FORMIC ACID AND DERIVATIVES VOL II

#97

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VOLUME 2

GRAS MONOGRAPH SERIES

FORMIC ACID AND DERIVATIVES

(COPIES OF ARTICLES CITED IN MONOGRAPH SUMMARY)

prepared for

THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION AND WELFARE

JANUARY 9, 1974

This publication was prepared under Contract Number FDA 72-100 with the Public Health Service, Food and Drug Administration, Department of Health, Education, and Welfare

prepared by

Tracor Jitco, Inc.

Biochem. J. (1962) **84**, 552

Formate Metabolism in Sheep

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(*Received* 15 *January* 1962)

The extensive participation of formate in intermediary metabolism is well established. Hartmann & Buchanan (1959) have reviewed the role of formate in purine metabolism, where interconversions involving C_1-unit metabolism are mediated by tetrahydrofolic acid derivatives. The incorporation of formate into the glucogenic amino acid serine (Sakami, 1948; Kruhoffer, 1951), which also involves activation of the C_1 compound by reaction with tetrahydrofolic acid (see Coon & Robinson, 1958), provides a pathway for formate incorporation into carbohydrate (Wood, 1949), and the entry of formate into acetoacetate (Plaut & Lardy, 1950) and choline (du Vigneaud, Verly & Wilson, 1950) has also been demonstrated. A substantial proportion of formate administered to animal tissues, however, is directly oxidized to carbon dioxide by a catalase–hydrogen peroxide complex (Nakada & Weinhouse, 1953; Oro & Rappoport, 1959).

The presence of formate in sheep blood was reported by Annison (1954), and McCarthy, Shaw, McCarthy, Lakshmanan & Holter (1958) demonstrated considerable formate production by a perfused goat-liver preparation. In the absence of information on the turnover of this substrate, however, no conclusions could be drawn concerning its metabolic significance. In the present studies the entry rates of formate have been measured by isotope dilution with the constant-infusion and single-injection methods.

Considerable incorporation of formate into plasma serine and plasma glucose occurred after the injection of labelled formate. The distribution

of radioactivity in serine and glucose was consistent with the hypothesis that the incorporation of formate into glucose occurred through serine, a well-established pathway in other animal tissues.

MATERIALS AND METHODS

Experimental animals. Merino wethers were maintained under animal-house conditions on diets of either lucerne chaff (500 g./day—diet 1), or lucerne chaff and maize (400 g./day and 500 g./day respectively—diet 2), as described by Annison & White (1961). Experiments on fed animals were started 60 min. after the end of the feeding period.

Measurement of the entry rates of formate in sheep. The rates of entry of formate were measured by isotope dilution with the continuous-infusion methods similar to those described for the entry rates of acetate (Annison & Lindsay, 1961) and of glucose (Annison & White, 1961). Labelled formate (1 mM, 1 μc/ml.) was infused at a rate of 0·33 ml./min. for 150 min. Constant specific activities of blood formate were achieved without an initial priming dose of [^{14}C]formate.

In single-injection experiments 50 or 100 μc of sodium [^{14}C]formate contained in 10 ml. of 1 mM-sodium formate was injected into the left jugular vein through a polythene catheter, and blood samples (10 ml.) were taken from the right-jugular catheter at intervals of 5–10 min. for 90 min.

Utilization or entry rates (Annison & Lindsay, 1961) are expressed as mg./hr./kg. body weight (mg./hr./kg.).

Labelled formate. [^{14}C]Formate was obtained from The Radiochemical Centre, Amersham, Bucks.

Leuconostoc mesenteroides. A culture of this organism (strain 39) was obtained from the culture collection of the Department of Bacteriology, Indiana University, Bloomington, Indiana, U.S.A.

Chemical methods

Methods used for: (*a*) the measurement of blood glucose, (*b*) the determination of the specific activity of blood CO_2, (*c*) the collection and assay of radioactive barium carbonate, (*d*) the isolation of plasma glucose as the osazone, and (*e*) the wet oxidation of glucosazones, were described by Annison & White (1961).

Measurement of the specific activity of blood formate. The low concentration of formate in blood (0·02–0·04 μmole/ml.) made it necessary to add carrier sodium formate (5 mg.) to blood (5 ml.) before deproteinization with metaphosphoric acid (Annison, 1954). The filtrate (about 30 ml.) was assayed for formate specific activity by the procedure described by Annison & White (1961) for the oxidation of formate produced by the periodate treatment of glucose. Control experiments established that CO_2 production from other substrates present in the filtrates was negligibly small relative to the concentration of added carrier formate. A possible source of error was the production of labelled formate from substances such as lactate or pyruvate derived from [^{14}C]glucose, which was invariably present in blood as a result of the incorporation of [^{14}C]formate into blood glucose. The examination of blood filtrates obtained during the infusion of [^{14}C]glucose (Annison & White, 1961), however, indicated that, even when the specific activity of blood glucose was ten times that observed during [^{14}C]-formate infusions, the CO_2 produced from added carrier formate was only slightly labelled. The concentration of formate in blood was determined on a second portion of the sample by steam-distillation (to measure the total volatile fatty acid concentration) followed by gas–liquid chromatography (Annison, 1954).

Isolation and degradation of plasma glucose. Plasma (50 ml.) containing labelled glucose was deproteinized with barium hydroxide and zinc sulphate after dilution with 200 ml. of water. The filtrate was concentrated *in vacuo* to about 5 ml. and added to a column (1 cm. × 20 cm.) of Dowex 50 (200 mesh; H^+ form) ion-exchange resin (California Foundation for Biochemical Research). Glucose (and labelled formate) was removed from the column with water (25 ml.) and isolated as the osazone after recrystallization in the presence of added unlabelled formate to minimize adsorption of labelled formate. The distribution of radioactivity in the glucose was examined by oxidation of the osazone with periodate (Topper & Hastings, 1949). In this procedure, 1 mol.prop. of glucosazone yields 1 mol.prop. of the 1,2-bisphenylhydrazone of mesoxaldehyde from glucose C-1, C-2 and C-3, 2 mol.prop. of formic acid from C-4 and C-5, and 1 mol.prop. of formaldehyde from C-6. The average specific activities of C-1, C-2 and C-3 were obtained by wet oxidation (Van Slyke & Folch, 1940) of the mesoxaldehyde derivative, the labelled CO_2 being collected and assayed as barium carbonate. Formaldehyde (from C-6) was separated from the reaction mixture as the dimedone derivative, recrystallized three times from aq. acetone, dissolved in acetone and precipitated with excess of water to give a fine suspension suitable for filtration and assay for radioactivity by the procedure described by Annison & White (1961) for barium carbonate. Counts were corrected for self-absorption by the method of Hendler (1959). Periodate oxidation of glucosazone of known specific activity was used to prepare an absorption curve for the dimedone complex of formaldehyde. Formic acid (from C-4 and C-5) was oxidized to CO_2 after treatment of the reaction mixture with strontium chloride (Greenberg & Rothstein, 1957), and collected and assayed as barium carbonate.

In two further experiments, glucose was isolated from plasma after the injection of labelled formate (100 μc) and completely degraded by procedures based on fermentation with *L. mesenteroides* (Bernstein & Wood, 1957). The results confirmed the labelling pattern established by periodate oxidation, most of the radioactivity occurring in the C-1 and C-6 of glucose.

Isolation of plasma amino acids. Plasma amino acids were removed from the ion-exchange column used in the isolation of glucose by elution with 2N-ammonia (30 ml.) and recovered by evaporation to dryness *in vacuo* over conc. H_2SO_4.

Degradation of serine. Serine was degraded by oxidation with periodate, which yields 1 mol.prop. of CO_2 (from C-1), 1 mol.prop. of formic acid (from C-2) and 1 mol.prop. of formaldehyde (from C-3) from 1 mol.prop. of amino acid. The CO_2 was collected and assayed as barium carbonate. Formic acid and formaldehyde were assayed for radioactivity as described above. The procedure is not specific for serine, since hydroxylysine also gives rise to formaldehyde, and the periodate oxidation of threonine produces acetaldehyde, formic acid and CO_2.

Paper chromatography of plasma amino acids. Amino acids were examined by one-dimensional chromatography on Whatman 3MM paper, with the phenol–borate system (pH 9·3) of Wade, Matheson & Hanes (1961), which separates serine from other groups of amino acids. Ninhydrin [0·2% (w/v) in butanol] was used as the spray reagent.

RESULTS

Formate 'tolerance' tests. Sodium formate (5 m-moles/kg. body wt.) contained in 30 ml. of solution (pH 7·0) was administered intravenously to five starved (24 hr.) sheep, and the rates of clearance of formate from blood were measured. Considerable differences in clearance rates were observed when concentrations of blood formate were plotted against time on a semilogarithmic scale, the slowest and most rapid rates obtained with the group of sheep being shown in Fig. 1. The half-times of formate clearance (i.e. the time taken for the formate concentration to decline to half of its initial value) were 67 and 23 min. respectively.

A marked diuresis was observed after the injection of formate, and urine analyses showed that 15–20% of the injected dose was excreted by this route. The sheep showed no signs of distress after administration of sodium formate.

Single-injection experiments with labelled formate. The specific activity of blood formate after the injection of sodium [^{14}C]formate was measured over a period of 90 min. The slow decline in specific activity is shown in Fig. 2, the half-times being about 20 min. Entry rates were calculated by using first-order reaction kinetics, and estimates of formate 'space' (the volume of distribution of the formate pool) and formate-pool size were made (Table 1). Changes in the specific activity of blood carbon dioxide, and the incorporation of formate into plasma glucose, were also followed, the results of a typical experiment being shown in Table 2.

Entry rates of formate measured by constant infusion. Constant specific activities of blood formate were achieved by the continuous infusion of [^{14}C]formate for about 60 min., and the results of a typical experiment are shown in Fig. 3. Measurements were made on fed and starved sheep (Table 3).

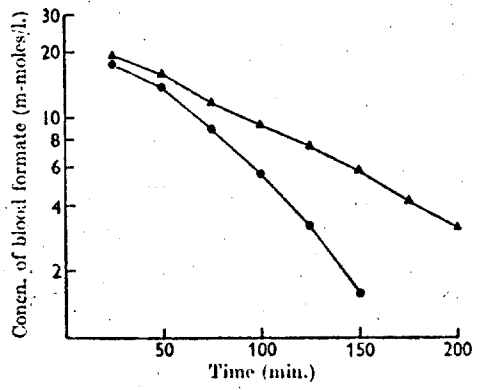

Fig. 1. Clearance of injected sodium formate (5 m-moles/kg.) in starved sheep. Results are shown for two of the animals examined which had the slowest and most rapid rates of removal of formate from blood.

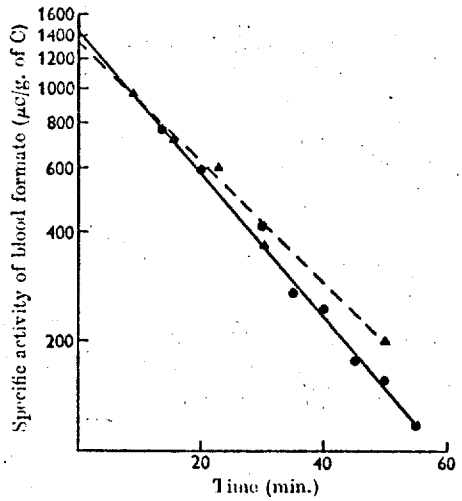

Fig. 2. Specific activity of blood formate after a single intravenous injection of 100 μc of sodium [^{14}C]formate ●, Starved sheep; ▲, fed sheep.

Table 1. *Entry rates of formate, formate 'space' and formate-pool size in fed and starved sheep*

The entry rates of formate, formate 'space' and formate-pool size were measured by a single injection of sodium [^{14}C]formate into fed sheep (F8 and F10) and into sheep starved for 24 hr. (F2) or for 4 days (F4 and F12).

Expt.	Wt. of sheep (kg.)	Concn. of plasma formate (mM)	Formate 'space' (% of body wt.)	Formate pool (mg.)	Formate entry rate (mg./hr./kg.)
F8	50·0	0·070	23·5	76	3·6
F10	45·2	0·121	11·5	64	3·8
F2	35·4	0·034	38·9	61	3·6
F4	35·8	0·060	21·3	59	9·2
F12	40·2	0·090	12·7	53	3·7

In further experiments on starved sheep, the concentration of blood formate was raised to 0·1–0·2 mM by the infusion of carrier formate (1·5 mg./min.) with labelled formate. Specific activities of blood formate were determined by the wet oxidation (Van Slyke & Folch, 1940) of formate fractions isolated by gas–liquid chromatography (Annison, 1954). The entry rates thus obtained were similar to those determined by the constant infusion of carrier-free [^{14}C]formate (Table 3).

Table 2. *Labelling of plasma glucose and blood carbon dioxide after the intravenous injection of sodium [^{14}C]formate into a starved sheep*

The amount of sodium [^{14}C]formate injected was 100 μc (3·4 mg.). Specific activities are expressed in terms of substrate carbon (C).

Time after injection (min.)	Sp. activity of blood formate (μc/g. of C)	Sp. activity of blood CO_2 (μc/g. of C)	Sp. activity of plasma glucose (μc/g. of C)
7·5	9800	11·61	0·79
15	4770	6·1	1·07
22·5	2840	5·31	1·19
30	2225	4·9	—
37·5	1653	4·9	1·00
45	—	4·7	0·98
55	980	3·6	1·17
65	611	—	0·91
75	471	3·4	0·84
90	204	1·9	0·57

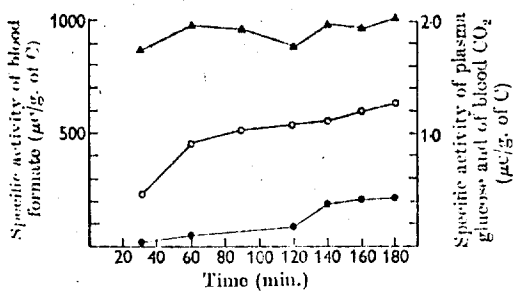

Fig. 3. Specific activities of blood formate, blood CO_2 and plasma glucose during the infusion of sodium [^{14}C]formate (0·33 μc/min.). ▲, Specific activity of blood formate; ○, specific activity of blood CO_2; ●, specific activity of plasma glucose.

Incorporation of formate into serine. The amino acid fraction isolated by ion-exchange chromatography from plasma obtained during the infusion of labelled formate contained appreciable radioactivity. Amino acids (equivalent to 2 ml. of plasma) were separated by paper chromatography and scanned for radioactivity with an end-window Geiger–Müller tube. Only one peak of radioactivity was observed which coincided with the position of serine. The association of most of the activity in the mixture with serine suggested that it was not necessary to isolate serine before applying the degradation procedure.

The amino acid fraction from 50 ml. of plasma was dissolved in 2 ml. of water and the serine content measured by oxidation of 0·1 ml. of solution with periodate and estimation of liberated formaldehyde with chromotropic acid (Boyd & Logan, 1942). The value obtained for the serine content (0·61 mg.) included any hydroxylysine in the mixture. Unlabelled serine (9 mg.) was now added to the mixture and the periodate degradation procedure carried out. About 90% of the total radioactivity recovered was associated with the C-3 of serine (Table 4). The specific activities of each carbon atom were calculated from the apparent serine content. Production of formic acid and carbon dioxide from unlabelled threonine introduced only slight errors in the presence of the relatively large amount of added carrier serine.

Incorporation of formate into glucose. Changes in the specific activity of plasma glucose after the injection or infusion of [^{14}C]formate are shown in Table 2 and Fig. 3 respectively. The specific activity of plasma glucose continued to rise

Table 3. *Entry rates of formate in fed and starved sheep*

Entry rates of formate were measured by continuous infusion of sodium [^{14}C]formate into fed sheep (F3 and F4) and into sheep starved for 24 hr. (F1 and F5) or for 48 hr. (F9). Carrier formate was negligible in Expts. F1, F3 and F4. In Expts. F5 and F9 carrier sodium formate was infused at a rate of 1·53 mg./min. Infusion rates of [^{14}C]formate were 0·33 μc/min. (Expts. F3, F4, F1 and F5) and 1·7 μc/min. (Expt. F9). The specific activities of plasma glucose and blood CO_2 measured at the end of the infusion (150 min.) are shown.

Expt.	Concn. of plasma formate (mM)	Concn. of plasma glucose (mg./100 ml.)	Sp. activity of formate (μc/g. of C)	Sp. activity of glucose (μc/g. of C)	Sp. activity of blood CO_2 (μc/g. of C)	Formate entry rate (mg./hr./kg.)
F3	0·029	66	970	0·40	1·01	2·5
F4	0·028	—	943	—	—	1·7
F1	0·039	52	934	0·93	0·93	2·7
F5	0·14*	62	290	0·77	0·90	7·2
F9	0·11*	64	1350	3·34†	7·28	9·2

* Carrier formate infused. † After infusion for 60 min.

are the precursors of the labelled serine (Sakami, 1948). Nadkarni, Friedmann & Weinhouse (1960) showed that L-[3-^{14}C]serine gave rise to glucose labelled largely at C-1, C-2, C-5 and C-6 in rats, but in the present studies the labelling was largely at C-6 and, by inference from the symmetrical labelling pattern, at C-1 (Table 5). This labelling pattern strongly suggests that the incorporation of formate into glucose in sheep occurs through serine, but the substantial absence of randomization of the terminal pairs of glucose carbon atoms suggests that the incorporation of serine into glucose occurs largely through hydroxypyruvate, and not pyruvate (Dickens & Williamson, 1959). The limited participation of pyruvate as an intermediate is not due to the absence of serine dehydratase in sheep tissues, since this enzyme was isolated from sheep liver by Sayre & Greenberg (1956). Chang, Hueter & Shaw (1959) demonstrated substantial incorporation of formate into glucose by cow-liver slices.

The failure to detect formate in the alimentary-tract contents of sheep (Annison, 1954) indicated that this substance is of endogenous origin. The source of body formate remains uncertain. McCarthy *et al.* (1958) observed considerable production of formate by a perfused goat liver, and using this preparation found a high degree of formate labelling during the perfusion of [1-^{14}C]propionate. Similar observations have been made in the whole sheep during the infusion of [1-^{14}C]propionate, although no elevation of concentrations of blood formate has been observed during the infusion of relatively large amounts (1–2 m-moles/min.) of sodium propionate (E. F. Annison & D. B. Lindsay, unpublished work). The possible production of formate during serine metabolism has not been checked.

SUMMARY

1. Isotope-dilution methods based on single-injection or continuous-infusion procedures indicate the relatively low turnover of formate in sheep, entry rates being in the range 2–4 mg./hr./kg. under normal feeding conditions.

2. Appreciable labelling of plasma serine and plasma glucose followed the injection or infusion of labelled formate. The radioactivity in plasma serine was almost entirely concentrated at C-3. Glucose was symmetrically labelled about C-3 and C-4 and 30–40% of the total radioactivity was present in C-6.

3. The labelling pattern in serine and glucose suggested that the incorporation of formate into glucose occurred through serine, a well-established pathway in other animal tissues.

4. Comparison of the specific activities of blood carbon dioxide and formate in infusion experiments indicated that the contribution of formate to total oxidative metabolism was about 0·1%.

This investigation was made possible by the generous support of the Australian Wool Research Committee. Mr J. Roberts and Mrs J. Musgrave gave valuable technical assistance.

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Rules and Regulations

Title 29—LABOR

Chapter I—National Labor Relations Board

PART 102—RULES AND REGULATIONS, SERIES 8

Subpart I—Service and Filing of Papers

PROOF OF SERVICE

Section 102.112 is amended to read as follows:

§ 102.112 Same; by parties; proof of service.

Service of papers by a party on other parties shall be made by registered mail, or by certified mail, or in any manner provided for the service of papers in a civil action by the law of the State in which the hearing is pending. Except for charges, petitions, exceptions, briefs, and other papers for which a time for both filing and response has been otherwise established, service on all parties shall be made in the same manner as that utilized in filing the paper with the Board, or in a more expeditious manner; however, when filing with the Board is accomplished by personal service the other parties shall be promptly notified of such action by telephone, followed by service of a copy by mail or telegraph. When service is made by registered mail, or by certified mail, the return post office receipt shall be proof of service. When service is made in any manner provided by the law of a State, proof of service shall be made in accordance with such law.

This section shall become effective upon date of publication (8-13-71).

OGDEN W. FIELDS,
Executive Secretary.

[FR Doc.71-11719 Filed 8-12-71;8:50 am]

Chapter XVII—Occupational Safety and Health Administration, Department of Labor

PART 1910—OCCUPATIONAL SAFETY AND HEALTH STANDARDS

Miscellaneous Amendments

On May 29, 1971, occupational safety and health standards were promulgated (36 F.R. 10466). Subsequent review of the standards, and comments and requests for information from the public have pointed up the need for some clarifications, corrections, and changes in effective dates of standards.

Reconsideration of "additional delay in effective date" provisions in various subparts of 29 CFR Part 1910 has shown that the scope of the provisions is too broad, and includes standards for which no delay is necessary for compliance. The purpose of some of the amendments is to limit the additional delay provisions to the specific standards that need additional time, so that the other standards may become effective sooner. On the other hand, Subpart J of Part 1910 is amended to give for the first time an additional delay to specified provisions.

Section 1910.179(b)(2) has been revised in order to give some time for the modification of existing overhead and gantry cranes, and to reflect accurately the American National Standards Institute (ANSI) standard from which the provision is derived. Similar changes have been made in § 1910.180 (crawler, locomotive, and truck cranes) and in § 1910.181 (derricks), for the same reasons.

Section 1910.217(a)(2) has been revised in order to give 36 months for the modification of presently installed mechanical power presses. The delay is given in the ANSI standard adopted in the section.

Section 1910.93 (air contaminants) has been revised in its entirety, in the interest of greater intelligibility and accuracy.

Several different dates have been changed to August 31, 1971, in order to achieve greater uniformity throughout Part 1910. Other amendments are made to correct mistakes, supply omissions, and to provide greater clarity.

Accordingly, pursuant to authority in section 6(a) and 8(g) of the Williams-Steiger Occupational Safety and Health Act of 1970 (84 Stat. 1593, 1600; 29 U.S.C. 655, 357), 29 CFR Part 1910 is hereby amended as set forth below. The provisions of 5 U.S.C. 553 concerning notice of proposed rule making, public participation therein, and delay in effective date are inapplicable by virtue of the exception to 5 U.S.C. Chapter 5 provided in section 6(a) of the Act. In addition, notice and public procedure are unnecessary because of the nature of the amendments, and impracticable, because of the need to give as much advance notice as possible of the changes before August 27, 1971, when many standards in Part 1910 become effective.

Part 1910 is amended as follows:

1. Section 1910.93 of Subpart G is revised to read as follows:

§ 1910.93 Air contaminants.

An employee's exposure to any material listed in table G-1, G-2, or G-3 of this section shall be limited in accordance with the requirements of the following paragraphs of this section.

(a) Table G-1:

(1) *Materials with names preceded by "C"—Ceiling Values.* An employee's exposure to any material in table G-1, the name of which is preceded by a "C" (e.g., C Boron trifluoride), shall at no time exceed the ceiling value given for that material in the table.

(2) *Other materials—8-hour time weighted averages.* An employee's exposure to any material in table G-1, the name of which is not preceded by "C", in any 8-hour work shift of a 40-hour work week, shall not exceed the 8-hour time weighted average given for that material in the table.

(b) Table G-2:

(1) *8-hour time weighted averages.* An employee's exposure to any material listed in table G-2, in any 8-hour work shift of a 40-hour work week, shall not exceed the 8-hour time weighted average limit given for that material in the table.

(2) *Acceptable ceiling concentrations.* An employee's exposure to a material listed in table G-2 shall not exceed at any time during an 8-hour shift the acceptable ceiling concentration limit given for the material in the table, except for a time period, and up to a concentration not exceeding the maximum duration and concentration allowed in the column under "acceptable maximum peak above the acceptable ceiling concentration for an 8-hour shift".

(3) *Example.* During an 8-hour work shift, an employee may be exposed to a concentration of Benzene above 25 p.p.m. (but never above 50 p.p.m.) only for a maximum period of 10 minutes. Such exposure must be compensated by exposures to concentrations less than 10 p.p.m. so that the cumulative exposure for the entire 8-hour work shift does not exceed a weighted average of 10 p.p.m.

(c) Table G-3: An employee's exposure to any material listed in table G-3, in any 8-hour work shift of a 40-hour work week, shall not exceed the 8-hour time weighted average limit given for that material in the table.

(d) Computation formulae:

(1) (i) The cumulative exposure for an 8-hour work shift shall be computed as follows:

$$E = \frac{C_aT_a + C_bT_b + \ldots C_nT_n}{8}$$

where:

E is the equivalent exposure for the working shift.

C is the concentration during any period of time T where the concentration remains constant.

T is the duration in hours of the exposure at the concentration C.

The value of E shall not exceed the 8-hour time weighted average limit in table G-1, G-2, or G-3 for the material involved.

(ii) To illustrate the formula prescribed in subdivision (i) of this subparagraph, note that isomyl acetate has an 8-hour time weighted average limit of 100 p.p.m. (table G-1). Assume that an

employee is subject to the following exposure:

Two hours exposure at 150 p.p.m.
Two hours exposure at 75 p.p.m.
Four hours exposure at 50 p.p.m.

Substituting this information in the formula, we have

$$\frac{2\times150+2\times75+4\times50}{8}=81 \text{ p.p.m.}$$

Since 81 p.p.m. is less than eight, the 8-hour time weighted average limit, the exposure is acceptable.

(2) (i) In case of a mixture of air contaminants an employer shall compute the equivalent exposure as follows:

$$E_m=\frac{C_1}{L_1}+\frac{C_2}{L_2}+\ldots\frac{C_n}{L_n}$$

Where:

E_m is the equivalent exposure for the mixture.

C is the concentration of a particular contaminant.

L is the exposure limit for that contaminant, from table G-1, G-2, or G-3.

The value of E_m shall not exceed unity (1).

(ii) To illustrate the formula prescribed in subdivision (i) of this subparagraph, consider the following exposures:

Material	Actual concentration of 8-hour exposure	8-hour time weighted average exposure limit
Acetone (Table G-1)	500 p.p.m.	1,000 p.p.m.
2-Butanone (Table G-1)	45 p.p.m.	200 p.p.m.
Toluene (Table G-2)	40 p.p.m.	200 p.p.m.

Substituting in the formula, we have:

$$E_m=\frac{500}{1,000}+\frac{45}{200}+\frac{40}{200}$$

$$E_m=0500+0225+0200$$

$$E_m=0925$$

Since E_m is less than unity (1), the exposure combination is within acceptable limits.

(e) To achieve compliance with paragraph (a) through (d) of this section, administrative or engineering controls must first be determined and implemented whenever feasible. When such controls are not feasible to achieve full compliance, protective equipment or any other protective measures shall be used to keep the exposure of employees to air contaminants within the limits prescribed in this section. Any equipment and technical measure use for this purpose must be approved for each particular use by a competent industrial hygienist or other technically qualified person. Whenever respirators are used, their use shall comply with § 1913.134 of this chapter.

TABLE G-I

Substance	p.p.m.[a]	mg./M³ [b]
Acetaldehyde	200	360
Acetic acid	10	25
Acetic anhydride	5	20
Acetone	1,000	2,400
Acetonitrile	40	70
Acetylene dichloride, see 1, 2-Dichloroethylene		
Acetylene tetrabromide	1	14
Acrolein	0.1	0.25
Acrylamide—Skin		0.3
Acrylonitrile—Skin	20	45
Aldrin—Skin		0.25
Allyl alcohol—Skin	2	5
Allyl chloride	1	3
**C Allyl glycidyl ether (AGE)	10	45
Allyl propyl disulfide	2	12
2-Aminoethanol, see Ethanolamine		
2-Aminopyridine	0.5	2
**Ammonia	50	35
Ammonium sulfamate (Ammate)		15
n-Amyl acetate	100	525
sec-Amyl acetate	125	650
Aniline—Skin	5	19
Anisidine (o, p-isomers)—Skin		0.5
Antimony and compounds (as Sb)		0.5
ANTU (alpha naphthyl thiourea)		0.3
Arsenic and compounds (as As)		0.5
Arsine	0.05	0.2
Azinphos-methyl—Skin		0.2
Barium (soluble compounds)		0.5
p-Benzoquinone, see Quinone		
Benzoyl peroxide		5
Benzyl chloride	1	5
Biphenyl, see Diphenyl		
Bisphenol A, see Diglycidyl ether		
Boron oxide		15
C Boron trifluoride	1	3
Bromine	0.1	0.7
Bromoform—Skin	0.5	5
Butadiene (1, 3-butadiene)	1,000	2,200
Butanethiol, see Butyl mercaptan		
2-Butanone	200	590
2-Butoxy ethanol (Butyl Cellosolve)—Skin	50	240
Butyl acetate (n-butyl acetate)	150	710
sec-Butyl acetate	200	950
tert-Butyl acetate	200	950
Butyl alcohol	100	300
sec-Butyl alcohol	150	450
tert-Butyl alcohol	100	300
C Butylamine—Skin	5	15
C tert-Butyl chromate (as CrO_3)—Skin		0.1
n-Butyl glycidyl ether (BGE)	50	270
*Butyl mercaptan	10	35
p-tert-Butyltoluene	10	60
Calcium arsenate		1
Calcium oxide		5
**Camphor	2	
Carbaryl (Sevin®)		5
Carbon black		3.5
Carbon dioxide	5,000	9,000
Carbon monoxide	50	55
Chlordane—Skin		0.5
Chlorinated camphene—Skin		0.5
Chlorinated diphenyl oxide		0.5
*Chlorine	1	3
Chlorine dioxide	0.1	0.3
C Chlorine trifluoride	0.1	0.4
C Chloroacetaldehyde	1	3
α-Chloroacetophenone (phenacylchloride)	0.05	0.3
Chlorobenzene (monochlorobenzene)	75	350
o-Chlorobenzylidene malononitrile (OCBM)	0.05	0.4
Chlorobromomethane	200	1,050
2-Chloro-1,3-butadiene, see Chloroprene		
Chlorodiphenyl (42 percent Chlorine)—Skin		1
Chlorodiphenyl (54 percent Chlorine)—Skin		0.5
1-Chloro,2,3-epoxypropane, see Epichlorohydrin		
2-Chloroethanol, see Ethylene chlorohydrin		
Chloroethylene, see Vinyl choride		
C Chloroform (trichloromethane)	50	240
1-Chloro-1-nitropropane	20	100
Chloropicrin	0.1	0.7
Chloroprene (2-chloro-1,3-butadiene)—Skin	25	90

TABLE G-I—Continued

Substance	p.p.m.[a]	mg./M³ [b]
Chromium, sol. chromic, chromous salts as Cr		0.5
Metal and insol. salts		1
Coal tar pitch volatiles (benzene soluble fraction) anthracene, BaP_1 phenanthrene, acridine, chrysene, pyrene		0.2
Cobalt, metal fume and dust		0.1
Copper fume		0.1
Dusts and Mists		1
Cotton dust (raw)		1
Crag® herbicide		15
Cresol (all isomers)—Skin	5	22
Crotonaldehyde	2	5
Cumene—Skin	50	245
Cyanide (as CN)—Skin		5
Cyclohexane	300	1,050
Cyclohexanol	50	200
Cyclohexanone	50	200
Cyclohexene	300	1,015
Cyclopentadiene	75	200
2, 4-D		10
DDT—Skin		1
DDVP, see Dichlorvos		
Decaborane—Skin	0.05	0.3
Demeton®—Skin		0.1
Diacetone alcohol (4-hydroxy-4-methyl-2-pentanone)	50	240
1,2-diaminoethane, see Ethylenediamine		
Diazomethane	0.2	0.4
Diborane	0.1	0.1
Dibutylphthalate		5
C o-Dichlorobenzene	50	300
p-Dichlorobenzene	75	450
Dichlorodifluoromethane	1,000	4,950
1,3-Dichloro-5,5-dimethyl hydantoin		0.2
1,1-Dichloroethane	100	405
1,2-Dichloroethylene	200	790
C Dichloroethyl ether—Skin	15	90
Dichloromethane, see Methylenechloride		
Dichloromonofluoromethane	1,000	4,200
C 1,1-Dichloro-1-nitroethane	10	60
1,2-Dichloropropane, see Propylenedichloride		
Dichlorotetrafluoroethane	1,000	7,000
Dichlorvos (DDVP)—Skin		1
Dieldrin—Skin		0.25
Diethylamine	25	75
Diethylamino ethanol—Skin	10	50
Diethylether, see Ethyl ether		
Difluorodibromomethane	100	860
C Diglycidyl ether (DGE)	0.5	2.8
Dihydroxybenzene, see Hydroquinone		
Diisobutyl ketone	50	290
Diisopropylamine—Skin	5	20
Dimethoxymethane, see Methylal		
Dimethyl acetamide—Skin	10	35
Dimethylamine	10	18
Dimethylaminobenzene, see Xylidene		
Dimethylaniline(N-dimethylaniline)—Skin	5	25
Dimethylbenzene, see Xylene		
Dimethyl 1,2-dibromo-2,2-dichloroethyl phosphate, (Dibrom)		3
Dimethylformamide—Skin	10	30
2,6-Dimethylheptanone, see Diisobutyl ketone		
1,1-Dimethylhydrazine—Skin	0.5	1
Dimethylphthalate		5
Dimethylsulfate—Skin	1	5
Dinitrobenzene (all isomers)—Skin		1
Dinitro-o-cresol—Skin		0.2
Dinitrotoluene—Skin		1.5
Dioxane (Diethylene dioxide)—Skin	100	360
Diphenyl	0.2	1
Diphenylmethane diisocyanate (see Methylene bisphenyl isocyanate (MDI)		
Dipropylene glycol methyl ether—Skin	100	600
Di-sec. octyl phthalate (Di-2-ethylhexylphthalate)		5
Endrin—Skin		0.1
Epichlorhydrin—Skin	5	19
EPN—Skin		0.5
1,2-Epoxypropane, see Propylene oxide		
2,3-Epoxy-1-propanol, see Glycidol		
Ethanethiol, see Ethylmercaptan		

See footnotes at end of table.

TABLE G-I—Continued

Substance	p.p.m.[a]	mg./M³ [b]
Ethanolamine	3	6
2-Ethoxyethanol—Skin	200	740
2-Ethoxyethylacetate (Cellosolve acetate)—Skin	100	540
Ethyl acetate	400	1,400
Ethyl acrylate—Skin	25	100
Ethyl alcohol (ethanol)	1,000	1,900
Ethylamine	10	18
Ethyl sec-amyl ketone (5-methyl-3-heptanone)	25	130
Ethyl benzene	100	435
Ethyl bromide	200	890
Ethyl butyl ketone (3-Heptanone)	50	230
Ethyl chloride	1,000	2,600
Ethyl ether	400	1,200
Ethyl formate	100	300
C Ethyl mercaptan	10	25
Ethyl silicate	100	850
Ethylene chlorohydrin—Skin	5	16
Ethylenediamine	10	25
Ethylene dibromide, see 1,2-Dibromoethane		
Ethylene dichloride, see 1,2-Dichloroethane		
C Ethylene glycol dinitrate and/or Nitroglycerin—Skin	[d] 0.2	1
Ethylene glycol monomethyl ether acetate, see Methyl cellosolve acetate		
Ethylene imine—Skin	0.5	1
Ethylene oxide	50	90
Ethylidine chloride, see 1,1-Dichloroethane		
N-Ethylmorpholine—Skin	20	94
Ferbam		15
Ferrovanadium dust		1
Fluoride (as F)		2.5
Fluorine	0.1	0.2
Fluorotrichloromethane	1,000	5,600
Formic acid	5	9
Furfural—Skin	5	20
Furfuryl alcohol	50	200
Glycidol (2,3-Epoxy-1-propanol)	50	150
Glycol monoethyl ether, see 2-Ethoxyethanol		
Guthion, ® see Azinphosmethyl		
Hafnium		0.5
Heptachlor—Skin		0.5
Heptane (n-heptane)	500	2,000
Hexachloroethane—Skin	1	10
Hexachloronaphthalene—Skin		0.2
Hexane (n-hexane)	500	1,800
2-Hexanone	100	410
Hexone (Methyl isobutyl ketone)	100	410
sec-Hexyl acetate	50	300
Hydrazine—Skin	1	1.3
Hydrogen bromide	3	10
C Hydrogen chloride	5	7
Hydrogen cyanide—Skin	10	11
Hydrogen peroxide (90%)	1	1.4
Hydrogen selenide	0.05	0.2
Hydroquinone		2
C Iodine	0.1	1
Iron oxide fume		10
Isoamyl acetate	100	525
Isoamyl alcohol	100	360
Isobutyl acetate	150	700
Isobutyl alcohol	100	300
Isophorone	25	140
Isopropyl acetate	250	950
Isopropyl alcohol	400	980
Isopropylamine	5	12
Isopropylether	500	2,100
Isopropyl glycidyl ether (IGE)	50	240
Ketene	0.5	0.9
Lead arsenate		0.15
Lindane—Skin		0.5
Lithium hydride		0.025
L.P.G. (liquified petroleum gas)	1,000	1,800
Magnesium oxide fume		15
Malathion—Skin		15
Maleic anhydride	0.25	1
C Manganese		5
Mesityl oxide	25	100
Methanethiol, see Methyl mercaptan		
Methoxychlor		15
2-Methoxyethanol, see Methyl cellosolve		
Methyl acetate	200	610
Methyl acetylene (propyne)	1,000	1,650
Methyl acetylene-propadiene mixture (MAPP)	1,000	1,800
Methyl acrylate—Skin	10	35
Methylal (dimethoxymethane)	1,000	3,100
Methyl alcohol (methanol)	200	260
Methylamine	10	12
Methyl amyl alcohol, see Methyl isobutyl carbinol		

TABLE G-I—Continued

Substance	p.p.m.[a]	mg./M³ [b]
Methyl (n-amyl) ketone (2-Heptanone)	100	465
C Methyl bromide—Skin	20	80
Methyl butyl ketone, see 2-Hexanone		
Methyl cellosolve—Skin	25	80
Methyl cellosolve acetate—Skin	25	120
Methyl chloroform	350	1,900
Methylcyclohexane	500	2,000
Methylcyclohexanol	100	470
o-Methylcyclohexanone—Skin	100	460
Methyl ethyl ketone (MEK), see 2-Butanone		
Methyl formate	100	250
Methyl iodide—Skin	5	28
Methyl isobutyl carbinol—Skin	25	100
Methyl isobutyl ketone, see Hexone		
Methyl isocyanate—Skin	0.02	0.05
C Methyl mercaptan	10	20
Methyl methacrylate	100	410
Methyl propyl ketone, see 2-Pentanone		
C α Methyl styrene	100	480
C Methylene bisphenyl isocyanate (MDI)	0.02	0.2
Molybdenum:		
Soluble compounds		5
Insoluble compounds		15
Monomethyl aniline—Skin	2	9
C Monomethyl hydrazine—Skin	0.2	0.35
Morpholine—Skin	20	70
Naphtha (coal tar)	100	400
Naphthalene	10	50
Nickel carbonyl	0.001	0.007
Nickel, metal and soluble cmpds, as Ni		1
Nicotine—Skin		0.5
Nitric acid	2	5
Nitric oxide	25	30
p-Nitroaniline—Skin	1	6
Nitrobenzene—Skin	1	5
p-Nitrochlorobenzene—Skin		1
Nitroethane	100	310
Nitrogen dioxide	5	9
Nitrogen trifluoride	10	29
Nitroglycerin—Skin	0.2	2
Nitromethane	100	250
1-Nitropropane	25	90
2-Nitropropane	25	90
Nitrotoluene—Skin	5	30
Nitrotrichloromethane, see Chloropicrin		
Octachloronaphthalene—Skin		0.1
*Octane	500	2,350
*Oil mist, mineral		[e] 5
Osmium tetroxide		0.002
Oxalic acid		1
Oxygen difluoride	0.05	0.1
Ozone	0.1	0.2
Paraquat—Skin		0.5
Petroleum distillates (naptha)	500	2,000
Propane	1,000	1,800
Silver, metal and soluble compounds		0.01
Sodium fluoroacetate (1080)—Skin		0.05
Sodium hydroxide		2
Stibine	0.1	0.5
*Stoddard solvent	500	2,950
Strychnine		0.15
Sulfur dioxide	5	13
Sulfur hexafluoride	1,000	6,000
Sulfuric acid		1
Sulfur monochloride	1	6
Sulfur pentafluoride	0.025	0.25
Sulfuryl fluoride	5	20
Systox, see Demeton ®		
2,4,5T		10
Tantalum		5
TEDP—Skin		0.2
Tellurium		0.1
Tellurium hexafluoride	0.02	0.2
TEPP—Skin		0.05
C Terphenyls	1	9
1,1,1,2-Tetrachloro-2,2-difluoroethane	500	4,170
1,1,2,2-Tetrachloro-1,2-difluoroethane	500	4,170
1,1,2,2-Tetrachloroethane—Skin	5	35
Tetrachloroethylene, see Perchloroethylene		
Tetrachloromethane, see Carbon tetrachloride		
Tetrachloronaphthalene—Skin		2
Tetraethyl lead (as Pb)—Skin		0.075
Tetrahydrofuran	200	590
Tetramethyl lead (as Pb)—Skin		0.075
Tetramethyl succinonitrile—Skin	0.5	3
Tetranitromethane	1	8

TABLE G-I—Continued

Substance	p.p.m.[a]	mg./M³ [b]
Tetryl (2,4,6-trinitrophenylmethylnitramine)—Skin		1.5
Thallium (soluble compounds)—Skin as Tl		0.1
Thiram		5
Tin (inorganic cmpds, except oxides		2
Tin (organic cmpds)		0.1
C Toluene-2,4-diisocyanate	0.02	0.14
o-Toluidine—Skin	5	22
Toxaphene, see Chlorinated camphene		
Tributyl phosphate		5
1,1,1-Trichloroethane see Methyl chloroform		
1,1,2-Trichloroethane—Skin	10	45
Parathion—Skin		0.1 [1]
Pentaborane	0.005	0.0
Pentachloronaphthalene—Skin		0.5
Pentachlorophenol—Skin		0.5
*Pentane	1,000	2,950
2-Pentanone	200	700
Perchloromethyl mercaptan	0.1	0.8
Perchloryl fluoride	3	13.5
Phenol—Skin	5	19
p-Phenylene diamine—Skin		0.1
Phenyl ether (vapor)	1	7
Phenyl ether-biphenyl mixture (vapor)	1	7
Phenylethylene, see Styrene		
Phenyl glycidyl ether (PGE)	10	60
Phenylhydrazine—Skin	5	22
Phosdrin (Mevinphos ®)—Skin		0.1
Phosgene (carbonyl chloride)	0.1	0.4
Phosphine	0.3	0.4
Phosphoric acid		1
Phosphorus (yellow)		0.1
Phosphorus pentachloride		1
Phosphorus pentasulfide		1
Phosphorus trichloride	0.5	3
Phthalic anhydride	2	12
Picric acid—Skin		0.1
Pival ® (2-Pivalyl-1,3-indandione)		0.1
Platinum (Soluble Salts) as Pt		0.002
Propargyl alcohol—Skin	1	
n-Propyl acetate	200	840
Propyl alcohol	200	500
n-Propyl nitrate	25	110
Propylene dichloride	75	350
Propylene imine—Skin	2	5
Propylene oxide	100	240
Propyne, see Methylacetylene		
Pyrethrum		5
Pyridine	5	15
Quinone	0.1	0.4
RDX—Skin		1.5
Rhodium, Metal fume and dusts, as Rh		0.1
Soluble salts		0.001
Rotenone		10
Rotenone (commercial)		5
Selenium compounds (as Se)		0.2
Selenium hexafluoride	0.05	0.4
Titanium dioxide		15
Trichloromethane, see Chloroform		
Trichloronaphthalene—Skin		5
1,2,3-Trichloropropane	50	300
1,1,2-Trichloro 1,2,2-trifluoroethane	1,000	7,600
Triethylamine	25	100
Trifluoromonobromomethane	1,000	6,100
2,4,6-Trinitrophenol, see Picric acid		
2,4,6-Trinitrophenylmethylnitramine, see Tetryl		
Trinitrotoluene—Skin		1.5
Triorthocresyl phosphate		0.1
Triphenyl phosphate		3
Turpentine	100	560
Uranium (soluble compounds)		0.05
Uranium (insoluble compounds)		0.25
C Vanadium:		
V_2O_5 dust		0.5
V_2O_5 fume		0.1
Vinyl benzene, see Styrene		
**C Vinyl chloride	500	1,300
Vinylcyanide, see Acrylonitrile		
Vinyl toluene	100	480
Warfarin		0.1
Xylene (xylol)	100	435
Xylidine—Skin	5	25
Yttrium		1
Zinc chloride fume		1
Zinc oxide fume		5
Zirconium compounds (as Zr)		5

See footnotes at end of table.

