradecyl	3×10^{-3}	149	100
Hexadecyl	4×10^{-4}	153	123
Octadecyl	8×10^{-5}	136	134
Docosyl	1×10^{-4}	130	134

* The heptyl homologue inhibited at 10^{-1}M.

These results further support the assumption that micelle formation is an important factor in the effect of Cetavlon on decarboxylation.

Detection of micelles by the use of indicators. Hartley (1923) showed that surface-active agents in concentrations above their critical points change the colour of certain indicator solutions although the pH remains unchanged. The colour change is presumably due to the dye being absorbed by, or dissolved in, the micelles of the detergent. Cetavlon and some other surface-active agents change the colour of solutions of methyl orange in buffer at pH 4·0 from orange to a bright canary yellow. When solutions of methyl orange in acetate buffer containing Cetavlon were compared in a Beckman spectrophotometer with solutions of the dye in anhydrous butanol, the adsorption curves of the solutions were almost identical and markedly different from the orange solutions of the dye in 0·1N-NaOH.

To test qualitatively for micelle formation, two drops of 0·4% aqueous methyl orange were added to the Warburg flasks after the completion of the incubation period. The flasks were then returned to the thermostat for 5 min. and the colour noted. The presence of a bright canary yellow colour was taken as an indication of the presence of micelles.

Corrin & Harkins (1946) used the colour change of indicators by surface-active agents to estimate the 'critical point' of micelle formation. To test the suitability of methyl orange for this estimation the homologous *n*-alkyltrimethylammonium bromides were titrated with the dye in the following manner. Methyl orange (10^{-4}M in 0·05M-acetate buffer, pH 4·1) was placed in a test tube in a comparator, and 2% Cetavlon was then added until the colour was bright yellow. This tube served as a colour control. To another tube, 2·0 ml. of the methyl-orange solution was added, and the solution of the homologue under examination added dropwise from a 2·0 ml. burette. The concentration of the homologue was adjusted to give a reading of from 1·0 to 1·5 ml. when the colour of the experimental tube matched that of the control tube. When the titrations were carried out quickly, in order to avoid the formation of a precipitate which developed on standing, the values for the critical point agreed to within ±5%. The results show approximate agreement with those obtained when 2:6-dichlorophenol-indophenol was used as in the method of Corrin & Harkins (1946) (Table 3). Both sets of results

Table 3. *Maximum acceleration of decarboxylation of glutamate and 'critical point' determined by titration with indicators*

(Maximum acceleration determined in experiments illustrated in Fig. 1; 'critical point' by titration with 2:6-dichlorophenolindophenol and with methyl orange as described in the text.)

n-Alkyltrimethyl-ammonium bromide added	Concentration causing maximum acceleration (M)	'Critical point' (M) 2:6-Dichlorophenolindophenol	Methyl orange
Decyl	$5{\cdot}0$–$7{\cdot}0 \times 10^{-2}$	$6{\cdot}0 \times 10^{-2}$	$6{\cdot}0 \times 10^{-2}$
Dodecyl	$1{\cdot}0$–$3{\cdot}0 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	$2{\cdot}0 \times 10^{-2}$
Tetradecyl	$1{\cdot}0$–$3{\cdot}0 \times 10^{-3}$	$1{\cdot}0 \times 10^{-3}$	$2{\cdot}0 \times 10^{-3}$
Hexadecyl	$2{\cdot}0$–$5{\cdot}0 \times 10^{-4}$	$5{\cdot}0 \times 10^{-4}$	$3{\cdot}0 \times 10^{-4}$
Octadecyl	$6{\cdot}0$–$8{\cdot}0 \times 10^{-5}$	$1{\cdot}0 \times 10^{-4}$	$1{\cdot}0 \times 10^{-4}$

obtained by indicator methods tended to be lower than those obtained by conductimetric methods by Scott & Tartar (1943), probably owing to the effect of acetate on the critical point (Corrin & Harkins, 1947).

Micelles and the acceleration of decarboxylation. As shown in Table 1, concentrations of Cetavlon which change the colour of methyl orange from orange to bright yellow, indicating the presence of micelles, accelerated decarboxylation. On the other hand, when the colour of methyl orange remained unchanged the rate of decarboxylation was not accelerated. Under a variety of experimental conditions with intact cells and with extracts, the colour change of methyl orange and the acceleration of decarboxylation ran parallel. Similar results to that given by Cetavlon were given by other members of the homologous series with chain length of more than ten carbons. The ethyl and heptyl homologues formed no micelles according to the methyl-orange test, nor did they accelerate decarboxylation.

Methyl orange added to suspensions of intact cells was absorbed on to the cells. When added to solutions containing extracts, the dye was absorbed on to a precipitate formed from the extract. The colour of the absorbed dye appeared to be similar to that of the dye in solution; that is to say when no Cetavlon was present the colour of the dye was orange, and when Cetavlon micelles were present it was yellow. It would appear, therefore, that Cetavlon micelles are able to change the colour of dye absorbed on to enzyme material as well as that of dye in solution. The critical point of Cetavlon in acetate buffer alone was $5{\cdot}0 \times 10^{-4}$M, and in buffer with enzyme material it ranged from 2·0 to $5{\cdot}0 \times 10^{-4}$M. Thus enzyme material has no major effect on the critical point, although some Cetavlon is probably bound by the enzymic material. Maximum acceleration for the *n*-alkyltrimethylammonium bromide occurs approximately at the critical point as titrated with indicators (see Table 3). Experiments with other types of cationic detergents showed that maximum acceleration occurred only when methyl orange was changed to bright yellow colour, suggesting that with these compounds maximum acceleration and critical point also coincide.

The effect of compounds other than n*-alkyltrimethylammonium bromides*

Quaternary ammonium compounds. Betaine, trigonelline, choline and acetylcholine were examined at various concentrations up to 0·05M; they did not accelerate or inhibit the decarboxylation of glutamate and glutamine by intact cells and extracts of *Cl. welchii*.

Quaternary nitrogen surface-active agents. All substances tested in this series were found to accelerate the decarboxylation of glutamate and glutamine. The concentration giving the maximum rate of decarboxylation is shown in Table 4. Methyl-orange tests indicated the presence of micelles at the concentration producing maximum acceleration.

Cetavlon and *n*-hexadecylpyridinium bromide, which have the same alkyl substituent, give maximum acceleration at approximately the same concentration, but the degree of acceleration is greater in the case of the pyridinium compound. The effect of chain length of the alkyl group in the pyridinium series appears to be the same as in the alkyltrimethylammonium series.

Some of the other compounds listed in Table 4 give a greater acceleration than Cetavlon although the chain length of their alkyl substituent is shorter.

Other surface-active agents. Other cationic long-chain salts (Table 5) accelerate the decarboxylation of both glutamate and glutamine. The following amidines, not listed in Table 5, were found ineffective: *N'*-*p*-chlorophenyl-N^5:N^5-di-*n*-butyldiguanide acetate, *N'*-α-naphthyl-N^5:N^5-dimethyldiguanide hydrochloride, *N*-(2'-diethylaminoethyl)-2-naphthamidine dihydrochloride (and dihydro-

Table 4. *Quaternary nitrogen surface-active agents and the acceleration of decarboxylation*

(Each flask contained 2·2 ml. solution (intact cells 3·5 mg. dry wt.; 0·1M-acetate buffer, pH 4·1; substance to be tested, 0·5 ml.; 0·0045M-glutamate or glutamine). All concentrations are final concentrations.)