*1970 Addition.

[a] Parts of vapor or gas per million parts of contaminated air by volume at 25° C. and 760 mm. Hg pressure.

[b] Approximate milligrams of particulate per cubic meter of air.

(No footnote "c" is used to avoid confusion with ceiling value notations.)

[d] An atmospheric concentration of not more than 0.02 p.p.m., or personal protection may be necessary to avoid headache.

[e] As sampled by method that does not collect vapor.

[f] For control of general room air, biologic monitoring is essential for personnel control.

TABLE G-2

Material	8-hour time weighted average	Acceptable ceiling concentration	Acceptable maximum peak above the acceptable ceiling concentration for an 8-hour shift.	
			Concentration	Maximum duration
Benzene (Z37.4-1969)	10 p.p.m	25 p.p.m	50 p.p.m	10 minutes.
Beryllium and beryllium compounds (Z37.29-1970).	2 μg./M³	5 μg./M³	25 μg./M³	30 minutes.
Cadmium fume (Z37.5-1970)	0.1 mg./M³	3 mg./M³		
Cadmium dust (Z37.5-1970)	0.2 mg./M³	0.6 mg./M³		
Carbon disulfide (Z37.3-1968)	20 p.p.m	30 p.p.m	100 p.p.m	Do.
Carbon tetrachloride (Z37.17-1967)	10 p.p.m	25 p.p.m	200 p.p.m	5 minutes in any 4 hours.
Ethylene dibromide (Z37.31-1970)	20 p.p.m	30 p.p.m	50 p.p.m	5 minutes.
Ethylene dichloride (Z37.21-1969)	50 p.p.m	100 p.p.m	200 p.p.m	5 minutes in any 3 hours.
Formaldehyde (Z37.16-1967)	3 p.p.m	5 p.p.m	10 p.p.m	30 minutes.
Hydrogen fluoride (Z37.28-1969)	do			
Fluoride as dust (Z37.28-1969)	2.5 mg./M³			
Lead and its inorganic compounds (Z37.11-1959).	0.2 mg./M³			
Methyl chloride (Z37.18-1969)	100 p.p.m	200 p.p.m	300 p.p.m	5 minutes in any 3 hours.
Methylene Chloride (Z37.3-1969)	500 p.p.m	1,000 p.p.m	2,000 p.p.m	5 minutes in any 2 hours.
Organo (alkyl) mercury (Z37.30-1969)	0.01 mg./M³	0.04 mg./M³		
Styrene (Z37.15-1969)	100 p.p.m	200 p.p.m	600 p.p.m	5 minutes in any 3 hours.
Trichloroethylene (Z37.19-1967)	do	do	300 p.p.m	5 minutes in any 2 hours.
Tetrachloroethylene (Z37.22-1967)	do	do	do	5 minutes in any 3 hours.
Toluene (Z37.12-1967)	200 p.p.m	300 p.p.m	500 p.p.m	10 minutes.
Hydrogen sulfide (Z37.2-1966)		20 p.p.m	50 p.p.m	10 minutes once only if no other measurable exposure occurs.
Mercury (Z37.8-1971)		1 mg./10M³		
Chromic acid and chromates (Z37.7-1971)		do[3]		

TABLE G-3—MINERAL DUSTS

Substance	Mppcf[a]	Mg/M³[b]
Silica:		
Crystalline:		
Quartz (respirable)	250[f]	10mg/M³[e]
	%SiO₂+5	%SiO₂+2
Quartz (total dust)		30mg/M³
		%SiO₂+2
Cristobalite: Use ½ the value calculated from the count or mass formulae for quartz.		
Tridymite: Use ½ the value calculated from the formulae for quartz.		
Amorphous, including natural diatomaceous earth	20	80mg/M³
		%SiO₂
Tremolite	5	20mg/M³
		%SiO₂
Silicates (less than 1% crystalline silica):		
Asbestos—12 fibers per milliliter greater than 5 microns in length[d] or	2	
Mica	20	
Soapstone	20	
Talc	20	
Portland cement	50	
Graphite (natural)	15	
Coal dust (respirable fraction less than 5% SiO₂)		2.4mg/M³ or 10mg/M³
For more than 5% SiO₂		
		%SiO₂+2
Inert or Nuisance Dust:		
Respirable fraction	15	5mg/M³
Total dust	50	15mg/M³

NOTE: Conversion factors—
mppcf×35.3=million particles per cubic meter
=particles per c.c.

[a] Millions of particles per cubic foot of air, based on impinger samples counted by light-field technics.

[b] The percentage of crystalline silica in the formula is the amount determined from air-borne samples, except in those instances in which other methods have been shown to be applicable.

[c] As determined by the membrane filter method at 430 × phase contrast magnification.

[e] Both concentration and percent quartz for the application of this limit are to be determined from the fraction passing a size-selector with the following characteristics:

Aerodynamic diameter (unit density sphere)	Percent passing selector
2	90
2.5	75
3.5	50
5.0	25
10	0

The measurements under this note refer to the use of an AEC instrument. If the respirable fraction of coal dust is determined with a MRE the figure corresponding to that of 2.4 Mg/M³ in the table for coal dust is 4.5 Mg/M³

2. Section 1910.98 of Subpart G is revised to read as follows:

§ 1910.98 Effective dates.

(a) The provisions of this Subpart G shall become effective on August 27, 1971, except as provided in the remaining paragraphs of this section.

(b) The following provisions shall become effective on February 15, 1972:

§ 1910.94 (a)(2)(iii), (a)(3), (a)(4), (b), (c)(2), (c)(3), (c)(4), (c)(5), (c)(6)(i), (c)(6)(ii), (d)(1)(ii), (d)(3), (d)(4), (d)(5), and (d)(7).

(c) Notwithstanding anything in paragraph (a), (b), or (d) of this section, any provision in any other section of this subpart which contains in itself a specific effective date or time limitation shall become effective on such date or shall apply in accordance with such limitation.

(d) Notwithstanding anything in paragraph (a) of this section, if any standard in 41 CFR Part 50-204, other than a national consensus standard incorporated by reference in § 50-204.2(a)(1), is or becomes applicable at any time to any employment and place of employment, by virtue of the Walsh-Healey Public Contracts Act, or the Service Contract Act of 1965, or the National Foundation on Arts and Humanities Act of 1965, any corresponding established Federal standard in this Subpart G which is derived from 41 CFR Part 50-204 shall also become effective, and shall be applicable to such employment and place of employment, on the same date.

3. Section 1910.114 of Subpart H is revised to read as follows:

§ 1910.114 Effective dates.

(a) The provisions of this Subpart H shall become effective on August 27, 1971, except as provided in the remaining paragraphs of this section.

(b) The following provisions shall become effective on February 15, 1972:

§ 1910.101(b).
§ 1910.102.
§ 1910.103 (a)(2), (b)(1)(i), (b)(1)(iii), (b)(2)(ii), and (b)(3).
§ 1910.105.
§ 1910.106 (b)(1), (b)(2), (b)(3), and (b)(4).
§ 1910.106 (b)(5), (c)(1), (c)(2), (c)(3), (c)(4), (c)(5), (c)(6), (d)(2), (d)(3)(ii), (d)(4)(i), (d)(4)(iii), (d)(4)(iv), (d)(5)(v), (d)(5)(vi)(a), (e)(3)(iii), (e)(3)(v), (e)(4), (e)(7), (f)(2)(iii), (f)(3)(i), (f)(4)(iii), (f)(4)(iv), (f)(4)(viii), (f)(5), (g), (h), and (i).
§ 1910.107 (b)(1), (b)(2), (b)(3), (b)(4), (b)(5)(i), (b)(5)(iii), (b)(5)(iv), (b)(6), (b)(7), (b)(8), (b)(10), (c)(1), (c)(4), (c)(5), (c)(6), (c)(7), (d), (e)(7), (e)(8), (f)(1), (f)(2), (h), (i), (j), (l)(2), (l)(3), (l)(5), (l)(6), (l)(7), and (m)(1).
§ 1910.108 (b), (c), (e)(1), (g)(2), (g)(3), (g)(4), and (g)(5).
§ 1910.108 (g)(6) and (h).
§ 1910.110.
§ 1910.111.

(c) Notwithstanding anything in paragraph (a), (b), or (d) of this section, any provision in any other section of this subpart which contains in itself a specific effective date or time limitation shall become effective on such date or shall apply in accordance with such limitation.

(d) Notwithstanding anything in paragraph (a), or (b) of this section, if any standard in 41 CFR Part 50-204, other than a national consensus standard incorporated by reference in § 50-204.2(a)(1), is or becomes applicable at any time to any employment and place of employment, by virtue of the Walsh-Healey Public Contracts Act, or the Service Contract Act of 1965, or the National Foundation on Arts and Humanities Act of 1965, any corresponding established

code of federal regulations

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Food and Drugs

PARTS 10 TO 129

Revised as of April 1, 1973

CONTAINING
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Published by
the Office of the Federal Register
National Archives and Records Service
General Services Administration

as a Special Edition of
the Federal Register

21 CFR 121.1096 (1973) only

(c) The use of the chemicals is followed by rinsing with potable water to remove, to the extent possible, residues of the chemicals.

(d) To assure safe use of the additive:

(1) The label and labeling of the additive container shall bear, in addition to the other information required by the act, the name of the additive or a statement of its composition.

(2) The label or labeling of the additive container shall bear adequate use directions to assure use in compliance with all provisions of this section.

[30 F.R. 15845, Dec. 23, 1965, as amended at 30 F.R. 16066, Dec. 24, 1965, 31 F.R. 7227, May 18, 1966, 31 F.R. 9216, July 6, 1966; 34 F.R. 12885, Aug. 8, 1969]

§ 121.1092 Acrylate-acrylamide resins.

Acrylate-acrylamide resins may be safely used in food under the following prescribed conditions:

(a) The additive consists of one of the following:

(1) Acrylamide-acrylic acid resin (hydrolyzed polyacrylamide) is produced by the polymerization of arcrylamide with partial hydrolysis, or by copolymerization of acrylamide and acrylic acid, with the greater part of the polymer being composed of acrylamide units.

(2) Sodium polyacrylate-acrylamide resin is produced by the polymerization and subsequent hydrolysis of acrylonitrile in a sodium silicate-sodium hydroxide aqueous solution, with the greater part of the polymer being composed of acrylate units.

(b) The additive contains not more than 0.05 percent of residual monomer calculated as acrylamide.

(c) The additive is used or intended for use as follows:

(1) The additive identified in paragraph (a)(1) of this section is used as a flocculent in the clarification of beet sugar juice or cane sugar juice in an amount not to exceed 5 parts per million by weight of the juice.

(2) The additive identified in paragraph (a)(2) of this section is used to control organic and mineral scale in beet sugar juice and liquor or cane sugar juice and liquor in an amount not to exceed 2.5 parts per million by weight of the juice or liquor.

[37 F.R. 330, Jan. 11, 1972]

§ 121.1093 DDT.

Tolerances are established for residues of the insecticide DDT (a mixture of 1, 1, 1-trichloro-2,2-bis(*p*-chlorophenyl) ethane and 1, 1, 1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl) ethane) in or on the following processed foods, when present therein as a result of the application of this insecticide to growing crops:

100 parts per million in or on peppermint oil and spearmint oil.

80 parts per million in or on dried hops.

6 parts per million in crude soybean oil.

If residues of toxaphene are also present from application to the growing crop, the total residue of both such chlorinated organic compounds shall not exceed 12 parts per million.

[31 FR 9454, July 12, 1966, as amended at 31 FR 12435, Sept. 20, 1966; 32 FR 11157, Aug. 1, 1967]

§ 121.1093a DDT and its related degradation products in manufactured dairy products.

Tolerances of 1.25 parts per million on the milk-fat basis are established for residues in manufactured dairy products for each or any combination of the following: DDT, DDD (1,1-dichloro-2,2-bis(p-chlorophenyl)ethane), and DDE (1,1 - dichloro - 2,2 - bis(*p*-chlorophenyl ethylene). Such residues may be present therein only as a result of permissible residues in milk.

[32 F.R. 4060, Mar. 15, 1967]

§ 121.1095 Nicotinamide-ascorbic acid complex.

Nicotinamide-ascorbic acid complex may be safely used in accordance with the following prescribed conditions:

(a) The additive is the product of the controlled reaction between ascorbic acid and nicotinamide, melting in the range 141° C. to 145° C.

(b) It is used as a source of ascorbic acid and nicotinamide in multivitamin preparations.

§ 121.1096 Ethyl formate.

The food additive ethyl formate may be safely used in or on specified dried fruits in accordance with the following prescribed conditions:

(a) It is used or intended for use in or on raisins and dried Zante currants as a bulk and package fumigant.

(b) It is used in accordance with directions registered with the United States Department of Agriculture, and so used that the total formic acid present, free and combined, in the finished

product shall not exceed 250 parts per million.

(c) To assure safe use of the additive, its label and labeling shall conform to that registered with the United States Department of Agriculture.

§ 121.1097 Safrole-free extract of sassafras.

The food additive safrole-free extract of sassafras may be safely used in accordance with the following prescribed conditions:

(a) The additive is the aqueous extract obtained from the root bark of the plant *Sassafras albidum* (Nuttall) Nees (Fam. *Lauraceae*).

(b) It is obtained by extracting the bark with dilute alcohol, first concentrating the alcoholic solution by vacuum distillation, then diluting the concentrate with water and discarding the oily fraction.

(c) The purified aqueous extract is safrole-free.

(d) It is used as a flavoring in food.

§ 121.1098 Rice bran wax.

Rice bran wax may be safely used in food in accordance with the following conditions:

(a) It is the refined wax obtained from rice bran and meets the following specifications:

Melting point 75° C. to 80° C.
Free fatty acids, maximum 10 percent.
Iodine number, maximum 20.
Saponification number 75 to 120.

(b) It is used or intended for use as follows:

Food	Limitation in food	Use
Candy	50 parts per million.	Coating.
Fresh fruits and fresh vegetables.	do	Do.
Chewing gum	2½ percent	Plasticizing material.

§ 121.1099 Defoaming agents.

Defoaming agents may be safely used in processing foods, in accordance with the following conditions:

(a) They consist of one or more of the following:

(1) Substances generally recognized by qualified experts as safe in food or covered by prior sanctions for the use prescribed by this section.

(2) Substances listed in this subparagraph, subject to any limitations imposed:

Substances	Limitations
Dimethylpolysiloxane (substantially free from hydrolyzable chloride and alkoxy groups; no more than 18 percent loss in weight after heating 4 hours at 200° C.; viscosity 300–600 centistokes at 25° C., refractive index 1.400–1.404 at 25° C.).	10 parts per million in food, or at such level in a concentrated food that when prepared as directed on the label the food in its ready-for-consumption state will have not more than 10 parts per million except as follows: Zero in milk; 110 parts per million in dry gelatin dessert mixes labeled for use whereby no more than 16 parts per million is present in the ready-to-serve dessert; 250 parts per million in salt labeled for cooking purposes, whereby no more than 10 parts per million is present in the cooked food.
Formaldehyde	As a preservative in defoaming agents containing dimethylpolysiloxane, in an amount not exceeding 1.0 percent of the dimethylpolysiloxane content.
Polyacrylic acid, sodium salt	As a stabilizer and thickener in defoaming agents containing dimethylpolysiloxane in an amount reasonably required to accomplish the intended effect.
Polyethylene glycol	As defined in § 121.1185.
Polyoxyethylene 40 monostearate	As defined in U.S.P. XVI.
Polysorbate 60	As defined in § 121.1030.
Polysorbate 65	As defined in § 121.1008.
Propylene glycol alginate	As defined in § 121.1015.
Silicon dioxide	As defined in § 121.1058.
Sorbitan monostearate	As defined in § 121.1029.
White mineral oil: Conforming with § 121.1146.	As a component of defoaming agents for use in wash water for sliced potatoes at a level not to exceed 0.008 percent of the wash water.

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Code of Federal Regulations

21 CFR 121.101 (h) only, cited; however 121.101 Introduction really needed also so included here

§ 121.72 Adoption of regulation on initiative of Commissioner.

(a) The Commissioner upon his own initiative may propose the issuance of a regulation prescribing, with respect to any particular use of a food additive, the conditions under which such additive may be safely used. Notice of such proposal shall be published in the FEDERAL REGISTER and shall state the reasons for the proposal.

(b) Action upon a proposal made by the Commissioner shall, after publication of the notice, proceed as provided in § 121.51 and section 409 of the act.

§ 121.73 Judicial review.

The Secretary of Health, Education, and welfare hereby designates the Assistant General Counsel for Food and Drugs of the Department of Health, Education, and Welfare as the officer upon whom copy of petition for judicial review shall be served. Such officer shall be responsible for filing in the court a transcript of proceedings and the record on which the order of the Secretary of Health, Education, and Welfare is based. The transcript and record shall be certified by the Secretary.

§ 121.74 Procedure for amending and repealing tolerances or exemptions from tolerances.

(a) The Commissioner or any interested person may propose the issuance of a regulation amending or repealing a regulation pertaining to a food additive or granting or repealing an exemption for such additive. Such a proposal by an interested person shall be in writing. If such proposal by an interested person furnishes reasonable grounds therefor, the Commissioner will publish a notice announcing the proposal. Proposals initiated by the Commissioner will likewise be published. Following such publication, the proceedings shall be the same as prescribed by section 409 of the act and the regulations in this part for the promulgation of a regulation.

(b) "Reasonable grounds" shall include an explanation showing wherein the person has a substantial interest in such regulation and an assertion of facts (supported by data if available) showing that new information exists with respect to the food additive or that new uses have been developed or old uses abandoned, that new data are available as to toxicity of the chemical, or that experience with the existing regulation or exemption may justify its amendment or repeal. New data should be furnished in the form specified in § 121.51 for submitting petitions.

§ 121.75 Exemption for investigational use and procedure for obtaining authorization to market edible products from experimental animals.

A food additive or food containing a food additive intended for investigational use by qualified experts shall be exempt from the requirements of section 409 of the act under the following conditions:

(a) If intended for investigational use in vitro or in laboratory research animals, it bears a label which states prominently, in addition to the other information required by the act, the warning:

Caution. Contains a new food additive for investigational use only in laboratory research animals or for tests in vitro. Not for use in humans.

(b) If intended for use in animals other than laboratory research animals and if the edible products of the animals are to be marketed as food, permission for the marketing of the edible products as food has been requested by the sponsor, and authorization has been granted by the Food and Drug Administration in accordance with § 135.3 of this chapter or by the Department of Agriculture in accordance with § 309.20 of Title 9 (9 CFR 309.20), and it bears a label which states prominently, in addition to the other information required by the act, the warning:

Caution. Contains a new food additive for use only in investigational animals. Not for use in humans.

Edible products of investigational animals are not to be used for food unless authorization has been granted by the U.S. Food and Drug Administration or by the U.S. Department of Agriculture.

[36 F.R. 18377, Sept. 14, 1971]

Subpart B—Exemption of Certain Food Additives From the Requirement of Tolerances

§ 121.101 Substances that are generally recognized as safe.

(a) It is impracticable to list all substances that are generally recognized as safe for their intended use. However, by way of illustration, the Commissioner regards such common food ingredients as salt, pepper, sugar, vinegar, baking powder, and monosodium glutamate as safe for their intended use. The lists in paragraph (d) of this section include addi-

tional substances that, when used for the purposes indicated, in accordance with good manufacturing practice, are regarded by the Commissioner as generally recognized as safe for such uses.

(b) For the purposes of this section, good manufacturing practice shall be defined to include the following restrictions:

(1) The quantity of a substance added to food does not exceed the amount reasonably required to accomplish its intended physical, nutritional, or other technical effect in food; and

(2) The quantity of a substance that becomes a component of food as a result of its use in the manufacturing, processing, or packaging of food, and which is not intended to accomplish any physical or other technical effect in the food itself, shall be reduced to the extent reasonably possible.

(3) The substance is of appropriate food grade and is prepared and handled as a food ingredient. Upon request the Commissioner will offer an opinion, based on specifications and intended use, as to whether or not a particular grade or lot of the substance is of suitable purity for use in food and would generally be regarded as safe for the purpose intended, by experts qualified to evaluate its safety.

(c) The inclusion of substances in the list of nutrients does not constitute a finding on the part of the Department that the substance is useful as a supplement to the diet for humans.

(d) Substances that are generally recognized as safe for their intended use within the meaning of section 409 of the act are as follows:

Product	Tolerance	Limitations or restrictions
(1) ANTICAKING AGENTS		
Aluminum calcium silicate	2 percent	In table salt.
Calcium silicate	5 percent	In baking powder.
Calcium silicate	2 percent	In table salt.
Magnesium silicate	do	Do.
*Sodium aluminosilicate (sodium silicoaluminate).	do	
*Sodium calcium aluminosilicate, hydrated (sodium calcium silicoaluminate).	do	
Tricalcium silicate	do	Do
(2) CHEMICAL PRESERVATIVES		
Ascorbic acid		
Ascorbyl palmitate		
Benzoic acid	0.1 percent.	
Butylated hydroxyanisole	Total content of antioxidants not over 0.02 percent of fat or oil content, including essential (volatile) oil content of food.	
Butylated hydroxytoluene	do	
Calcium ascorbate		
Calcium propionate		
*Calcium sorbate		
Caprylic acid		
Dilauryl thiodipropionate	Total content of antioxidants not over 0.02 percent of fat or oil content, including essential (volatile) oil content of the food.	In cheese wrape.
Erythorbic acid		
Gum guaiac	0.1 percent (equivalent antioxidant activity 0.01 percent).	In edible fats or oils:
*Methylparaben (methyl-p-hydroxybenzoate).	0.1 percent	
Potassium bisulfite		Not in meats or in food recognized as source of vitamin B_1.
Potassium metabisulfite		Do.
Potassium sorbate		
Propionic acid		
Propyl gallate	Total content of antioxidants not over 0.02 percent of fat or oil content, including essential (volatile) oil content of the food.	
*Propylparaben (propyl p-hydroxybenzoate).	0.1 percent	
Sodium ascorbate		
Sodium benzoate	0.1 percent	

See footnotes at end of table.

(f) *Trace minerals added to animal feeds.*[1] These substances added to animal feeds as nutritional dietary supplements are generally recognized as safe when added at levels consistent with good feeding practice.

Element	*Source compounds*
Cobalt	Cobalt acetate.
	Cobalt carbonate.
	Cobalt chloride.
	Cobalt oxide.
	Cobalt sulfate.
Copper	Copper carbonate.
	Copper chloride.
	Copper gluconate.
	Copper hydroxide.
	Copper orthophosphate.
	Copper oxide.
	Copper pyrophosphate.
	Copper sulfate.
Iodine	Calcium iodate.
	Calcium iodobehenate.
	Cuprous iodide.
	3,5-Diiodosalicylic acid.
	Ethylenediamine dihydroiodide.
	Potassium iodate.
	Potassium iodide.
	Sodium iodate.
	Sodium iodide.
	Thymol iodide.
Iron	Iron ammonium citrate.
	Iron carbonate.
	Iron chloride.
	Iron gluconate.
	Iron oxide.
	Iron phosphate.
	Iron pyrophosphate.
	Iron sulfate.
	Reduced iron.
Manganese	Manganese acetate.
	Manganese carbonate.
	Manganese citrate (soluble).
	Manganese chloride.
	Manganese gluconate.
	Manganese orthophosphate.
	Manganese phosphate (dibasic).
	Manganese sulfate.
	Manganous oxide.
Zinc	Zinc acetate.
	Zinc carbonate.
	Zinc chloride.
	Zinc oxide.
	Zinc sulfate.

(g) Synthetic flavoring substances and adjuvants that are generally recognized as safe for their intended use, within the meaning of section 409 of the act, are as follows:

Acetaldehyde (ethanal).
Acetoin (acetyl methylcarbinol).
Aconitic acid (equisetic acid, citridic acid, achilleic acid).
Anethole (parapropenyl anisole).
Benzaldehyde (benzoic aldehyde).
N-Butyric acid (butanoic acid).
d- or *l*-Carvone (carvol).
Cinnamaldehyde (cinnamic aldehyde).
Citral (2,6-dimethyloctadien-2,6-*al*-8, geranial, neral).
Decanal (*N*-decylaldehyde, capraldehyde, capric aldehyde, caprinaldehyde, aldehyde C-10).
Diacetyl (2,3-butandeione).
Ethyl acetate.
Ethyl butyrate.
3-Methyl-3-phenyl glycidic acid ethyl ester (ethyl-methyl-phenyl-glycidate, so-called strawberry aldehyde, C-16 aldehyde).
Ethyl vanillin.
Eugenol.
Geraniol (3,7-dimethyl-2,6 and 3,6-octadien-1-*ol*).
Geranyl acetate (geraniol acetate).
Glycerol (glyceryl) tributyrate (tributyrin, butyrin).
Limonene (*d*-, *l*-, *and dl*-).
Linalool (linalol, 3,7-dimethyl-1,6-octadien-3-*ol*).
Linalyl acetate (bergamol).
1-Malic acid.
Methyl anthranilate (methyl-2-aminobenzoate).
Piperonal (3,4-methylenedioxy-benzaldehyde, heliotropin).
Vanillin.

(h) Substances migrating to food from paper and paperboard products used in food packaging that are generally recognized as safe for their intended use, within the meaning of section 409 of the act, are as follows:

Acetic acid.
Alum (double sulfate of aluminum and ammonium potassium, or sodium).
Aluminum hydroxide.
Aluminum oleate.
Aluminum palmitate.
Ammonium chloride.
Ammonium hydroxide.
Calcium chloride.
Calcium hydroxide (lime).
Calcium sulfate.
Casein.
Cellulose acetate.
Clay (kaolin).
Copper sulfate.
Cornstarch.
Corn sugar (sirup).
Dextrin.
Diatomaceous earth filler.
Ethyl cellulose.
Ethyl vanillin.
Ferric sulfate.
Ferrous sulfate.
Formic acid or sodium salt.
Glycerin.
Guar gum.
Invert sugar.

[1] All substances listed may be in anhydrous or hydrated form.

Iron, reduced.
Locust bean gum (carob bean gum).
Magnesium carbonate.
Magnesium chloride.
Magnesium hydroxide.
Magnesium sulfate.
Methyl and ethyl acrylate.
Mono- and diglycerides from glycerolysis of edible fats and oils.
Oleic acid.
Oxides of iron.
Potassium sorbate.
Propionic acid.
Propylene glycol.
Silicon dioxides.
Pulps from wood, straw, bagasse, or other natural sources.
Soap (sodium oleate, sodium palmitate).
Sodium aluminate.
Sodium carbonate.
Sodium chloride.
Sodium hexametaphosphate.
Sodium hydrosulfite.
Sodium hydroxide.
Sodium phosphoaluminate.
Sodium silicate.
Sodium sorbate.
Sodium sulfate.
Sodium thiosulfate (additive in salt).
Sodium tripolyphosphate.
Sorbitol.
Soy protein, isolated.
Sulfamic acid.
Sulfuric acid.
Starch, acid modified.
Starch, pregelatinized.
Starch, unmodified.
Sucrose.
Talc.
Urea.
Vanillin.
Zinc hydrosulfite.
Zinc sulfate.

(l) Substances migrating to food from cotton and cotton fabrics used in dry food packaging that are generally recognized as safe for their intended use, within the meaning of section 409 of the act, are as follows:

Acacia (gum arabic).
Acetic acid.
Beef tallow.
Calcium chloride.
Carboxymethylcellulose.
Coconut oil, refined.
Corn dextrin.
Cornstarch.
Fish oil (hydrogenated).
Gelatin.
Guar gum.
Hydrogen peroxide.
Japan wax.
Lard.
Lard oil.
Lecithin (vegetable).
Locust bean gum (carob bean gum).
Oleic acid.
Peanut oil.
Potato starch.
Sodium acetate.
Sodium bicarbonate.
Sodium carbonate.
Sodium chloride.
Sodium hydroxide.
Sodium sulfate.
Sodium silicate.
Sodium tripolyphosphate.
Sorbose.
Soybean oil (hydrogenated).
Stearic acid.
Talc.
Tall oil.
Tallow (hydrogenated).
Tallow flakes.
Tapioca starch.
Tartaric acid.
Tetrasodium pyrophosphate.
Urea.
Wheat starch.
Zinc chloride.

(Secs. 201(s), 409, 701(a), 52 Stat. 1055, 72 Stat. 1784, 1785 et seq., as amended; 21 U.S.C. 321(s), 348, 371(a)) [30 F.R. 15845, Dec. 23, 1965, as amended at 33 F.R. 5619, Apr. 11, 1968; 35 F.R. 1049, Jan. 27, 1970; 37 F.R. 2437, Feb. 1, 1972]

§ 121.102 Adjuvants for pesticide chemicals.

Adjuvants, identified and used in accordance with 40 CFR 180.1001 (c) and (d), which are added to pesticide use dilutions by a grower or applicator prior to application to the raw agricultural commodity, are exempt from the requirement of tolerances under section 409 of the act.

(Sec. 409, 72 Stat. 1785; 21 U.S.C. 348)

Subpart C—Food Additives Permitted in Feed and Drinking Water of Animals or for the Treatment of Food-Producing Animals

AUTHORITY: The provisions of this Subpart C issued under sec. 409, 72 Stat. 1785; 21 U.S.C. 348, unless otherwise noted.

§ 121.200 Definitions and interpretations applicable to Subpart C.

(a) Regulations prescribing conditions under which additives may be safely used in animal feed, animal feed supplements, concentrates, or premixes or in animals intended for food use shall not be construed to relieve such additives from the provisions of sections 505 and 507 of the act, where applicable, and § 121.7 and § 121.9 of the food additive regulations.

(b) For the purposes of this Subpart C:

84-069—73——20

Transfer of carbon from formate to amino acids of milk protein in the intact cow[1]

A. L. BLACK, M. KLEIBER, J. R. LUICK, AND J. J. KANEKO
University of California School of Veterinary Medicine and College of Agriculture, Davis, California

BLACK, A. L., M. KLEIBER, J. R. LUICK, AND J. J. KANEKO. *Transfer of carbon from formate to amino acids of milk protein in the intact cow.* Am. J. Physiol. 203(6): 1067–1070. 1962.—C^{14}-formate was injected intravenously into two lactating cows or infused into the cistern of a quarter of the udder in a third cow. Several amino acids were recovered from milk protein collected at intervals during a 36-hr period after the C^{14}-formate was injected. The amino acids were assayed for C^{14} and, in the case of serine and methionine, degraded to establish the intramolecular C^{14} distribution. The C^{14} was found to be confined largely to the hydroxymethyl carbon of serine and to the methyl carbon of methionine. Relatively little C^{14} was present in other positions of these amino acids or in the other amino acids normally synthesized by the lactating cow, showing that formate carbon was confined to a very limited pool during utilization. The incorporation of formate carbon into amino acids apparently takes place largely outside the udder, presumably in the liver.

THE SHORT-CHAIN FATTY ACIDS are quantitatively important metabolites in the ruminant and are readily utilized in the synthesis of all the major organic constituents of milk in cows. Earlier studies in our laboratory have dealt with precursor-product relationships in the lactating cows and the relative efficiency of utilization of fatty acids for milk formation (1). One of the interesting observations in these comparisons was the preferential utilization of formate for the synthesis of milk protein. It was found that 50% of the C^{14}-formate transferred to milk products was recovered in casein.

The present study reports on the distribution of C^{14}-formate among amino acids from milk protein. The results show that formate carbon was rapidly incorporated into serine and methionine and that these two amino acids accounted for most of the C^{14} in casein. The results with C^{14}-formate are discussed in relation to studies reported earlier on amino acid synthesis in lactating cows injected with C^{14}-acetate (2), C^{14}-propionate (3), C^{14}-butyrate (4), and C^{14}-glucose (5).

Received for publication 9 April 1962.
[1] This work was supported by grants from the Public Health Service (RG 8183) and the National Science Foundation (G 9893).

METHODS

Table 1 lists some of the experimental conditions used in the present study. In *trials 1* and *2* the animals were injected intravenously with C^{14}-formate through plastic tubing inserted into the jugular vein prior to the trial. Milk was collected at 3, 10, 24, and 36 hr after the injection of the labeled compound and was fractionated into organic components. The casein or whey proteins were hydrolyzed and the amino acids separated with ion-exchange columns, crystallized, combusted, and degraded as reported earlier (2–5). In *trial 3* C^{14}-formate was infused into the cistern of the right front quarter of a third cow to obtain information on the site of utilization of formate carbon for synthesis of amino acids. The formate was administered via a metal cannula that passed through the teat canal into the cistern. The cannula was removed from the teat canal immediately after the C^{14}-labeled compound was infused. Three hours later milk was collected separately from the right front quarter and from the other three quarters. Casein was prepared from each milk sample, and amino acids were recovered as described above.

The amount of pure methionine recovered from protein hydrolysates on the ion-exchange columns was sufficient only for total combustion to measure the specific activity of the samples. For the degradation of methionine it was necessary to use samples containing varying amounts of isoleucine that were eluted from the column together with methionine. Carrier methionine was added to these overlapping fractions to ensure sufficient material for the degradation procedure, but since the dilution was not known accurately results were not corrected for carrier addition. Because of this, there is a discrepancy between the amount of C^{14} in methionine measured by complete combustion and the C^{14} recovered in the degradation. The dilution does not affect the relative C^{14} distribution, however, since the degradation and assay were carried out on the methyl carbon and the "homocysteine" moiety from the same diluted sample.

Methionine was degraded according to the method described by Sakami (6). At the end of the reaction, the

TABLE 1. *Experimental conditions*

Formate Trial No.	Route of Isotope Administration	Cow No.	Body Wt., kg	Dose	
				mc	μc/kg Body wt.
1	Intravenous	860	524	5.0	9.54
2	Intravenous	941	454	5.0	11.0
3	Cistern of mammary	147	453	3.30	7.3

HI was distilled off, the residue brought to pH of about 10 by addition of dilute NaOH, and the cyclic thioether derived from the homocysteine moiety of methionine was extracted into ether. The extract was transferred to the combustion flask, the solvent removed by distillation, and the residue wet-combusted with Van Slyke reagent.

All samples used in this study were converted to $BaCO_3$ and counted at infinite thickness on an end-window gas-flow Geiger counter. Results are expressed in microcuries per gram atom carbon as fraction of microcuries injected per kilogram body weight. Specific activities expressed in this manner provide a basis for comparing results among cows of different weight receiving different amounts of carbon 14.

RESULTS

The specific activities of amino acids from milk proteins are listed in Table 2 for samples collected at different times after intravenous injection of C^{14}-formate. The specific activities were greatest in the earliest sample, 3 hr after injection of the C^{14}-formate, and decreased rapidly in samples collected at later intervals. Serine and methionine had the greatest specific activities in all samples of milk protein. In casein these two amino acids accounted for 90–95% of the total C^{14} present in the protein. Even at 36 hr the specific activities of serine and of methionine were 10–30 times greater than that of any other amino acids. This would indicate that the formate carbon remained closely confined within a "C_1 pool" during the experimental period and did not become widely distributed among metabolic pools utilized by the cow for the synthesis of other amino acids. A close metabolic relationship between formate carbon and the amino acids, serine and methionine, have been demonstrated also in studies with the intact rat (7) and with liver extracts from pigeons and sheep (8–10).

There was no appreciable amount of carbon 14 in valine or lysine at any time during the experimental period. These results with formate are consistent with our earlier observation, after using acetate, propionate, and butyrate (11), that amino acids which are dietary essentials in other mammalian species do not become labeled after intravenous injections of cows with C^{14}-labeled compounds.

Table 3 gives the intramolecular C^{14} distribution in samples of serine and methionine recovered from 3 to 36 hr after injection of C^{14}-formate. In each case the C^{14} in the hydroxymethyl carbon of serine or the methyl carbon of methionine accounted for most of the isotope in the amino acids. This selective labeling by formate of specific carbon atoms in serine and methionine is known from studies with other species (7, 8); the present results demonstrate that formate carbon has a similar metabolic fate in the intact lactating cow. In this respect formate stands apart from the other short-chain fatty acids, since carbon from the latter becomes more widely dispersed among several amino acids. In earlier studies with lactating cows injected with C^{14}-acetate (2), C^{14}-propionate (3), or C^{14}-butyrate (4) the specific activities of glutamic and aspartic acids, alanine, glycine, proline, and arginine were greater than in the present study with C^{14}-formate. In addition, the isotope was found to be more uniformly distributed intramolecularly in the amino acids than was the case with formate. Also, after injection of cows with C^{14}-glucose (5), or C^{14}-fructose (12), the C^{14} was widely distributed among all the nonessential amino acids.

DISCUSSION

Formate occurs in the blood of several animal species in amounts ranging from 5 to 50 M of total volatile fatty acids (13). In cattle the concentration of formate was reported to be 0.1–0.3 mmoles liter of blood. This would amount to 3.5–10 mmoles circulating in total blood of cows similar in weight to those used in this study. Since the amount of C^{14}-formate injected (0.3–0.5 mmoles) represents only 5–15% of the amount normally circulating in the blood, these experiments would not be expected to create unphysiological levels of formate.

The origin of blood formate is still uncertain but it apparently is not derived from the rumen as are the

TABLE 2. *Specific activities* of amino acids from milk protein after intravenous injection of C^{14}-formate into lactating cows*

Amino Acid	Formate Trial 1		Formate Trial 2				
	Sample time, hr†						
	3		3		10	24	36
	Casein	Whey protein	Casein	Whey protein	Casein	Casein	Casein
Serine	46.2	21.6	64.5	43.3	20.7	3.57	0.99
Methionine	55.0		57.2		13.9	2.68	0.93
Glutamic	1.12	0.67	0.68	0.54	0.36	0.1	0.04
Aspartic	0.96	0.71	1.04	1.07	0.26	0.06	0.03
Alanine	1.85	0.79	1.75	0.96	0.70	0.15	0.07
Glycine	1.94	0.31	1.55		0.79	0.14	0.06
Proline	0.18	0.21	0.09	0.14	0.06	0.02	0.02
Arginine	0.15		0.13		0.29	0.04	0.003
Cystine		0.23		0.77			
Valine	0.008	0.02	0.02				
Lysine					0.003	0.01	0.009

* Specific activities expressed as microcuries C^{14} per gram atom of carbon as fraction of microcuries C^{14} injected per kilogram body weight. † Time of sample refers to interval between isotope injection and time when milk samples were collected from the cows.

TABLE 3. *Specific activity of individual carbon atoms of serine and methionine from milk protein after administering C^{14}-formate*

Trial No.	Sample		Amino Acid Degraded	Carbon Atom				
	Time, hr	Source		C-1	C-2	C-3	C-4	C-5
1	3	Casein	Serine	1.26	6.30	124.0		
1	3	Whey	Serine	0.99	1.77	58.4		
2	3	Casein	Serine	2.93	8.0	172.0		
	3	Whey	Serine	1.3	1.9	114.5		
2	10	Casein	Serine	0.46	5.5	54.4		
2	24	Casein	Serine	0.14	0.61	8.7		
2	36	Casein	Serine	0.07	0.29	3.0		
3	3	Casein	Serine*	0.3	0.8	74.8		
2	3	Casein	Methionine	0.18†				20.4
2	24	Casein	Methionine	0.36†				4.64

* This serine sample was recovered from the right front quarter of the mammary gland, into which C^{14}-formate was infused. † Carbons (C-1, -2, -3, -4) measured together (see METHODS).

other volatile fatty acids, which are largely the product of rumen microbial fermentation. Annison found only insignificant amounts of formate in all parts of the sheep alimentary tract, including the rumen, except for small amounts in intestinal contents (13). Additional evidence that the rumen has a small role, if any, in formate production comes from studies with perfused goat rumen. McCarthy et al. (14) found a net decrease of formate in blood perfusing the rumen whereas all other short-chain fatty acids increased appreciably. In contrast to these results with a perfused rumen, the level of formate increased severalfold in the fluid perfusing goat liver during a period of 1 hr (15). This study provided direct evidence that the liver may be an important source of blood formate in the ruminant.

Several compounds are known to contribute carbon to the C_1 pool, some of which could appear as blood formate. Studies with rats have shown that serine (16, 17), glycine (18), histidine (19), methionine (20), choline (21), and acetone (22) contribute carbon to formate synthesis. In addition, other amino acids that give rise to ketone bodies [e.g., tyrosine and phenylalanine or leucine (23, 24)] are potential sources of formate carbon via acetone formation. Thus it appears that the metabolic products of many compounds may contribute to the production of formate and that the liver may be an important site for this activity.

The rapid decrease in the specific activities of amino acids from casein with time suggests that the C_1 pool was rapidly turning over in the cow. An indication of the rate of this process is apparent in the plot of the log of the specific activity of methionine carbon and the hydroxymethyl carbon of serine (Fig. 1) as a function of time after injection of C^{14}-formate. The results for both amino acids deviate from linearity, indicating that the turnover of the C_1 pool that contributes carbon to the synthesis of these amino acids does not follow simple first-order kinetics. Thus this C_1 pool would not be among

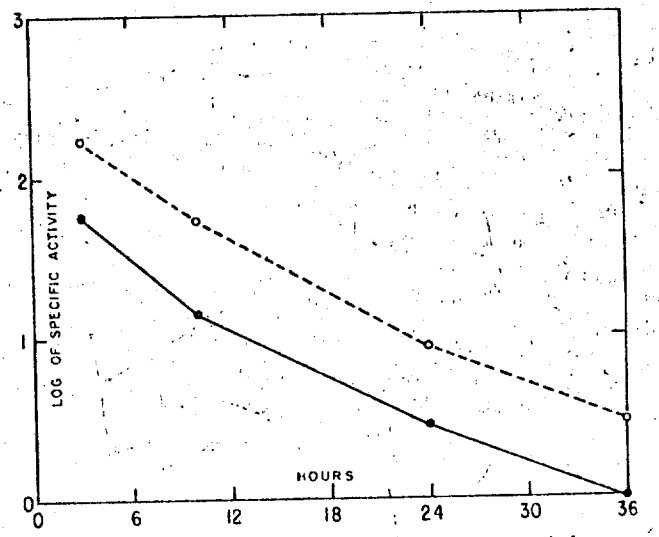

FIG. 1. Log of specific activity vs. time for hydroxymethyl group of serine (○— —○) and for methionine (●——●) recovered from casein after intravenous injection of C^{14}-formate into lactating cows.

the limited number of pools, such as that for plasma phospholipid (25), in which simple dilution determines the kinetics. However, the fact that the data for serine and methionine follow similar curves would indicate that carbon was utilized from a single pool during synthesis of these amino acids.

It was of interest to determine whether the transfer of C^{14} from formate to serine or methionine might be taking place in the mammary gland where milk protein is synthesized. This possibility was studied by infusing C^{14}-formate into the cistern of the right front quarter of the udder. If formate were directly utilized in the udder, then the products synthesized in the infused quarter would be provided with carbon of very high specific activity. On the other hand, if formate were utilized outside the udder (e.g., in the liver) the trace amounts of

TABLE 4. *Specific activities* of amino acids from casein after infusion of C^{14}-formate into udder of intact cow*

Amino Acid	Udder Quarter From Which Casein was Recovered		Ratio of RFQ to 3Q
	RFQ†	3Q†	
Serine	26.3	25.9	1.02
Methionine	5.8	5.1‡	1.1
Glutamic	0.41	0.19	2.1
Aspartic	0.26	0.19	1.4
Alanine	0.7	0.8	0.9
Glycine	0.2	0.1	2.0

* See footnote to Table 2. † RFQ refers to right front quarter of udder. C^{14}-formate was infused into cistern of RFQ at start of experiment. Milk was collected separately from RFQ and from the other three quarters (3Q), and the casein prepared from each milk sample was the source of the amino acids studied. ‡ Contaminated with some isoleucine.

C^{14}-formate injected would be diluted by the blood formate pool prior to its utilization. This would result in a lower specific activity in the formate carbon but, more important, the products synthesized from this carbon would be returned to the udder without distinction among the quarters. In this latter case, there should be no difference in the specific activity of amino acids from the infused quarters and the same amino acid from the other three noninfused quarters.

The specific activities of amino acids from the right front quarter and from the other three quarters are given in Table 4. Serine and methionine had the greatest specific activities among the amino acids, as was also the case after intravenous injections of C^{14}-formate. However, the specific activities were approximately the same in these amino acids from the injected quarter and from the noninjected quarters and were only one-half to one-tenth as great as the specific activities found after intravenous injection of C^{14}-formate. These results would be consistent with the transfer of carbon from formate to serine and methionine outside the mammary gland.

In glycine, glutamic acid, aspartic acid and alanine, all of which had very low specific activities, there were small differences between results for the injected and noninjected quarters. These differences may reflect one or more of the following: minor utilization of formate carbon by mammary gland; the influence of fixation of $C^{14}O_2$ derived from oxidation of C^{14}-formate in the infused quarter; and experimental error associated with measurement of small amounts of C^{14}. In any case the results indicate that the major utilization of formate carbon (for synthesis of serine and methionine) occurred outside the mammary gland, presumably in the liver.

In contrast to these results with formate were those obtained when 2-C^{14}-acetate was infused into the mammary gland. Amino acids from the infused quarters, except for glycine and arginine, had specific activities that were two to five times greater than the specific activities of corresponding amino acids from the noninfused quarters (26). Similar results to those described for acetate were observed when U-C^{14}-glucose, U-C^{14}-fructose, 2-C^{14}-propionate, and 2-C^{14}-butyrate were infused into one quarter of the mammary gland of intact cows (27).

These results show that the mammary gland has the capacity to synthesize amino acids to some extent at least, but that the utilization of carbon from formate in the synthesis of amino acids occurs largely outside of the mammary gland.

The authors acknowledge the valuable technical assistance of J. A. Nakagiri, Edna Yeh, and T. E. Chapman.

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SILAGE AND MILK PRODUCTION, A COMPARISON BETWEEN WILTED AND UNWILTED GRASS SILAGES MADE WITH AND WITHOUT FORMIC ACID

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ABSTRACT

Three grass silages, all made in mid-June from the same herbage, were compared in a 12-week winter-feeding experiment with 12 Ayrshire cows. The silages were fed *ad lib.* with a supplement of moist barley and groundnut cake. Silage A was made from unwilted herbage with no additive, silage B from identical herbage treated with ½ gal of formic acid/ton (2·24 l./tonne) of herbage, and silage C from the herbage after wilting for 28 h. The DM contents of silages A, B and C were 20·5, 21·1 and 31·8%, respectively, and the contents of digestible organic matter in the DM were 58·9, 68·8 and 67·8%. Silage and total DM intake were highest on the treatment containing silage B. The mean daily milk yields on the treatments with silages A, B and C were 35·1, 38·0 and 34·3 lb (15·9, 17·2 and 15·6 kg), respectively and the solids-not-fat (S.N.F.) contents of the milk averaged 8·53, 8·54 and 8·50%. It is concluded that silage B, the unwilted silage with formic-acid additive, was superior to both the untreated and the wilted silage as a feed for dairy cows.

INTRODUCTION

The results from a recent experiment at this Institute showed clearly that unwilted grass silage made with formic acid was superior to untreated silage as a feed for dairy cows (8). American workers have also demonstrated the nutritional advantages of formic acid as an additive for unwilted silage (16). From these experiments it is apparent that the addition of formic acid improves the fermentation of the silage, lowers the pH and ultimately increases the DM and nutrient intake of the animal. It is, however, well known that if herbage is wilted before ensiling, the fermentation characteristics will be improved (12, 13) and the intake of DM by ruminants increased when compared with unwilted silage (1, 12). Wilting could therefore be a possible alternative to the use of formic acid and the technique would require no special equipment or materials. To compare these methods, silages were made from unwilted herbage either with or without formic acid and from wilted herbage and the silages fed to dairy cows.

EXPERIMENTAL

Experimental silages

The herbage for the experimental silages was cut from two leys consisting mainly of cocksfoot, timothy, meadow fescue and rough-stalked meadow grass. The leys were given a dressing of compound fertilizer supplying 80 lb N, 40 lb P_2O_5 and 40 lb K_2O/ac in mid-April and the herbage was cut and ensiled on 10, 11 and 12 June 1969. At this period the heads of the cocksfoot and the meadow fescue had emerged fully, but no timothy heads were visible. The yield of herbage DM was approximately 48 cwt/ac. The three silages were: A. Unwilted, with no additive; B. Unwilted, plus formic acid; C. Wilted, with no additive.

All the herbage for the three silages was cut with a reciprocating-blade mower. The herbage for silage A was picked up immediately from the swathe with a forage harvester, before any wilting had occurred, and ensiled. Alternate loads of herbage for silage B were collected in exactly the same way, but an additive (Add-F) containing 80–85% w/w formic acid was applied at the rate of ½ gal/ton of herbage. The two herbages were put in two separate 30-ton vacuum silos. A third swathe for silage C was left undisturbed until the following day when it was picked up with the forage harvester and ensiled, with no additive, in a third vacuum silo. Air from the three silos was evacuated immediately

after they were filled. The weather during the period of silage making was dry and sunny with maximum air temperatures of 73 and 75°F on 11 and 12 June, respectively. Thermistors were placed in the centre of each silo to measure the temperature of the silage.

Feeding

There were three periods of 4 weeks each during the feeding experiment, which lasted from 24 Nov. 1969 to 15 Feb. 1970. The three silages were given as the variable feed on the three experimental treatments A, B and C. The cows were offered the silage at 7 a.m., noon and 5 p.m. in sufficient quantities to ensure that a residue was available for weighing 3 h later. In addition to the silage, rolled moist barley with an average DM content of 80·2% was fed at the rate of 3·9 lb per 10 lb milk. Each cow also received 1 lb groundnut cake and 5 oz mineral mixture (Boots Super Mindif) per day.

Experimental design and methods

Nine Ayrshire cows and three first-calf heifers, which had calved on average 10 weeks before the start of the experiment, were used. The experimental methods were the same as those in previous feeding experiments (7, 8), except that the animals in the balanced groups were allotted to the treatment sequences in two pairs of 3×3 Latin square (9).

RESULTS

Grass and silage composition

The DM contents of the herbage for silages A and B were virtually identical with values of 19·6 and 19·8%, respectively (Table 1), whereas that of the herbage for silage C averaged 35·2% after wilting. The average length of the wilting period was 28 h and this wilting clearly increased the variability of the DM values of the herbage, as indicated by the high standard deviation attached to the mean value (Table 1). As expected, the digestibility and the total soluble sugar content of the wilted herbage for silage C were lower than those in the two unwilted herbages (Table 1).

The temperature data given in Table 1 and Fig. 1 show that the acid-treated silage was cooler than either the untreated silage A or the wilted silage C for 10–11 weeks after the silos were filled. From late August onwards there was little difference in temperature between silages A and B, but the higher temperature of silage C was maintained until the silo was opened in November.

The DM content of silage B was consistently higher than that of silage A throughout the winter-feeding period (Table 2), but both were markedly lower than the value for the wilted silage C which averaged 31·8%. The acid-treated silage (silage B) had a higher crude-protein content, and lower crude-fibre content, in the DM than the other two silages. The amounts of the organic acids in the silages (Table 3) indicate a better pattern of fermentation in the acid-treated silage B than in the untreated silage A, but the highest lactic-acid content was found in the wilted silage C. The pH values (Table 3) show a satisfactory low value for silage B, whereas silages A and C had higher values. The wilted silage C had a much wider range of pH values than silage A.

TABLE 1. Details of the herbage ensiled (1969) and the silage temperature

Herbage for silage treatment	DM of herbage (%)	Percentage of DM: Digestible organic matter†	Percentage of DM: Crude protein	Percentage of DM: Total soluble sugars	Maximum temperature in silo (°F)
A	19·6±0·3*	65·5	13·1	12·8	102
B	19·8±0·4	65·1	13·6	12·1	91
C	35·2±5·0	62·9	13·0	10·6	106

*Standard deviation.
†Determined by *in vitro* method (2)

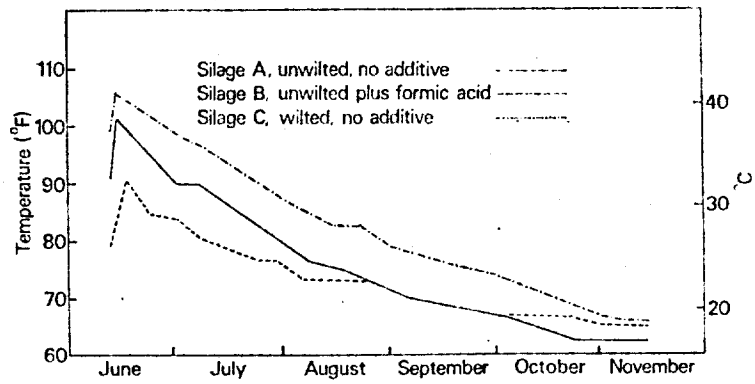

Fig. 1. The temperature of the silages.

The digestibilities of the three silages were determined by the *in vitro* technique (3) and by using the total collection method with three Ayrshire cows (Table 3). These results indicate that the DM of silage A contained less digestible nutrients than the DM of the other two silages. Silage B, the acid-treated silage, had the highest content of digestible organic matter and the highest digestible CP value.

Feed and water consumption

The intakes of barley and of groundnut were identical in the three treatments, but the silage intake varied between the treatments (Table 4).

TABLE 2. The composition of the grass silages

Silage fed on treatment	DM of silage (%) †	Percentage of DM: Crude protein	Crude fibre	Ether extract	Ash	N-free extract	Total soluble sugars
A	20·5±1·3*	12·6	35·1	3·8	8·4	40·1	1·1
B	21·1±1·3	14·5	32·3	4·1	7·4	41·7	0·7
C	31·8±3·9	13·1	33·7	3·7	7·9	41·6	0·7

*Standard deviation.
†Determined by toluene distillation method (10)

TABLE 3. The digestibility and organic acid content of the silages

Silage fed on treatment	Digestible organic matter in DM (%): *In vivo*	*In vivo* predicted from *in vitro* data†	Digestible crude protein in DM (%)	Percentage of DM: Lactic	Acetic	Propionic	Butyric	Silage pH
A	58·9	58·2	7·0	0·4	3·8	0·8	2·8	5·0±0·1*
B	68·8	65·1	10·4	0·6	0·7	0·1	0·1	3·9±0·2
C	67·8	61·2	8·6	2·8	1·2	0·2	1·2	4·8±0·3

*Standard deviation.
†From regression equation (3).

TABLE 4. Feed intake on the three feeding treatments

Treatment	DM (lb/cow per day) Silage	Barley	Groundnut cake	Total	Daily DM intake as % of liveweight
A	16·7	11·2	0·9	28·8	2·7
B	18·3	11·2	0·9	30·4	2·8
C	16·9	11·2	0·9	29·0	2·7
Standard error of a difference between two means	±0·49	±0·08	—	±0·46	±0·03

The highest intake occurred in treatment B which contained the acid-treated silage where the amount of silage DM eaten by the cows was significantly higher than in treatments A and C ($p<0·01$ and $<0·05$, respectively). The difference in intake of silage DM between treatments A and C was not significant. These differences in silage intake were reflected in the total intake of DM (Table 4) which was significantly higher ($p<0·01$) in treatment B than in the other two treatments. Regressions of the mean daily silage DM intake on the DM percentage of the silage were significant ($p<0·05$) for each silage, but the differences between the silages were not significant.

The total amounts of water consumed by the cows on the different treatments did not differ significantly, although the amount drunk and the amount in the feed varied widely (Table 5). The amount of water drunk in treatment C was significantly ($p<0·01$) higher than that in treatments A and B but, conversely, the amount of water in the feed in treatment C was significantly lower than that in A and B. When measured as the weight of water per lb of DM consumed there was no significant difference between treatments A and B, i.e., between the untreated and the acid-treated silages, but the value in treatment C was higher than that in treatment B ($p<0·05$). The average temperature in the byre was 9°C.

Milk yield and composition

The average daily milk yields per cow in each treatment are shown in Table 6 as uncorrected values and when corrected to 4% fat content. Milk yields were significantly higher ($p<0·01$) in treatment B than in treatments A and C. The uncorrected yield in treatment A was significantly higher ($p<0·05$) than the yield in treatment C, but when the two yields were adjusted to the same fat content treatments A and C were not significantly different.

The effect of the treatments on milk composition (Table 6) was small and not statistically significant, although there was a tendency for the lowest S.N.F. and protein values to be found in treatment C.

The mean liveweights of the cows (Table 6) in treatment B were significantly ($p<0·05$)

TABLE 5. The water intake on the three feeding treatments

Treatment	Drinking water (lb/day)	Water in feed (lb/day)	Drinking water and water in feed (lb/day)	lb water/lb DM consumed*
A	58·0	66·8	124·8	3·25
B	58·1	70·4	128·5	3·13
C	86·7	40·1	126·8	3·32
Standard error of a difference between two means	±2·73	±0·42	±3·28	±0·08

*Allowance made for the water content of the milk.

TABLE 6. The mean daily milk yield, chemical composition of the milk and the mean liveweight of the cows

Treatment	Milk yield (lb/cow per day)		Milk composition (%)					Liveweight (lb)
	Uncorrected	Corrected to 4% fat content	Fat	S.N.F.	Total solids	Crude protein*	Lactose	
A	35·1	37·5	4·48	8·53	13·01	3·07	4·83	1052
B	38·0	40·2	4·40	8·54	12·94	3·07	4·85	1070
C	34·3	37·2	4·56	8·50	13·06	3·06	4·82	1060
Standard error of a difference between two means	±0·29	±0·51	±0·08	±0·02	±0·09	±0·03	±0·02	±7

*N×6·38

higher than those in treatment A, but other differences between treatments were not significant. The mean daily loss of liveweight was 0·26 lb throughout the period of the experiment and in this respect the differences between the treatments were not significant.

DISCUSSION

The results of this experiment confirm the findings from the previous experiment which showed that silage made from unwilted herbage treated with formic acid was superior to untreated silage as a feed for dairy cows (8). As in the previous experiment, the silage treated with formic acid had the following advantages over the untreated silage; a lower pH, an improved fermentation, a higher DM content and a higher nutritive value, which resulted in higher milk yields. The increase in daily milk yield attributable to the acid-treated silage was 2·9 lb per cow in the present experiment and 2·5 lb per cow from the June-cut silage in 1968 (8). The similarity of the increases, 7% in 1968 and 8% in 1969, is of particular interest as the type of herbage and its treatment in the field during silage making were different in the two years. In 1968 the herbage consisted of timothy and perennial ryegrass, whereas in 1969 the herbage was a mixture of cocksfoot, timothy, meadow fescue, rough-stalked meadow grass and only a little perennial ryegrass. A further difference was that the herbage in 1968 was cut and collected with a forage harvester which chopped and lacerated the material, but in 1969 the herbage was cut with a reciprocating blade mower and picked up with a forage harvester with a minimum of laceration. Norwegian work has suggested that formic acid is less efficient as a silage additive when the grass is long rather than lacerated, but in the present experiment formic acid gave a good fermentation with herbage that was only slightly lacerated (14).

Wilting did not have the same beneficial effects as did the use of formic acid. The pH of the wilted silage was only 0·2 units lower than that of the untreated silage, and the intake of silage DM was virtually the same with the two silages. The content of digestible organic matter in the DM of the wilted silage, determined *in vivo*, was apparently higher than that of the untreated silage (Table 3) but it was clear that the lowest milk yields occurred in the treatment containing the wilted silage (Treatment C, Table 6). It would appear, therefore, that the value of the wilted silage for milk production was without any doubt lower than the value of the untreated silage. The *in vivo* values of the digestible organic matter in the DM predicted from the *in vitro* data, using a bivariate regression (3) which incorporates a correction for the DM content of the silage (Table 3), suggests that the wilted silage had only a slightly higher value than the untreated silage. If these predicted values are then used to calculate the starch equivalent (SE) of the silage DM (4) the values found for silages A, B and C are 42, 48 and 44, respectively. These estimates of SE values are subject to error and an SE value of 40 for the wilted silage is suggested from calculations based on the known response in milk yield to changes

in the SE intake of cows (6). From the combined evidence of the feeding experiment and the digestibilities it is concluded that the acid-treated silage had the highest value for milk production, and the wilted silage the lowest. The daily intake of digestible CP in the treatment containing the wilted silage was calculated to be 105% of the Woodman standard (11) so protein intake was presumably not the limiting factor. The treatment containing the formic-acid silage gave higher yields of milk than the treatment with the wilted silage and this confirms the results of a similar experiment conducted in Norway (15).

Although the wilted silage gave the lowest milk yields, it should be noted that the wilting of the herbage was done rapidly and with a minimum loss of nutrients in the swathe. The DM content of the cut herbage increased from 19·7 to 35·2% in 28h with a reduction of only 2·4 units in the content of digestible organic matter in the herbage DM. Losses in the field were not measured, but no rain fell on the cut herbage and no visible losses occurred during the collection operation. The maximum temperature in the wilted silage was 106°F, which is not considered to be excessively high. The wilting of silage will reduce, and even eliminate, the production of effluent and in some circumstances increase the speed of the silage-making process, but the effects of feeding the silage to milking animals is variable. A recent experiment with silages of high digestibility showed no advantage in terms of milk yield and milk composition as a result of feeding a wilted silage containing 29% DM, compared with an unwilted silage with only 20% DM (1). Another experiment comparing silages with 22 and 32% DM showed only a small and non-significant difference in milk yield between the treatments (5). Experimental and practical experience at the Hannah Institute supports the view of Alder *et al.* (1) that wilting should be adopted if it helps the ensiling process, rather than as a necessary technique to ensure maximum voluntary intake with the doubtful anticipation of higher animal production.

In addition to the nutritional advantage of using formic acid for preserving unwilted silage, it was evident from the experiments and from observations of silage made for 3 years on a commercial scale at this Institute that formic-acid silage was much pleasanter to handle than untreated silage. This is attributed to the lower pH and the lower content of butyric acid in the treated silage, and results from this Institute agree with those of Waldo *et al.* (16) who found formic acid silage more aesthetically acceptable than many untreated unwilted silages.

ACKNOWLEDGEMENTS

The authors thank N. H. Strachan and Mrs C. A. Carter for the chemical analyses of the feeds and the milk, R. Waite for the acid analyses, and R. H. Alexander and Miss M. McGowan, West of Scotland Agricultural College, for the *in vitro* digestibility determinations.

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(*No. 823. Received for publication 31 July 1970*)

Vol. LXXXV, No. 821 The American Naturalist March-April, 1951

A SURVEY OF CHEMICALS FOR MUTAGENIC ACTION ON E. COLI[1]

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INTRODUCTION

At present we have available two good methods, using *Escherichia coli*, for the quantitative study of spontaneous mutability and of the mutagenic action of various agents such as radiations and chemicals. The older of the two methods deals with mutational changes from phage sensitivity to phage resistance, and the more recently developed one (Demerec, 1951; Bertani, 1951) with back-mutations from streptomycin dependence to nondependence. In both cases the mutants can be easily classified; very large numbers of bacteria may be tested; and slight increases in mutation rate can be easily detected.

After the streptomycin method had been developed we undertook extensive tests of a number of chemicals for mutagenicity, using mainly this method because it is the simpler of the two.

SELECTION OF CHEMICALS

Previous work by Witkin with *E. coli* (Witkin, 1947; Demerec, Wallace, and Witkin, 1948) had indicated that compounds exhibiting quite different properties may all be mutagenic. Using the phage method, she found that sodium desoxycholate, pyronin, acriflavine, caffeine, and colchicine acted as mutagens, but detected no mutagenic effect in tests with methyl green, basic fuchsin, and methylcholanthrene endosuccinate.

In planning the present survey, therefore, we prepared a list of chemicals, including representatives of widely separated groups, from simple inorganic and organic compounds to complex organic chemicals. Before the scheduled tests had been completed, however, we found that certain ferrous and manganous salts are very potent mutagens, and our attention was thus diverted to a detailed study of these compounds. Since it will require some time to

[1]This study was conducted in part under grants from the American Cancer Society, through the Committee on Growth of the National Research Council, and from the Jane Coffin Childs Memorial Fund for Medical Research. The assistance of Miss Eileen Yongen in carrying out the experiments is acknowledged.

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carry this out, the data so far available from the survey are being summarized here.

MATERIALS AND METHODS

The "streptomycin" method for detection of induced mutations has been fully described by Bertani (1951). Only technical details will be given here.

Strains B/Sd-4/1,3,4,5 and B/Sd-4/3,4 of *E. Coli* were used throughout this work. For every experiment, bacteria were grown for 24 hours at 37°C in an aerated broth culture containing 10 micrograms of streptomycin per milliliter. They reached a saturation titer of approximately 2 to 3 × 10^9 cells per milliliter. Each culture was started from an inoculum, usually large, taken from streptomycin-agar slants kept in a refrigerator. Before treatment the bacteria were washed in saline and resuspended in distilled water. A sample of the new suspension was added to the desired solution of chemical in distilled water, and incubated at 37°C for a certain period of time; no growth occurs under these conditions. Another sample of the same suspension was added to an equal amount of distilled water and incubated for the same period of time, as a control. At the end of the treatment period, both treated and control suspensions were assayed by plating suitable dilutions on streptomycin-agar plates. At the same time they were plated (0.1 ml per Petri dish), either undiluted or diluted not more than 1:10 in plain broth, onto a number of streptomycin-free plates, using a glass spreader and a turntable. The assay plates were incubated for 48 hours, after which it was possible to count the colonies and calculate the titers of the two suspensions at the end of treatment, and the percentage of survivors. The streptomycin-free plates (mutant plates) were incubated for at least six days. After this time the colonies were scored, and the frequency of mutants calculated by dividing the number of colonies by the number of (viable) bacteria plated.

The length of the period of incubation in distilled water does not appreciably affect the proportion of mutations in the controls. In all but a few exceptional cases, nevertheless, the control suspensions were incubated for the same period of time as the chemically treated suspensions.

The degree of crowding of the dependent bacteria on the mutant plates would be expected to affect the observed frequency of mutants, as it influences the extent of residual growth and thus the chance that a mutant cell will develop into a visible colony. This effect is slight, however, when the bacteria plated have been raised in 10 micrograms of streptomycin per milliliter.

The chemical treatment also may limit the extent of residual growth, causing a spurious lowering of the frequency of mutants in the treated series. This may explain why, in experiments with some chemicals, the treated series showed frequencies lower than those of the controls.

Several of the chemicals used cause the formation of clumps of bacteria during treatment. If these clumps have not dissolved by the time the treated suspension is assayed to determine survival, the observed survival value

will be too low, and consequently the calculated mutation frequency too high. Therefore, except in the very first experiments done (*i.e.*, those with sulfuric, nitric, and hydrochloric acids, which in any case were not found to be mutagenic), the treated suspensions were always examined under the microscope before plating, and discarded if clumping was detected. By varying the concentration of chemical and the length of treatment, it was possible to eliminate clumping in many cases.

In a few scattered experiments (and in most of those done with phenol and formaldehyde) the dependent bacteria were grown in 25 instead of 10 micrograms of streptomycin per milliliter. It is known that this results in greater residual growth and therefore a larger number of spontaneous mutants per plate. This explains why in some experiments the spontaneous mutant frequencies were higher than average.

RESULTS

The results of a number of experiments for each chemical are reported in table 1. The concentration of the chemical shown in column 2 is the effective concentration at the time of treatment (*i.e.*, corrected for the addition of the bacterial suspension in distilled water). Number of plates (column 5) is the number of streptomycin-free plates used for the scoring of mutants. Total number of bacteria (column 6) is the number of viable dependent bacteria put on each plate, multiplied by the number of plates used. Number of mutants per 10^8 bacteria (column 7) is the mutant frequency, calculated by dividing the total number of colonies scored in an experiment by the total number of bacteria. The total number of mutant colonies is not given in the table, but can easily be calculated by the inverse operation. From it, one can determine the average number of mutants per plate, which in the experiments reported here was rarely larger than 10–20. Assuming a Poisson distribution, the variance can be estimated and the statistical significance of the results evaluated. Sometimes the same controls were common to several treated series; and in these cases the number of plates and total number of bacteria are reported only once for the controls. Different experiments done with each chemical are listed in order of decreasing survival.

Inorganic acids. Tests made with sulfuric, orthophosphoric, nitric, and hydrochloric acids gave negative results. Under certain conditions, all four of these compounds produced clumping of the treated bacteria, and this explains why in some experiments the values obtained for mutant frequency were higher in the treated series than in the controls. Negative results for hydrochloric acid had also been obtained in earlier experiments with Drosophila (Geldat and Beliaieva, 1935).

Boric acid was the only inorganic acid tested that gave positive results. In all experiments but one, the treated series showed an increase in mutation frequency. No clumping was ever observed among bacteria treated with boric acid.

Alkalies. Sodium hydroxide and potassium hydroxide were tested, with inconclusive results. Under the conditions of these experiments they never produced clumping.

Ammonia. Ammonia had been used in experiments with Drosophila by Lobashov and Smirnov (1934) and Lobashov (1937), and found to have mutagenic action. In the tests reported here, ammonia showed definite mutagenic activity in experiments where the proportion of survivors was lower than 2 per cent. Apparently similar positive results were obtained with ammonium chloride; they are not reported here, however, because clumping could not be avoided after treatment with this chemical.

Hydrogen peroxide. Attention was first drawn to the mutagenic activity of peroxides by the work of Stone, Wyss, and Haas (1947) on the indirect effect of mutation-inducing radiations on bacteria. Dickey, Cleland, and Lotz (1949), using Neurospora, studied the mutagenic potency of hydrogen peroxide and of a number of organic peroxides, and obtained positive results. In our experiments also, hydrogen peroxide was clearly shown to be mutagenic.

Metal salts. Mercuric chloride, silver nitrate, copper sulfate, and sodium silicate were tested. Only copper sulfate showed indications of mutagenic effect in the lower range of survival. Mutagenic potency had already been demonstrated for this salt by Magrzhikovskaja (1936) and Law (1938) in experiments with Drosophila. The results reported for mercuric chloride and silver nitrate do not exclude the possibility of their having mutagenic activity at the lowest survival levels, because it was extremely difficult to obtain survivals below 1.0 per cent and yet not so low as to fall beyond the range of sensitivity of the method.

Organic acids. Lobashov and Smirnov (1934) and Lobashov (1937) had treated Drosophila with acetic acid, but failed to detect any mutagenic activity of this substance. In our experiments, acetic, formic, and lactic acids were tested. The first two showed mutagenic activity. Some of the experiments with lactic acid indicated a weak mutagenic effect; but this is rendered doubtful by the fact that experiments with sodium lactate consistently gave negative results.

Formaldehyde. The mutagenic potency of this substance had been discovered by Rapoport (1946) and confirmed by W. D. Kaplan (1948) and Auerbach (1949), working with Drosophila. Clear-cut positive results were obtained in our experiments also. Auerbach's hypothesis that production of mutations by formaldehyde can occur only through some intermediate effect on substances contained in Drosophila food is not supported by our experiments, in which the treatment was carried out on washed bacteria.

Phenol. The mutagenic action of phenol had been discovered by Hadorn and Niggli (1946), working on Drosophila with a very special treatment technique. Other techniques had failed to give positive results. Negative results had been obtained by Dickey, Cleland, and Lotz (1949) with Neurospora. Our tests gave clear-cut positive results for this substance, and also for two closely related substances, alpha-dinitrophenol and trinitro-

TABLE 1

RESULTS OF EXPERIMENTS TESTING INDUCTION OF BACK-MUTATIONS IN Sd-4 (STREPTOMYCIN-DEPENDENT) *E. COLI* TREATED WITH CERTAIN CHEMICALS

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Sulfuric acid	0.003	3	13.	10	1.2×10^8	11.8	4	8.5×10^7	8.3
	0.002	3	12.	8	8.0×10^7	70.0	4	7.0×10^7	147.
	0.002	3	11.	8	1.1×10^8	28.7	4	8.4×10^7	35.4
	0.005	3	7.0	9	4.6×10^7	10.9	4	5.4×10^7	33.1
	0.002	3	1.9	8	2.2×10^7	74.	4	9.2×10^7	98.1
Phosphoric acid (ortho-)	0.008	3	29.	8	1.9×10^8	29.4	4	6.4×10^7	35.9
	0.008	3	17.	8	1.0×10^8	16.5	4	7.3×10^7	12.3
	0.010	3	1.2	8	9.3×10^7	2.2	4	8.9×10^7	21.3
Nitric acid	0.008	3	8.8	10	8.1×10^7	11.1	4	8.5×10^7	8.3
	0.005	3	8.3	8	5.8×10^7	90.2	4	7.0×10^7	147.
	0.005	3	6.9	8	6.8×10^7	16.2	4	8.4×10^7	35.4
	0.006	3	3.5	10	2.6×10^7	11.7	4	5.4×10^7	33.1
	0.005	3	2.7	8	2.2×10^7	111.1	4	9.2×10^7	98.1
Hydrochloric acid	0.0015	3	100.	5	6.0×10^7	6.7	4	4.8×10^7	10.4
	0.0015	3	33.	5	4.0×10^8	5.8	4	9.6×10^7	24.0
	0.00375	3	21.	5	2.5×10^8	6.8			24.0
	0.0075	3	11.	10	1.3×10^7	30.1			10.4
	0.00075	3	0.8	3	1.1×10^7	0.0	2	9.2×10^7	3.6
	0.0015	3	0.2	4	4.0×10^6	0.0			3.6
	0.0015	3	0.2	4	3.8×10^6	25.0	5	2.5×10^8	26.2
Boric acid	4.0	3	50.	8	1.0×10^9	27.3	6	7.6×10^7	6.6
	1.8	24	33.	8	6.0×10^8	28.9	5	1.1×10^8	7.9
	2.0	24	25.	8	4.6×10^8	28.7			7.9
	2.0	24	24.	8	3.3×10^8	82.3	6	1.0×10^8	5.8

TABLE 1—*continued*

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Boric acid *(continued)*	2.0	24	17.	8	1.1×10^8	6.6	6	4.6×10^8	9.8
	2.0	24	11.	8	1.6×10^8	64.1	6	1.0×10^8	13.6
	1.5	24	11.	8	8.3×10^7	31.2	4	8.9×10^7	21.3
	1.7	24	10.	9	5.9×10^7	20.3	6	9.0×10^7	6.7
	2.0	48	9.7	8	9.2×10^7	16.4	4	7.0×10^7	5.7
	1.9	24	3.3	10	2.2×10^7	18.6			6.7
	1.8	24	2.8	8	2.1×10^7	56.6	4	7.5×10^7	30.6
Sodium hydroxide	0.013	3	86.	10	2.6×10^9	11.7	5	3.0×10^8	18.9
	0.015	3	37.	10	3.0×10^8	32.4	4	6.4×10^7	35.9
	0.02	3	27.	7	5.4×10^8	13.8	5	1.4×10^8	16.8
	0.011	3	24.	10	3.4×10^8	3.3	6	1.9×10^8	3.6
	0.0145	3	20.	10	1.9×10^8	20.2	4	8.5×10^7	8.3
	0.015	3	16.	10	1.2×10^8	16.3	4	7.3×10^7	12.3
	0.0125	3	12.	8	1.2×10^8	28.2	4	1.0×10^8	38.0
	0.0125	3	11.	8	1.0×10^8	130.7	4	9.2×10^7	98.1
	0.014	3	5.7	8	5.3×10^7	43.0	4	6.8×10^7	43.8
	0.022	3	1.3	8	2.3×10^7	21.6	5	1.1×10^8	12.1
Potassium hydroxide	0.00945	3	44.	10	2.8×10^8	10.3	6	9.0×10^7	6.7
	0.015	3	42.	8	3.8×10^8	55.5	4	9.2×10^7	98.1
	0.016	3	32.	8	3.0×10^8	43.0	4	6.8×10^7	43.8
	0.015	3	29.	8	2.0×10^8	79.2	4	7.0×10^7	147.
	0.019	3	20.	10	3.6×10^8	12.5	6	1.8×10^8	5.5
	0.018	3	18.	10	1.4×10^8	9.9	6	1.1×10^8	9.7
	0.018	3	19.	10	3.4×10^8	20.1			5.5
	0.0185	3	13.	10	1.9×10^8	6.4	6	1.9×10^8	3.6
	0.0165	3	13.	10	1.8×10^8	4.4			3.6
	0.017	3	10.	10	7.9×10^7	12.7			9.7

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TABLE 1 (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Potassium hydroxide (*continued*)	0.019	24	8.5	10	1.5×10^8	17.8			5.5
	0.018	24	8.3	10	1.5×10^8	9.4			5.5
	0.0175	3	2.9	8	2.9×10^7	37.6	4	1.0×10^8	38.0
Ammonia	0.025	3	66.	6	2.4×10^9	4.9	2	1.8×10^9	6.8
	0.100	3	26.	9	1.1×10^8	2.6	5	2.5×10^8	3.7
	0.250	3	13.	9	3.9×10^8	10.3	5	1.7×10^8	10.0
	0.250	3	12.	8	3.1×10^8	1.6	4	1.4×10^9	2.8
	0.300	3	9.6	5	2.4×10^8	3.4	5	2.5×10^9	2.6
	0.400	3	2.3	10	1.1×10^8	8.0			2.6
	0.250	3	1.2	8	2.4×10^7	37.5	5	1.3×10^9	12.9
	0.500	3	1.0	4	1.2×10^7	41.6	5	1.5×10^9	13.3
	0.500	3	0.4	10	2.0×10^7	121.			2.6
	0.500	3	0.2	10	5.6×10^6	90.			12.9
Hydrogen peroxide	0.150	4	78.	9	1.9×10^9	19.9	8	2.2×10^9	5.0
	0.195	4	59.	8	1.3×10^9	28.7			5.0
	0.195	½	45.	6	5.2×10^8	124.	8	1.5×10^9	6.9
	0.225	½	32.	8	4.9×10^8	121.			6.9
	0.195	½	3.1	8	5.4×10^7	175.	6	1.3×10^9	4.7
	0.240	4	1.4	9	3.5×10^7	120.			5.0
	0.195	½	0.6	8	1.1×10^7	26.7			4.7
Mercuric chloride	0.00005	6	61.	9	5.8×10^8	1.7	5	1.5×10^9	0.7
	0.00030	3	40.	10	4.1×10^8	4.2	4	4.1×10^8	12.3
	0.00025	3	21.	4	3.1×10^8	5.9	4	1.5×10^8	2.0
	0.00030	3	18.	3	2.5×10^8	2.9	2	9.2×10^7	3.3
	0.00005	9	11.	9	1.1×10^8	3.7			0.7
Silver nitrate	0.000005	24	84.	9	3.3×10^8	4.8	6	2.6×10^8	2.3
	0.000100	3	79.	10	1.6×10^8	2.5	5	1.0×10^8	8.0
	0.000050	3	75.	9	1.1×10^9	6.7	7	1.1×10^9	8.6

TABLE 1 (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Silver nitrate (*continued*)	0.000025	3	59.	9	2.3×10^8	2.1			2.3
	0.000100	3	26.	9	3.8×10^8	7.4			8.6
	0.000100	24	4.3	9	6.1×10^7	8.2			8.6
Copper sulfate	0.00020	3	88.	9	1.4×10^9	5.7	6	1.0×10^9	5.1
	0.00040	3	34.	9	4.7×10^8	3.6	8	1.2×10^9	8.8
	0.00050	3	22.	10	5.0×10^8	4.4	5	1.1×10^9	8.9
	0.00050	3	14.	9	3.8×10^8	10.6	5	1.5×10^9	2.1
	0.00050	3	12.	3	1.1×10^8	20.7	4	1.2×10^8	20.8
	0.00075	3	2.4	9	3.7×10^7	40.5			5.1
	0.00100	3	0.36	9	7.2×10^6	55.5			8.9
	0.00125	3	0.22	10	4.9×10^6	0.0			8.9
Sodium silicate	0.025	3	66.	10	1.7×10^8	5.9	5	1.3×10^8	6.3
	0.100	3	33.	5	4.2×10^8	2.4	5	1.3×10^9	5.3
	0.050	3	27.	10	6.9×10^7	8.7			6.3
	0.100	3	16.	7	3.8×10^8	6.6	5	1.7×10^9	6.1
	0.100	3	4.6	9	7.9×10^7	11.4	7	1.3×10^9	6.2
	0.150	3	3.0	9	5.1×10^7	2.0			6.2
	0.300	3	0.11	5	1.3×10^6	0.0	5	1.2×10^9	7.0
Acetic acid	0.03	3	79.	10	1.3×10^9	8.3	5	1.6×10^8	10.0
	0.03	3	36.	8	9.9×10^8	36.4	5	1.7×10^8	9.2
	0.03	3	11.	10	8.5×10^7	15.3			10.0
	0.01	24	7.2	8	7.6×10^7	19.8	6	7.9×10^7	10.1
	0.03	3	6.0	10	9.5×10^7	25.2			10.0
	0.03	3½	0.9	8	8.8×10^6	56.8	6	7.6×10^7	6.6
Lactic acid	0.010	3	40.	10	6.4×10^8	12.8	5	1.6×10^8	10.0
	0.010	3	36.	10	2.9×10^8	10.7			10.0
	0.019	3	14.	8	2.0×10^8	16.7	5	8.9×10^7	4.5

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TABLE 1 (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Lactic acid (*continued*)	0.021	3	7.5	8	1.1×10^8	40.2			4.5
	0.020	3	6.8	8	1.7×10^8	17.0	5	1.6×10^8	15.3
	0.020	3	4.6	8	6.7×10^8	32.9	5	9.2×10^7	27.3
	0.020	4	2.5	8	2.9×10^7	34.7	6	8.5×10^7	9.4
	0.020	3	1.9	8	2.6×10^7	11.4	6	1.0×10^8	4.8
Sodium lactate	2.5	3	85.	8	1.5×10^9	7.3	5	1.1×10^8	12.1
	3.0	3	61.	8	1.7×10^9	16.4	5	1.6×10^8	15.3
	2.0	24	8.3	8	8.7×10^7	5.8	6	7.9×10^7	10.1
	2.5	24	1.4	8	3.8×10^7	5.2	5	1.7×10^8	9.2
Formic acid	0.0070	3	100.	8	1.5×10^9	18.1	6	1.1×10^8	2.7
	0.0060	3	93.	8	1.4×10^9	18.4			2.7
	0.0050	3	77.	8	1.3×10^9	20.2	5	1.1×10^8	12.1
	0.0065	3	30.	8	8.2×10^8	44.0	5	1.7×10^8	9.2
	0.0060	3	20.	8	5.0×10^8	43.3	5	1.6×10^8	15.3
	0.0065	3	19.	8	4.6×10^8	24.4	5	1.5×10^8	3.3
	0.0060	3	15.	8	1.4×10^8	27.7	6	7.1×10^7	2.8
	0.0075	3	3.4	8	8.2×10^7	38.0			3.3
	0.0070	3	2.8	8	2.6×10^7	38.5			2.8
Formaldehyde	0.01	3	61.	2	7.6×10^7	400.	2	1.3×10^8	26.2
	0.01	3	58.	5	6.8×10^8	133.	4	9.4×10^8	15.5
	0.02	3	58.	5	6.8×10^8	157.			15.5
	0.01	3	48.	4	3.7×10^7	245.	4	7.6×10^7	79.
	0.01	3	48.	4	3.7×10^6	470.	4	7.6×10^6	68.
	0.01	3	21.	8	1.3×10^8	120.	4	7.3×10^7	12.3
	0.02	3	16.	8	1.0×10^8	129.			12.3
	0.04	3	9.0	7	1.5×10^8	326.			15.5
	0.05	3	1.7	10	3.2×10^8	560.			79.