Detergent added	Approximate concentration of detergent causing maximum acceleration (M)	Q_{CO_2} Glutamate	Q_{CO_2} Glutamine
None	—	362	146
Cetavlon	5×10^{-4}	740	406
N-*n*-Dodecylpyridinium bromide	2×10^{-3}	815	435
N-*n*-Hexadecylpyridinium bromide	2×10^{-4}	900	540
N-*n*-Dodecoxymethylpyridinium dithionite	5×10^{-4}	750	368
n-Dodecyl ester of triethylcarboxymethylammonium chloride	2×10^{-4}	865	420
2-(4:6-diaminopyrimidino)-methyldimethyl-*n*-dodecylammonium bromide	1×10^{-3}	805	480
n-Hexadecyl ester of *p*-carboxy-*N*:*N*:*N*-trimethylanilinium hydrogen sulphite	4×10^{-4}	685	485

Table 5. *Non-quaternary surface-active agents and the acceleration of decarboxylation*

(Each flask contained 2·2 ml. (intact cells 3·5 mg. dry wt.; 0·1M-acetate buffer, pH 4·1; substance to be tested in 0·5 ml.; 0·0045M-glutamate or glutamine). All concentrations are final concentrations.)

Compound added	Approximate concentration for maximum effect (M)	Q_{CO_2} Glutamate	Q_{CO_2} Glutamine
None	—	362	146
Cetavlon	5×10^{-4}	740	406
Ethanolamine ester of dodecane-1-carboxylic acid (hydrochloride)	3×10^{-3}	990	359
Ethanolamine ester of stearic acid (hydrochloride)	$2{\cdot}6 \times 10^{-4}$	1005	540
Stearamidine hydrochloride	4×10^{-4}	900	460
Ethyl-*n*-hexadecylamine	1×10^{-3}	750	406
Methyldihexylamine	$2{\cdot}5 \times 10^{-2}$	190	200
Methyl-*n*-octylamine	8×10^{-2}	740	406

bromide). Active compounds showed the methyl-orange reaction for micelles but inactive compounds did not.

Anionic surface-active agents. Sodium dodecyl sulphate and dioctyl sodium sulphosuccinate (Aerosol OT) were found to be powerful inhibitors of the decarboxylation of glutamate and glutamine, at concentrations above (10^{-2}M) and below (10^{-5}M) their critical points. Ammonia formation from glutamine was inhibited at concentrations below the inhibitory range for the decarboxylation of glutamate.

Non-ionic surface active agents. Tween 80 and Tween 20 (described by the makers, Atlas Powder Co. London, as 'polyoxyalkalene sorbitans') were purified by the method of Archibald (1946). They did not accelerate decarboxylation of glutamate or of glutamine by extracts of *Cl. welchii*, *Pr. morganii* and *Esch. coli*; they slightly accelerated (20%) the decarboxylation of glutamine, but not glutamate, by intact cells of *Cl. welchii* and *Pr. morganii*. This effect was not investigated further. Both compounds changed the colour of methyl orange to a bright yellow colour indicating the presence of micelles under the conditions of test.

n-*Fatty acids.* Solutions of the sodium salts of butyric, valeric and pentane-1-carboxylic acids were adjusted to pH 6·0 with acetic acid. These solutions did not appreciably alter the pH of the decarboxylation system. Higher fatty acids prepared in this way altered the pH of the decarboxylation system because of the low solubility of the free acid, e.g. 0·5 ml. of 0·1M-heptane-1-carboxylate added to 1·0 ml. of 0·2M-acetate buffer at pH 4·0 changed the pH to 4·3. In order to prevent this change in pH, heptane-1-carboxylic, octane-1-carboxylic, nonane-1-carboxylic, lauric, palmitic and oleic acids were emulsified in 0·2M-acetate buffer, pH 4·0. These emulsions, when added to the decarboxylation system in quantities sufficient to give a concentration of acid of 3×10^{-2}M, did not appreciably change the pH.