TABLE 1 (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Phenol	0.10	3	90.	10	4.4×10^8	84.5	4	2.0×10^8	74.5
	0.10	3	69.	9	1.2×10^9	2.5	5	9.5×10^8	0.6
	0.08	3	48.	8	7.4×10^8	3.4			0.6
	0.20	3	43.	9	7.5×10^8	2.5			0.6
	0.10	12	1.7	10	7.3×10^8	124.	5	2.2×10^8	74.8
	0.10	24	1.1	10	3.1×10^8	192.	4	1.1×10^8	59.5
	0.20	3	0.5	9	2.3×10^6	820.			74.5
Alpha-dinitro-phenol	0.005	3	87.	3	9.6×10^8	2.7	2	7.4×10^7	4.1
	0.020	3	72.	3	1.1×10^8	8.1	5	2.5×10^8	5.5
	0.050	3	58.	10	1.7×10^8	33.5	5	1.5×10^8	5.4
	0.020	3	46.	10	9.5×10^7	51.5	4	8.4×10^7	8.5
	0.005	3	37.	10	2.3×10^9	25.1	5	3.1×10^8	12.5
	0.050	3	32.	10	6.6×10^7	56.0			8.5
	0.025	3	31.	9	1.7×10^9	14.2			12.5
	0.040	3	23.	10	4.8×10^7	25.0			8.5
	0.050	3	18.	10	9.1×10^7	37.4			5.5
	0.100	3	3.0	9	8.2×10^7	44.0	5	1.5×10^9	7.2
	0.100	3	2.6	10	7.3×10^7	97.0	5	1.4×10^9	7.3
	0.100	3	2.5	9	1.2×10^8	6.0			5.5
	0.100	3	2.4	7	3.5×10^7	65.5	4	8.4×10^8	7.6
	0.100	3	1.7	8	4.8×10^7	79.0	4	1.4×10^9	2.8
Trinitro-phenol	0.010	3	94.	8	1.9×10^9	14.9	5	1.3×10^8	14.3
	0.018	3	34.	8	4.9×10^8	97.3	6	1.1×10^8	9.3
	0.017	3	7.4	8	5.8×10^7	132.	6	5.8×10^7	3.4
	0.017	3	4.2	8	6.2×10^7	27.4	5	8.9×10^7	4.5
	0.018	3	1.9	8	1.9×10^7	68.4	6	7.8×10^7	7.7

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TABLE 1 (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Sodium salicylate	2.0	3	62.	5	4.2×10^7	16.7	5	7.0×10^7	20.8
	1.0	3	38.	3	9.5×10^7	5.3	5	4.0×10^8	5.8
	2.0	3	10.	8	3.2×10^8	0.0	5	2.0×10^9	0.7
	3.0	3	0.03	10	2.2×10^6	0.0	5	4.0×10^8	5.8
Carbamate (methyl)	4.0	48	100.	7	3.0×10^8	8.0	5	1.7×10^7	6.1
	8.0	3	69.	8	1.4×10^9	14.4	6	7.8×10^7	7.7
	7.0	25	10.	8	1.8×10^8	5.5	6	1.3×10^8	10.6
	8.0	24	5.5	8	1.1×10^8	9.1	6	8.6×10^7	19.9
	8.0	24	4.6	8	8.6×10^7	4.7	6	7.0×10^7	5.7
	7.0	29	2.2	5	2.4×10^7	12.6			10.6
Carbamate (ethyl) =Urethane	5.0	3	62.	10	1.4×10^9	18.6	5	1.1×10^8	7.9
	6.0	3	56.	8	1.3×10^9	26.5	5	1.4×10^8	16.8
	4.0	3	27.	10	4.8×10^8	8.4	6	1.8×10^8	5.5
	3.5	3	25.	10	4.5×10^8	6.6			5.5
	7.0	3	10.	8	1.7×10^8	41.4	5	1.1×10^8	12.1
	7.0	3	8.3	8	1.7×10^8	53.3	5	1.3×10^8	14.3
	7.0	4	8.2	8	2.3×10^8	22.5	5	1.7×10^8	9.2
	7.0	4	4.7	7	5.6×10^7	98.5	5	8.5×10^7	8.3
	7.0	4½	2.7	8	2.0×10^7	40.0	6	5.8×10^7	3.4
Carbamate (*n*-propyl)	2.15	3	52.	7	4.6×10^8	23.0	6	7.6×10^7	6.6
	2.00	3	50.	8	7.0×10^8	11.9	6	1.1×10^8	7.5
	2.00	3	35.	8	3.6×10^8	29.9	6	7.8×10^7	6.4
	2.00	3½	32.	8	6.6×10^8	8.3	6	1.6×10^8	3.9
	2.00	3½	30.	8	7.2×10^8	10.6	6	1.8×10^8	2.2
	2.10	3½	29.	6	5.2×10^8	16.4			2.2
	2.10	3	24.	8	2.7×10^8	23.0	6	8.5×10^7	9.4
	2.10	3½	4.1	8	9.8×10^7	17.4	5	1.5×10^8	3.3

TABLE 1 (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Carbamate (*n*-propyl) (*continued*)	2.10	3½	3.9	8	8.1×10^7	9.9			3.9
	2.10	3	2.9	8	7.0×10^7	24.2			3.3
	2.10	3½	2.5	8	3.2×10^7	34.8	6	9.7×10^8	12.3
	2.10	4	1.4	8	3.4×10^7	23.8			3.3
Carbamate (*n*-butyl)	0.50	3	85.	8	8.6×10^8	21.4	6	7.8×10^7	7.7
	0.76	3½	44.	7	9.2×10^8	5.3	6	1.8×10^8	2.2
	0.75	3½	29.	8	7.0×10^8	5.6	5	1.5×10^8	3.3
	0.75	3½	20.	8	4.2×10^8	8.3	6	1.6×10^8	3.9
	0.75	3½	19.	7	1.7×10^8	20.9	6	7.6×10^7	6.6
	0.75	3½	17.	8	2.6×10^8	7.4	6	1.1×10^8	2.7
	0.75	3	16.	8	1.6×10^8	10.0	7	7.6×10^7	9.2
	0.80	3½	4.9	8	1.2×10^8	5.9			3.3
	0.75	3½	3.4	8	4.5×10^7	8.9	6	9.7×10^7	12.3
	0.75	3½	1.4	8	1.5×10^7	0.0	6	7.8×10^7	6.4
Carbamate (isoamyl)	0.25	3	100.	8	2.0×10^9	8.4	6	1.6×10^8	3.9
	0.30	3	100.	8	1.6×10^9	11.3	6	1.1×10^8	2.7
	0.35	3	57.	8	8.5×10^8	8.0			2.7
	0.35	3	54.	8	1.1×10^8	9.4			3.9
	0.36	3	26.	8	6.2×10^8	6.3	6	1.8×10^8	2.2
	0.38	3	6.3	8	1.5×10^8	7.2			2.2
	0.37	3	5.8	8	1.4×10^8	7.0			2.2
Neutral red (in light)	0.00025	3	50.	9	7.6×10^8	57.3	9	1.5×10^8	6.6
	0.00100	3	43.	9	6.6×10^8	118.			6.6
	0.00050	3	24.	8	3.2×10^8	122.			6.6
	0.00050	12	0.2	8	1.2×10^6	178.	8	6.7×10^8	10.8
Neutral red (in the dark)	0.00050	24	90.	9	4.2×10^8	17.2	6	4.6×10^8	10.1
	0.00050	12	86.	8	9.6×10^7	10.6	7	9.8×10^7	9.8

TABLE 1 (*continued*)

Chemical	Concentration %	Treatment (hours)	Survival %	Treated			Control		
				No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria	No. of plates	Total no. of bacteria	Mutants per 10^8 bacteria
Neutral red (in the dark) (*continued*)	0.00050	24	83.	12	6.5×10^7	15.4	10	6.5×10^7	15.4
	0.00100	24	71.	10	4.6×10^7	17.4			15.4
	0.00050	12	70.	15	9.0×10^8	10.8	8		10.8
Acriflavine	0.0015	3	39.	10	4.8×10^8	78.3	4	1.0×10^9	14.4
	0.0030	3	28.	10	3.4×10^8	59.1			14.4
Caffeine alkaloid	2.10	3	72.	7	1.3×10^9	15.5	6	7.6×10^7	6.6
	0.60	48	44.	7	7.8×10^8	31.1	6	1.5×10^9	9.8
	2.10	24	34.	8	1.7×10^8	30.3	6	3.7×10^7	10.9
	0.90	48	15.	8	3.9×10^8	21.8	5	1.1×10^8	7.9
	1.00	48	15.	8	1.3×10^9	32.1	6	6.7×10^7	17.9
	0.50	48	12.	10	7.0×10^7	13.0	6	7.1×10^7	5.6
	0.60	48	5.1	8	9.3×10^7	34.6			7.9
	1.09	48	3.8	8	2.0×10^7	20.4	6	3.9×10^7	2.5
Necrosin	0.20	3	100.	8	4.6×10^8	23.6	6	1.4×10^7	6.9
	0.05	3	100.	8	4.2×10^8	26.0			6.9
	0.40	3	100.	8	1.2×10^9	16.4	6	1.0×10^8	9.9
	1.30	3	44.	8	8.5×10^8	17.8	6	1.4×10^8	8.4
	0.65	3½	3.4	7	3.9×10^7	172.	6	9.8×10^7	4.1

phenol (picric acid). The mutagenicity of phenol, however, was evident only after treatments that left very few survivors.

Sodium salicylate. Not mutagenic.

Carbamates. It was known from Oehlkers' work (1943) that some carbamates were able to induce chromosomal aberrations. Vogt (1948) had discovered that ethyl carbamate (urethane) showed mutagenic action in Drosophila. Since then, carbamates had been used by Bryson (unpublished) to induce mutations to phage resistance in *E. coli.* In our experiments, methyl carbamate gave negative results; ethyl and *n*-propyl carbamate gave good positive results; and *n*-butyl carbamate and isoamyl carbamate showed much weaker mutagenic effect.

Neutral red. Luther (1939) had found that neutral red was able to produce chromosomal aberrations. Buzzati (1947), treating Drosophila eggs with this substance, had observed an increase in frequency of mosaic flies. In our tests it seemed at first impossible to obtain low survival ranges, because treatment with concentrations higher than 0.001 per cent produced heavy clumping, and increases in length of treatment did not appreciably affect the number of survivors. Therefore no clear-cut positive results were obtained with the standard procedure. Since neutral red is a photodynamic substance, however, we then tried exposing the bacteria to light during treatment (a 60-watt bulb at a distance of about 10 cm), and finally got the desired survival ranges and with them a very large increase in frequency of mutants. Light had no effect on the controls. Similar results were reported by Kaplan (1948, 1949, 1950a, 1950b), treating *Bacterium prodigiosum* and *E. coli* with erythrosine.

Acriflavine. This substance was known to be a mutagenic agent for both bacteria and Drosophila (Witkin, 1947). Our tests also showed clear-cut positive results.

Caffeine. Caffeine had been found to be mutagenic for Ophiostoma (biochemical mutations) by Fries and Kihlman (1948) and for *E. coli* (mutations to phage resistance) by Witkin (Demerec, Wallace, and Witkin, 1948). Our experiments confirmed these results.

Necrosin. Necrosin is the name given by Menkin (1943) to the potent injurious euglobulin fraction of the exudate of inflamed tissues of vertebrates. A similar substance has subsequently been found by him in severely damaged tissues of some invertebrates (Menkin, 1949). The sample of necrosin used in our experiments was received from Dr. Menkin. It was found to be mutagenic, particularly in experiments where the proportion of survivors was low.

DISCUSSION

Streptomycin-dependent (Sd-4) bacteria, when plated on broth-agar medium without any streptomycin, undergo several divisions (residual growth) and then stop growing. The concentration of streptomycin in the medium in which the parent bacteria are grown is the most significant of the known factors influencing the number of divisions. For example, bacteria grown

in broth medium containing 10 micrograms of streptomycin per milliliter, when plated on medium without streptomycin, will pass through 2-3 divisions; whereas bacteria grown in medium containing 25 micrograms of streptomycin will pass through 3-4 divisions. It has been found that very few, if any, nondependent back-mutants (background mutants) are present in cultures grown in broth with streptomycin, and therefore almost all such back-mutants appearing on the streptomycin-free plates can be assumed to originate during the residual growth. The almost complete absence of background mutants reduces the possibility that differential killing may be responsible for the increased proportion of back-mutants found among the survivors of treatment with certain chemicals; and this, together with the facts that residual growth can be dependably regulated in untreated material and that it allows expression of the back-mutations induced by treatment, makes the Sd-4 method especially suitable for studies of induced mutability.

Since spontaneous back-mutations occur during residual growth, any factor that increases this growth should increase the number of mutants. Therefore, in experiments such as those described here, it is necessary to be sure that any observed increase in mutant frequency is an effect of the treatment on the treated bacteria and not an effect of the treatment on the extent of residual growth. It is not likely that the chemicals used in these experiments would increase the residual growth. In the first place, they were usually applied in nonphysiological, toxic doses. Furthermore, the streptomycin requirement of the dependent bacteria is highly specific (Demerec, 1950), and ability to substitute for it would be quite unexpected in an unrelated chemical. However, even supposing that this had occurred, it would have been detected, either by the appearance of heavy residual growth on the plates of treated bacteria or through the microscopic check of the extent of residual growth that was made in all doubtful cases.

The analysis of data on the mutation-producing chemicals, presented in table 2, indicates that there were considerable differences of behavior among them. Some (ammonia, copper sulfate, and necrosin) were mutagenic only in treatments that left less than 5 per cent survivors; whereas others (hydrogen peroxide, formaldehyde, alpha-dinitrophenol, and neutral red in light) showed effects in treatments that allowed more than 50 per cent survival. Formaldehyde appears to be the most potent mutagen on the list. A treatment that left 61 per cent survivors resulted in 400 mutants per 10^8 treated bacteria; and two other treatments, each leaving 58 per cent survivors, gave 133 and 157 mutants, whereas in the controls there were only 25.2 and 15.5 mutants per 10^8.

Of the 31 chemicals listed in table 1, 19 were found to be mutagenic (table 2), and the following 12 were not: sulfuric, phosphoric, nitric, and hydrochloric acids; sodium and potassium hydroxides; mercuric chloride; silver nitrate; sodium silicate; sodium lactate; sodium salicylate; and methyl carbamate. Since the potency of the different mutagenic chemicals ranges from slight to strong, and, at least in the case of one mutagen that has been studied intensively (manganous chloride), can be shifted from one

TABLE 2

SUMMARY OF THE DATA ON MUTAGENIC CHEMICALS, SHOWING HIGHEST AND LOWEST FREQUENCIES OF MUTANTS OBSERVED IN EXPERIMENTS USING DIFFERENT TREATMENTS (EXPRESSED IN PERCENTAGES OF SURVIVORS)

	Survivors (per cent)			Controls
	50–100	5–50	less than 5	
	No. of mutants per 10^8 bacteria			
Boric acid	27	16–82	19–57	6–31
Ammonia	5	2–10	38–121	3–13
Hydrogen peroxide	20–29	121–124	27–175	5–7
Copper sulfate	6	4–21	40–56	2–21
Acetic acid	8	15–36	57	7–10
Lactic acid	...	11–40	11–35	5–27
Formic acid	18–20	24–44	38–39	3–15
Formaldehyde	133–400	120–326	560	12–79
Phenol	3–85	...	124–820	1–75
Alpha-dinitrophenol	3–34	14–56	6–97	3–13
Trinitrophenol	15	9–132	27–68	3–14
Carbamate, ethyl	19–27	7–53	40–99	6–20
n-propyl	12–28	8–30	10–35	2–12
n-butyl	21	5–21	6–9	2–12
isoamyl	8–11	6	7	2–4
Neutral red, light	57	118–122	178	7–11
dark	11–17	...	...	7–15
Acriflavine	...	59–78	...	14
Caffeine	16	13–35	20	3–13
Necrosin	16–26	18	172	4–10

extreme to the other by simple pretreatments of the bacteria, it seems very likely that the chemicals listed above as nonmutagens might become mutagenic if the treatment were made in a certain way. At any rate, examination of the list of mutagens in the table shows that mutagenicity does not belong to any one group of chemicals but is distributed widely among the different groups tested. Chemicals with such different properties as boric acid, ammonia, hydrogen peroxide, copper sulfate, acetic acid, formaldehyde, and phenol are on the list of mutagens. This fact, together with the indicated possibility that chemicals now considered noneffective would become mutagenic if appropriate conditions of treatment were found, leads us to conclude that genetic changes may be induced by most agents that are able to enter a living cell, upsetting some of its metabolic functions. It seems very likely that the genetic effect of a chemical is not direct but induced through changes in the cytoplasm, which in turn affect the genes. If this is so, it should be very difficult to establish what is the final reaction producing a change in a gene. It may well be that similar final reactions may be initiated by very different primary changes.

In this study we were considering back-mutations from Sd–4; and there is evidence to indicate that a majority of these may occur at one gene locus (Demerec, 1950; Newcombe and Nyholm, 1950). Several experiments by

others, however, based on observation of mutations to T1 phage resistance, have tested the mutagenicity of a few of the chemicals we used. Witkin (Witkin, 1947; Demerec, Wallace, and Witkin, 1948) found that acriflavine and caffeine were mutagenic; and Bryson (in manuscript), in extensive experiments with several carbamate compounds, showed that ethyl carbamate (urethane) is mutagenic. It therefore appears very probable that the mutagenic effect observed by us was not specific, but that treatment with these chemicals increases the mutation rate of the whole genome.

It should be kept in mind that we are dealing here with genetic changes that occur spontaneously with a rate of the order of one per 10^9 cell generations, a considerably lower rate than could be detected directly in germ cells of multicellular organisms. Since each cell of a multicellular organism is a genetic entity, however, in which mutations may occur spontaneously or be induced by various agents, the likelihood of mutation in some of the somatic cells of a higher organism is good, if a large number of these cells are exposed to the action of a mutagenic chemical. This likelihood is further increased by the fact that each cell contains several thousand genes, in any one of which a mutation may be induced.

SUMMARY

Thirty-one chemicals, representing various organic and inorganic groups, were tested for ability to induce back-mutations from streptomycin dependence (Sd-4) to nondependence in *E. coli*. Nineteen were found to be mutagens. It was demonstrated that mutagenicity is not a specific property of any one group of chemicals, but appears among widely different groups. Chemicals having such different properties as boric acid, ammonia, hydrogen peroxide, copper sulfate, acetic acid, formaldehyde, and phenol were found to be mutagenic, indicating that genetic changes may be induced by many agents that are able to enter the living cell and upset its metabolic functions.

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Vitamin E Deficiency in the Monkey

III. THE METABOLISM OF SODIUM FORMATE-C^{14}*

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(Received for publication, February 10, 1958)

Monkeys deprived of vitamin E have been shown to develop nutritional muscular dystrophy with metabolic aberrations resembling those observed in other species (1, 2). Vitamin E deficiency in rabbits and rats has been shown to result in deranged formate metabolism (3, 4), and the present report describes some effects of vitamin E deficiency in monkeys on formate metabolism.

EXPERIMENTAL

A total of eight young rhesus monkeys, weighing between 2 and 3 kilos each, were used in these experiments. All were given the basal vitamin E-deficient diet previously described (1). The diet of two of the animals was supplemented with 20 mg. of α-tocopherol acetate daily throughout the experiment and these animals will be referred to as "normal." The other six monkeys were fed the deficient diet until the previously described signs of vitamin E deficiency appeared (1). This required approximately 10 months of feeding. Four of these animals were then taken for experimentation and will be referred to as "E-deficient." The remaining two animals, fed the unsupplemented diet, were permitted to develop the usual deficiency signs and were then treated with vitamin E. These animals were each given two injections of 20 mg. of α-tocopherol phosphate intraperitoneally, and their diet was supplemented with 20 mg. of α-tocopherol acetate daily. After approximately 3 months of this therapy, the animals appeared normal and were taken for experimentation. These animals will be referred to as "recovered."

Each monkey was injected intraperitoneally with 10^7 c.p.m. of sodium formate-C^{14} per kilo of body weight. The specific activity of the formate was approximately 10^6 c.p.m. per μmole. Each animal was then placed in a large jar with a sealed lid connected to a gas train. Air was bubbled through the system and expired CO_2 was collected in 5 N NaOH and later precipitated as barium carbonate. The barium carbonate was counted with an end window Geiger tube and the counts were corrected to infinite thinness.

4 hours after the injections of the C^{14}-formate, the animals were killed and samples of various tissues were taken and immediately frozen with dry ice-acetone.

The tissues were fractionated according to the procedure of Schneider (5). An aliquot of the nucleic acid fraction was evaporated for counting and a separate aliquot was taken for phosphorus determination. The protein fractions were dried and counted and the counts were corrected to infinite thinness. All C^{14} assays were made with an end window Geiger tube with a window thickness of 2 mg. per sq. cm.

Nucleic acids from bone marrow were fractionated into RNA[1] and DNA by the alkaline digestion method (6) as previously described for rat liver (4).

In order to determine the specific activities of acid-soluble and nucleic acid purines, samples of skeletal muscle were homogenized in cold 10 per cent TCA. The residue was reextracted with cold TCA and the fractions combined for the acid-soluble fraction. Nucleic acids were extracted from the residue with hot 5 per cent TCA. These extracts were hydrolyzed with H_2SO_4, as described by Heinrich and Wilson (7), and purines were precipitated as copper salts (8). Free purines were regenerated with H_2S. An aliquot of the purine solution was evaporated for counting. Purine concentration was calculated from optical density measurements at 260 mμ.

The specific activities of creatine from skeletal muscle and cardiac muscle were determined as previously described (9).

RESULTS

Vitamin E deficiency did not significantly influence the oxidation of formate to CO_2, as shown by the data in Table I. Similar observations have been made on rabbits (3). These results indicate that vitamin E deficiency does not seriously affect the size of the formate pool since the specific activity of expired CO_2 after the injection of C^{14}-formate should reflect differences in pool size. Approximately 40 per cent of the injected C^{14} was expired as CO_2 over the 4 hour collection period.

Specific activities of tissue nucleic acids and proteins are given in Table II. The only tissues significantly affected by vitamin E deficiency were skeletal muscle and bone marrow. In both these tissues vitamin E deficiency led to an increased incorporation of formate into nucleic acids. Treatment of the animals with tocopherol seemed to reverse completely this metabolic lesion in bone marrow but only partially to reverse it in skeletal muscle, as judged by comparison of recovered animals with normal animals. Vitamin E deficiency had no consistent effect on the incorporation of formate into protein.

The data in Table III show that the effects of vitamin E

* This investigation was aided by a grant from the Muscular Dystrophy Association of America, Inc. and Research Grant No. A-741 *et seq.* from the National Institutes of Health.

† Studies carried out during the tenure of a Lederle Medical Faculty Award.

[1] The abbreviations used are: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; and TCA, trichloroacetic acid.

TABLE I

Specific activities of expired CO_2 after injection of sodium formate-C^{14} into monkeys

Results are expressed as counts per minute per mg. of barium carbonate

Interval after injection	Normal animals	E-deficient animals	Recovered animals
hrs.			
0–1	377	500	473
1–2	350	364	476
2–3	145	177	194
3–4	61	112	168

TABLE II

Specific activities of nucleic acids and protein in monkey tissues after injection of sodium formate-C^{14}

Tissue	Animals	Specific activity of:	
		Nucleic acids	Protein
		c.p.m./μmole P	*c.p.m./mg.*
Liver	Normal	31.1	30.3
	E-deficient	24.5	26.4
	Recovered	32.0	16.4
Spleen	Normal	8.0	18.5
	E-deficient	10.8	10.5
	Recovered	11.9	8.8
Kidney	Normal	23.1	19.7
	E-deficient	21.4	19.7
	Recovered	28.2	10.4
Skeletal muscle	Normal	2.3	3.0
	E-deficient	10.5	4.9
	Recovered	6.0	3.4
Heart	Normal	15.4	10.2
	E-deficient	12.3	5.4
	Recovered	22.3	5.6
Small intestine	Normal	32.4	24.6
	E-deficient	39.3	23.2
	Recovered	33.6	23.2
Bone marrow	Normal	38.1	26.8
	E-deficient	96.4	32.6
	Recovered	26.4	13.7

TABLE III

Specific activities of monkey bone marrow RNA and DNA after injection of sodium formate-C^{14}

Results are expressed as counts per minute per μmole of phosphorus

Animals	RNA	DNA
Normal	75	18
E-deficient	96	102
Recovered	54	12

TABLE IV

Specific activities of purines of monkey skeletal muscle

Results are expressed as counts per minute per μmole of purine

Animals	Acid-soluble purines	Nucleic acid purines
Normal	9.2	8.3
E-deficient	105.2	35.7
Recovered	18.8	5.8

TABLE V

Specific activities of monkey muscle creatine after injection of sodium formate-C^{14}

Results are expressed as counts per minute per μmole of creatine

Animals	Skeletal muscle	Cardiac muscle
Normal	3.3	21.5
E-deficient	8.3	9.1
Recovered	2.2	17.8

deficiency on the metabolism of nucleic acid from bone marrow were confined principally to DNA. The incorporation of formate into marrow DNA was elevated approximately 6-fold in the vitamin E-deficient animals and was restored to normal in the recovered animals.

Vitamin E deficiency resulted in a greatly increased incorporation of formate into the acid-soluble purines of skeletal muscle, as shown by the data in Table IV. This is quite similar to the finding in vitamin E-deficient rabbits (3).

The data in Table V show that the incorporation of formate into skeletal muscle creatine was increased in vitamin E-deficient monkeys. This is similar to findings with vitamin E-deficient rabbits (9) and suggests that the creatinuria of vitamin E deficiency in the monkey may be due in part to an inability of the skeletal muscle to retain creatine. Vitamin E-deficient monkeys incorporated less formate into heart creatine than did normal animals. The explanation for this finding is not apparent.

DISCUSSION

It is of interest that by all criteria available the tissues most drastically affected by vitamin E deficiency in the monkey are skeletal muscle and bone marrow. The most obvious clinical signs of the deficiency are muscular dystrophy, anemia, and leukocytosis (1). Concentrations of nucleic acid in the deficient animals are elevated only in bone marrow and skeletal muscle (2). The present experiments indicate that the turnover rates of the nucleic acids are increased in skeletal muscle and bone marrow of the deficient animals but are unchanged in the other tissues. The results of these experiments again indicate the important role of vitamin E in regulating the metabolism of nucleic acid.

SUMMARY

Vitamin E deficiency in monkeys led to an increased incorporation of formate into the nucleic acids of skeletal muscle and bone

marrow, but it did not influence the incorporation of formate into the nucleic acids of the other tissues tested. The increased incorporation of formate into nucleic acids from bone marrow by deficient monkeys was confined mainly to deoxyribonucleic acid. Vitamin E deficiency did not affect the incorporation of formate into protein or the oxidation of formate to CO_2.

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TOXICITY OF SELECTED CHEMICALS TO CERTAIN ANIMALS

Bobby F. Dowden and Harry J. Bennett

The median tolerance limit, defined as that concentration of a substance which is lethal to 50 percent of test animals in an arbitrary time period and abbreviated TL_m, has many uses in physiological studies. These values are especially useful as a starting point for studies of sublethal effects of chemicals on the metabolic processes of animals. Other uses may be made of these values depending on the nature of the research involved.

During the past 19 years the Industrial Research Laboratory at Louisiana State University has measured, for various purposes, the median tolerance limits of a large number of organic and inorganic chemicals for various animals. This paper presents the TL_m values of 86 of these chemicals as tested for 9 species of animals in 3 different support media. Some of the TL_m values were determined in conjunction with other research projects and, thus, were reported with that work (1) (2) (3). All the values reported herein have been gleaned from the Progress Reports of the Industrial Research Laboratory to the Louisiana Petroleum Refiners' Waste Control Council which have a limited circulation in the Council minutes to member organizations only. Because of the varied purposes for obtaining them, there can be little correlation of these data.

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Materials and Methods

The animals used for bioassay were amphipoda (probably *Hyallela* sp.); frog eggs, *Rana* sp.; three species of fish, *Carassius carassius, Lepomis marochirus,* and *Mollienesia latipinna;* mosquito larvae, *Culex* sp.; planaria worms, *Dugesia* sp.; snail eggs, *Lymnaea* sp.; and water fleas, *Daphnia magna.* The amphipods, planaria, and frogs were obtained from University Lake on the campus of the University. The snails from which eggs were obtained were found in a ditch near Fountainebleau State Park, 60 miles (96.6 km) east of Baton Rouge. The goldfish, *C. carassius,* and the mollies, *M. latipinna,* were obtained from a local pet shop. The bluegills, *L. macrochirus,* were obtained from state and federal fish hatcheries. The mosquito larvae were obtained during the summer in mixed culture of mostly *Culex pipiens* from puddles in a ditch on the campus. The daphnids were cultured in the laboratory from a starting culture originally obtained from Put-In-Bay, Ohio.

Bioassays with the fish were conducted by methods outlined by Freeman (4). Bioassays with the invertebrates and the frog eggs were conducted with the methods of Anderson *et al.* (5). These methods are basically similar to each other—they differ only in the dilution series utilized.

The support media employed were Standard Reference Water (4), Reference Dilution Water (6), and water from University Lake filtered through glass wool. Standard Reference Water is described as a laboratory-prepared medium, free from organics, and containing all the major ions in concentrations and proportions of a mean surface water of the United States. Reference Dilution Water is a more easily formulated organic-free medium which will support aquatic animals longer than other artificial media tested.

Results and Discussion

The median tolerance limits determined are shown in Table I according to the chemicals tested. The animals on which the chemicals were tested, the support media employed, and the time for which the TL_m's were determined also are shown. In certain instances in the table, the same animal may be listed more than once under one chemical. This is because more than one support medium was employed. A different support medium may evoke a very different TL_m (7).

It may be noted in Table I that in the testing of combinations of chemicals, more than one concentration is given at one TL_m time period. These TL_m values are the test solution concentrations of each of the chemicals, separated by a hyphen, given in the order listed at the left side of the column.

Some values are given with the sign > (greater than) and < (less than). The TL_m values shown with either of these symbols preceding a single number, e.g., <3,750, represent either the upper or lower limit (whichever is applicable) of the most definitive test. A general rule used in the laboratory is that with TL_m values greater than 3,000 mg/l the toxicity is due to hypertonicity of the test solution. The values with two numbers and two symbols, e.g., >100 <1,000, designate the limits or "brackets" determined from preliminary tests. The TL_m of the chemical lies between the values given. The TL_m values are presented in mg/l. If, at room temperature, the chemical were a solid, the measure of TL_m was by weight. If the chemical tested were a liquid at room temperature, the measure was by volume.

It generally is expected that shorter-term TL_m values will be greater than those for longer periods of time. In Table I, it may be noted that some TL_m values for longer time periods are greater than those for shorter periods. This has occurred possibly because the TL_m values for one TL_m time period may have been tested by one worker at one time and the value for another time period tested by another worker at another time. Because the methods used by all the workers in the laboratory have been the same, these differences are considered to be due to differences in the test animals obtainable at the different times of testing.

Another consequence of having different workers determine the different TL_m's at different times is a lack of strict comparability of the data presented. In most cases, these data were obtained as background material for other research problems, e.g., effects of certain chemicals on the taste of fish flesh, effects on adults emerging from larvae exposed to various chemicals, effects on the young after sublethal exposure of the eggs to certain chemicals, and toxicity levels of common solvents used for transport or dilution of other chemicals. It is felt that the use of these data lies in their being utilized for the same purpose for which they were obtained, i.e., background material for more detailed studies.

The TL_m values given for the *Lymnaea* sp. eggs are the concentrations at which 50 percent of the eggs hatched. The *Lymnaea* snails used herein lay their eggs in an orderly arrangement of 8 to 12 eggs per egg case. This egg case is more or less rigid—having the consistency of a gela-

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TABLE I. Median Tolerance Limits of Certain Chemicals for Various Animals in Different Dilution Media

Compound and Animal Tested	Medium	TL_m (mg/l) 24 hr	48 hr	72 hr	96 hr
Acetic acid					
Culex sp. larvae	RDW	1,500	1,500	—	—
D. magna	SRW	47	—	—	—
L. macrochirus	RDW	>100<1,000	—	—	—
Acetone					
D. magna	RDW	10	10	—	—
Adipic acid					
L. macrochirus	RDW	<330	—	—	—
Ammonium chloride					
C. carassius	SRW	640	—	—	—
D. magna	ULW	202	161	67	50
D. magna	SRW	—	—	—	139*
L. macrochirus	SRW	725	725	725	725
Lymnaeae sp. egg	ULW	241	173	73	70
Ammonium hydroxide					
D. magna	SRW	60*	32*	—	20*
Ammonium sulfate					
D. magna	SRW	423*	433*	—	292*
Ammonium sulfite					
D. magna	SRW	299*	273*	—	203*
Barium chloride					
Rana sp. eggs	ULW	244.3 mg/l—91.67% development 2,443.0 mg/l—89.55% development 24,430.0 mg/l— 0.00% development			
Butyric acid					
D. magna	SRW	—	61	—	—
L. macrochirus	RDW	200	—	—	—
Calcium chloride					
D. magna	SRW	3,526*	3,005*	—	—
D. magna	ULW	1,838	759	759	649*
L. macrochirus	SRW	8,350	—	—	—
Lymnaea sp. eggs	ULW	4,485	3,094	3,308	2,573
Capric acid					
L. macrochirus	RDW	I			
Caproic acid					
L. macrochirus	RDW	>150<200	—	—	—
Caprylic acid					
L. macrochirus	RDW	I	—	—	—
Chromic sulfate					
D. magna	ULW	0.1	0.03	—	—
Chromic sulfate plus sodium dichromate					
Lymnaea sp. eggs	ULW	0.17—0.22	0.17—0.22	—	—

* Denotes 25 hr when placed after a value in the 24-hr column, 50 hr when in the 48-hr column, and 100 hr when in the 96-hr column.

Note:
TL_m (mg/l)—median tolerance limit.
SRW—Standard Reference Water (4).
RDW—Reference Dilution Water (3).
ULW—glass-wool filtered University Lake water.
I—chemical too insoluble in water to be toxic.

TABLE I.—*Continued*

Compound and Animal Tested	Medium	TL_m (mg/l) 24 hr	48 hr	72 hr	96 hr
Ferric chloride					
D. magna	SRW	36*	21*	—	15*
Formaldehyde					
D. magna	RDW	>100<1,000	—	—	—
Formic acid					
L. macrochirus	RDW	175	—	—	—
Glutaric acid					
L. macrochirus	RDW	330	—	—	—
Magnesium chloride					
D. magna	SRW	3,391*	3,699*	—	3,484*
Magnesium sulfate					
D. magna	ULW	963	929	861	788
D. magna	SRW	—	—	—	3,803
L. macrochirus	SRW	19,000	—	—	—
Lymnaea sp. eggs	ULW	10,530	6,525	6,300	6,250
Malonic acid					
L. macrochirus	RDW	150	—	—	—
Phenol					
D. magna	RDW	100	100	—	—
D. magna young	SRW	17*	7*	—	—
D. magna adult	SRW	61*	21*	—	—
L. macrochirus	SRW	>10<15*	—	—	—
M. latipinna	SRW	63*	22*	—	—
Potassium chloride					
D. magna	SRW	—	—	—	679*
D. magna	ULW	343	337	117	29
L. macrochirus	SRW	5,500	—	—	—
Lymnaea sp. eggs	ULW	1,941	1,492	1,018	1,100
Potassium cyanide					
D. magna	ULW	2	2	0.7	0.4
Lymnaea sp. eggs	ULW	796	796	147	130
Potassium dichromate					
C. carassius	SRW	705	—	—	—
D. magna	SRW	—	—	—	0.4*
L. macrochirus	SRW	739	—	—	—
Potassium ferricyanide					
D. magna	ULW	905	549	0.6	0.1
Potassium nitrate					
D. magna	ULW	490	490	226	39
D. magna	SRW	—	—	—	900*
L. macrochirus	SRW	5,500	—	—	—
Lymnaea sp. eggs	ULW	1,941	1,492	1,018	1,100
Propionic acid					
Culex sp. larvae	RDW	>1,000	>1,000	—	—
D. magna	SRW	—	50	—	—
L. macrochirus	RDW	188	—	—	—
Pyridine					
D. magna	RDW	2,114	944	—	—
Sodium acetate					
L. macrochirus	RDW	5,000	—	—	—
Culex sp. larvae	RDW	7,500	7,425	—	—
Sodium anthraquinone alpha-sulfonate					
D. magna	ULW	186	186	186	50
D. magna	SRW	—	—	—	12*
Lymnaea sp. eggs	ULW	186	186	186	186

TABLE I.—*Continued*

Compound and Animal Tested	Medium	TL_m (mg/l)			
		24 hr	48 hr	72 hr	96 hr
Sodium bicarbonate					
Culex sp. larvae	RDW	2,000	2,000	—	—
Sodium bisulfate					
Culex sp. larvae	RDW	300	300	—	—
Sodium bisulfite					
D. magna young	SRW	116*	81*	—	—
D. magna adult	SRW	—	—	—	102
D. magna adult	ULW	171	119	97	82
Dugesia sp.	ULW	179	179	179	179
Lymnaea sp. eggs	ULW	179	59	59	59
M. latipinna	SRW	241*	220*	—	—
Sodium bisulfite plus sodium carbonate					
D. magna	ULW	1,484–665	1,484–665	1,060–475	99–45
D. magna	SRW	—	—	—	436–85
Sodium bisulfite plus sodium chromate					
D. magna	SRW	—	—	—	67–0.278*
Sodium bisulfite plus sodium silicate					
D. magna	SRW	14,210–950	11,723–784	222–15	222–15
Sodium bisulfite plus sodium sulfate					
D. magna	SRW	—	—	—	82–3,654
Sodium bisulfite plus sodium carbonate and sodium chromate					
D. magna	SRW	—	—	—	86–441–0.354*
Sodium bisulfite plus sodium carbonate and sodium silicate					
D. magna	SRW	—	—	—	39–198–93*
Sodium bisulfite plus sodium carbonate and sodium sulfate					
D. magna	SRW	—	—	—	57–296–2,569*
Sodium bisulfite plus sodium chromate and sodium silicate					
D. magna	SRW	—	—	—	224–0.86–506*
Sodium bisulfite plus sodium chromate and sodium sulfate					
D. magna	SRW	—	—	—	78–0.32–3,443*
Sodium bisulfite plus sodium silicate and sodium sulfate					
D. magna	SRW	—	—	—	52–126–2,326*
Sodium p-bromobenzene sulfonate					
D. magna	ULW	2,347	1,943	971	809
D. magna	SRW	—	—	—	523*
L. macrochirus	SRW	<1,560	—	—	—
Lymnaea sp. eggs	ULW	2,590	2,590	2,590	2,590
Sodium butyl sulfonate					
D. magna	ULW	8,000	8,000	5,400	2,700

TABLE I.—*Continued*

Compound and Animal Tested	Medium	TL_m (mg/l)			
		24 hr	48 hr	72 hr	96 hr
Sodium butyrate					
L. macrochirus	RDW	5,000	—	—	—
Sodium carbonate					
Amphipoda	ULW	360	176	67	67
Culex sp. larvae	RDW	1,820	600	—	—
D. magna	ULW	347	265	—	—
D. magna	SRW	607*	565	—	524
Dugesia sp.	ULW	384	360	360	341
L. macrochirus	ULW	385	—	—	—
Lymnaea sp. eggs	ULW	403	403	395	411
M. latipinna	SRW	405*	297*	—	—
Sodium carbonate plus sodium chromate					
D. magna	SRW	—	—	—	420–0.34*
Sodium carbonate plus sodium chromate and sodium silicate					
D. magna	SRW	—	—	—	187–0.15–88
Sodium carbonate plus sodium chromate and sodium sulfate					
D. magna	SRW	—	—	—	240–0.19–2,078*
Sodium carbonate plus sodium silicate					
D. magna	ULW	265–1,776	265–1,776	265–1,776	265–1,776
Sodium carbonate plus sodium silicate and sodium sulfate					
D. magna	SRW	—	—	—	161–76–1,396*
Sodium carbonate plus sodium sulfate					
D. magna	ULW	198–666	172–577	172–577	66–222
Sodium chloride					
C. carassius	SRW	13,750	—	—	—
Culex sp. larvae	RDW	10,500	10,200	—	—
D. magna	SRW	6,447*	5,874*	—	3,114*
D. magna	ULW	3,412	3,310	—	—
L. macrochirus	SRW	14,125	—	—	—
Lymnaea sp. eggs	ULW	3,412	3,388	—	—
M. latipinna	SRW	18,735*	16,595	—	—
Sodium p-chlorobenzene sulfonate					
D. magna	ULW	8,600	7,659	3,964	2,150
D. magna	SRW	—	—	—	2,394*
L. macrochirus	SRW	<3,219	—	—	—
Lymnaea sp. eggs	ULW	8,600	7,633	6,343	5,053
Sodium 2-chlorotoluene-4-sulfonate					
L. macrochirus	SRW	<1,374	—	—	—
Sodium 2-chlorotoluene-5-sulfonate					
D. magna young	SRW	0.8*	0.6*	—	0.4*
D. magna adult	SRW	3.3*	1.3*	—	—
Lymnaea sp. eggs	SRW	30*	—	—	—
M. latipinna	SRW	115.2*	66.1*	—	—

TABLE I.—*Continued*

Compound and Animal Tested	Medium	TL_m (mg/l)			
		24 hr	48 hr	72 hr	96 hr
Sodium chromate plus sodium silicate					
D. magna	SRW	—	—	—	0.21–130*
Sodium chromate plus sodium silicate and sodium sulfate					
D. magna	SRW	—	—	—	0.28–122–2,255*
Sodium chromate plus sodium sulfate					
D. magna	SRW	—	—	—	0.28–3,044*
Sodium 2, 5-dichloro-benzene sulfonate					
D. magna	ULW	4,931	4,931	2,490	938
D. magna	SRW	—	—	—	1,468*
L. macrochirus	SRW	<3,750	—	—	—
Lymnaea sp. eggs	ULW	4,981	4,513	3,984	3,144
Sodium dichromate					
D. magna	ULW	22	10	—	—
Sodium formate					
L. macrochirus	RDW	5,000	—	—	—
Sodium nitrate					
C. carassius	SRW	12,150	—	—	—
D. magna	ULW	5,980	3,581	2,125	665
D. magna	SRW	—	—	—	4,206
L. macrochirus	SRW	12,800	—	—	—
Lymnaea sp. eggs	ULW	6,375	6,460	5,950	3,251
Sodium m-nitrobenzene sulfonate					
D. magna	ULW	8,665	8,665	6,017	5,067
D. magna	SRW	—	—	—	2,235*
L. macrochirus	SRW	<1,350	—	—	—
Sodium 4-nitrochloro-benzene-2-sulfonate					
D. magna	ULW	4,698	3,483	948	948
D. magna	SRW	—	—	—	1,474
L. macrochirus	SRW	6,375	—	—	6,375*
Lymnaea sp. eggs	ULW	3,532	4,439	3,736	3,208
Sodium 4-nitrotoluene-2-sulfonate					
L. macrochirus	SRW	<1,440	—	—	—
Sodium oxalate					
L. macrochirus	RDW (without $CaCl_2$)	4,000	—	—	—
Sodium p-phenolsulfonate					
D. magna	ULW	13,510	13,510	3,494	1,471
L. macrochirus	SRW	—	—	—	19,616*
Lymnaea sp. eggs	ULW	10,700	9,122	8,828	8,828
Sodium mono-hydrogen phosphate					
D. magna	SRW	1,154*	1,089*	—	426*
Sodium mono-hydrogen phosphate plus sodium pyrophosphate					
D. magna	ULW	3,580–433	3,580–391	—	—
L. [illegible] sp. eggs	ULW	2,685–63	2,954–223	2,828–60	2,685–248

TABLE I.—*Continued*

Compound and Animal Tested	Medium	TL_m (mg/l)			
		24 hr	48 hr	72 hr	96 hr
Sodium phosphate					
D. magna	SRW	237*	177*	—	126*
Sodium propionate					
Culex sp. larvae	RDW	—	2,320	—	—
L. macrochirus	RDW	5,000	—	—	—
Sodium pyrophosphate					
D. magna	ULW	433	391	—	—
Sodium silicate					
Amphipoda	ULW	895	263	261	160
D. magna	ULW	575	494	413	216
D. magna	SRW	—	—	—	247
Lymnaea sp. eggs	ULW	632	630	630	632
Sodium sulfate					
Amphipoda	ULW	2,380	1,110	880	880
Culex sp. larvae	RDW	11,430	13,350	—	—
D. magna	ULW	8,384	2,564	725	630
D. magna adult	SRW	—	—	—	4,547
D. magna young	SRW	6,800	6,100	—	—
L. macrochirus	SRW	17,500	—	—	—
Lymnaea sp. eggs	ULW	5,401	5,400	5,400	3,553
M. latipinna	SRW	20,040	15,996	—	—
Sodium sulfide					
D. magna	SRW	16*	13*	—	9*
Sodium sulfite					
D. magna	SRW	299*	273*	—	203*
Sodium thiosulfate					
D. magna	SRW	2,245*	1,334*	—	805*
Sodium valerate					
L. macrochirus	RDW	5,000	—	—	—
Valeric acid					
D. magna	SRW	—	45	—	—
Xylene					
D. magna	RDW	>100<1,000	—	—	—
Zinc stearate					
L. macrochirus	RDW	1			

tin dessert which has "set." It is felt that the more or less rigid case of *Lymnaea* may offer a small amount of protection from the chemicals tested, thereby giving the TL_m values almost equivalent to the values for adults of other forms.

One very significant effect of some of the chemicals tested herein has been largely unexplored. This is the effect of pH on the TL_m values. In one of the few definitive tests of pH effect conducted it was found that a 0.1 unit increase in pH increased the toxicity of ammonium chloride tenfold and that a 0.1 unit decrease reduced the toxicity by tenfold. The concentration of ammonium chloride was held at the TL_m concentration. This variation in toxicity probably is due to the logarithmic basis for pH. The pH and some other effects of some weak acids have been studied and reported to be due to the concentration of the undissociated portion of the molecule (7).

Summary and Conclusions

The median tolerance limits (TL_m) of 86 different chemicals and combinations of chemicals, both organic and inorganic, are given for 9 different animals in 3 different support media. Determination of the TL_m of a chemical at a different time may show vari-

ations from the expected because of differences in test animals available at the different times of testing. *Lymnaea* sp. eggs show TL_m values comparable to those of adults of other forms because of protection afforded by the egg case. Increasing or decreasing the pH of a concentration of a chemical can cause an increase or decrease in toxicity commensurate to the logarithmic basis for pH.

Acknowledgment

This research was conducted under a grant by the Louisiana Petroleum Refiners' Waste Control Council administered by the faculty of the Department of Zoology of Louisiana State University. Laboratory facilities were provided by the Division of Water Pollution Control of the Louisiana Wild Life and Fisheries Commission. Appreciation is expressed to each of the workers in the Industrial Research Laboratory who have aided in obtaining these data over the past 19 years.

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MONTHLY CONTRACT AWARDS—WASTEWATER TREATMENT WORKS AND COLLECTING SEWERS AS REPORTED BY THE PUBLIC HEALTH SERVICE (MILLIONS OF DOLLARS)

Month	Treatment		Sewers		Total	
	Monthly	Cumulative	Monthly	Cumulative	Monthly	Cumulative
(1965)						
January	41.2	41.2	22.9	22.9	64.1	64.1
February	40.4	81.6	22.0	44.9	62.4	126.5
March	43.4	125.0	38.3	83.2	81.7	208.2
April	38.8	163.8	28.2	111.4	67.0	275.2
May	33.5	197.3	38.6	150.0	72.1	347.3
June	64.8	262.1	56.6	206.6	121.4	468.7
July	41.6	303.7	37.0	243.6	78.6	547.3

Current Food Additives Legislation

GERMANY (Federal Republic)

Orders of 25.IV.1968 and 15.XI.1968 fixing the date for the revocation of the authorization of formic acid as an additive to foodstuffs.

(Bundesgesetzblatt I No. 26, 7.V.1968, p.339; and No. 79, 13.XI.1968, p.1 183)

Under the Third Ordinance amending the Preservatives Ordinance (see 107 CFAL 1635), formic acid and its sodium, potassium and calcium compounds were to be no longer permitted as additives to foodstuffs. However, no date had been specified for the entry into force of this revocation.

The date in question, after having been fixed at 1.XI.1968 by the Order of 25.IV.1968, is now, pursuant to the Order of 15.XI.1968, set at 1.I.1970.

Food Protection Committee. 1972
Comprehensive GRAS Survey
National Academy of Sciences, National Research Council,
Washington, D.C.

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National Academy of Sciences, National Research Council,
Washington, D.C.
Publication 1274

THE CORRELATION OF PROMOTION OF TUMOUR GROWTH AND OF INDUCTION OF HYPERPLASIA IN EPIDERMAL TWO-STAGE CARCINOGENESIS

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Received for publication October 30, 1967

MOTTRAM (1944) and Berenblum and Shubik (1949) have demonstrated the cocarcinogenic potency of croton oil applied topically to mouse skin which had been pretreated once with a dose of a carcinogenic polycyclic hydrocarbon too low to induce tumours. The active principles of croton oil responsible for this effect were more recently identified by the groups of Hecker (1962), Hecker and Kubinyi (1965) and of Van Duuren *et al.* (1966). The mechanism of action of these active principles or of their parent mixture is not yet known, though the work of Setälä *et. al.* (1959) and Merenmies (1959) on similarly cocarcinogenic Tween 60 and allied agents suggests that stimulation of cell proliferation may be an important feature of their action.

In this paper we examine the cocarcinogenic and cell proliferation stimulating actions of croton oil, of Tween 60 and of other agents, as well as the changes induced by these agents in the tissue, changes which may be part of their cocarcinogenic action.

MATERIALS AND METHODS

The mice used in the experiments were 6–10 week old Swiss mice randomly bred in our laboratory. In one experiment inbred Swiss mice (Ball, Huh and McCarter, 1964) originally obtained from J. A. McCarter were used. The mice were kept 10 to a cage. The chemicals obtained commercially were used without additional purification. They were 7, 12-dimethylbenz (*a*) anthracene (DMBA) (Eastman Organic Chemicals, Rochester, N.Y.), croton oil (Boots Pure Drugs, Nottingham, U.K.), Tween 60 (Atlas Powder Co. Canada Ltd., Brantford, Ontario), toluene (Shell Oil Canada Ltd., Montreal), turpentine (Record Chemical Co., Inc., Montreal), oil of sweet orange (R. D. Webb & Co., Inc., Linden, New Jersey), d-limonene (Eastman Organic Chemicals, Rochester, N.Y.), silver nitrate (Johnson, Matthey, & Mallory Ltd., Montreal), acridine (Eastman Organic Chemicals, Rochester, N.Y.), formic acid (Fisher Scientific Co., Montreal) and mineral oil (Nujol, Plough, Canada Ltd., Toronto). Some agents were used undiluted, most were dissolved in mineral oil, and formic acid and silver nitrate were dissolved in distilled water.

All the experiments were performed on male mouse ears. The test solutions were applied to the ears by means of a paint brush dipped into the solution. Both

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sides of both ears were painted. No attempt was made to control the spreading of the solution to the adjacent skin, nor to determine the exact amount of material delivered to the ears. Nevertheless, brushes of the same design and constant conditions of painting were used throughout all the experiments.

Tumours were induced by painting the ears of groups of 30 or 60 mice once with 1·5% DMBA (initiation) followed beginning 1 week later by twice weekly paintings with the test solution (promotion) for 20 weeks. Any lesion larger than 1 mm. in diameter which was seen for at least 3 consecutive weeks, was considered to be a tumour. Tumours appearing towards the end of the experiment were observed for an additional 1 or 2 weeks to satisfy this criterion. Lesions which were first seen but later failed to be noted (" regressing " tumours) were not considered to be tumours.

For histological studies groups of 10 ears were removed in a standard manner close to the head of the anaesthetised animal, fixed in 10% formalin, and embedded in paraffin. Sections were cut as perpendicularly to the surface of the ear as possible and stained with haematoxylin and eosin. Measurements of epidermal and dermal changes were made on the outer surface of the ears, no closer than one high-power field from the edge of the ears. On each ear 10 areas of constant standard length of interfollicular epidermis were examined. By the use of an ocular counting grid, the number of nuclei in each area, the width of the area including the strata basale, spinosum and granuosulm, and the number of inflammatory cells under the area down to the auricular cartilage were determined separately. The values thus obtained were compared to those obtained for animals treated with mineral oil alone using the *t* test.

Autoradiography was performed on ears of mice given 2 μCi of tritiated thymidine (Schwarz BioResearch) per g. body weight intraperitoneally 1 hour before sampling. The time of sampling was 10 a.m. Sheets of epidermis were removed by sharp dissection from the ears soaked for 5 hours in 0·5% acetic acid at 4° C. The sheets were glued to slides basal cell layer up and dipped in Ilford L4 emulsion. After a month's exposure the slides were developed, stained with haematoxylin and coverslipped. The proportion of labelled nuclei among 1000 nuclei of interfollicular epidermis was determined in each specimen. Only nuclei covered by more than 4 grains were considered to be labelled.

The uptake of the dyes Lissamine Green B or Evan's Blue was measured in treated ears by mounting whole ears in an Evelyn colourimeter. Lissamine Green B was injected intraperitoneally 30 minutes before sampling, whereas Evan's Blue was injected by the same route 1 hour before sampling. The ears were removed by the standard method described above and mounted carefully over an ear-shaped opening in a specially made sheet metal screen which fitted into the colorimeter. The opening was somewhat smaller than the ear. Optical density was then read against appropriate blanks. Ten ears were examined in each treatment group and compares for significance with respect to ears treated with mineral oil by the *t* test.

RESULTS

Table I shows the result of a tumour-promotion experiment. Five per cent croton oil, full strength Tween 60 and full strength turpentine promoted significantly more tumours than appeared in the control group promoted with mineral oil. The yield of tumours with croton oil and with Tween 60 was almost identical

but that with turpentine was considerably less. In these experiments full strength toluene and 10% of sweet orange promoted a yield of tumours no different from the controls.

A larger list of agents was tested for their ability to induce histologically measurable changes. Groups of 10 inbred Swiss mice were treated twice a week with the various agents, but without a preliminary DMBA treatment, except where specified. A set of 10 ears was removed, 2, 5, 10, 20 or 50 days from the beginning of the treatments and perpendicular sections were examined for increased epidermal cell number (Table II), for variations in thickness of the epidermis (Table III), and for the presence of a cellular inflammatory exudate (Table IV). It should be noted that in each case statistical analysis using the *t* test was done by comparing the treated ears to the appropriate groups treated with the solvent mineral oil alone.

TABLE I.—*Tumour Promotion*

Initiation with 1·5% DMBA	Promotion twice a week	Results at 20 weeks of promotion: No. of survivors	Tumour-bearing survivors	Tumours present	Tumours per survivor
Yes*	No	23	4%	1‖	0·04
Yes†	5% croton oil	33	88%	352‡	10·7
Yes†	100% Tween 60	41	95%	345‡	8·4
Yes*	100% turpentine	21	71%	46‡	2·2
Yes†	100% toluene	35	11%	7‖	0·2
Yes†	10% oil of sweet orange	52	8%	7‖	0·13
Yes†	100% mineral oil	53	11%	8	0·15
No*	5% croton oil	20	5%	1‖	0·05
No*	100% Tween 60	25	0%	0‖	0·0
No*	100% turpentine	18	7%	1‖	0·06
No*	100% toluene	14	0%	0‖	0·0
No*	10% oil of sweet orange	24	0%	0‖	0·0

* 30 mice at the start.
† 60 mice at the start.
‡ Yield significantly different at the 1% level from group promoted with mineral oil.
‖ Yield not significantly different.

Hyperplasia was defined by Virchow (1860) as growth of tissue by "numerical hypertrophy", i.e. by an increase in the number of the constituent cells. The marked hyperplasia following repeated applications of croton oil or Tween 60 (Table II) is much like that seen by Merenmies (1959) with Tween 60. The number of cells rises rapidly and by the 10th day becomes stable at a new level. There is little difference between the effect of croton oil and Tween 60, or between croton oil with DMBA pretreatment and croton oil without it. Significant but less pronounced and less lasting hyperplasia was induced also by turpentine and by 20% limonene. We did not test limonene for promoting power and there is no record of its use as such in the literature.

The accompanying changes in the thickness of the epidermis seen in Table III are similar to those in hyperplasia. The thickness increases strikingly and markedly during the treatment with croton oil with or without DMBA pretreatment and during the treatment with Tween 60. The increase in thickness is about twice as large as the increase in the number of cells, so that true increase in cell size occurred as well. This is "true hypertrophy" as defined by Virchow (1860).

TABLE II.—*Hyperplasia*

Number of nuclei per standard length of a perpendicular cross-section of epidermis

Agent		Days after treatment 2	5	10	20	50
Control	Mean	136				
	S.D.	20·2				
5% croton oil	Mean	197	255	286	283	286
	S.D.	25·7	46·9	46·9	37·5	63·1
100% Tween 60	Mean	154	228	213	204	217
	S.D.	25·7	22·2	19·0	31·0	28·8
100% turpentine	Mean	141	141	174	169	212
	S.D.	20·2	17·0	21·4	18·2	37·7
20% limonene	Mean	127	131	172	146	198
	S.D.	16·8	10·5	15·2	12·8	51·9
0·3% acridine	Mean	146	168	151	180	228
	S.D.	17·5	29·1	19·6	28·1	54·7
100% toluene	Mean	149	138	137	158	167
	S.D.	21·1	19·4	18·4	26·9	16·8
10% oil of sweet orange	Mean	161	160	148	159	187
	S.D.	22·5	27·3	20·3	22·9	40·7
10% silver nitrate	Mean	126	142	148	128	142
	S.D.	19·3	20·6	41·2	14·1	23·9
8% formic acid	Mean	127	130	120	159	144
	S.D.	11·9	14·4	22·4	33·9	14·5
100% mineral oil	Mean	135	152	132	151	171
	S.D.	13·6	36·8	14·9	14·5	27·4
1·5% DMBA once +5% croton oil	Mean	205	273	262	305	354
	S.D.	48·7	70·3	39·9	54·4	77·5

Underlined values have a $p < 0{\cdot}01$ compared by the *t* test with the values obtained with mineral oil. Based on 10 measurements per ear, 10 ears per value given.

Electron micrographs of Setälä *et al.* (1960) using Tween 60 show that less than about one fifth of the area during hyperplasia is due to the opening up of the extracellular space. Similar observations can be made on our electron micrographs of treated epidermis (Frei and Sheldon, 1961*a*, 1961*b*).

Of the other agents only turpentine and acridine gave significant increase in the thickness of the epidermis at 2 of the times examined each and oil of sweet orange and limonene at 1 time each. We had not tested acridine for promoting power. Of the agents so tested, the weekly promoting turpentine gives the highest score both in terms of hyperplasia and in terms of increased thickness of the epidermis.

The results obtained in measuring the cellular inflammatory exudate (Table IV) are similar. The exudated cells were mostly neutrophils in all instances, but differential counts were not attempted. Croton oil with or without DMBA pre-treatment and Tween 60 gave a significantly greater formation of cellular exudate at all 3 times measured than the mineral oil controls. Turpentine and limonene gave a single significantly high value each and of the 2 agents turpentine was tested for promoting power as noted and proved to have some.

The next set of experiments was done using a shorter list of agents, which were applied *once only*. The uptake of tritiated thymidine (Table V) was increased

TABLE III.—*Thickness of Epidermis*

Arbitrary units of an ocular counting grid

		Days after treatment				
		2	5	10	20	50
Control	Mean	25·1				
	S.D.	7·4				
5% croton oil	Mean	60	114	123	122	106
	S.D.	10·2	28·8	20·2	27·6	35·3
100% Tween 60	Mean	43	91	82	67	64
	S.D.	10·2	14·2	10·7	9·1	9·6
100% turpentine	Mean	26	33	37	42	71
	S.D.	4·9	11·4	10·7	13·7	20·5
20% limonene	Mean	25	27	39	30	46
	S.D.	4·8	7·3	6·7	5·7	18·9
0·3% acridine	Mean	38	41	35	44	73
	S.D.	8·3	10·1	8·9	11·0	26·2
100% toluene	Mean	27	26	26	31	38
	S.D.	6·9	6·6	7·1	7·6	6·5
10% oil of sweet orange	Mean	37	35	36	34	45
	S.D.	6·2	10·1	5·6	8·5	13·4
10% silver nitrate	Mean	24	28	32	24	25
	S.D.	6·2	9·0	17·0	3·0	5·4
8% formic acid	Mean	20	22	24	28	26
	S.D.	3·8	5·2	6·2	9·6	5·4
100% mineral oil	Mean	30	36	25	33	43
	S.D.	6·9	22·0	5·2	6·5	16·8
1·5% DMBA once +5% croton oil	Mean	76	127	103	107	139
	S.D.	19·5	27·8	21·5	33·7	29·9

Underlined values have a $p < 0{\cdot}01$ compared by the t test with the values obtained with mineral oil. Based on 10 measurements per ear, 10 ears per value given.

by croton oil and by Tween 60 after a lag period of about 10 hours. DMBA had the same effect. The other agents tested did not increase thymidine uptake for at least 20 hours following treatment to a significant degree compared with mineral oil. This observation does not exclude the possibility that significantly increased uptakes may be observed by these other agents at other times following an application. Nevertheless, the 2 powerful promoting agents croton oil and Tween 60 were found to stimulate DNA synthesis markedly 20 hours following an application, as their effectiveness in inducing hyperplasia suggested they should.

The dye Lissamine Green B may be used to measure the distribution of extracellular water (Goldacre and Sylvén, 1962). Table VI demonstrates that extracellular water is markedly but transiently increased soon after the application of turpentine and toluene, but that it is increased late and for a longer period of time following the application of croton oil with or without DMBA pretreatment or of Tween 60. The early transient rise does not correlate with the promoting activity of the 2 agents that caused it. The delayed rise, on the other hand, is present only with the powerful promoters croton oil and Tween 60.

The dye Evan's Blue is thought to become attached to albumin and to measure approximately albumin distribution (Threefoot, 1960; Table VII). We found

TABLE IV.—*Cellular Inflammatory Exudate*

The number of inflammatory cells under a standard area of ear epidermis

Agent		Days after treatment 2	5	10
Control:	Mean	3		
	S.D.	2		
5% croton oil	Mean	149	246	176
	S.D.	80	102	142
100% Tween 60	Mean	69	285	92
	S.D.	77	148	55
100% turpentine	Mean	17	21	41
	S.D.	10	18	37
20% limonene	Mean	13	7	29
	S.D.	9	6	14
0·3% acridine	Mean	64	40	14
	S.D.	64	28	14
100% toluene	Mean	18	13	17
	S.D.	15	12	18
10% oil of sweet orange	Mean	32	31	11
	S.D.	27	47	13
10% silver nitrate	Mean	14	15	17
	S.D.	12	12	27
8% formic acid	Mean	7	5	3
	S.D.	4	4	5
100% mineral oil	Mean	15	47	6
	S.D.	14	109	4
1·5% DMBA once	Mean	208	164	98
	S.D.	84	96	39

Statistical analysis was done after a logarithmic transformation of the data. The underlined values had a $p < 0{\cdot}01$ compared to the values obtained with mineral oil by the t test. Based on 10 measurements per ear, 10 ears per value given.

TABLE V.—*Uptake of Tritiated Thymidine*

Per cent of labelled cells in the epidermis after a single treatment

Treatment	10 hours after treatment Mean %	S.D.	20 hours after treatment Mean %	S.D.
Control: Mean 3·9% S.D. 2·2				
5% croton oil	3·5	2·0	32·1*	4·5
100% Tween	3·5	1·9	11·8	7·4
100% turpentine	2·9	0·9	5·0	4·2
100% toluene	2·8	2·0	4·2	2·2
10% oil of sweet orange	3·4	1·4	8·9	6·1
100% mineral oil	3·6	1·1	8·3	10·2
1·5% DMBA	3·1	1·4	28·2	15·4
5% croton oil 1 week after 1·5% DMBA	14·8	4·2	est. 30–50†	

* 22 hours after treatment.

† These samples were too thick to be evaluated in flat mounts of epidermis. Ten samples were measured for each value shown in the table. Underlined values had a $p < 0{\cdot}01$ by the t test as compared to 100% mineral oil.

TABLE VI.—*Permeability to Lissamine Green B*

Optical density of whole ears in arbitrary units

Treatment	Time after treatment: 5 min.	25 min.	2 hrs.	10 hrs.	2 d.	10 d.
Untreated 0·35; 0·31	0·20	0·41	0·45	0·54	0·48	0·32
5% croton oil	0·23	0·37	0·32	0·42	0·46	0·41
100% Tween 60	0·33	0·81	0·39	0·40	0·34	0·38
100% turpentine	0·44	0·60	0·47	0·38	0·37	0·35
100% toluene	0·24	0·32	0·23	0·25	0·24	0·35
10% oil of sweet orange	0·26	0·39	0·24	0·34	0·35	0·33
100% mineral oil	0·29	0·36	0·28	0·32	0·35	0·40
1·5% DMBA	0·29	0·37	0·45	0·68	0·51	0·51
5% croton oil 1 week after DMBA						

Dye injected intraperitoneally 30 minutes before ear removed. A single application of an irritant used. Optical density was measured on whole mounted ears in an Evelyn spectrophotometer. Ten samples were measured for each value shown in table. Underlined values had a $p < 0{\cdot}01$ by the t test as compared to 100% mineral oil values.

TABLE VII.—*Permeability to Evan's Blue*

Optical density of whole ears in arbitrary units

Treatment	Time after treatment: 5 min.	25 min.	2 hrs.	10 hrs.	2 d.	10 d.
Untreated 0·15; 0·17; 0·13	0·12	0·15	0·24	0·29	0·34	0·21
5% croton oil	0·12	0·14	0·15	0·15	0·24	0·18
100% Tween 60	0·19	0·28	0·24	0·21	0·21	0·15
100% turpentine	0·32	0·34	0·24	0·17	0·18	0·16
100% toluene	0·10	0·10	0·13	0·17	0·20	0·18
10% oil of sweet orange	0·07	0·11	0·10	0·14	0·14	0·17
100% mineral oil	0·16	0·18	0·16	0·15	0·19	0·43
1,5% DMBA	0·36	0·41	0·48	0·54	0·60	0·43
5% croton oil 1 week after DMBA						

Dye injected intraperitoneally 1 hour before ear removed. A single application of the irritant used. Optical density was measured on whole mounted ears in an Evelyn spectrophotometer. Ten samples were measured for each value shown in table. Underlined values had a $p < 0{\cdot}01$ by the t test as compared to 100% mineral oil.

differences in this response to the test agents, but no distinction in response between agents with promoting power and those without it.

The exudation of water and protein and the emigration of leukocytes are generally thought to be a part of the inflammatory response. Of these 3 elements of the inflammatory response, cellular exudation and the increase in extracellular water correlated well with promoting power of the agents that provoked them. The exudation of protein (Evan's Blue tagged albumin) did not.

DISCUSSION

The usefulness of the two-stage model of carcinogenesis in the mouse introduced by Mottram (1944) and by Berenblum and Shubik (1947, 1949) hinges on the

possibility of analysing and defining the mechanism of each of the 2 stages. Neither of the 2 stages has as yet been completely defined. The most commonly cited theory for the mechanism of the first stage, or the action of a single painting with a subliminal dose of a potent carcinogen, is that it induces latent tumour cells by a mutation-like process as stated originally by Berenblum and Shubik (1949). Berenblum and Shubik have not formed a theory for the second stage, the mechanism of which has, however, been examined repeatedly. The present work tends to support the theory that the mechanism of the second stage is the induction of a marked and sustained hyperplasia.

The same conclusion was reached by the workers in Setälä's group, working mainly with Tween and Span detergents (Setälä, 1956; Dammert, 1961; Merenmies 1959; and Holsti, 1960). As in the present work, these authors have performed statistically analysed cell counts at various times during treatment with the detergents. The results of other workers, who measured the degree of hyperplasia induced at 1 or 2 times during treatment and expressed it in terms of 1 + to 4+, are not as clear cut. Those of Salaman (1961) using various plant oils on the whole agree with the hyperplasia theory, while those of Roe and Pierce (1961) using the lattices of *Euphorbiaceae* contain one exception to it among 9 substances tested, and those of Shubik (1950) contain 2 exceptions in 9 instances.

The present authors have retested the exceptions to the hypothesis described by Shubik (1950), namely the effect of 0·3% acridine in mineral oil and of 10% silver nitrate in water. In more extensive experiments using Swiss mice these two agents were found to conform to the general proposition. There remains therefore, only the one latex tested by Roe and Pierce (1961; latex of *E. obovalifolia*) which was not available to the authors for retesting, and which in the original work induced marked (4+) hyperplasia but which promoted only a small number of tumours.

The overwhelming evidence of the literature and of the work presented here therefore, supports the theory that the mechanism of action of substances capable of promoting the growth of "latent tumour cells", resides in their ability to stimulate a marked and lasting hyperplasia. It may be hoped that as the recently identified active principles of croton oil will become available for this purpose, this matter will not only be definitely settled, but that the mechanism by which the hyperplasia is induced will be studied more accurately.

During the testing of the active croton oil principles, Hecker and his group have used as a measure with predictive value the reddening of mouse ears to test their fractions for potential promoting power (Hecker, 1963; Hecker *et al.*, 1966). The present work, albeit using the unpurified parent croton oil, has examined the induction of the inflammatory response by it as well as by other promoting and non-promoting agents in more detail. Of the various elements of the inflammatory response, three were studied: the extravasation of inflammatory cells, the increase in water content of the test tissue as measured by the accumulation of the dye Lissamine Green B, and the increase in albumin content of the test tissue, as measured by the accumulation of the dye Evan's Blue. A correlation with the induction of hyperplasia was obtained only for the first 2 parameters (exudation of inflammatory cells and of water), but not for the third (exudation of albumin). In the absence of any additional information in the available literature on these correlations, these results are only suggestive of a possible relationship of the inflammatory response to hyperplasia. The matter requires further experimental

examination before any relationship between an inflammatory stimulus and hyperplasia and hence tumour promotion can be discounted or established.

SUMMARY

Tumours were induced in adult Swiss male mouse ears by giving a single painting with 7,12-dimethylbenz(*a*)anthracene followed by paintings with various tumour-enhancing and other substances twice a week for 20 weeks.

The rate of tumour enhancement was compared with the stimulation of epidermal thickening and with the induction of hyperplasia by the same agents.

These phenomena were found in good correlation, an observation which supports the hypothesis that tumour enhancement is due to the stimulation of hyperplasia.

The enhancing agents were further studied for the rate of induction of the inflammatory response. The outpouring of water and the emigration of leucocytes into the site correlated well with the rate of induction of hyperplasia, but the exudation of albumin did not.

The relationship of the inflammatory response to the induction of hyperplasia was therefore not fully clarified.

Discussions of this work with Professor J. A. McCarter of the Cancer Research Laboratory, University of Western Ontario, and with Professor A. C. Ritchie, Department of Pathology, University of Toronto, were helpful to the authors. Technical assistance was given by Misses H. Pineau, B. Hershon, K. Wolever and by Messrs. W. Kingsley, S. Muity and L. Sales.

This work was supported by grants from the National Cancer Institute of Canada.

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Report from the Commission on Food on the Use of Formic Acid and Formates as Preservatives in Food Products

by Pierre Gley and Jean-Emile Courtois

The Minister of Agriculture, by his letter of October 4, 1967 asked for the opinion of the Academy on the use of formic acid and formates for the preservation of food products.

One of the scientific commissions of the European Economic Community is in the process of studying the use of formic acid as a food preservative. Although adoption by the Community of a new preservative does not require the member states to authorize the use of this substance in their respective countries, the French delegation represented by Professor Truhaut, expressed a waiting reservation to allow them to consult the scientific authorities in their country before the next meeting of this commission.

Formic acid, like benzoic acid, has been used in Germany for a long time for the preservation of concentrated fruit juices to be used in the production of fancy drinks. In some respects, it is considered to be less active than benzoic acid. The useful concentration of the latter being 1 p. 1000, that of formic acid must attain 2.5 p. 1000 in order to be equivalent. In addition, in this country formic acid passes for "gesundheitunschadlich" in other words, harmless to the health; these facts raise the question precisely, and we are going to examine whether formic acid should be permitted in food.

Before looking at the toxicology of formic acid, it would not hurt to glance at the fate of this substance in the organism. Let us note

first of all that formic acid is not completely foreign to the organism. It is produced physiologically in small quantities, and 13 to 120 mg are eliminated each day in the urine. This endogenous formic acid comes neither from the oxidation of glucosides nor lipids. It is produced by the metabolism of certain amino acids, histidine in particular, which is decomposed in the liver with the formation of glutamic acid, formic acid and ammonia. It is then used in the synthesis of purines. With ribose phosphoric acid, glycine, ammonia and carbonic acid, it contributes to the building of inosinic acid from which other nucleotides are derived, its incorporation being catalyzed by folic acid.

When formic acid is introduced into an organism, it does not disappear completely, and a more or less large proportion of the quantity absorbed is eliminated naturally through the urine. While absorption is significant, the undestroyed quantity may attain 2/3. This is because formic acid, containing only one carbon atom cannot enter into the Krebs cycle which utilizes acetic acid coming from the β-oxidation of fatty acids. It is only by other means that it can be transformed. According to research in which rats were given glycine marked with carbon C^{13} in the carboxylic group, and at the same time, formic acid marked with carbon C^{14}, the two substances came together in the liver to form serum containing carbon C^{13} of glycine in its carboxylic group and carbon C^{14} of formic acid in its alcoholic group. Thus, formic acid can disappear in the organism, but this process is limited by the disposable quantity of glycine. It is thus understood why it is only partially destroyed.

The question then arises of knowing whether the untransformed fraction of the absorbed formic acid exerts a pharmacological effect. This problem

has been studied for a long time, and since 1907, Fleig has examined the effect of this substance on different organs. He found a notable effect only on the kidney. Formic acid has a diuretic effect, which while being inferior to that of theobromine, is not negligiable, and which increases, as the latter does, the vasodilatation of the renal vessels. Physiology confirmed the clinical data because Huchard just discovered in formic acid and its salts, besides the debatable tonic muscular properties, this diuretic effect which physiology experiments have confirmed. Lately, new research has been done. It has been found that formic acid exerts an inhibiting effect on enzymes. This explains a singular particularity of the toxicology of this substance. A minimal amount (6 mg/kg in the rabbit) causes methemoglobinemia, and the alteration of the blood pigment is persistant. After a single dose of formic acid, the effect lasts about 10 days. It is possible that the slowness with which the abnormal pigment disappears is due to the inhibition of the catalase. In other research, a comparative study of formic, sorbic and benzoic acids which are all food preservatives, on the formation of vitamin A was recently done (M. Kramer and R. Trajan, Intern Z. Vitaminforschung 32: 149-175, 1962). This study showed that in the rat, an animal which normally transforms carotene from its diet into vitamin A, this transformation was slowed by formic acid. It can therefore be seen that we are not yet completely knowlegeable about the long term toxicity of this substance.

The fact that formic acid does not enter into the cycle of reactions which take place in the organism (except the combination with glycine), allows us to predict that this substance is slightly toxic. Actually, according to the experiments of Fleig, the immediate toxic dose of sodium

formate is 3 g/kg given intravenously. If the substance is ingested, the amount surpasses 4 g. But, in relation to its use as a food preservative, it is more interesting to study its chronic toxicity rather than the immediate toxicity. However, unfortunately, studies on this are not numerous. We have found only in Sollmann's manual on pharmacology information that a dose of 240 mg/kg would be tolerated by the rat on a long term basis, while a dose double that amount would bring about the stunting of growth. From other information kindly furnished by Professor Truhaut which he recently found by G. Malorny, Director of the Pharmacology Institute at Hamburg, it was disclosed that the rat would tolerate long-term doses of 200 to 400 mg/kg of calcium formate.

Information relating to humans is not lacking, but is not very precise. Formic acid or substances in which it constitutes the base has been used for a long time in human therapy. Without going back to Ambroise Paré who recommended extracts from ants as aphrodisiacs, in the 17th century, they made an elixir with red ants called electuarium magnanimitatis which was reputed to be a cordial and diuretic. In Germany, formic acid, or spiritus formicarum was still used at the beginning of this century. It is still sometimes used in Swiss pharmacopoeia. In our own country, in 1902, it found renewed usage, and this old medication was promoted by several therapists, the most notable of whom was Huchard. His work was previously cited. What interests us here is the dosage utilized: the amounts reached 3 to 4 g per day of sodium formate.

What can be concluded from these data?

The committee of Experts on Food Additives of the World Health Organization examined this question in 1961. It concluded that in view of

the lack of studies on long-term toxicity, it was impossible to give indications for the daily permissible amounts for humans, without any reservations. Under the section on directions for use and with certain reservations, it gave a dose of 5 mg/kg. (Technical Report #228 of June 1961, confirmed by Technical Report #309 in 1965).

The Scientific Commission of the European Economic Community which has asked for the opinion of the Academy of Medicine expressed the following opinion which Professor Truhaut has communicated to us:

"The Scientific Commission is of the opinion that the evaluation of formic acid should not be made only on the basis of routine experiments, but also on biochemical considerations. It emphasizes that in the fast growing field of toxicology experimentation, formic acid is not of much interest, since formic acid and its salts exist as intermediate products of metabolism.

In conclusion, the Scientific Commission sees no harm in allowing the use of formic acid and its salts as long as the daily absorption does not exceed 5 mg/kg body weight. It is furthermore of the opinion that it is necessary to determine as a second step the food in which the use of formic acid is authorized as well as the respective concentrations of its utilization.

The Commission further recommends the exclusive use of formate salts because of the irritating nature of the acid to those who handle it."

We have tried, besides, to get the opinions of manufacturers, and we heard from two large companies which make fruit juices. The fruit juice and lemonade industries in our country know how to prepare products of high quality and how to preserve them well without resorting to the

addition of preservatives. They have obtained these results by observing strict regulations in order to avoid all bacterial or parasitic contamination. The authorization for the use of formic acid and formates, as in the case of every other chemical preservative, does not come from us as a result of necessity.

Your Food Commission has carefully examined these data. It has decided that there is no reason to abandon the consistent policy of the Academy in opposing the addition of foreign substances to natural foods. This policy seems particularly valid in the cases of lemonade and fruit juices which are largely consumed by children. As a result, it proposes to give an unfavorable opinion on the addition of formic acid or formates to food products.

The conclusions proposed by the Commission are unanimously adopted.

RAPPORT

Au nom de la Commission de l'alimentation sur l'emploi de l'acide formique et des formiates comme agents de conservation des denrées alimentaires

par MM. **Pierre Gley** et **Jean-Emile Courtois**

Monsieur le Ministre de l'Agriculture, par lettre du 4 octobre 1967 demande l'avis de l'Académie sur l'emploi de l'acide formique et des formiates pour la conservation des denrées alimentaires.

Une des commissions scientifiques de la Communauté économique européenne est en train d'étudier l'admission de l'acide formique comme agent de conservation des aliments. Bien que l'adoption par la Communauté d'un nouveau conservateur n'entraîne pas l'obligation pour les états membres d'autoriser cette substance dans leurs pays respectifs, la délégation française, représentée par le professeur Truhaut, a exprimé une réserve d'attente pour lui permettre de consulter les autorités scientifiques de son pays avant la prochaine réunion de cette commission.

L'acide formique est employé, comme l'acide benzoïque, depuis longtemps en Allemagne pour la conservation des jus concentrés de fruits destinés à la fabrication des boissons de fantaisie. Il est d'ailleurs considéré comme moins actif que l'acide benzoïque. La concentration utile de ce dernier étant de 1 p. 1 000 celle d'acide formique doit atteindre 2,5 p. 1 000 pour être équivalente. L'acide formique passe d'ailleurs dans ce pays pour être « gesundheitunschädlich » c'est-à-dire inoffensif pour la santé. Ces données posent la question d'une manière précise et nous allons examiner si l'acide formique peut être admis dans les aliments.

Avant de regarder la toxicologie de l'acide formique il ne sera pas inutile de jeter un coup d'œil sur le sort de ce corps dans l'organisme. Notons d'abord que l'acide formique n'est pas complètement étranger à l'organisme. Il y est produit physiologiquement en petite quantité et les urines en éliminent de 13 à 120 mg par jour. Cet acide formique d'origine endogène ne provient ni de l'oxydation des glucides ni de celle des lipides. Il est produit par le métabolisme de certains acides aminés, l'histidine notamment, qui est décomposée dans le foie avec formation d'acide glutamique, d'acide formique et d'ammoniaque. Il est utilisé ensuite pour la synthèse des purines. Avec l'acide ribose-phosphorique, la glycine, l'ammoniaque et l'acide carbonique, il concourt à l'édification de l'acide inosinique d'où dérivent les autres nucléotides, son incorporation étant catalysée par l'acide folique.

Lorsqu'on introduit dans l'organisme de l'acide formique, il ne disparaît pas complètement et une proportion plus ou moins grande de la quantité absorbée est éliminée en nature par les urines. Lorsque l'absorption est importante, la quantité non détruite peut atteindre les 2/3. C'est que l'acide formique, ne contenant qu'un seul atome de carbone, ne peut entrer dans le cycle de Krebs qui utilise l'acide acétique provenant de la β-oxydation des acides gras. C'est seulement par d'autres voies qu'il peut être transformé. D'après des recherches dans lesquelles on a fait absorber à des rats de la glycine marquée avec du carbone C^{13} dans le groupe carboxylique et, en même temps, de l'acide formique marqué avec du carbone C^{14} ces deux corps s'unissent dans le foie pour former de la sérine contenant le carbone C^{13} de la glycine dans son groupe carboxylique et le carbone C^{14} de l'acide formique dans son groupe alcoolique. Ainsi l'acide formique peut disparaître dans l'organisme, mais ce processus est limité par la quantité disponible de glycine. On comprend pourquoi il n'est détruit que partiellement.

La question se pose alors de savoir si la fraction non transformée de l'acide formique absorbé exerce une action pharmacologique. Ce problème a été étudié depuis longtemps et, dès 1907, Fleig examina l'action de ce corps sur les différents appareils. Il ne reconnut d'effet notable que sur le rein. L'acide formique a une action diurétique qui, pour être inférieure à celle de la théobromine, n'est pas négligeable et qui relève, comme cette dernière, de la vaso-dilatation des vaisseaux rénaux. La physiologie confirmait les données de la clinique car Huchard venait de reconnaître à l'acide formique et à ses sels, à côté de propriétés toni-musculaires discutables, cette action diurétique que les expériences de physiologie confirmaient. Depuis cette époque, de nouvelles recherches ont été faites. On a trouvé que l'acide formique exerce une action inhibitrice sur les enzymes. Cela explique une particularité singulière de la toxicologie de ce corps. Une dose minime (6 mg/kg chez le lapin) est méthémoglobinisante et l'altération du pigment sanguin est persistante. Après une seule dose d'acide formique, elle dure une dizaine de jours en effet. Il est possible que la lenteur avec laquelle le pigment anormal disparaît soit due à l'inhibition de la catalase. Dans un autre ordre de recherches, une étude comparative des acides formiques, sorbique et benzoïque, qui tous trois sont des agents conservateurs des aliments, sur la formation de la vitamine A a été faite récemment (M. Kramer et R. Trajan, *Intern. Z. Vitaminforschung*, 1962, **32**, 149-175). Cette étude a montré que chez le rat, animal qui normalement transforme le carotène de la ration alimentaire en vitamine A, cette transformation est ralentie par l'acide formique. On voit donc que nous ne sommes pas complètement informés encore sur la toxicité à long terme de ce corps.