Butyric, valeric and pentane-1-carboxylic, acids had no effect on the rate of decarboxylation of glutamate or glutamine in intact cells of *Cl. welchii*. Heptane-1-carboxylic, octane-1-carboxylic and nonane-1-carboxylic acids slightly (10–15%) accelerated the decarboxylation of both substrates in intact cells. In cell-free extracts fatty acids had no effect.

DISCUSSION

A parallelism has been established between the capacity of cationic surface-active agents to accelerate the decarboxylation of glutamate and glutamine by *Cl. welchii* and other bacteria on the one hand, and the capacity to form micelles on the other. It was suggested in a previous paper (Hughes, 1949) that Cetavlon acts by removing a competitive inhibitor accompanying the enzymes in cells and extracts, either by solubilization or by chemical complex formation. The experiments reported in this paper show that anionic and non-ionic detergents do not accelerate the enzymic reactions although they form micelles. Thus the charge on the micelle is an essential factor in the activity of the detergents, and this favours the view that the inhibitor is removed by complex formation. The nature of such complexes has been reviewed by Putman (1948).

The presence of an inhibitor together with the enzymes is not improbable, as both glutamate and glutamine take part in other enzymic reactions for which they must to some extent be protected against the action of the decarboxylase and glutaminase.

SUMMARY

1. An acceleration of the decarboxylation of glutamate by extracts and intact cells of *Clostridium welchii*, previously reported for Cetavlon, is given by homologous *n*-alkyltrimethylammonium bromides. The minimum number of carbon atoms in the alkyl substituent needed to give the effect is ten for both

intact cells and extracts. The number of carbon atoms causing maximum acceleration is sixteen in the case of cells and from sixteen to eighteen in the case of extracts.

2. A parallelism was observed between the ability of cationic detergents to form micelles in aqueous solution, and their ability to accelerate the decarboxylation of glutamate and glutamine. Maximum acceleration of the decarboxylase within the homologous series approximately coincided with the 'critical point of micelle formation' as determined by the colour change of indicators.

3. Anionic surface-active agents inhibited the decarboxylation of glutamate and glutamine. Non-ionic surface-active agents and fatty acids were without effect.

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Studies in Detoxication

30. THE METABOLISM OF BENZENE. (*a*) THE DETERMINATION OF BENZENE. (*b*) THE ELIMINATION OF UNCHANGED BENZENE BY RABBITS

By D. V. PARKE and R. T. WILLIAMS
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

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In general, the metabolism of benzene proceeds along four paths, namely, (1) oxidation to phenols, (2) elimination unchanged, (3) formation of phenylmercapturic acid, and (4) ring fission to muconic acid and other products. The first of these was quantitatively studied by Porteous & Williams (1949), who concluded that in the rabbit an average of 21% or, at most, about 30% of an oral dose of benzene at the level of 500 mg./kg. was metabolized to phenols. At least 70% of the administered benzene must take other paths. The present work deals with the second path, the elimination unchanged. It is found that at a level of 500 mg./kg. about 40% of orally administered benzene is eliminated unchanged through the lungs. Thus the first two paths can account for at least 60% or, taking the higher values of some experiments, up to 80–90% of the dose.

That orally administered benzene might be excreted unchanged through the lungs was first suggested by Schultzen & Naunyn (1867), but Munk (1876) doubted this possibility. In 1883, Nencki & Sieber, after experiments on men, dogs and rabbits, expressed the view that approximately one-third of benzene was excreted as phenol conjugates, one-third as catechol and quinol and the remaining third must come out unchanged. The first quantitative experiments on the elimination of unchanged benzene were carried out by Lehmann, Stohr, Kleiner & Gundermann (1910). Tracheotomized rabbits were allowed to inhale benzene-air mixtures, and from an analysis of the inspired and expired air, the amount of benzene absorbed was ascertained. During 1 hr. after benzene ceased to be inhaled, 25% of the absorbed benzene was exhaled unchanged. When the period was increased to 4 hr. then 34% was exhaled,