Le fait que l'acide formique n'entre pas dans le cycle des réactions qui se produisent dans l'organisme (sauf la combinaison avec la glycine) laisse prévoir que ce corps est peu toxique. En effet, d'après les expériences de Fleig, la dose toxique immédiate du formiate de sodium est, pour le chien, de 3 g/kg par voie intraveineuse. Si le corps est ingéré, elle passe à 4 g. Mais en ce qui concerne l'emploi comme conservateur alimentaire, la toxicité

chronique est plus intéressante à connaître que la toxicité immédiate. Or, malheureusement, les études ne sont pas nombreuses sur ce point. Nous avons seulement trouvé dans le manuel de pharmacologie de Sollmann une indication d'après laquelle la dose de 240 mg/kg serait tolérée par le rat à long terme tandis que la dose double entraînerait l'arrêt de la croissance. D'après une autre indication, obligeamment fournie par le professeur Truhaut, il a été trouvé récemment par G. Malorny, Directeur de l'Institut de Pharmacologie de Hambourg, que le rat supporte à long terme la dose de 200 à 400 mg/kg de formiate de calcium.

Les données relatives à l'homme ne manquent pas mais sont peu précises. L'acide formique, ou des préparations dont il constitue la base, a été utilisé en thérapeutique humaine depuis fort longtemps. Sans remonter à Ambroise Paré, qui recommandait les extraits de fourmi comme aphrodisiaque, on fabriquait au XVIIe siècle, avec les fourmis rouges, un élixir connu sous le nom d'*electuarium magnanimitatis* qui était réputé comme cordial et diurétique. En Allemagne l'acide formique, ou *spiritus formicarum* était encore employé au début de notre siècle. Il figure d'ailleurs encore dans la pharmacopée helvétique. Chez nous il connut, en 1902, un renouveau et cette vieille médication fut prônée par quelques thérapeutes dont le plus marquant fut Huchard. Les travaux de celui-ci ont été cités précédemment. Ce qui nous intéresse ici, c'est la dose utilisée. Or, elle atteignait 3 à 4 g par jour pour le formiate de sodium.

Que conclure de ces données ?

Le Comité d'Experts des Additifs alimentaires de l'Organisation Mondiale de la Santé a examiné la question en 1961. Il a conclu que, vu l'absence d'études de toxicité à long terme, il était impossible de donner une indication sur les doses quotidiennes admissibles sans réserves chez l'homme. A titre d'indication, et sous certaines réserves, il a donné la dose de 5 mg/kg. (Rapport technique n° 228 de juin 1961, confirmé par le rapport technique n° 309 de 1965.)

La Commission scientifique de la Communauté économique européenne qui est à l'origine de la demande d'avis à l'Académie de Médecine a exprimé l'avis suivant que le professeur Truhaut a bien voulu nous communiquer :

« La Commission scientifique est d'avis que l'évaluation de l'acide formique ne doit pas seulement se faire sur la base des expériences de routine mais également sous l'aspect biochimique. Elle souligne que, dans l'espèce, l'expérimentation toxicologique très poussée ne présente pas beaucoup d'intérêt, étant donné que l'acide formique et ses sels existent en tant que produits intermédiaires du métabolisme.

En conclusion, la Commission scientifique ne voit pas d'inconvénient à admettre l'acide formique et ses sels, *à condition qu'il n'en résulte pas une absorption journalière dépassant 5 mg/kg de poids corporel*. Elle est, en outre, d'avis qu'il est indispensable de fixer dans un second stade les aliments dans lesquels l'emploi de l'acide formique est autorisé ainsi que les concentrations respectives de leur utilisation.

La Commission recommande, en outre, l'emploi exclusif des formiates en raison du caractère irritant de l'acide pour les manipulateurs. »

Nous avons tenu, en outre, à recueillir l'avis des fabricants et nous nous sommes adressés pour cela à deux grandes marques d'eau minérale qui fabriquent des jus de fruit. L'industrie des limonades et des jus de fruit dans notre pays sait préparer des produits de haute qualité et de conservation parfaite sans recourir à l'addition de conservateurs. Elle est parvenue à ce résultat en observant des règles très strictes afin d'éviter toute contamination bactérienne ou parasitaire. L'autorisation de l'acide formique et des formiates, comme d'ailleurs celle de tout autre conservateur chimique, ne répondrait par conséquent chez nous à aucune nécessité.

Votre Commission de l'Alimentation a examiné attentivement ces données. Elle a estimé qu'il n'y avait pas lieu d'abandonner la doctrine constante de l'Académie qui est de s'opposer à l'addition aux aliments naturels de toute substance étrangère. Cette doctrine lui a paru particulièrement valable dans le cas des limonades et des jus de fruit qui sont surtout consommés par les enfants. Elle vous propose, en conséquence, de donner un avis défavorable sur l'addition d'acide formique ou de formiates aux denrées alimentaires.

Les conclusions proposées par la Commission sont adoptées à l'unanimité.

ÉLECTION

d'un Membre titulaire dans la VIIIe Section

(Membres non résidants)

M. le Président : Messieurs, il va être procédé à l'élection d'un Membre titulaire dans la VIIIe Section (*Membres non résidants*), en remplacement de M. Maurice Patel, décédé.

Sont présentés :

En première ligne : M. Huriez (de Lille).

En seconde ligne *ex æquo* et par ordre alphabétique, MM. Boulanger (de Lille), Lazorthes (de Toulouse), Morin (de Marseille), Tayeau (de Bordeaux), Turchini (de Montpellier).

Adjoint par l'Académie : M. Weiss (de Strasbourg).

Il est procédé à l'élection.

Votants : 99. — Suffrages exprimés : 97. — Majorité : 50.

Fd Cosmet. Toxicol. Vol. 5, pp. 141–157. Pergamon Press 1967. Printed in Great Britain

Research Section

Food Flavourings and Compounds of Related Structure. II. Subacute and Chronic Toxicity

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(*Received 7 November 1966*)

Abstract—The subacute and/or chronic toxicity of 48 food flavourings was studied in rats. The toxicity of five of these flavourings (coumarin, 6-methylcoumarin, dihydrocoumarin, methyl salicylate and safrole) was also studied in dogs. A summary of the studies is presented. The loss of 21 flavourings from laboratory animal diets during a 7-day period was also determined.

INTRODUCTION

For a number of years the Food and Drug Administration has been investigating the toxicity of food flavourings, both natural and synthetic. The toxicity studies consist of (1) determination of the acute oral effects, (2) subacute studies in which the flavouring agents are mixed in the diet or administered by stomach tube, and (3) chronic feeding studies on those flavouring agents having wide use, having chemical structures implying toxicity and/or showing toxic effects in the subacute studies. The data on the acute oral toxicity of these substances have been reported (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The present report is a summary of the subacute and chronic studies. Full reports on some of the chronic studies have been published for methyl salicylate (Webb & Hansen, 1963), safrole (Long, Nelson, Fitzhugh & Hansen, 1963; Hagan, Jenner, Jones, Fitzhugh, Brouwer & Webb, 1965) and dihydrosafrole (Long & Jenner, 1963; Hagan *et al.* 1965).

Commercially-available materials, rather than pure chemicals, were used since the purpose of these studies was to evaluate the toxicity of these materials in relation to their use as food additives.

EXPERIMENTAL

Rat studies. Weanling Osborne–Mendel rats were used in the chronic and most of the subacute experiments. In a few subacute experiments young adult rats of the same strain were used. Litter mates were used except for a few subacute studies in which the weanlings were randomized by weight (every level having animals of equal weight). A control group containing the same number of rats as the test groups was included with each test compound except in the following studies in which a common group was used for a number of test compounds:

(1) Allyl caproate, allyl cyclohexane propionate, anisaldehyde, benzaldehyde, carvone, dimethylbenzyl carbinol, ethyl sebacate, geraniol extra, piperonal and vanillin fed at 1000 ppm for 27–28 wk (control group 10 males and 10 females).

(2) Anethole, anisaldehyde, benzaldehyde, carvone, dimethylbenzyl carbinol, Dolcourin, ethyl methylphenyl glycidate, ethyl pelargonate, ethyl sebacate, geraniol extra, isoeugenol, piperonal and vanillin fed at 10,000 ppm for 16 wk (control group 10 males and 10 females).

(3) Benzyl cinnamate, eugenol and thymol fed at 1000 and 10,000 ppm for 19 wk (control group 5 males and 5 females).

(4) Anethole, carvone and ethyl methylphenyl glycidate fed at 2500 ppm for 1 yr (control group 5 males and 5 females).

(5) Allyl caproate and allyl cyclohexane propionate fed at 2500 ppm for 1 yr (control group 5 males and 5 females).

(6) Ethyl vanillin and vanillin fed at 5000, 10,000 and 20,000 ppm for 2 yr (control group 12 males and 12 females).

(7) Ethyl vanillin and vanillin fed at 20,000 and 50,000 ppm for 1 yr (control group 5 males).

(8) Dihydrosafrole, isosafrole and safrole fed at 1000, 2500, 5000 and 10,000 ppm for 2 yr (control group 35 males and 35 females).

The animals were housed individually in wire cages and had access to food and water at all times. Most of the compounds were fed in the diet, but a few were administered by stomach tube. Fresh diets were made and distributed weekly except in the study with allyl isothiocyanate in which the diet was prepared and fed daily*. When the compound was administered by stomach tube, corn oil solutions of the flavouring were prepared. The concentration of the solution was adjusted for each level so that all rats received a constant volume of 1 ml of solution/kg daily. The control animals were given an equal volume of corn oil. The rat's weight, food intake and general condition were recorded every week. Haematological examinations were made at termination of the subacute studies, and at 3, 6, 12 and 22 months in the chronic experiments. These examinations included white cell counts, red cell counts, haemoglobins and haematocrits.

At the termination of the experiments the rats were sacrificed and exsanguinated. The tissues of all the rats were examined macroscopically at the time of sacrifice. The viscera were removed and the liver, kidneys, spleen, heart, and testes were weighed. These organs, the remaining abdominal and thoracic viscera, and one hind leg, for bone, bone marrow, and muscle, were preserved in 10% buffered formalin-saline solution for histopathological examination. For routine histopathology, sections were embedded in paraffin wax and stained with haematoxylin and eosin. Tissues from rats dying during the experiment were examined for gross changes and were preserved if autolysis was not advanced. Organs were not weighed but abnormalities and the suspected reason for death were noted.

Detailed microscopic examinations in the subacute studies were generally done on 6 or 8 rats, evenly divided by sex, from the high dose group and the control group. If changes attributable to the test compound were found in the high dose group, additional animals on lower dosage levels were examined as indicated. For animals from the long-term studies, more extensive histopathological study was done.

Dog studies. Pure-bred beagles were used in the studies with safrole and methyl salicylate. Mongrels and beagles were used in the coumarin, dihydrocoumarin, and 6-methylcoumarin studies. The dogs were wormed and immunized against distemper, rabies, and infectious

*Two other exceptions were the safrole rat study (Long *et al.* 1963) and the chronic rat feeding study of methyl salicylate (Webb & Hansen, 1963) in which the diets were prepared every other week and stored under refrigeration in sealed containers.

hepatitis before treatment was begun. They were housed in individual cages with food (commercial dry dog food) and water available at all times. The test compound was administered by capsule daily for 6 days a week. The animals were weighed weekly and the dosages were recalculated at this time. Haematological examinations were made three times prior to the start of treatment and at 2 wk, 1 month, 3 months, 6 months and 1 yr after treatment began and then at yearly intervals until termination of the study.

At the end of a study, survivors were killed with an overdose of an intravenous barbiturate and exsanguinated. The major organs were weighed. Sections of brain (four levels), pituitary, salivary gland, thyroid, parathyroid, thymus, lymph nodes, lung, heart, stomach, spleen, pancreas, kidney, adrenal, liver, gall bladder, small intestine (three levels), large intestine, urinary bladder, rib, skeletal muscle, and testis and prostate (or ovary and uterus) were preserved in 10% buffered formalin–saline for histological examination. In addition liver, kidney and adrenal sections and a femoral marrow plug were fixed in Zenker's solution. The sections were embedded in paraffin wax and stained with haematoxylin and eosin. Frozen sections of the liver and kidney were stained with Oil Red O for fat. A rib marrow smear was air-dried and stained with the Wright–Giemsa stain. Dogs found in a moribund condition during the experiment were sacrificed and necropsied. Dogs dying during the experiment were necropsied and tissue sections preserved if autolysis was not too advanced. All the tissues that were preserved were examined microscopically.

RESULTS

Table 1 shows the compounds tested; the dose levels; number, sex and species of test animals; duration; and effects. When the dose level is given in ppm, the test compound was mixed in the diet. When the dose is given in mg/kg, the test compound was administered daily by stomach tube to rats and (6 days/wk) by capsule to dogs. Only those effects believed to be due to the administration of the compound are listed. The designation "no effect" indicates that there was no effect on growth or haematology, and no macroscopic or microscopic change in the tissues. The designation "no macroscopic effect" indicates that there was no effect on growth or haematology, and no macroscopic change in the tissues, and that microscopic examination of the tissues was not performed. Pathological changes attributable to disease or age have not been included. For those compounds on which the toxicity studies have been reported previously the reference is given in Table 1 with the name of the compound.

In the evaluation of the data from the feeding studies with flavouring agents, the possibility of loss of the test compound from the experimental diet must be considered. Many of the flavouring agents used in these studies were quite volatile. Studies in our laboratory have shown that a decrease in the concentration of some of the flavourings occurred when the experimental diet was exposed in the open feed cups (Jones, Taylor, & Hagan, 1962). In order to determine the actual amount of the flavouring consumed by the animals, many of the experimental diets were analysed for the flavouring immediately after preparation and again after being exposed to laboratory conditions for 7 days. The flavourings on which analytical studies have been made and the percentage loss calculated over a 7-day period are presented in Table 2.

Table 1. *Summary of subacute and chronic toxicity of food flavourings and related compounds*

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Acetophenone (methyl 1-phenyl ketone)	10,000	Rat 10M & 10F	17 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
	90 mg/kg	do.	18 wk	Growth retardation in males. Liver: macroscopic—rough or granular surface, firm consistency, nutmeg appearance; microscopic—slight to moderate bile-duct proliferation and fibrosis with pseudolobule formation. Necrosis with polymorphonuclear infiltration and swollen, foamy liver cells in 2 of 8 rats examined
Allyl butyrate (allyl butanoate)	50 mg/kg	do.	17 wk	Lung—slight to marked peribronchial lymphocytic infiltration[b]. Liver—no effect
Allyl caproate (allyl hexanoate)	2500	5M & 5F	1 yr	No effect
	1000	do.	28 wk	do.
	100 mg/kg	10M & 10F	18 wk	Liver: macroscopic — somewhat nodular and wrinkled with granular or rough surface; microscopic—slight to moderate bile-duct proliferation, some lobular architectural disarrangement, slight fibrosis, pigment in macrophages; necrotic foci in 2 of 8 rats examined
	65 mg/kg	do.	do.	Liver: microscopic—very slight bile-duct proliferation in 2 of 8 rats examined[c]
	15 mg/kg	do.	do.	No effect[c]
Allyl cyclohexane propionate (allyl 3-cyclohexylpropionate)	2500	5M & 5F	1 yr	No effect
	1000	do.	27–28 wk	do.
Allyl isothiocyanate[d]	10,000	do.	26 wk	do.
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.

[a]Unless otherwise stated.
[b]Controls had slight to marked peribronchial lymphocytic infiltration not as severe as with the 50 mg/kg/day dose.
[c]Microscopic examinations were made on the liver only.
[d]The diet was prepared daily because of the extreme volatility of this compound. A control group pair-fed with the 10,000 ppm group was included in this study in addition to the usual untreated control group.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Allyl isothiocyanate[d]	50 mg/kg	Rat 5M & 5F	20 days	Stomach: macroscopic—non-glandular part of the stomach was thickened, with lining occasionally roughened; microscopic—slight to moderate epithelial hyperplasia of the non-glandular part of the stomach, with acute to subacute ulcers from 2 to 6 1/2 mm across observed in all animals. Liver: microscopic—minor inflammatory foci
	20 mg/kg	do.	20 days	Stomach: macroscopic—same as seen in the 50 mg/kg animals; microscopic—the same type of ulceration as seen in the higher dose, occurring in 50% of the animals
Amyl butyrate (pentyl butyrate)	10,000	10M & 10F	16 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Amyl valerianate (pentyl pentanoate)	10,000	do.	13 wk	No effect
	1000	do.	do.	No macroscopic effect
Anethole (1-methoxy-4-propenylbenzene)	10,000	5M & 5F	15 wk	Liver: microscopic—slight hydropic changes of hepatic cells in males only
	2500	do.	1 yr	No effect
Anisaldehyde (*p*-methoxybenzaldehyde)	10,000	do.	15 wk	No effect
	1000	do.	27–28 wk	do.
Benzaldehyde	10,000	do.	16 wk	do.
	1000	do.	27–28 wk	do.
Benzyl cinnamate (cinnamein, benzyl β-phenylacrylate)	10,000	do.	19 wk	do.
	1000	do.	do.	No macroscopic effect
Calamus oil	10,000[e]	10M & 10F	18 wk	Dose-related growth depression, greater in males than in females. Increased mortality. Large amounts of clear fluid in the abdominal cavity. Liver: macroscopic—variable in appearance with some lobes reduced in size with rough surfaces and others smooth, abnormally rounded, and enlarged; microscopic—variation in cell size, capsular thickening, proliferation of bile duct epithelium, portal area fibrosis with haemosiderin deposition (females more severely affected than males). Heart—minimal to slight myocardial degeneration characterized by varying degrees of necrosis of muscle fibres, early fibrosis, and infiltration with mononuclear cells

[a]Young adult animals were used.

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Calamus oil	5000	Rat[e] 10M & 10F	18 wk	Growth depression. Moderate amounts of clear fluid in the abdominal cavity. Liver—same changes as with 10,000 ppm but less severe. Heart—same changes as with 10,000 ppm but lower incidence
do.	2500	do.	do.	Growth depression. Liver—same changes as with 5000 and 10,000 ppm but less severe. Heart—same changes seen with the 10,000 ppm dose were seen in 1 of 6 rats examined
	1000	5M & 5F	do.	Growth depression
	10,000	do.	16 wk	Growth retardation, testicular atrophy
Carvone (*p*-mentha-6,8-dien-2-one)	2500	do.	1 yr	No effect
	1000	do.	27–28 wk	do.
Cinnamic aldehyde (cinnamaldehyde)	10,000	10M & 10F	16 wk	Liver—slight hepatic cell swelling. Stomach—slight hyperkeratosis of squamous portion
	2500	do.	do.	No effect[f]
	1000	do.	do.	No macroscopic effect
Citral (3,7-dimethyl-2,7-octadienal)	10,000	do.	13 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Coumarin[g,h] (2*H*-1-benzopyron-2-one)	10,000[e]	3M & 3F	4 wk	Marked growth retardation, testicular atrophy. Liver: microscopic—slight to moderate damage consisting of dead and dying cells, decrease in oxyphilia and cytoplasm in the centrolobular cells, and proliferation of bile ducts
	10,000	10M & 10F	1–8 wk	Marked growth retardation. No animal survived beyond 8 wk. Liver: microscopic—slight to moderate damage of the type seen with the 10,000 ppm dose for 4 wk

[f]Microscopic examinations were made on the stomach only.
[g]Toxicity study will be presented in a separate report.
[h]Not acceptable for food use because of toxic effects observed in chronic feeding studies.

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Calamus oil	5000	Rat[c] 10M & 10F	18 wk	Growth depression. Moderate amounts of clear fluid in the abdominal cavity. Liver—same changes as with 10,000 ppm but less severe. Heart—same changes as with 10,000 ppm but lower incidence
do.	2500	do.	do.	Growth depression. Liver—same changes as with 5000 and 10,000 ppm but less severe. Heart—same changes seen with the 10,000 ppm dose were seen in 1 of 6 rats examined
	1000	5M & 5F	do.	Growth depression
Carvone (*p*-mentha-6,8-dien-2-one)	10,000	do.	16 wk	Growth retardation, testicular atrophy
	2500	do.	1 yr	No effect
	1000	do.	27–28 wk	do.
Cinnamic aldehyde (cinnamaldehyde)	10,000	10M & 10F	16 wk	Liver—slight hepatic cell swelling. Stomach—slight hyperkeratosis of squamous portion
	2500	do.	do.	No effect[f]
	1000	do.	do.	No macroscopic effect
Citral (3,7-dimethyl-2,7-octadienal)	10,000	do.	13 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Coumarin[g,h] (2*H*-1-benzopyron-2-one)	10,000[e]	3M & 3F	4 wk	Marked growth retardation, testicular atrophy. Liver: microscopic—slight to moderate damage consisting of dead and dying cells, decrease in oxyphilia and cytoplasm in the centrolobular cells, and proliferation of bile ducts
	10,000	10M & 10F	1–8 wk	Marked growth retardation. No animal survived beyond 8 wk. Liver: microscopic—slight to moderate damage of the type seen with the 10,000 ppm dose for 4 wk

[f]Microscopic examinations were made on the stomach only.
[g]Toxicity study will be presented in a separate report.
[h]Not acceptable for food use because of toxic effects observed in chronic feeding studies.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Coumarin[g,h] (2*H*-1-benzopyron-2-one)	1000	Rat 10M & 10F	14 wk	No effect
	1000	5M & 5F	28 wk	do.
	2500	6M & 6F	29 wk	Growth retardation. Liver: macroscopic—slight mottling in the males; microscopic—slight mid-zonal fatty change in both sexes but more marked in the males
	5000[i]	do.	2 yr	Growth retardation. Normal food utilization (measured for first year). Haemogram-decrease in haemoglobin. Liver: macroscopic—enlargement, distortion by poorly to well delineated masses, pale pin-point foci; microscopic—focal proliferation of bile ducts of atypical appearance with associated fibrosis (cholangiofibrosis) situated in the masses described grossly and not clearly delineated from the surrounding hepatic parenchyma; slight degree of fatty metamorphosis, very slight to slight architectural irregularity, very slight to slight variation in cell size, very slight proliferation of bile ducts of the normal type, minimal focal necrosis
	2500[i]	5M & 7F	2 yr	Liver—slight damage of the same type as seen with the 5000 ppm dose; cholangiofibrosis was not seen
	2500	6M & 6F	do.	Liver—minimal damage of the same type as seen with 5000 ppm dose; cholangiofibrosis was not seen
	1000[i]	do.	do.	No effect

[i]Corn oil added to diet at a level of 3% (w/w). Two control groups were included in the study. Corn oil was added to the diet of one control group; the diet of the other group was untreated.

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Coumarin[g,h] (2*H*-1-benzopyron-2-one)	100 mg/kg	Dog 1M & 1F	9–16 days	Male killed in extremis after 9 days. Female found dead on day 16. Marked emaciation, slight dehydration, and slight jaundice. Liver: macroscopic—moderate to marked yellow colour and moderate "nutmeg" appearance; microscopic—marked disorganization of the lobular pattern, moderate increase in size of liver cells (with variation ranging from slight atrophy to a great increase), slight to moderate vacuolation, moderate to large amount of diffusely distributed fat, slight to moderate focal necrosis, fibrosis ranging from slight (centrolibular) to moderate to marked (portal) and very slight to moderate bile-duct proliferation. Spleen—macroscopic moderately pale. Gastro-intestinal tract—intestinal contents thick, dark, and tarry. Bone marrow—thin and fatty. Gall bladder—moderately distended
	50 mg/kg	2M & 1F	35–277 days	Emaciation; slight to moderate jaundice in the two female dogs. One death at 35 days; remaining dogs sacrificed at intervals between 45 and 277 days. Liver: macroscopic—moderately yellow colour with "nutmeg" appearance; microscopic—the same changes as with the 100 mg/kg dose except that the liver cell vacuolation was greater. Spleen—large amount of haemosiderin. Bone marrow—pale
	25 mg/kg	do.	133–330 days	Moderate emaciation and slight jaundice in one female. Weight gain in remaining dogs. Liver—slightly pale in two dogs, dark red in a third and yellow, markedly "nutmeg" in the fourth; microscopic—liver changes like those seen on the 50 mg/kg dose to the same degree in one dog and less severe in the other three. Spleen—moderate haemosiderosis. Gall bladder—moderately distended in two animals
	10 mg/kg	2M & 2F	297–350 days	No definite effect
Dihydroanethole (1-methoxy-4-propylbenzene)	10,000	Rat 10M & 10F	19 wk	Bone—slight osteoporosis of the tibia and femur (6/10)
	2500	do.	do.	Bone—slight to very slight osteoporosis (6/10)
	1000	do.	do.	Bone—very slight osteoporosis[j] (4/10)

[j]In the control group slight osteoporosis was seen in one of 10 animals.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Dihydroanethole (1-methoxy-4-propylbenzene)	Maximal dose 5000 mg/kg	Rat 20M	32 days	Initial dose 2000 mg/kg increased gradually to 5000 mg/kg. Seven of the animals survived the full 32 days of treatment and 16 lived long enough to receive the maximal daily dose of 5000 mg/kg Stomach: macroscopic—mucosa of the forestomach showed coalescent areas covered by thick flaky white material punctuated by minute ulcers; microscopic—moderately severe degree of hyperkeratosis of the stratified squamous epithelium in the forestomach. Bone—moderate degree of osteoporosis
Dihydrocoumarin (1,2-benzohydropyrone)	10,000	10M & 10F	14 wk	No effect
	1000	do.	do.	do.
	150 mg/kg	Dog 1F	2 yr	do.
	50 mg/kg	1M & 1F	do.	do.
Dihydrosafrole[b] (1,2-methylenedioxy-4-propylbenzene) (Long & Jenner, 1963) (Hagan *et al.* 1965)	10,000	Rat 10M & 10F	do.	Growth retardation in both sexes. Oesophagus—benign epidermoid papillomas and malignant papillary epidermoid carcinomas; 75% of the rats showed oesophageal tumours, 50% of the tumours were malignant. Liver: macroscopic—enlargement; microscopic—slight damage of the same type as that seen in the safrole-treated rats (see safrole rat—2-yr feeding study). Spleen—moderate follicular atrophy
	5000	25M & 25F	do.	Growth retardation in both sexes. Oesophagus—74% of the rats showed oesophageal tumours, 32% of the tumours were malignant. Liver: macroscopic—enlargement; microscopic—slight damage of the same type as seen in the safrole-treated rats. Spleen—moderate follicular atrophy
	2500	10M & 10F	do.	Growth retardation in the females. Oesophagus—20% of the rats showed oesophageal tumours, 5% of which were malignant. Liver: macroscopic—enlargement; microscopic—slight liver damage of the same type as seen in the safrole-treated rats. Kidney—moderate increase in chronic nephritis

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Dihydrosafrole[h] (1,2-methylenedioxy 4-propylbenzene) (Long & Jenner, 1963) (Hagan *et al.*1965)	1000	Rat 10M & 10F	2 yr.	Growth retardation in the females. Liver: macroscopic—slight enlargement; microscopic—slight liver damage of the same type as that seen in the safrole-treated rats. Kidney—moderate increase in chronic nephritis
Dimethylbenzyl carbinol (1,1-dimethyl-2-phenylethanol)	10,000	5M & 5F	16 wk	No effect
	1000	10M & 10F	28 wk	do.
Dolcourin[k]	10,000	5M & 5F	16 wk	do.
Ethyl caprylate (ethyl penanoate)	10,000	10M & 10F	17 wk	do.
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Ethyl formate	10,000	do.	17 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Ethyl methylphenyl glycidate (ethyl ester of 2,3-epoxy-3-methyl-3-phenylpropionic acid)	10,000	5M & 5F	16 wk	Growth retardation most obvious in males Testes—marked atrophy
	2500	do.	1 yr	No effect
Ethyl oenanthate (ethyl heptanoate)	10,000	10M & 10F	13 wk	do.
	1000	do.	do.	No macroscopic effect
Ethyl oxyhydrate[l]	10,000	do.	17–18 wk	Liver: microscopic—very slight swelling of hepatic cells in males
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Ethyl pelargonate (ethyl nonanoate)	10,000	5M & 5F	16 wk	No effect
Ethyl sebacate	10,000	do.	17–18 wk	do.
	1000	do.	27–29 wk	do.
Ethyl vanillin (3-ethoxy-4-hydroxybenzaldehyde)	50,000[m]	5M	1 yr	do.
	20,000[m]	do.	do.	do.
	20,000[n]	12M & 12F	2 yr	do.
	10,000[n]	do.	do.	do.
	5000[n]	do.	do.	do.
Eugenol (1-hydroxy-2-methoxy-4-allylbenzene)	10,000	10M & 10F	19 wk	do.
	1000	do.	do.	No macroscopic effect

[k]Dolcourin is a trade mark name for a substance supplied by Dodge and Olcott, Inc. as a coumarin substitute.
[l]Ethyl oxyhydrate is a mixture whose composition varies with the method of manufacture. The material used in this study was obtained from Florasynth Labs., Inc., N.Y.
[m]3% (w/w) corn oil added to control and test diets as a binder to reduce evaporation of the flavouring.
[n]3% (w/w) propylene glycol added to control and test diets as a binder to reduce evaporation of the flavourings.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Eugenol (1-hydroxy-2-methoxy-4-allylbenzene)	Maximal dose 4000 mg/kg	Rat 20M	34 days	Initial dose 1400 mg/kg increased gradually to 4000 mg/kg. Eight of the animals survived the full 34 days of treatment and 15 lived long enough to receive the maximal dose of 4000 mg/kg Stomach: macroscopic—mucosa of the forestomach showed coalescent areas covered by thick flaky white material punctuated by minute ulcers; microscopic—moderately severe degree of hyperkeratosis of the stratified squamous epithelium in the forestomach. Bone—small degree of osteoporosis
Eugenyl acetate (1-acetoxy-2-methoxy-4-allylbenzene)	10,000	10M & 10F	19 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Geraniol extra (3,7-dimethyl-2,6-octadienol) (3,7-dimethyl-1,6-octadienol)	10,000	5M & 5F	16 wk	No effect
	1000	do.	27–28 wk	do.
Geranyl acetate	10,000	10M & 10F	17 wk	do.
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Ionone standard [60% α-ionone, 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one and 40% β-ionone, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one]	10,000	do.	do.	Liver: microscopic—slight to moderate swelling of parenchymal cells
	2500	do.	do.	Liver: microscopic—slight amount of swelling of parenchymal cells[c]
	1000	do.	do.	Liver: microscopic—very slight amount of swelling of the parenchymal cells[c]
Isoeugenol (1-hydroxy-2-methoxy-4-propenylbenzene)	10,000	5M & 5F	16 wk	No effect
Isosafrole[b]	10,000	10M & 10F	11 wk	Growth retardation in both sexes. No rats on this dose survived beyond 11 wk of treatment. Liver: macroscopic—enlargement; microscopic—slight damage of the same type as seen with safrole
(Hagan *et al.* 1965)	5000	25M & 25F	2 yr	Growth retardation in both sexes. Testes—increased number of interstitial cell tumours. Liver: macroscopic—enlargement; microscopic—slight damage of the same type as seen with safrole. Five rats had primary hepatic tumours (two of these had hepatic cell adenomas and three had hepatic cell carcinomas). Kidney—increased incidence of chronic nephritis. Thyroid—slight hyperplasia

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Isosafrole[b] (Hagan *et al.* 1965)	2500	Rat 10M & 10F	2 yr	Slight growth retardation in females. Liver: macroscopic—slightly enlarged; microscopic—slight damage of the same type as seen with safrole, no primary hepatic tumours. Thyroid—slight hyperplasia
	1000	do.	do.	Slight growth retardation in females. Liver: microscopic—slight damage of the same type as seen with safrole
Linalyl cinnamate	10,000	10M & 10F	17 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Linalyl isobutyrate	10,000	do.	18 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Methyl anthranilate (*o*-aminobenzoic acid, methyl ester)	10,000	do.	13 wk	No effect
	1000	do.	do.	No macroscopic effect
6-Methylcoumarin (6-methyl-2*H*-1-benzopyran-2-one)	15,000	25M & 25F	2 yr	Depression of growth, severe in males and moderate in females, and paralleled by decrease in food intake. Liver: microscopic—slight fatty metamorphosis, and very slight bile-duct proliferation and focal telangiectasia. Testes—moderate atrophy, probably due to growth retardation
	7500	do.	do.	Depression of growth in males
	3500	do.	do.	No effect
	1000	do.	do.	do.
	500	do.	do.	do.
	10,000	10M & 10F	14 wk	do.
	1000	do.	do.	do.
	150 mg/kg	Dog 1M	39 days	Sacrificed because of weakness, emaciation, dehydration. Liver: microscopic—moderate to severe hepatitis. Skeletal muscle—slight to moderate atrophy
	50 mg/kg	1M & 1F	2 yr	No effect

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Methyl salicylate (*o*-hydroxybenzoic acid, methyl ester) (Webb & Hansen, 1963)	20,000	Rat 24M & 26F	49 wk	Growth retardation, rough coats. All dead by wk 49. Bone: microscopic—excess of cancellous tissue
	10,000	25M & 25F	2 yr	Growth retardation, rough coats. Bone: microscopic—slight excess of cancellous tissue
	5000	do.	do.	Bone: microscopic—slight excess of cancellous tissue
	1000	do.	do.	No effect
	10,000	10M & 10F	17 wk	Growth retardation
	1000	do.	do.	No macroscopic effect
	1200 mg/kg	Dog 1M & 1F	3 days	Died, weight loss. Liver: macroscopic—slightly pale, "nutmeg" in appearance; microscopic—moderate to marked amount of fat
	800 mg/kg	1M	29 days	Sacrificed, weight loss. Liver: microscopic—trace of fat
	800 mg/kg	1F	4 days	Died, weight loss. Liver: macroscopic—slightly pale, "nutmeg" in appearance; microscopic—moderate to marked amount of fat
	500 mg/kg	1M & 1F	8–9 days	Sacrificed—No effect
	250 mg/kg	do.	51–52 days	do.
	100 mg/kg	do.	52–53 days	do.
	50 mg/kg	do.	53–59 days	do.
	350 mg/kg	2M & 1F	2 yr	Weight loss. Liver: macroscopic—enlarged; microscopic—enlarged hepatic cells
	150 mg/kg	2M & 2F	do.	Growth retardation. Liver: macroscopic—enlarged; microscopic—enlarged hepatic cells
	50 mg/kg	do.	do.	No effect
Phenylethyl phenylacetate	10,000	Rat 10M & 10F	17 wk	do.
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Piperonal (3,4-methylenedioxybenzaldehyde)	10,000	5M & 5F	15 wk	No effect
	1000	do.	27–28 wk	do.

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Safrole[b] (1,2-methylenedioxy-4-allylbenzene) (Hagan *et al.* 1965)	10,000	Rat 10M & 10F	62 wk	Growth retardation in both sexes. All rats dead by wk 62. Testes—atrophy. Stomach—atrophy and atypical regeneration of the mucosa glands with associated fibrosis and hyalinization of the surrounding stroma most severe at the base. Liver: macroscopic—enlarged (2 to 3 times the normal size), mottled and irregularly nodular, with single or multiple tumour masses; microscopic—hepatic cell enlargement, which was usually focal and resulted in the formation of nodules. The enlargement was chiefly of the cytoplasm, which appeared normal in some cells but showed fatty metamorphosis or peripheral migration of the basophilic cytoplasmic granules in other cells. The nodules tended to progress in one of 3 ways (sometimes all 3 were seen in the same liver): (1) cystic necrosis: (2) cirrhosis; and (3) adenomatoid hyperplasia, leading to the formation of both benign and malignant tumours (hepatic cell adenoma, hepatic cell carcinoma, hepatocholangioma, hepatocholangiocarcinoma)
	5000	25M & 25F	2 yr	Growth retardation in both sexes. Increased mortality in the males. Liver—changes of the same type and severity as seen with the 10,000 ppm dose except for the occurrence of macroscopic cysts and a higher incidence of tumours in the lower dose, statistically-significant increase in malignant primary hepatic tumours. Stomach—same type of changes as with 10,000 ppm dose. Kidney—slight increase of chronic nephritis in the females. Thyroid—mild hyperplasia
	2500	10M & 10F	do.	Growth retardation in both sexes. Liver—moderate liver damage of the same type as seen with the 10,000 ppm dose with the exception of cirrhosis. Kidney—moderate increase of incidence of chronic nephritis.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Safrole[b] (1,2-methylenedioxy-4-allylbenzene) (Hagan *et al.* 1965)	1000	Rat 10M & 10F	2 yr	Growth retardation in the females. Liver—slight liver damage of the type seen with the 10,000 ppm dose, except that malignant tumours and cirrhosis were not seen with this dose. Kidney—moderate increase in chronic nephritis in the males.
(Long *et al.* 1963)	5000	25M & 25F	do.	Growth retardation in both sexes. Haemograms—mild anaemia, slight leucocytosis. Liver—marked liver changes (including malignant tumours but only rare cirrhosis) of the type seen with the 10,000 ppm dose. Statistically-significant increase in benign and malignant primary liver tumours.
	1000	25M & 25F	do.	Liver—slight to moderate damage of the same type seen with the 10,000 ppm dose except that malignant tumours and cirrhosis were not seen
	500	do.	do.	Liver—slight damage of the same type seen with the 10,000 ppm dose except that cirrhosis was not seen
	100	do.	do.	Liver—very slight damage of the same type seen with the 10,000 ppm dose except that cirrhosis was not seen
	80 mg/kg	Dog 2M & 2F	26–39 days	One dog died; the remaining dogs were sacrificed in a moribund condition. Marked weight loss. Skeletal muscle—moderate to severe atrophy in 2 of 4 dogs. Liver: macroscopic—slight enlargement in 2 of 4 dogs; microscopic—heavy infiltration of fat, occasional bile-duct proliferation and inflammatory-cell infiltration, slight to moderate streaking with necrobiosis and liver cell atrophy
	40 mg/kg	do.	91–116 days	Emaciation, general weakness. Liver: macroscopic—slight enlargement in 3 of 4 dogs; microscopic—slight fat infiltration, moderate hepatic cell atrophy and necrobiosis as at 80 mg/kg

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Safrole[h] (1,2-methylenedioxy-4-allylbenzene (Long *et al.* 1963)	20 mg/kg	Dog 2M & 2F	6 yr	Liver: macroscopic—slight to moderate enlargement, nodular surface; microscopic—mild post-necrotic cirrhosis characterized by focal or generalized nodules, consisting of enlarged hepatic cells with slight focal fatty vacuolization, separated by bands of atrophied hepatic cells and collagen; slight lymphocytic, Kupffer cell and bile-duct proliferation; minimal focal necrosis. Thyroid—hyperplasia
	5 mg/kg	do.	do.	Liver: microscopic—minimal focal necrosis, bile-duct proliferation, fatty metamorphosis, hepatic cell atrophy and leucocytic infiltration
Terpinyl acetate (*p*-menth-1-en-8-ol ester of acetic acid)	10,000	Rat 10M & 10F	20 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Thymol (*p*-cymen-3-ol; hydroxy-*p*-cymene)	10,000	5M & 5F	19 wk	No effect
	1000	do.	do.	No macroscopic effect
γ-Valerolactone (4-hydroxypentanoic acid lactone)	10,000	10M & 10F	13 wk	No effect
	5000	do.	do.	do.
	2500	11M & 9F	do.	do.
	1000	10M & 10F	do.	do.
Vanillin (4-hydroxy-3-methoxybenzaldehyde)	50,000[m]	5M	1 yr	do.
	20,000[m]	do.	do.	do.
	20,000[n]	12M & 12F	2 yr	do.
	10,000[n]	do.	do.	do.
	5000[n]	do.	do.	do.
	10,000	5M & 5F	16 wk	do.
	1000	do.	27–28 wk	do.

Table 2. *Loss of food flavourings from laboratory animal diets during a 7-day period*

Flavouring	Percentage loss*	Flavouring	Percentage loss*
Acetophenone	31	Eugenyl acetate†	6
Allylcaproate	31	Geranyl acetate†	4
Amyl butyrate†	35	Ionone†	1
Anethole	6	Isosafrole	5
Calamus oil	0	Linalyl cinnamate	4
Cinnamic aldehyde	14	Linalyl isobutyrate	7
Citral	58	Methyl salicylate†	3 & 5§
Dihydroanethole†	16	Phenylethyl phenylacetate	0
Dihydrocoumarin	—‡	Safrole	12
Dihydrosafrole	6	Terpinyl acetate	7
Ethyl caprylate†	33		

*100−(100×day 7 recovery/day 0 recovery).
†Reported by Jones *et al.* (1962). Recovery data on the remaining compounds were supplied by W. I. Jones.
‡Dihydrocoumarin was partially converted to melilotic acid when mixed with the laboratory diet; the test animals ingested a mixture of the two compounds.
§The two values for percentage loss indicate two different analytical procedures.

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Substances d'assaisonnement et composés de structure connexe. II. Toxicité subaiguë et chronique

Résumé—On a étudié sur des rats la toxicité subaiguë et chronique de 48 substances d'assaisonnement. On a également étudié sur des chiens la toxicité de cinq d'entre elles (coumarine, 6-méthylcoumarine, dihydrocoumarine, salicylate de méthyle et safrol). On a présenté un résumé de ces études. On a déterminé aussi la depérdition de 21 substances d'assaisonnement en 7 jours, dans des régimes pour animaux de laboratoire.

Lebensmittel-Geschmacksstoffe und Verbindungen verwandter Struktur. II. Subakute und chronische Toxizität

Zusammenfassung—Die subakute und bzw. oder chronische Toxizität von 48 Lebensmittel-Geschmackszusätzen wurde an Ratten untersucht. Die Toxizität von fünf dieser Geschmacksstoffe (Cumarin, 6-Methylcumarin, Dihydrocumarin, Methylsalicylat und Safrol) wurde auch an Hunden untersucht. Eine Zusammenfassung der Versuchsergebnisse wird vorgelegt. Der Verlust von 21 Geschmacksstoffen aus dem Futter von Laboratoriumstieren während einer 7-Tage-Periode wurde ebenfalls bestimmt.

Folic Acid Coenzymes

Metabolic reactions involving "active formate" and "active formaldehyde" are surveyed.

F. M. Huennekens, M. J. Osborn, H. R. Whiteley

For a great variety of cells of animal, plant, and microbial origin, the vitamin, folic acid, serves as a growth factor by controlling the metabolism of the one-carbon (C_1) compounds, formate and formaldehyde. These C_1 compounds serve as building blocks in the biosynthesis of purines, certain pyrimidines, and certain amino acids. An increased capacity to incorporate formate into cellular nucleic acids (*1*), and also an elevated level of folic acid (*2*) and its coenzyme forms (*3*), has been found to characterize at least one type of abnormal growth, namely that of the leukemic white cell.

A description of C_1 metabolism is provided by the following generalized equations for the mobilization of *X*, the formaldehyde or formate group.

$$D—X + C \rightleftharpoons C—X + D \qquad (1a)$$

$$C—X + A \rightleftharpoons A—X + C \qquad (1b)$$

In these equations, *C* is the folic acid coenzyme, *D* is a donor molecule containing a potential C_1 unit (serine, purine, histidine, and so on), and *A* is an acceptor molecule (glycine, carboxamide ribotide, and so on). When the C_1 unit is bound to the folic acid coenzyme (*C—X*), the complex may be considered as "active formate" or "active formaldehyde," respectively; the latter complex is more frequently referred to as "active hydroxymethyl." The analogy between these complexes and the well-known "active acetate" (acetyl coenzyme *A*) is apparent.

Although the early recognition of various metabolites as donors and acceptors of formyl and hydroxymethyl units was achieved by tracer and nutritional studies (*4, 5*), recent work has been directed toward studies with isolated enzyme systems, which permit a greater degree of understanding of reaction mechanisms. Study of the detailed mechanisms of the various reactions, represented by Eqs. 1a and 1b, has been hampered by the multiplicity of the C_1 fragments (formyl, hydroxymethyl, and in some instances, methyl) and by uncertainty concerning the chemical structure of the intermediates, *C—X*, since, as is discussed in subsequent paragraphs, folic acid has several potential sites of attachment for a C_1 group.

In this article (*6*) we shall attempt to summarize the current information regarding the nature of "active formyl" and "active hydroxymethyl" complexes and to survey the various metabolic reactions in which these compounds participate (*7*).

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Reactions Involving "Active Formaldehyde"

Serine hydroxymethylase. Numerous investigators (summarized in reference *8*) have studied the enzymatic interconversion of serine and glycine. In early work (*9*), in which tissue slices or intact animals were used, sensitive tracer techniques were required to detect the small conversions. More recently, larger conversions have been achieved by use of purified enzymes from avian and mammalian livers (*3; 10–13*).

The over-all reaction (Eq. 2) for the biosynthesis of serine from glycine and HCHO

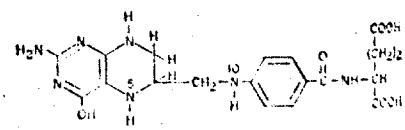
$$\text{HCHO} + \text{glycine} \rightleftharpoons \text{serine} \qquad (2)$$

is actually the sum of two separate reactions

$$\text{HCHO} + \text{FH}_4 \rightleftharpoons \text{hFH}_4 \qquad (2a)$$

$$\text{hFH}_4 + \text{glycine} \rightleftharpoons \text{serine} + \text{FH}_4 \qquad (2b)$$

where FH_4 is the abbreviation (*11*) for 5,6,7,8-tetrahydrofolic acid (I)



I

the coenzyme form of folic acid, and hFH_4 symbolizes hydroxymethyl FH_4 without specifying the position of the C_1 group on the folic acid coenzyme.

Reactions 2a and 2b are catalyzed by the formaldehyde-activating enzyme and serine hydroxymethylase [or serine aldolase (*11*)], respectively.

In the over-all reaction (Eq. 2) a requirement for pyridoxal phosphate has been observed in all systems studied (*8*; *10–13*; *16*); an additional requirement for Mn^{++} has been demonstrated only with a bacterial enzyme (*15*). The site of action of both cofactors is undoubtedly in reaction 2b, as would be anticipated from the chemical studies of Snell and his co-workers (*16*).

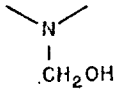
Formaldehyde-activating enzyme. Since spectroscopic evidence indicates that HCHO and FH_4 interact rapidly in the absence of any enzyme to form one or more complexes, it was not immediately clear whether reaction 2a was enzyme-catalyzed or not. However, during purification of the enzyme system that catalyzes reaction 2, it was found that the enzymes for reactions 2a and 2b could be partially separated (*13*). The formaldehyde-activating enzyme can be assayed in the following manner. When FH_4 ($\lambda_{max.}$ at 300 mμ, $\epsilon \cong 22 \times 10^6$ cm²/mole) is mixed with a slight excess of HCHO at *p*H 7, no reaction occurs. When the purified activating enzyme is added, the spectral peak shifts to 291 mμ, and ε increases to 26×10^6 cm²/mole. This shift is due to the formation of hFH_4 according to Eq. 2a. When hFH_4 is synthesized enzymatically, it is a reasonably stable substance and can be separated from unreacted FH_4 by column chromatography or by paper chromatography (*17*).

The authenticity of hFH_4 is established by the following enzymatic assays.

i. In the presence of serine hydroxymethylase and glycine, hFH_4 disappears and FH_4 reappears (see reactions 2b).

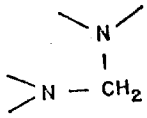
ii. In the presence of the hFH_4 dehydrogenase (see below) and TPN, hFH_4 is oxidized to formyl FH_4, and an equivalent amount of TPNH is formed. Nonenzymatically, hFH_4 may be converted to FH_4 by the addition of hydroxylamine in acid; in a basic medium the material is stable to H_2NOH.

Structure of "active formaldehyde." Formaldehyde is known to react with –NH_2 or –SH groups, forming equilibrium adducts of the type represented by II.



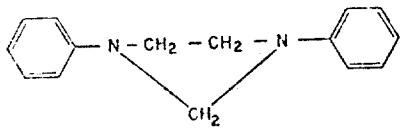
II

When a second ligand is possible with the bound HCHO, as in III,



III

much more stable complexes are formed, especially if both ligands are on the same molecule. An example of this is the reaction of HCHO with cysteine to form the stable heterocycle, thiazolidine carboxylic acid. An important clue to the structure of hFH_4, was provided by Blakley (*18*), who prepared the HCHO adduct (IV) of N,N-diphenyl ethylenediamine:



IV

Structure IV is quite stable, and, like thiazolidine carboxylic acid, it does not release its bound HCHO to aldehyde-binding agents such as chromotropic acid and acetyl acetone. Although hFH_4 in acid behaves like a type II adduct in releasing HCHO, its stability in base strongly suggests a type III structure, very probably $h^{5-10}FH_4$ (*19*). Confirmatory evidence for this proposed structure comes from a study of the substrate specificity of the hFH_4 dehydrogenase, as described below.

While HCHO and FH_4 react rapidly in the absence of enzyme to form a product which is active in systems requiring hFH_4, the reaction is not confined to a single product as in the enzymatic synthesis. Various spectroscopic complexes, whose $\lambda_{max.}$ range between 290 and 300 mμ, are formed when increasing amounts of HCHO are added to FH_4. This result is not unexpected, for FH_4 contains several potential sites, namely, the N^3, N^5, N^8, and N^{10} positions, for binding HCHO.

Reactions Involving "Active Formate"

Hydroxymethyl tetrahydrofolic acid (hFH_4) dehydrogenase. The metabolic equivalence of formyl and hydroxymethyl donors in early tracer studies of C_1 reactions (that is, the labeling of the C-2 position of the purine ring both by formate and by the β-carbon atom of serine) presupposed the existence of a system for the interconversion of the two oxidation states. A TPN-linked dehydrogenase, carrying out this interconversion, was first described by Jaenicke (*20*) and by Greenberg *et al.* (*21*) and was later obtained in purified form in our laboratory (*22*). The over-all reaction is shown in Eq. 3.

$$hFH_4 + TPN^+ \rightleftharpoons fFH_4 + TPNH + H^+. \quad (3)$$

For the moment, the precise nature of hFH_4 and fFH_4 is not specified. Reaction 3 is best studied in conjunction with reactions 2b or 2a, serine or HCHO being used to generate hFH_4. When crude dehydrogenase preparations are used, the accumulated reaction product, fFH_4, is the N^{10}-isomer, $f^{10}FH_4$. However, when dehydrogenase preparations are freed from the enzyme, cyclohydrolase (*23*), which carries out reaction 4,

$$(f^{5-10}FH_4)^+ + H_2O \rightleftharpoons f^{10}FH_4 + H^+ \quad (4)$$

the end product of reaction 3 is $f^{5-10}FH_4$. With purified dehydrogenase preparations, $f^{5-10}FH_4$, but not $f^{10}FH_4$ or f^5FH_4, serves as a substrate for reaction 3 in reverse. Similarly, $f^{5-10}FH_4$ (but not f^5FH_4 or $f^{10}FH_4$) can be reduced quantitatively with borohydride, and the product is identical to authentic hFH_4 as measured by absorption spectrum and enzymatic assays. The experiments cited above provide support for the hypothesis that both hFH_4 and fFH_4 in reaction 3 are the N^5, N^{10}-bridge compounds. Greenberg and Jaenicke (*7*) and Kisliuk (*24*) have also suggested that hFH_4 is $h^{5-10}FH_4$.

The equilibrium constant for reaction 3 is near unity at *p*H 8.0 (the *p*H optimum for the enzyme), and the extent of the reaction is dependent, of course, upon H^+. This can be demonstrated by starting with $f^{10}FH_4$ (in the presence of cyclohydrolase) and following the disappearance of TPNH spectrophotometrically at 340 mμ. After the equilibrium position has been established, the reaction may be driven further to the left, as evidenced by further disappearance of TPNH, by the addition of H^+, or by the addition of glycine (to "trap" hFH_4 via reaction 2b).

In addition to facilitating the interconversion of C_1 units at the levels of formyl and hydroxymethyl, the dehydrogenase can be used as a spectroscopic "marker" for following a variety of re-

actions (see Fig. 1) that involve hFH_4 or fFH_4. Furthermore, the equilibrium of the dehydrogenase itself (and, of course, any coupled system involving the dehydrogenase) can be manipulated in either direction—for example, by trapping TPNH via the glutathione reductase system or by trapping TPN with glucose-6-phosphate dehydrogenase.

N^{10}-formyl tetrahydrofolic acid ($f^{10}FH_4$) deacylase. Studies with the dehydrogenase in beef and pigeon liver preparations led to the discovery of a deacylase (25) specific for $f^{10}FH_4$:

$$f^{10}FH_4 + H_2O \rightarrow HCOOH + FH_4 \quad (5)$$

This enzyme is present in beef liver preparations but absent from pigeon liver preparations. When the dehydrogenase was studied with the latter tissue, the amounts of TPNH and $f^{10}FH_4$ formed during the reaction were equal. With the former tissue, the amount of accumulated $f^{10}FH_4$ was much less than the TPNH because of the concomitant action of the deacylase. The deacylase has been partially purified from beef liver preparations; it can be added to pigeon liver dehydrogenase to produce the beef liver pattern (that is, TPNH ≫ $f^{10}FH_4$). A more simplified assay for the deacylase consists of incubating $f^{10}FH_4$ (absorption maximum at 258 mμ) directly with the enzyme and measuring the increase in optical density at 300 mμ as FH_4 is produced. Since small quantities of formate are difficult to estimate, the stoichiometry of reaction 5 has been studied with respect to FH_4. 5,6,7,8-Tetrahydrofolic acid (FH_4) has been identified by its activity with the formate-activating enzyme (see below) and by its activity in the coupled hydroxymethylase-dehydrogenase system:

$$\text{Serine} + FH_4 + TPN^+ \rightleftharpoons \text{glycine} + f^{10}FH_4 + TPNH + H^+ \quad (6)$$

The deacylase for $f^{10}FH_4$ probably serves to regenerate FH_4 in the absence of actively functioning acceptors for "active formyl," much as the deacylase for acetyl coenzyme A provides a means for regenerating coenzyme A in the absence of acceptors for "active acetate."

Formate-activating enzyme. Since free formate has long been known to enter metabolic systems involving "active formyl" and "active hydroxymethyl" groups, it was anticipated that there would be a formate-activating enzyme. Formate activation was first reported (21, 26) in connection with studies of the biosynthesis of purines, and the following stoichiometry was proposed:

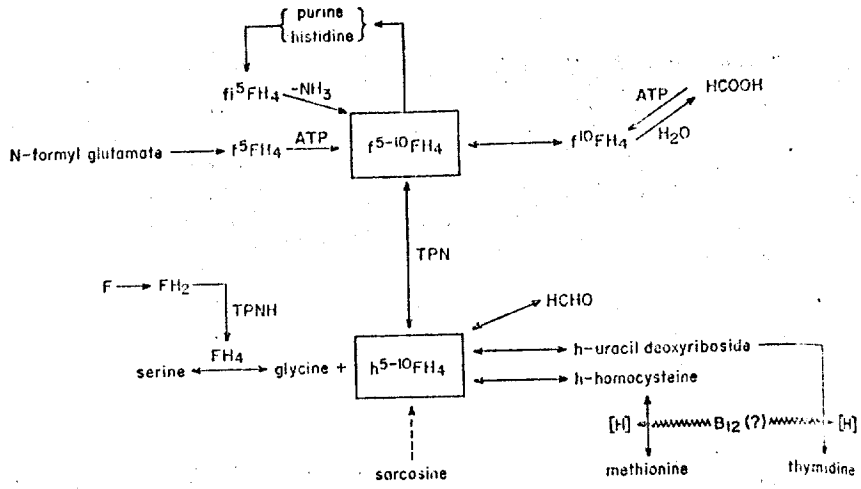


Fig. 1. Metabolic interrelationships of active C_1 units.

$$HCOOH + ATP + FH_4 \xrightarrow{Mg^{++}} f^{10}FH_4 + ADP + P_i \quad (7)$$

The formation of ADP and P_i as end products makes this reaction similar to the activation of glutamate or succinate. In contrast, the activation of other carboxylic acids, such as acetate or butyrate, produces AMP and PP as end products. Using a highly purified activating enzyme from *Micrococcus aerogenes*, we have shown (27) that the sequence in reaction 7 is

$$ATP + FH_4 \rightleftharpoons ADP + pFH_4 \quad (7a)$$
$$pFH_4 + HCOOH \rightleftharpoons f^{10}FH_4 + P_i \quad (7b)$$

The mechanism thus resembles that proposed for the formation of succinyl-CoA, where S-phosphoryl CoA is the analogous intermediate (28). Spectroscopic evidence has been adduced for reaction 7a: when enzyme and ATP are added to FH_4, the peak at 300 mμ shifts to shorter wavelengths, and ε decreases about 15 percent. "pFH_4," labeled with P^{32}, has also been isolated from reaction mixtures by use of ATP^{32}. Reaction 7a is also followed conveniently by measuring the liberated ADP with the coupled system employing pyruvic kinase and lactic dehydrogenase:

$$ADP + \text{phospho-enol pyruvate} \rightleftharpoons ATP + \text{pyruvate} \quad (8)$$

$$\text{Pyruvate} + DPNH + H^+ \rightleftharpoons \text{lactate} + DPN^+ \quad (9)$$

The observed reaction product in Eq. 7 is $f^{10}FH_4$. Since the purified bacterial enzyme contains no cyclohydrolase, it seems probable that $f^{5-10}FH_4$ is not an intermediate in this reaction. Consequently, it would seem most likely that the intermediate in reactions 7a and 7b, pFH_4, is N^{10}-phosphoryl FH_4 ($p^{10}FH_4$ in our symbols), but the verification of this assumption must await the unambiguous chemical synthesis of this compound.

N^5-formyl tetrahydrofolic acid (f^5FH_4) isomerase. In all of the studies mentioned above, $f^{10}FH_4$ or $f^{5-10}FH_4$ has been involved as the "active formyl" group. The question may be raised, therefore, about the relationship of the classical formyl isomer, f^5FH_4 (folinic acid), to "active formate." Except for the formylation of glutamate (29), where f^5FH_4, rather than $f^{10}FH_4$ or $f^{5-10}FH_4$, is the formyl donor, folinic acid is curiously inert metabolically. There is, however, an enzyme system (30) present in many tissues which brings about the apparently unidirectional isomerization of f^5FH_4 to $f^{10}FH_4$ via the following reaction:

$$f^5FH_4 + ATP \xrightarrow{Mg^{++}} f^{10}FH_4 + ADP + P_i \quad (10)$$

Recent studies by Peters and Greenberg (31) have suggested that AMP and PP rather than ADP and P_i are the reaction products. It has been established (31, 32) that reaction 10 in certain tissues may be separated into two separate steps:

$$f^5FH_4 + ATP \rightarrow f^{5-10}FH_4 + ADP + P_i \quad (10a)$$
$$\text{(or AMP + PP)}$$

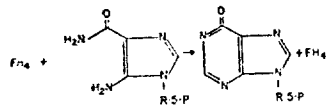
and

$$f^{5-10}FH_4 + H_2O \rightarrow f^{10}FH_4 \quad (10b)$$

Reaction 10b is catalyzed by cyclohydrolase, while the enzyme responsible for reaction 10a may be termed *cyclodehydrase*, by analogy to the *cyclodeaminase* reaction studied by Rabinowitz and Pricer (*23*):

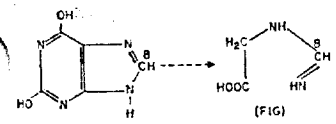
$$f^5FH_4 \rightarrow f^{5\text{-}10}FH_4 + NH_3 \qquad (11)$$

Biosynthesis and breakdown of purine and histidine. The laboratories of Buchanan (*33*), and G. R. Greenberg (*26*) have shown that both the 2- and 8-carbon atoms of the purine ring arise by the action of a specific transformylase with $f^{10}FH_4$ (or $f^{5\text{-}10}FH_4$) as the formyl donor.



(12)

In contrast, the *breakdown* of purines, at least in certain bacterial systems, is much more complicated. Cleavage of xanthine gives rise to CO_2 from the C-2 position, but the C-8 carbon emerges as the formimino group, H—C=NH, of formiminoglycine (FIG) (*23, 34*):



(13)

Formiminoglycine is then decomposed according to the sequence:

$$FIG + FH_4 \rightarrow f^5FH_4 + \text{glycine} \qquad (14)$$

followed by reactions 11 and 10b. Similarly, in mammalian liver (*35*), histidine is degraded to the analogous intermediate, formiminoglutamic acid (FIGlu), which, in turn, is metabolized by a series of reactions analogous to 14, 11, and 10b. The pathway of histidine biosynthesis has not yet been elucidated fully, although the involvement of a formyl or formimino group has been demonstrated.

Formate exchange into pyruvate. Recent reports (*36*) indicate that the exchange of $HC^{14}OOH$ into pyruvate by crude extracts of *Escherichia coli* is stimulated by the addition of FH_4. Similar results have been obtained with extracts of *Micrococcus aerogenes* and *Micrococcus lactilyticus* (*37*). However, the identity of the C_1 complex participating in the exchange reaction is not known, and the mechanism of the reaction has not been established. In the anaerobic micrococci the exchange reaction does *not* occur via serine as an intermediate—that is, by synthesis of serine from formate and endogenous glycine followed by deamination to pyruvate.

Structure of "active formate." Because of its early recognition, folinic acid (f^5FH_4) was assumed to be the "active formate" in C_1 metabolism. Subsequent work with purified enzyme systems, however, has revealed that f^5FH_4 functions only in the formylation of glutamate (*29*) and in the isomerase reaction (Eq. 10). As shown in Fig. 1, $f^{5\text{-}10}FH_4$ or $f^{10}FH_4$ is the formylating agent in all other reactions.

Interconversion of Formyl and Hydroxymethyl Groups with Methyl Groups

Although the details of the enzymatic pathways concerned with thymine and methionine synthesis are not fully known, the methyl groups of these compounds have been shown to be interconvertible with formyl and hydroxymethyl groups (*5*). It seems probable also that the transfer of the C_1 group occurs at the hydroxymethyl level rather than at the formyl level (*38*). In thymidine synthesis, uracil deoxyriboside (*39*) [or ribotide (*40*)], rather than free uracil, has been implicated as the C_1 acceptor. In methionine synthesis, homocysteine serves as the acceptor. The above reactions may be generalized in the following sequence, where *A* represents either uracil deoxyriboside or homocysteine, *h-A* the corresponding hydroxymethyl derivatives, and *m-A* the methyl derivatives:

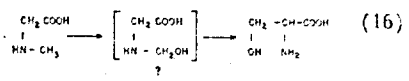
$$hFH_4 + A = h\text{-}A + FH_4 \qquad (15a)$$

$$h\text{-}A \underset{(2H)}{\rightleftharpoons} m\text{-}A \qquad (15b)$$

Both thymidine (*39, 40*) and methionine (*41*) syntheses have been studied in crude extracts, and it has been shown that the required components are (i) the acceptor (uracil deoxyriboside or homocysteine); (ii) a hydroxymethyl source and FH_4; (iii) a reducing source, usually DPN, although this may not be the actual [2H] shown in reaction 15b; and (iv) ATP, perhaps to promote the condensation in reaction 15a.

Reaction 15b is of great interest when uracil deoxyriboside is the acceptor because it seems most probable that the over-all requirement for vitamin B_{12} in thymine synthesis can be narrowed down to this step. The possibilities that the porphyrin-bound cobalt of vitamin B_{12} passes through an oxidoreduction cycle during reaction 15b deserve investigation.

The sarcosine-to-serine conversion (*42*) is also of interest, for it represents the hydroxymethyl → methyl sequence in reverse and is carried out intramolecularly:


(16)

This reaction has been demonstrated in intact mitochondria (*42*) or digitonin extracts of mitochondria (*43*), but the cofactor requirements or intermediates are presently unknown.

Biosynthesis of Folic Acid Coenzymes

The above reactions emphasize the role of reduced folic acid (FH_4) as the coenzyme, or carrier, of the C_1 groups. The formation of FH_4 from the vitamin, folic acid, has been the subject of recent investigations. Wright *et al.* (*44*) have shown in a bacterial system that folic acid is reduced to dihydrofolic acid during operation of the pyruvic oxidase. In chicken liver extracts both steps in the reduction of folic to tetrahydrofolic acid require TPNH (*45, 46*).

In our laboratory (*47*), a TPN-linked, dihydrofolic reductase has been purified from avian liver and shown to catalyze the final step in FH_4 biosynthesis:

$$FH_2 + TPNH + H^+ \rightarrow FH_4 + TPN^+ \qquad (17)$$

The equilibrium at *p*H 7 for reaction 17 lies far to the right. At extremely low concentrations (about $10^{-7}M$), the folic acid antagonists, aminopterin and amethopterin, are extremely potent competitive inhibitors of FH_2 in the reaction (*48*).

Summary

The above reactions involving "active formyl" and "active hydroxymethyl" groups, their interconversion, their connections with free HCOOH and HCHO, and their interdigitation with methyl groups are diagrammed in Fig. 1.

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Research Section

Food Flavourings and Compounds of Related Structure
I. Acute Oral Toxicity

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(*Received* 11 *May* 1964)

Abstract—Oral dosages of 107 synthetic and natural flavourings and structurally-related compounds were administered by intubation to the mouse, rat or guinea-pig. Animals were observed usually for 2 weeks during which time the development of toxic signs was followed and time of death recorded. The acute oral LD_{50} of each compound was determined.

INTRODUCTION

Substances used as food flavourings have received little attention from the toxicological viewpoint. Because of their extensive use as food additives, the Food and Drug Administration has been investigating their toxicity.

The initial step in our toxicity studies was the determination of the acute oral effects. This paper presents data on acute toxicity for a large number of flavouring matters. Similar data are reported for additional compounds, not necessarily flavourings, but included as a means of correlating structure with toxicity. These relationships have been discussed by Taylor, Jenner & Jones (1964) and Hagan, Jenner, Jones & Fitzhugh—*Toxicology;* Long, Brouwer & Webb—*Pathology* (1964).

Flavour additives include compounds with a wide variety of chemical structures, and mixtures of variable composition derived from plants and other natural sources. Some of the substances are synthetic, others are isolates or extracts of natural products. Since the purpose of these studies was to evaluate the toxicity of these materials in relation to their use as food additives, a commercially available material was used. No attempt was made to secure chemically pure compounds.

METHODS

Groups of 10 young adult Osborne–Mendel rats evenly divided by sex were fasted for approximately 18 hr prior to treatment. Groups of guinea-pigs consisting of both males and females were fasted for the same period. Mice were treated on full stomachs. Animals had access to water at all times, and the food was replaced in cages as soon as animals received their respective doses. All doses were given by intubation.

All animals were maintained under close observation for recording toxic signs and time of death. Such observation was continued until animals appeared normal and showed weight gain. The usual observation period was 2 weeks; in a few cases, where no acute toxic signs were seen, the animals were observed for only one week. LD_{50}'s were computed by the method of Litchfield & Wilcoxon (1949).

RESULTS

The LD_{50}'s, slope function, and their confidence limits, together with toxic signs and times of death, are recorded for each compound in Table 1. The species tested, solvent, and solution concentration (w/v) are also listed. Where no solvent is indicated the substance was a liquid and it was administered undiluted.

Table. 1 *Acute oral toxicity of food flavourings and compounds of related structure*

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Acetophenone (methyl-1-phenyl ketone)		Rat	3200 (2460–4160)	1·9 (1·3–2·8)	Coma within 5 min persisting in some rats for 24 hr D.T. 1 hr–4 days
Aldehyde C-10 decyl (decanal)		Rat Mouse	>33,320* >41,750	— —	Excitation, diarrhoea, wet fur on stomach and posterior in both species
Aldehyde C-14 (γ-undecalactone)		Rat	18,500 (16,930–20,260)	1·2 (1·0–1·3)	Depression within 10 min. Wet fur D.T. 4 hr–5 days
Aldehyde C-18 prunolide (lactone of 4-hydroxy-nonanoic acid)		Rat	9780 (7480–12,810)	1·6 (1·4–1·8)	Depression, coma D.T. 4–18 hr
do.		Guinea-pig	3440 (2890–4100)	1·5 (1·2–1·8)	Depression, salivation D.T. 4 hr–6 days
Allyl acetate†	A‡—10%	Rat	142 (116–175)	1·6 (1·3–2·0)	Depression soon after treatment. Rough fur, scrawny appearance for several days D.T. 4 hr–6 days
do.	A—2 & 5%	Mouse	170 (152–190)	1·2 (1·1–1·3)	Depression D.T. 4–18 hr
Allylacetic acid† (pentenoic acid)	A—15%	Rat	470 (385–573)	1·5 (1·3–1·8)	Depression within 30 min. High dose caused convulsions. Most rats that recover appear normal the day following treatment D.T. 30 min–18 hr
do.	A—15%	Mouse	610 (460–808)	2·2 (1·5–3·1)	Depression D.T. 1–18 hr
Allyl alcohol†	B—2%	Rat	70 (63–79)	1·6 (1·2–2·0)	Depression, colourless secretion from eyes, diarrhoea, scrawny appearance for several days D.T. 4 hr–4 days

*Highest dose administered

†Toxicity was studied because of structural relation to a flavouring agent.

‡A = corn oil; B = water.

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
p-Allylanisole (1-methoxy-4-allylbenzene)		Rat	1820 (1670–1980)	1·2 (1·1–1·3)	Marked depression, some rats in coma for 24 hr, rough fur, wet posterior, porphyrin-like deposit around eyes D.T. 4 hr–8 days
do.	A—10, 20%	Mouse	1250 (812–1920)	1·6 (0·9–2·8)	Depression soon after treatment, coma on higher doses D.T. 1 hr–4 days
Allylbenzene†		Rat	5540 (4620–6650)	1·5 (1·2–2·0)	Depression, some rats comatose for 2–3 days, wet posterior, scrawny appearance for several days D.T. 4 hr–5 days
Allyl butyrate (allyl butanoate)		Rat	250 (216–290)	1·5 (1·2–1·8)	Depression, wet posterior, scrawny appearance for several days D.T. 4 hr–5 days
Allyl caproate (allyl hexanoate)		Rat	218 (186–255)	1·3 (1·1–1·5)	Depression. Scrawny appearance D.T. 4–18 hr
do.		Guinea-pig	280 (246–319)	1·3 (1·1–1·5)	Depression, salivation D.T. 4 hr–3 days
Allyl cinnamate (allyl 3-phenylacrylate)		Rat	1520 (1290–790)	1·4 (1·1–1·8)	Scrawny appearance D.T. 4 hr–8 days
Allyl cyclohexane propionate (allyl 3-cyclohexyl-propionate)		Rat	585 (480–714)	1·5 (1·0–2·2)	Depression, rough fur D.T. 4–hr 6 days
do.		Guinea-pig	380 (172–834)	1·3 (0·5–3·8)	Depression, salivation, haemorrhage in small intestine D.T. 1 hr–6 days
Allyl formate†	A—5%	Rat	124 (107–144)	1·3 (0·5–3·6)	Depression, scrawny appearance for several days D.T. 4 hr–5 days
do.	A—2%	Mouse	136 (122–151)	1·2 (1·1–1·3)	Depression D.T. 4–18 hr
Allyl heptylate (allyl heptanoate)	A—50%	Rat	500 (392–638)	1·7 (1·2–2·2)	Ataxia D.T. 2–18 hr
do.		Guinea-pig	444 (363–541)	1·6 (1·2–2·3)	Depression D.T. 2–18 hr

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
do.	A—25%	Mouse	630 (514–772)	1·5 (1·2–1·8)	Depression D.T. 2–18 hr
Allyl isothiocyanate	A—10%	Rat	339 (318–361)	1·6 (1·2–2·1)	Scrawny appearance, porphyrin-like deposit around eyes and nose, rough fur D.T. 4 hr–15 days
Amyl acetate (pentyl acetate)		Rat	16,550 (14,370–19,030)	1·2 (1·1–1·4)	Depression, coma, rough fur D.T. 4 hr–2 days
Amyl alcohol (pentanol)		Rat	3030 (1440–6360)	1·2 (1·0–1·4)	Depression D.T. few min–18 hr
Amyl butyrate (pentyl butyrate)		Rat	12,210 (10,260–14,560)	1·6 (1·3–1·8)	Depression, rough fur, wet posterior D.T. few min–2 hr
do.		Guinea-pig	11,950 (8530–16,730)	3·0 (1·2–7·2)	Depression, ataxia D.T. 2 hr–6 days
Amyl cinnamic aldehyde (α-*n*-pentyl-β-phenyl-acrolein)		Rat	3730 (3190–4370)	1·4 (1·2–1·6)	Depression, porphyrin-like deposit around eyes and nose D.T. 4 hr–5 days
Amyl valerianate (pentyl pentanoate)		Rat	>35,420*	—	No effect in 2 weeks observation
do.		Guinea-pig	>17,260*	—	Wet fur D.T. 2–6 days
Amyris oil		Rat	5580 (4540–6860)	1·5 (1·4–1·6)	Ataxia, coma within 1 hr, porphyrin-like deposit around eyes and nose. Wet posterior D.T. 1–3 days
Anethole (1-methoxy-4-propenyl-benzene)		Rat	2090 (1420–3070)	1·8 (1·3–2·4)	Low doses—depression, high doses—coma D.T. 4 hr–4 days
do.		Guinea-pig	2160 (1920–2450)	1·3 (1·2–1·5)	Depression D.T. 1–7 days
do.		Mouse	3050 (2330–4000)	1·6 (1·2–2·1)	Depression, coma within 15 min D.T. 2–4 hr
Anisaldehyde (*p*-methoxybenzaldehyde)		Rat	1510 (1360–1700)	1·2 (1·1–1·3)	Depression D.T. 4–18 hr
do.		Guinea-pig	1260 (937–1700)	1·6 (1·2–2·0)	Depression within 2 hr D.T. 1–3 days

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Anisole (methoxybenzene)		Rat	3700 (3240–4220)	1·2 (1·1–1·4)	Depression, porphyrin-like deposit around eyes, salivation, bloody urine, rough fur D.T. 4 hr–8 days
β-naphthyl *iso*butyl ether		Rat	5930 (4750–7420)	1·9 (1·4–2·5)	Wet posterior, coma within 1 hr, rough fur and black, soft stools D.T. 1–6 days
Benzaldehyde		Rat	1300 (1110–1540)	1·4 (1·2–1·6)	Depression, coma on higher doses D.T. 4–18 hr
do.		Guinea-pig	1000 (800–1250)	1·4 (1·2–1·8)	Diuresis, tremors, intestinal irritation and haemorrhage D.T. 1 hr–4 days
Benzene†		Rat	4080 (3260–5100)	1·7 (1·4–2·1)	Tremors, loss of equilibrium and comatose on high doses, scrawny appearance for several days D.T. 1 hr–4 days
Benzyl acetate		Rat	2490 (2040–3040)	1·6 (1·1–2·4)	Depression D.T. 4 hr–3 days
Benzyl alcohol		Rat	1230 (1130–1330)	1·2 (1·1–1·3)	Depression, coma within 10–15 min. Excitable for 3–4 days D.T. 1 hr–4 days
do.	A—25%	Mouse	1580 (1410–1770)	1·2 (1·1–1·3)	Depression D.T. 2–18 hr
Benzyl *n*-butyrate (benzyl butanoate)		Rat	2330 (1940–2800)	1·3 (1·1–1·6)	Depression, scrawny appearance, tremors with higher doses D.T. 4 hr–4 days
Benzyl cinnamate (cinnamein, benzyl β-phenylacrylate)		Rat	5530 (3100–7740)	2·2 (0·9–3·6)	Depression, coma persisting in some rats for 24 hr D.T. 4 hr–5 days
do.		Guinea-pig	3760 (2340–6055)	4·2 (1·3–13·8)	Depression, gastro-intestinal tract irritation, rectal bleeding D.T. 4 hr–6 days
n-Butyl alcohol (butanol)		Rat	2510 (2220–2840)	1·4 (1·2–1·7)	Depression, coma D.T. 4–18 hr

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Cajeput oil		Rat	3870 (3360–4450)	1·2 (1·1–1·3)	Scrawny appearance, wet posterior. Gross pathology shows pale, nutmeg livers. Depression, persisting in some animals for as long as 3 days D.T. 4 hr–9 days
Calamus oil		Rat	777 (612–987)	1·7 (1·3–2·3)	Severe tremors from 30 min–2 hr after treatment. Scrawny appearance, weight loss for several days D.T. 4 hr–8 days
n-Caprylic acid (octanoic acid)		Rat	10,080 (8190–12,370)	1·6 (1·3–2·0)	Depression, diarrhoea D.T. 4 hr–9 days
Carvacrol (2-methyl-5-*iso*propylphenol)		Rat	810 (710–920)	1·2 (1·1–1·3)	Depression within 10 min, coma within 1 hr D.T. 1 hr–3 days
Carvone (*p*-mentha-6, 8-dien-2-one)		Rat	1640 (1260–2130)	1·8 (1·3–2·5)	Depression, ataxia (loss of use of hind legs) D.T. 4 hr–5 days
do.		Guinea-pig	766 (603–845)	1·3 (1·1–1·6)	Extreme depression D.T. 4–18 hr
Cedryl acetate		Rat	44,750 (33,650–59,520)	1·6 (1·3–2·0)	Depression, scrawny appearance, rough, wet fur D.T. 4 hr–11 days
Cinnamic aldehyde (cinnamaldehyde)		Rat	2220 (1910–2600)	1·4 (1·2–1·6)	Depression, diarrhoea, scrawny appearance D.T. 2–3 hr
do.		Guinea-pig	1160 (955–1420)	1·5 (1·2–1·9)	Coma with higher doses D.T. 2 hr–4 days
Citraconic acid (*cis*-methylbutenedioic acid)	B—25%	Rat	1320 (1070–1640)	1·6 (1·1–2·4)	Depression, scrawny appearance, tremors, stomach haemorrhage D.T. 4 hr–3 days
do.	B—25%	Mouse	2260 (1930–2640)	1·4 (1·2–1·6)	Depression, laboured respiration, haemorrhage in gastro-intestinal tract D.T. few min–3 days

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
do.	B—25%	Guinea-pig	1350 (1040–1755)	1·9 (1·1–3·1)	Scrawny appearance, gastro-intestinal tract irritation D.T. few min–3 days
Citral (3,7-dimethyl-2,7-octadienal)		Rat	4960 (3940–6240)	1·5 (1·2–2·0)	Depression D.T. 4 hr–4 days
Coumarin§ (2 H-1-benzopyran-2-one)	A—5%	Rat	680 (505–920)	1·8 (1·0–3·5)	Depression soon after treatment, mottled livers D.T. 4 hr–8 days
do.	C‡—10%	Guinea-pig	202 (179–228)	1·2 (1·1–1·4)	Depression, ataxia, severe gastro-intestinal tract irritation D.T. 1–6 days
Cuminaldehyde (*p-iso*propylbenzaldehyde)		Rat	1390 (1140–1700)	1·5 (1·3–1·8)	Depression within 1 hr after treatment, scrawny appearance, porphyrin-like deposit around eyes and nose. Gross pathology showed discoloured liver, irritated gastro-intestinal tract, stomach haemorrhage, yellowish material attached to the intestines D.T. 4 hr–10 days (most deaths within 2 days)
Cyclamen aldehyde (*p-iso*propyl-α-methylhydrocinnamaldehyde)		Rat	3810 (3080–4730)	1·7 (1·3–2·2)	Ataxia soon after treatment, coma within 1 hr. Wet fur, porphyrin-like deposit around eyes and nose D.T. 1–6 days
p-Cymene (4-*iso*propyl-1-methyl benzene)		Rat	4750 (3720–6060)	1·7 (1·5–2·0)	Depression soon after dosing, coma, bloody lacrimation, diarrhoea. Irritable, scrawny appearance for as long as 2 weeks D.T. 4 hr–12 days
Diacetyl (2,3-butanedione)		Rat	1580 (1310–1920)	1·5 (1·2–1·9)	Depression followed by convulsions within 10–15 min after treatment D.T. few min–2 hr

‡C=Propylene glycol

§Not acceptable for food use because of toxic effects observed on chronic feeding.

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
do.		Guinea-pig	990 (728–1350)	2·4 (1·2–4·6)	Ataxia, gasping, coma D.T. few min–4 days
Diallylacetic acid†	B—2%	Rat	570 (467–695)	1·6 (1·3–1·9)	Depression soon after treatment, weight loss, scrawny appearance D.T. 3–5 days
Dihydroanethole (1-methoxy-4-propylbenzene)		Rat	4400 (3380–5720)	1·9 (0·6–5·6)	Depression, wet posterior D.T. 4 hr–3 days
do.		Mouse	7300 (5930–9000)	1·6 (1·4–1·8)	Porphyrin-like deposit around eyes and nose, rough fur, scrawny appearance D.T. 1–4 days
Dihydrocoumarin (1,2-benzohydropyrone)		Rat	1460 (1180–1820)	1·6 (1·0–2·6)	Depression D.T. 2 hr–2 days
do.		Guinea-pig	1760 (1460–2170)	1·6 (1·2–2·1)	Depression D.T. 4 hr–4 days
Dihydrosafrole§ (1,2-methylenedioxy-4-propylbenzene)		Rat	2260 (1840–2780)	1·7 (1·4–2·0)	Depression soon after treatment, scrawny appearance, lacrimation D.T. 2–5 days
do.		Mouse	4300 (3420–5420)	1·8 (1·2–2·7)	Severe ataxia, coma D.T. 4 hr–5 days
Dimethylbenzyl carbinol (1,1-dimethyl-2-phenyl-ethanol)		Rat	1280 (934–1770)	2·3 (1·0–5·1)	Depression, coma D.T. 1–24 hr
do.		Guinea-pig	988 (705–1380)	1·7 (1·4–2·0)	Diuresis, coma. Severe gastro-intestinal tract irritation D.T. 1 hr–4 days
Dipropylacetic acid† (2-propylvaleric acid)	B—2%	Rat	670 (598–750)	1·2 (1·0–1·4)	Depression, scrawny appearance, diarrhoea in 1 hr D.T. 2 hr–2 days
Dolcourin‖		Rat	924 (825–1030)	1·2 (1·1–1·3)	Depression, abdominal cavity filled with bloody fluid D.T. 2 hr–5 days
Ethyl butyrate (ethyl butanoate)		Rat	13,050 (12,210–13,940)	1·2 (1·0–1·3)	Depression within a few min, coma on higher doses D.T. 4–18 hr

‖Dolcourin is a trademark name for a substance supplied by Dodge & Olcott, Inc. as a coumarin substitute.

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Ethyl caprylate (ethyl pentanoate)		Rat	25,960 (22,190–30,370)	1·4 (1·2–1·6)	Depression, coma, wet posterior D.T. 4 hr–4 days
Ethyl formate		Rat	1850 (1520–2240)	1·6 (1·3–1·9)	Depression within 5–10 min. Laboured respiration D.T. 15 min–2 hr
do.		Guinea-pig	1110 (887–1390)	2·0 (1·3–3·1)	Depression. Irritated gastro-intestinal tract D.T. 10 min–2 hr
Ethyl methylphenyl glycidate (ethyl ester of 2,3-epoxy-3-methyl-3-phenylproprionate)		Rat	5470 (4670–6410)	1·3 (1·1–1·5)	Depression, rough fur, porphyrin-like deposit around eyes and nose D.T. 4 hr–8 days
do.		Guinea-pig	4050 (3540–4620)	1·5 (1·2–1·8)	Depression in varying degrees, rectal bleeding on high doses D.T. 4 hr–7 days
Ethyl oenanthate (ethyl heptanoate)		Rat	>34,640*		Depression, coma, rough and wet fur
Ethyl oxyhydrate¶		Rat	14,700 (13,300–16,240)	1·1 (0·9–1·4)	Coma within 5 min D.T. 4 hr–5 days
Ethyl pelargonate (ethyl nonanoate)		Rat	>43,000*	—	Depression, irritated gastro-intestinal tract. Appeared normal 24 hr after treatment
do.		Guinea-pig	24,190 (19,350–30,240)	1·5 (1·2–2·0)	Depression, irritated gastro-intestinal tract D.T. 4 hr–6 days
Ethyl sebacate (ethyl decandioate)		Rat	14,470 (12,805–16,350)	1·4 (1·3–1·5)	Wet fur, depression D.T. 4 hr–4 days
do.		Guinea-pig	7280 (5970–8900)	1·3 (1·1–1·4)	Depression, diuresis D.T. 8 hr–6 days
Ethyl vanillin (3-ethoxy-4-hydroxy-benzaldehyde)	C—20%	Rat	>2000**	—	Depression, coma on high doses D.T. 3–18 hr

¶Ethyl oxyhydrate is a mixture whose composition varies with the method of manufacture. The material used in this study was obtained from Florasynth Labs., Inc., N.Y.

**Toxicity of the propylene glycol solvent prohibited administration of higher doses.

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Eucalyptol (1,8-epoxy-*p*-menthane)		Rat	2480 (2100–2930)	1·4 (1·2–1·6)	Depression, coma on high doses, scrawny appearance for 3–4 days. Recovery within 7 days D.T. 2 hr–4 days
Eugenol (1-hydroxy-2-methoxy-4-allylbenzene)		Rat	2680 (2420–2970)	1·2 (1·1–1·4)	Coma soon after treatment D.T. approximately 1 hr
do.		Mouse	3000 (2400–3750)	1·8 (1·3–2·3)	Severe depression immediately after treatment D.T. few min–2 days
do.		Guinea-pig	2130 (1860–2430)	1·3 (1·2–1·5)	Depression D.T. 4 hr–3 days
Eugenol acetate (1-acetoxy-2-methoxy-4-allylbenzene)		Rat	1670 (1265–2200)	1·9 (1·4–2·5)	Rough fur, depression D.T. 4 hr–2 days
Eugenol methyl ether (1,2-dimethoxy-4-allylbenzene)		Rat	1560 (1170–2070)	2·6 (1·6–4·3)	Coma within 1 hr after treatment D.T. less than 8 hr
Fenchone (*d*,1,3,3-trimethyl-2-norcamphanone)		Rat	6160 (4400–8630)	2·7 (1·1–6·5)	Depression, scrawny appearance, porphyrin-like deposit around eyes and nose for a week after treatment D.T. 4 hr–9 days
2-Furaldehyde	A—5%	Rat	127 (110–147)	1·3 (1·2–1·5)	Depression soon after treatment. Scrawny appearance, porphyrin-like deposit around eyes and nose. Gross pathology showed lung haemorrhage on the higher doses D.T. 4 hr–4 days
Geraniol extra (3,7-dimethyl-2,6-octadienol) (3,7-dimethyl-1,6-octadienol)		Rat	3600 (2840–4570)	1·7 (1·3–2·2)	Depression, coma, wet fur D.T. 4–18 hr
Geranyl acetate		Rat	6330 (5450–7340)	1·3 (1·2–1·4)	Depression, coma D.T. 4 hr–3 days
Geranyl butyrate		Rat	10,660 (8020–14,180)	1·6 (0·7–3·7)	Marked depression and coma on higher doses D.T. few min–4 days

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Guaiac gum	A—20%	Rat	>5000*	—	Rough fur, scrawny appearance D.T. less than 18 hr
Ho leaf oil		Rat	3270 (2780–3830)	1·6 (1·3–2·0)	Coma on higher doses within 30 min. Scrawny appearance, rough fur, porphyrin-like deposit around eyes and nose D.T. 1–4 days
Hydratropic aldehyde (2-phenylpropanal)		Rat	2800 (2390–3280)	1·3 (1·2–1·4)	Coma within 20 min after treatment. Rough fur D.T. 1–5 days
Ionone [60% α-ionone, 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one and 40% β-ionone, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one]		Rat	4590 (3880–5400)	1·4 (1·3–1·5)	Depression, tremors D.T. 4 hr–4 days
Isoamyl formate		Rat	9840 (7720–12,550)	1·7 (1·3–2·3)	Depression soon after treatment, scrawny appearance D.T. 4 hr–4 days
Isoeugenol (1-hydroxy-2-methoxy-4-propenylbenzene)		Rat	1560 (1290–1880)	1·4 (1·3–1·5)	Coma soon after treatment, scrawny appearance D.T. 1 hr–7 days
do.		Guinea-pig	1410 (1130–1780)	2·0 (1·5–2·7)	Depression, coma D.T. 3–6 days
Isosafrole§ (1,2-methylenedioxy-4-propenylbenzene)		Rat	1340 (1140–1590)	1·4 (1·2–1·7)	Depression, coma, rough fur, scrawny appearance D.T. 4 hr–8 days
do.	A—25%	Mouse	2470 (2010–3040)	1·7 (1·3–2·2)	Depression soon after treatment. Severe ataxia within 24 hr. Recovery from ataxia but scrawny in appearance within 48 hr D.T. 1 hr–4 days

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Juniper tar		Rat	8014 (6550–9770)	1·5 (1·2–1·9)	Depression soon after treatment, scrawny appearance for several days. Irritated gastro-intestinal tract D.T. 4 hr–4 days
Linalool (3,7-dimethyl-1,6-octadien-3-ol or 3,7-dimethyl-1,7-octadien-3-ol)		Rat	2790 (2440–3180)	1·3 (1·2–1·4)	Ataxia soon after treatment D.T. 4–18 hr
Linalyl acetate		Rat	14,550 (12,300–17,170)	1·8 (1·3–2·6)	Depression soon after treatment, coma, wet posterior D.T. 4 hr–4 days
do.		Mouse	13,360 (11,920–15,000)	1·2 (1·1–1·3)	Depression within 10–15 min after treatment D.T. 1–3 days
Linalyl cinnamate		Rat	9960 (8230–12,050)	1·4 (1·2–1·7)	Depression. Scrawny appearance, porphyrin-like deposit around eyes and nose, wet fur on posterior 24 hr after treatment D.T. 4 hr–5 days (most deaths in 48 hr)
Linalyl *iso*butyrate		Rat	>36,300*	—	Depression, wet fur, diarrhoea. Appeared normal after 1 week
do.		Mouse	15,100 (12,330–18,500)	1·6 (1·1–2·2)	Depression soon after treatment. Excitable after 1 hr. Rough fur D.T. 4 hr–3 days
Mace oil		Rat	3640 (3170–4190)	1·4 (1·0–2·0)	Depression. Scrawny appearance for several days after treatment D.T. 4–18 hr
Melilotic anhydride (anhydride of *o*-hydroxy-cinnamic acid)		Rat	1510 (1310–1750)	1·3 (1·1–1·6)	Depression D.T. 1 hr–2 days
Menthol (*p*-menthan-3-ol)	A—50%	Rat	3180 (2790–3620)	1·3 (1·1–1·5)	Ataxia, scrawny appearance D.T. 4 hr–3 days

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Methyl anthranilate (*o*-aminobenzoic acid, methyl ester)		Rat	2910 (2500–3400)	1·4 (0·9–2·0)	Depression, coma D.T. 1–2 days
do.		Mouse	3900 (3260–4680)	1·5 (1·3–1·7)	Depression D.T. 4–18 hr
do.		Guinea-pig	2780 (2210–3500)	1·8 (1·4–2·3)	Depression, gasping, rapid respiration, irritated gastro-intestinal tract D.T. 4 hr–4 days
Methyl benzoate		Rat	1350 (1290–1410)	1·1 (0·7–1·5)	Depression, porphyrin-like deposit around nose, rough fur, wet posterior. Survivors excitable D.T. 2–18 hr
do.	A—50%	Mouse	3330 (2920–3800)	1·2 (1·1–1·4)	Excitation, tremors D.T. few min–18 hr
Methylenedioxybenzene†		Rat	580 (487–690)	1·4 (1·2–1·7)	Depression and coma on high doses, rough fur, porphyrin-like deposit around eyes and nose D.T. 1–6 days
do.	A—25%	Mouse	1220 (976–1520)	1·4 (1·2–1·7)	Depression and coma on high doses, rough fur, porphyrin-like deposit around eyes and nose D.T. 1–5 days
Methyl salicylate (*o*-hydroxybenzoic acid, methyl ester)		Rat	887 (715–1100)	1·5 (1·2–1·8)	Depression soon after treatment D.T. 4–18 hr
do.		Guinea-pig	1060 (873–1300)	1·6 (1·3–1·9)	Convulsions. Irritated gastro-intestinal tract D.T. 1 hr–3 days
Musk ambrette (artificial) (6-*tert*-butyl-3-methyl-2, 4-dinitroanisole)	A—25%	Rat	339 (283–408)	1·4 (1·2–1·7)	Increased respiration and hypersensitivity 24 hr after treatment. Scrawny appearance, wet posterior, rough fur D.T. 1–3 days

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Nutmeg oil		Rat	2620 (2200–3120)	1·3 (1·2–1·5)	Depression soon after receiving treatment, scrawny appearance for several days D.T. 4–18 hr
Phenylethyl alcohol (2-phenylethanol)		Rat	1790 (1580–2020)	1·2 (1·1–1·3)	Coma within 15 min. Gross pathology shows irritation of the lower half of the stomach on the higher doses D.T. 4–18 hr
Phenylethyl phenylacetate		Rat	15,390 (12,830–18,470)	1·5 (1·4–1·7)	Depression soon after treatment, scrawny appearance for several days D.T. 4 hr–5 days
Piperonal (3,4-methylenedioxybenzaldehyde)	A—25%	Rat	2700 (2350–3100)	1·5 (1·1–2·0)	Tremors for several hr followed by depression and ataxia D.T. 2 hr–5 days
n-Propanol		Rat	6500 (5800–7280)	1·2 (1·1–1·3)	Coma soon after treatment, scrawny appearance D.T. 2–18 hr
Propenylbenzene†		Rat	3600 (2650–4900)	2·2 (1·3–3·8)	Scrawny appearance, weight loss, wet posterior for 5–7 days after treatment D.T. 4 hr–9 days
n-Propyl acetate		Rat	9370 (7670–11,430)	1·5 (1·1–1·8)	Depression soon after treatment, rough fur, scrawny appearance D.T. 4–18 hr
do.		Mouse	8300 (7280–9460)	1·2 (1·1–1·4)	Depression soon after treatment D.T. few min–18 hr
Propylbenzene†		Rat	6040 (4830–7550)	1·5 (1·2–2·0)	Depression soon after receiving treatment. Scrawny appearance D.T. 1–3 days
n-Propyl *n*-butyrate		Rat	15,000 (12,600–17,850)	1·4 (0·8–2·5)	Depression soon after treatment followed by coma on higher doses. Rough fur, diarrhoea D.T. 1–3 days
n-Propyl formate		Rat	3980 (3350–4740)	1·5 (1·2–1·7)	Depression soon after treatment D.T. 4–18 hr

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
do.		Mouse	3400 (3060–3780)	1·7 (1·3–2·2)	Depression soon after treatment D.T. few min–6 hr
Safrole§ (1,2-methylenedioxy-4-allylbenzene)		Rat	1950 (1760–2160)	1·3 (0·6–3·0)	Depression, ataxia, diarrhoea D.T. 1–5 days
do.	A—50%	Mouse	2350 (2010–2750)	1·4 (1·1–1·6)	Ataxia, depression, scrawny appearance. D.T. 4 hr–7 days
Terpinyl acetate (*p*-menth-1-en-8-ol ester of acetic acid)		Rat	5075 (4160–6190)	1·5 (1·3–1·7)	Depression, scrawny appearance, porphyrin-like deposits around eyes and nose D.T. 4 hr–5 days
Thymol (*p*-cymen-3-ol,3-hydroxy-*p*-cymene)	C—20%	Rat	980 (817–1180)	1·6 (1·3–2·0)	Depression, ataxia, coma on high doses D.T. 4 hr–5 days
do.	C—20%	Guinea-pig	880 (740–1050)	1·6 (1·2–2·1)	Irritated gastro-intestinal tract, tremors, coma, respiratory failure D.T. 1 hr–10 days
Vanillin (4-hydroxy-3-methoxy-benzaldehyde)	C—20%	Rat	1580 (1390–1810)	1·3 (1·2–1·5)	Coma soon after treatment D.T. 4 hr–4 days
do.	C—20%	Guinea-pig	1400 (1310–1500)	1·1 (1·09–1·2)	Depression within 1 hr D.T. 1–3 days
Veratrole (1,2-dimethoxybenzene)		Rat	1360 (980–1870)	1·6 (1·3–1·9)	Coma within 10 min after treatment. Salivation, porphyrin-like deposit around eyes, diarrhoea, scrawny appearance for 3–4 days D.T. 1–4 days
do.	A—25%	Mouse	2020 (1650–2480)	1·7 (1·4–2·0)	Rapid, laboured respiration; lacrimation; pawing about mouth; hyperactivity followed by coma within 15 min D.T. few min–4 hr

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Condiments et Complexes de Structure Voisine. I. Toxicité Aiguë par Voie Buccale

Résumé—On administra par intubation des doses de complexes faits de 107 condiments synthétiques et naturels, de structure chimique voisine, à des souris, des rats et des cobayes. On observa habituellement les animaux pendant 2 semaines, durant lesquelles on suivit le développement de signes toxiques et on nota la date de la mort. Pour chaque complexe on détermina la dose orale limite au-delà de laquelle commence l'intoxication aiguë.

Lebensmittelgeschmackszusatze und Verbindungen verwandter Struckturen I. Akute Oraltoxititat

Zusammenfassung—107 synthetischen un natürliche Geschmackszusätze und strukturverwandte Verbingdungen wurden durch Intubation an Mäuse, Ratten und Meerschweinchen verabreicht. Die Tiere wurden gewöhnlich 2 Wochen lang unter Beobachtung gehalten, während welcher Zeit die Entwicklung toxischer Symptome verfolgt und die Zeit des Todeseintritts registriert wurde. Die akute orale mittlere tödliche Dosis jeder Verbindung wurde festgestellt.

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From the Department of Pharmacology, University of Copenhagen.
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Metabolism of Methanol and Formic Acid in Dogs.

By

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(Received December 5, 1947)

The excretion of formic acid in the urine of rabbits after administration of methanol was discussed in a previous paper (LUND 1948). It was shown that administration of methanol to rabbits elicits a very small increase only in the formic acid excretion, no doubt because these animals are able to oxidise formic acid almost completely. In dogs, as will be shown in the present paper, the conditions are very different. Dogs, like methanol-poisoned humans, show a very considerable excretion of formic acid with a characteristic protracted course.

For the methods of analysis used reference may be made to the above mentioned paper.

Previous Investigations.

The excretion of formic acid after administration of methanol to dogs was first studied by POHL (1893), who showed that the content of formic acid in urine rises to nearly 100 times the normal value, and that the excretion is typically protracted, the maximal concentration being reached on the 2nd or 3rd day after administration. The formic acid concentration generally became normal again after 6 days. By *normal* formic acid excretion of a dog we should understand an amount of up to 50 mg within 24 hours. Other somewhat different indications (POHL: 10 mg; DAKIN (1913): 30 mg and KAPELLER-ADLER and LAUDA (1932): 40 mg) are no doubt due to the fact that animals — and humans as

well — excrete varying amounts of formic acid depending on variations in the diet.

POHL's results were borne out in all main essentials by various other investigators, a. o. KROL (1913), BENEDICT and HARROP (1922), and LEO (1927).

Statements concerning the proportion of excreted formic acid to the amount of methanol administered vary a great deal. POHL found 2 to 3% of the alcohol as formic acid in the urine, while ASSER (1914) found 7 to 9%, BENEDICT and HARROP 14%, VOIT (1929) 10%, and LEO in the same animal 7,6 to 2,8%, decreasing with increasing habituation. By administration of large amounts of methanol (50 g) a comparatively high percentage (10 to 15%), while after smaller doses under 10% is recovered as formic acid.

Present Investigations.

1. Excretion of Methanol and Formic Acid after Administration of Methanol.

The experiments were carried out on two full-grown male dogs, "S" and "H".

Within the experimental period the daily diet consisted of 200 g minced beef and boiled potatoes, with water *ad lib.* The animals were placed in cages with a device for collecting urine; but by frequent catheterisations we soon succeded in preventing them from excreting urine in the cage. The blood samples were obtained by puncture of the cephalic vein, which is freely accessible along 5 cm of the anterior surfaces of the forelegs.

Some preliminary experiments were made with peroral administration of 1 gram methanol per kilogram body weight; this amount elicited no signs of poisoning. Thereby the dogs got accustomed to the various disturbances, and it was also ascertained that the excretion of formic acid corresponded in the main to that observed by previous investigators. It was aremarkable thing that the excretion reached maximal values already within the first 24 hours. Previous investigators found the excretion to reach its peak on the 2nd or 3rd day after the administration of alcohol. The divergence is, however, only apparent, since earlier investigators always estimated the formic acid on 24-hour urine.

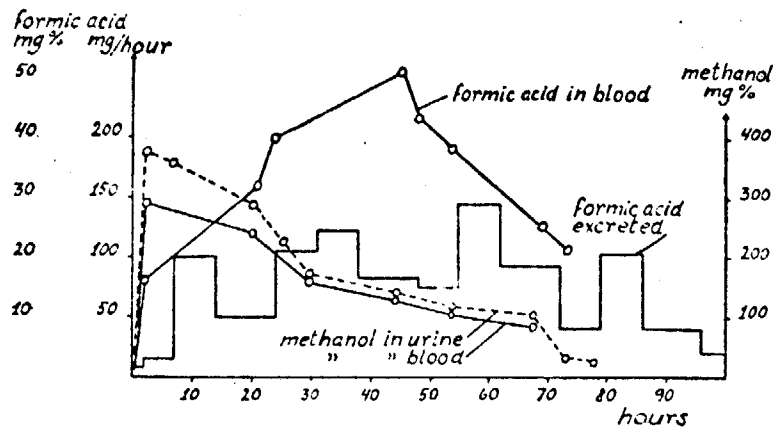

Fig. 1. *The excretion of formic acid; the concentration of formic acid in blood and the concentration of methanol in urine after peroral administration of methanol.* Dog "H". Weight 10.0 kg. Oct. 21, 14 o'clock: 25 ml of methanol plus 75 ml of water (1.97 g methanol/kg). Abscissa: Number of hours after administration of methanol. Ordinate: mg formic acid excreted per hour; mg formic acid per 100 ml of blood and mg methanol per 100 ml of urine.

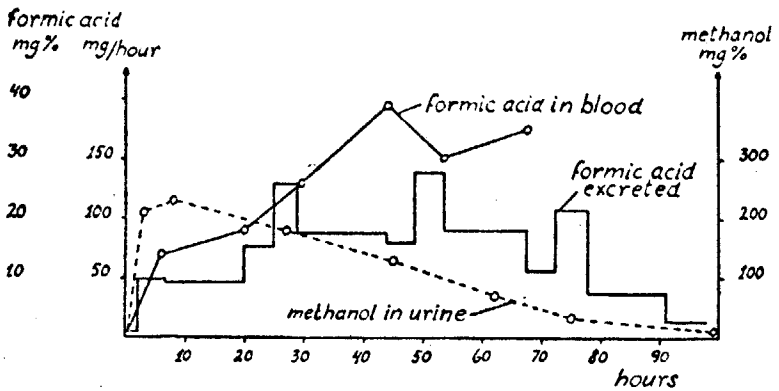

Fig. 2. *The excretion of formic acid; the concentration of formic acid in blood and the concentration of methanol in urine and blood after peroral administration of methanol.* Dog "S". Weight 14.0 kg. July 1, 8 o'clock: 30 ml of methanol plus 120 ml of water (1.70 g methanol/kg). Abscissa: Number of hours after administration of methanol. Ordinate: mg formic acid excreted per hour; mg formic acid per 100 ml of blood and mg methanol per 100 ml (g) of urine (blood).

The low initial values would thus shift the maximum towards a later time.

In two experiments (Figs. 1 and 2) methanol and formic acid

were estimated in both blood and urine after administration of 1.7 to 2 g methanol/kg.

The methanol excretion was even more protracted than in rabbits. Methanol could still be demonstrated in the urine 100 hours after administration. A curve illustrating the methanol concentration in the blood presented a steady fall, as shown by previous investigators (FABRE 1943).

The concentration of formic acid in the blood rose and fell steadily on each side of a maximum lying almost in the middle of the experimental period; the formic acid concentration of the urine corresponded fairly closely to this course.

A survey of the two experiments gives the following result. After administration of 19.8 g methanol to dog "H" (23.8 g) — the figures in brackets indicate the results from the corresponding experiment with dog "S" — the dog excreted 1.722 g methanol (1.420 g) or 8.7 % (6.0 %) and 6.726 g formic acid (7.652g), or — calculated as methanol — 23.7 % (22.4 %) of the administered amount, in other words nearly twice as much as indicated in previous papers. Thus a total of 32.4 % (28.4 %) of the alcohol, converted as well as non-converted, was recovered in the urine. The amount of alcohol eliminated in the expired air can roughly be estimated at 50 % of the administered amount. (According to VÖLTZ and DIETRICH (1912) well over 20 % of the administered methanol is eliminated in this manner within 48 hours, while ASSER recovered over half of the alcohol in the expired air over the entire experimental period). About 20 % is, however, still needed to make the account balance. As will be shown later, this may be explained by the fact that a certain amount of the methanol is oxidised completely, i. e. the formic acid formed is in part converted into carbon dioxide and water.

Apart from an uncertain gait for a few hours after the alcohol administration, there were observed no signs of poisoning despite the fairly large amounts administered (1.7 to 2 g/kg).

In another experiment 2.08 g Methanol or 10.5 % of the amount administered (19.8 g) were excreted. Of formic acid 4.11 g or 14.5 % were excreted. In other words 25 % of the alcohol was recovered in the urine, which accords with the results obtained in the two former experiments.

2. Methanol and Formic Acid Concentration in Organs after Administration of Methanol.

The series of experiments was concluded by killing the dogs after administration of about 3 grams methanol per kilogram body weight, after which methanol and formic acid estimations were made on different organs. The killing took place at a time when the formic acid concentration in the organism must be supposed to have been at its highest, that is, 1 or 2 days after the administration of the alcohol.

In these experiments there were seen rather severe signs of poisoning, increasing to narcosis. Not until from 12 to 24 hours after the alcohol administration did the dogs begin to take food and seem fully restored, though not quite so lively as under normal conditions.

The animals were killed by intravenous injection of 10 to 20 ml of a 50% magnesium sulphate solution. The carotid arteries were opened after death and the blood was collected, after which the organs were removed. Sections of kidneys and liver were fixed immediately in formol-alcohol for histological examination; this revealed slight or no changes in the hepatic and renal tissues. Hence the renal function of the animals must be supposed to have been normal throughout.

The analysis of the organs were made after mincing in a meat chopper. A weighed portion was stirred with water to a paste, which was submitted to the same treatment as urine: distillation in steam for the formic acid estimations (BANNICKE 1939) and acid-base distillation for the methanol estimations.

The course of the excretion prior to the killing was followed in the usual way by urine analysis. The results of the analysis are shown in Table 1.

Both the methanol and the formic acid concentrations were highest in serum, decreasing in the order liver, muscle, brain. The concentration of formic acid in the kidneys was, however, just as high as in the serum, but the presence of a few drops of urine may have been the cause of the high concentration, so that no importance can be attached to this finding.

The recovered amounts of methanol and formic acid have been entered in table 2 for the sake of clearness. The values have, more-

Table 1.

The content of methanol and formic acid in the tissues after peroral administration of methanol.

Dog "S". Weight 14.2 kg. Sept. 18, 12 o'clock: 50 ml of methanol plus 150 ml of water (2.80 g methanol/kg). Killed Sept. 20, 14 o'cl. — Dog "H". Weight 10,1 kg. Nov. 25, 11 o'clock: 40 ml of methanol plus 150 ml of water (3.14 g methanol/kg). Killed Nov. 26, 15 o'cl.

Dog	Tissue	Weight g	Formic acid content mg/100 g	Formic acid content mg total	Methanol content mg/100 g	Methanol content mg total
"S"	Serum*)	350	38.4	134	280	980
	Liver	275	15.8	43.5	130	358
	Muscle**) ...	5600	11.9	670	153	8570
	Brain	78	10.1	7,9	124	96
Total in tissues				0.86 g		10.00 g
Excreted before death				3.655 g		3.71 g
"H"	Serum	250	24.6	62	349	870
	Liver	300	13.0	39	221	663
	Muscle	4000	6.9	275	206	8240
	Brain	80	4.0	3.2	248	198
	Kidneys	55	23.6	13.0	—	—
Total in tissues				0.39 g		9.97 g
Excreted before death				2.14 g		2.11 g

*) Serum constituting half of the blood which is estimated to constitute 5% of the body weight.

**) The weight of muscles is estimated to constitute 40% of the body weight.

over, been indicated as per cent of the alcohol administered, for formic acid after conversion to the equivalent amount of methanol.

The excreted amounts of methanol and formic acid as well as the contents of formic acid in the organs were greater for dog "S" than for dog "H". This is due, of course, to the fact that dog "S" lived for 50 hours after the alcohol administration, while dog "H" lived for 28 hours only. When this difference is taken into consideration, there is agreement between the results.

3. Estimation of the Renal Clearance of Methanol and Formic Acid.

An attempt has been made at elucidating the mechanism of

excretion of methanol and formic acid by means of renal clearance estimations based on creatinine tolerance tests:

3 to 5 g creatinine dissolved in 100 to 200 ml of water were administered by stomach tube. The urinary bladder was emptied about 3 hours later, and the blood sample was taken after another 30 minutter. The bladder was emptied again 1 hour after the first catheterisation, so that the clearance period extended over 1 hour. The estimations of creatinine in serum and blood were carried out according to VAN SLYKE's method in the modification indicated by NIELSEN (1933). It should be emphasized that both formic acid and methanol were estimated in serum, and that accordingly it is here a question of *serum*-clearance. The clearance estimations are in Table 3, marked with the date of their performance.

The formic acid clearance amounted to an average of 6.5 (ranging from 4 to 9). There seems to be no interrelation between the magnitude of the clearance and the formic acid concentration in the serum. Formic acid must thus be a no threshold substance which is abundantly reabsorbed at any concentration. The percentage excretion was fairly constant, about 13, but the last value found (8/11) was considerably lower. The reabsorption takes place not only in the kidneys, but also, as will be shown later, from the urinary bladder, from which about 5 % of the formic acid present is reabsorbed per hour. As the clearance period extended over 1 hour only, the reabsorption of formic acid in the kidneys must be predominant.

The percentage excretion of methanol was found to be almost the same as that for water, which means that methanol in passing through the kidneys (and the bladder) behaves exactly like the water.

4. *Excretion of Formic Acid after Administration of Formic Acid.*

It was shown in section 1 that dogs excrete about 30 % of administered methanol as methanol and formic acid. Since hardly more than about 50 % of the alcohol is eliminated with the expired air, dogs may *a priori* be supposed — like rabbits — to be able to oxidise formic acid. Various writers have already found this to be so. Thus LEO (1927) showed that about 70 % of an injected amount of sodium formate is oxidised, while KNOOP and JOST

Table 2.

The relations between the amounts of methanol and formic acid found in the urine and the tissues and the administered amount of methanol expressed as per cent of the dose.

Dog	Admin. g methanol	Formic acid in urine		Formic acid in tissues		Methanol in urine		Methanol in tissues		found formic acid		found methanol		found total	
		g	%	g	%	g	%	g	%	g	%	g	%	%
"S"	39.7	3.65	6.4	0.86	1.5	3.71	9.5	10.00	25.2	4.51	7.9	13.7	34.5	42.4
"H"	31.7	2.14	4.7	0.39	0.8	2.11	6.7	9.97	31.4	2.53	5.6	12.1	38.2	43.8

Table 3.

The renal clearance of methanol and formic acid after peroral administration of methanol.

Excretion of water:

Date	Diuresis ml/min.	Creatinine mg/100 ml serum	Creatinine mg/100 ml urine	Conc. index	Filtration ml/min.	Reabsorb. ml/min.	Excretion %
3/9	0.565	16.7	1200	71.8	45.0	44.4	1.25
4/9	0.672	18.6	1380	74.0	49.7	49.0	1.35
5/9	3.25	23.2	371	16.1	52.3	49.0	6.2
19/9	0.763	19.7	1240	62.9	48.0	47.2	1.6
6/11	0.400	19.7	2750	140	56.0	55.6	0.72
8/11	0.500	21.8	2770	127	63.5	63.0	0.79

Excretion of formic acid.

Date	Formic acid mg/100 ml serum	Formic acid mg/100 ml urine	Conc. index	Clearance ml/min.	Filtrat. mg/min	Excret. mg/min	Reabs. mg/min	Excretion %
3.9	33.2	331	10.0	5.65	14.9	1.9	13.0	12.7
4.9	25.0	248	9.9	6.67	12.4	1.7	10.7	13.7
5.9	12.4	31.7	2.56	8.31	6.5	1.0	5.5	15.4
19.9	25.3	250	8.8	6.74	13.6	1.9	11.7	14.0
6.11	14.1	319	22.6	9.05	7.9	1.3	6.6	16.1
7.11	7.8	63	8.1	4.05	5.0	0.3	4.7	6.3

Injection of methanol

Date	Methanol mg/100 ml serum	Methanol mg/100 ml urine	Conc. index	Clearance ml/min.	Filtrat. mg/min.	Excret. mg/min.	Reabs. mg/min.	Excretion %
19.9	320	345	1.05	0.82	1.53	2.64	160	1.7
6.11	184	169	0.92	0.37	103	0.68	102	0.7

(1923), in accordance with ASSER (1914), found that about 80 % is oxidised no matter whether the sodium formate is administered subcutaneously, intravenously, or perorally.

In some preliminary experiments with peroral administration of sodium formate, from 42 to 56 % of the amount of formic acid administered was recovered in the urine. In the experiment recorded in Table 4 the formic acid excretion was estimated after subcutaneous injection of 2 g sodium formate dissolved in 40 ml of water. In addition the formic acid concentration in the blood was estimated at suitable intervals.

Table 4.

The excretion of formic acid after subcutaneous administration of formic acid.

Dog. "H". Weight 10.1 kg. Oct. 30, 10 o'clock: Subcutaneous injection of 40 ml of a 5% solution of sodium formate (~ 1.35 g formic acid).

Hours after injection	Diuresis ml	Formic acid in urine mg/100 ml	Formic acid in urine mg total	Formic acid in blood mg/100 ml
0	2	—	—	14.2
1	—	—	—	15.5
3.7	48	720	345	8.1
9.5	46	466	215	—
23	148	7.5	11	—
27	22	0.0	0.0	

Total excretion: *0.571 g formic acid.*

Of the injected amount of formic acid (1.35 g) 0.57 g or 42 % was excreted, so that 58 % was oxidised. This is rather low in comparison with the figures given above. However, the writers there quoted used exclusively 24-hour urine for their estimations and still found an increased formic acid excretion on the second day after administration. In the present experiment the bladder was emptied at shorter intervals and the formic acid concentration in the urine was found to be normal already 23 hours after the injection. It is natural to suppose that formic acid can be reabsorbed from the bladder and then be oxidised. The experiment recorded in Table 5 confirmed this hypothesis.

After evacuation of 15 ml of urine and washing of the dog's bladder with water, 50 ml of a solution containing 1.00 g sodium formate were injected through the catheter. The dog was then placed in the urine-collecting cage where it remained for 5 hours without emptying the bladder. Blood samples were taken after 1, 3, and 5 hours. The bladder was emptied 5 hours after the injection and washed with 3 times 20 ml of water. The formic acid concentration was estimated in the samples collected.

Table 5.

The excretion of formic acid after injection of formic acid in the bladder.

Dog "H". Weight 10.1 kg. Nov. 15, 10 o'clock: injection in the urine bladder of 50 ml of a solution containing 1.00 g sodium formate (~ 678 mg formic acid).

Hours after injection	Total fluid emptied from bladder	Formic acid content mg/100 ml	Formic acid content mg total	Formic acid in serum mg/100 ml
0	15 urine	5.4	0.8	1.0
1	"			4.9
3	"			5.2
5	85 urine + inject. liq.	596	507	5.7
5	60 Wash. liq. ...	6.2	3.7	—
12	70 urine	9.9	6.9	—

After evacuation and washing of the dog's bladder the "urine" and the washing water were found to contain 511 mg formic acid. The urinary output amounted to 35 ml within the 5 hours, namely the evacuated 85 ml of fluid minus the injected fluid. If we put the normal formic acid concentration in the urine at 5 mg %, no more than 2 mg formic acid can have been normally excreted. Thus 509 mg of the injected 678 mg formic acid were recovered, and 169 mg formic acid or *25 % of the injected amount* must, therefore, have been reabsorbed from the bladder. The formic acid concentrations found in the serum, which presented a slight rise in the course of the experimental period, prove that it really was a question of reabsorption and not of destruction. Since the urine

evacuated 7 hours after the end of the experiment contained no more than 6.9 mg formic acid, it must be justifiable to conclude that the reabsorbed formic acid was oxidised almost completely.

It is thus to be supposed that the amount of formic acid excreted after *subcutaneous* injection of sodium formate would have been much reduced if the bladder had been emptied at a later time, e. g. 24 hours after the injection. The experimental technique applied is accordingly of essential importance for the reproducibility of the results.

Discussion.

It seems justifiable to infer from the experiments with subcutaneous and peroral administration of sodium formate that dogs after administration of methanol excrete half the amount of formic acid formed in the organism by conversion of the alcohol, while the other half is oxidised (in rabbits, as shown in the preceding paper, formic acid is oxidised almost completely, no matter whether it is introduced as sodium formate or as methanol). On the basis of this inference the calculations made on p. 111 give the following result.

23.7 % (22.4 %) of the alcohol was excreted as formic acid. As, however, a corresponding amount of formic acid must be supposed to have been oxidised, 47 % (45 %) of the alcohol must have been converted into formic acid. About 9 % (6 %) of the methanol was excreted unchanged with the urine. Thus it was possible to account for 56 % (51 %) of the administered methanol. The results of this calculation are naturally approximate only, but they seem to bear out the theory advanced in the literature that dogs eliminate about half of any administered methanol with the expired air.

The fact that the nocturnal excretion of formic acid after methanol administration (the long, horizontal parts of the curves Figs. 1 and 2) was far lower than the diurnal excretion through the entire experimental period can be explained on the basis of the re-absorption, demonstrated in section 4, of formic acid from the bladder with ensuing oxidation. At least the greatest difference between day and night from the point of view of experimen-

tal technique was that by day the animals were catheterized several times, while by night they were left for more than 10 hours with a (partially) filled urinary bladder.

From 30 to 50 hours after peroral administration of about 3 g methanol/kg the dogs organism contains a considerable store of methanol (25 to 30 % of the administered amount), whereas the formic acid content is very low (about 1 % of the amount of alcohol). This fact — compared with the conditions of excretion — goes to show that formic acid is excreted or oxidised gradually as it is formed having thus no chance of being stored.

Summary.

1) After administration to dogs of a single dose of methanol about 10 % is excreted unchanged with the urine. The excretion is not concluded till after about 100 hours.

The methanol concentration in the organs is nearly half as high as in the serum.

2) It is shown, through estimations of the renal clearance, that the excretion of methanol through the kidneys follows that of water.

3) After administration of a single dose of methanol to dogs about 20 % of the alcohol is excreted as formic acid with the urine. The increased formic acid excretion does not come to an end till after 100 hours.

The formic acid concentration in the blood reaches its maximum 40 to 50 hours after the administration of alcohol.

The formic acid concentration in the organs is from ½ to ¼ that in the serum.

4) It is shown through clearance estimations that formic acid should probably be regarded as no threshold substance (excretion percentage about 13).

5) Dogs oxidise about 50 % of an administered amount of sodium formate no matter how it is administered. The excretion of the remaining 50 % with the urine is concluded after about 10 hours.

6) After injection into the urinary bladder of a certain amount of sodium formate about 25 % of the injected amount is re-absorbed in the course of 5 hours, and the formic acid concentration in the blood rises.

7) Peroral administration of 1 g methanol per kilogram body weight elicits no signs of poisoning in dogs. 2 g methanol/kg elicit light and 3 g/kg rather severe signs of poisoning.

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FROM THE PHARMACOLOGICAL INSTITUTE OF THE UNIVERSITY OF HAMBURG

THE ACUTE AND CHRONIC TOXICITY OF FORMIC ACID AND ITS FORMATES*

By G. Malorny

With 1 Illustration and 6 tables

(Entered 19 February, 1969)

Formic Acid and formates have been already used in different ways as preservatives for certain foodstuffs. However up to now very little has been known with certainty regarding their acute and chronic toxicity as well as their relationship to metabolism.

In the "Eight [sic] Report of the Joint FAO/WHO Expert Committee on Food Additives, Geneva ~~Genf~~ 1965" [in English] (1) there are only scanty data regarding the effects of formic acid. It is stated there: "Exact LD_{50} values are not available." And in another place: "Since long-term toxicity studies are lacking, it is not possible to give guidance on an unconditional acceptable daily intake level in man."

1. Acute Toxicity

Free formic acid exerts ~~on all tissue~~ a strong irritation on all tissue especially on mucus membranes. Therefore it is mostly used in the form of one of its salts in preservation techniques. In order to ~~unde~~ be able to understand the share of toxicity which is alloted to the free hydrogen ions, we have determined, on the white mouse, the acute toxicity (LD_{50}) both of pure formic acid as well as of ~~for~~ its salts. In Table 1, the LD_{50} data are summarized after ~~according~~ oral application and intravenous injection.

*Report to the Commission on Foreign Substances of the German Research Society.

Tab. 1. LD_{50} for formic acid and its salts (mouse).

Tab. 1. LD_{50} für Ameisensäure und ihre Salze (Maus) calculated according to formate

Substance	LD_{50} mg/kg	LD_{50} Range mg/kg	LD_{50} berechnet auf Formiat	Anzahl der Tiere No. of animals
per os				
Formic acid — Ameisensäure	1,100	1,000– 1,200	1,076	55
Na-Formiat formate	11,900	9,000–12,800	7,410	45
K-Formiat "	5,700	5,000– 6,000	2,050	50
NH_4-Formiat "	2,250	2,050– 2,400	1,610	50
Ca-Formiat "	1,220	1,250– 2,500	1,830	45
intravenous				
Formic acid — Ameisensäure	145	138–151	142	50
Na-Formiat formate	807	800–813	534	50
K-Formiat "	95	93– 97	51	55
NH_4-Formiat "	410	408–412	293	50
Ca-Formiat "	154	150–158	107	55

(Potassium)
Kalium formate has the highest toxicity in intravenous feeding, free formic acid follows in order and thereafter calcium formate. (Sodium) Natrium formate has the most indifferent effect; its toxicity is eight times less than that of kalium formate [mistakenly written as kalzium in the German text]. The toxicity of the cations themselves is to ~~ge~~ be regarded as the cause for these distinctions. It is known that K+ and Ca++ for example have a toxic effect on the heart in greater dosages.

We find similar relationships in the ~~values for~~ oral LD_{50} values. Free formic acid (1.1 g/kg) shows the highest toxicity. Its toxicity is conditioned, in the first line, through the free H-ions, less through the formate ions. This becomes clear from the comparison with natrium formate. Here the LD_{50} is ~~based on is~~ at 11.2 g/kg or based on the formate, at 7.41 g/kg. For purposes of comparison the LD_{50} for cooking salt amounts to 3.096 g/kg in intraperitoneal application.

2. Chronic Toxicity

Chronic feeding experiments on Wistar rats with calcium formate (0.2% in the drinking water) have been going on ~~running~~ for more than 3 years. In the experiment, 8 male and 24 female animals with a body weight of 160-210 g were taken at the beginning as well as 8 controls.

According to our measurements, the animals receive daily 150-200 mg of calcium formate per kilogram. We now have the fifth generation without anything ~~extraordinary with~~ extraordinary with regard to growth and fertility having been shown. The organic functions were not disturbed. In particular no disturbances of the eyeground were observed either in vivo or in the later neuropathological ~~investigations~~ examination. In Table 2, the course of growth is given for the first weeks of the test; in Table 3, the fertility of the mother animals from the I calcium formate generation; in Table 4, the course of growth of the I up to the III generation.

Tab. 2. The weight curves for the 4 test and 2 control groups with 4 Wistar rats in each group (160-210g). Data for the mean values and standard deviations

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Tab. 2. Gewichtskurven bei 4 Versuchs- und 2 Kontrollgruppen mit je 4 Wistar-Ratten (160–210 g). Angabe der Mittelwerte und Standardabweichungen

Weeks Wochen	0,2% $Ca(HCOO)_2$ in the drinking water Trinkwasser				Kontrollen Controls	
	I ♀	II ♀	III ♀	IV ♂	V ♀	VI ♂
1	175 ± 9,9	170 ± 12,5	170 ± 11,0	195 ± 9,4	170 ± 11,2	200 ± 9,8
2	205	190	185	250	195	235
3	220	190	195	252	210	250
4	235	200	200	270	225	275
5	240	205	210	285	235	288
6	240	215	220	300	238	295
7	240	220	237	310	240	305
8	257	230	250	320	260	318
10	262	235	255	340	262	340
12	275	240	258	358	270	360
	± 10,6	± 9,8	± 8,5	± 12,4	± 9,0	± 14,6
15	290	245	270	365	280	370
	± 8,5	± 10,5	± 12,0	± 11,8	± 10,6	± 12,7

A further series of tests has been going on for two years in the same course of experiments with a doubled calcium formate dosage (0.4% in drinking water). Our test animals are in the second generation. Here also ~~there are~~ no disturbances in comparison with the control groups have appeared as the pathological-histological research--carried out by Professor Lindner (the Pathological Institute of the University of Hamburg)--~~have yielded as results.~~ has shown.

Tab. 3. The fertility of rats, which have received daily, as did their parents, 200 mg/kg calcium formate. Body weight and length of the fetuses with data regarding the standard deviation.

Tab. 3. Fertilität von Ratten, die täglich – ebenso wie ihre Elterntiere – 200 mg/kg Calciumformiat erhalten hatten. Körpergewicht und Länge der Feten mit Angabe der Standardabweichung

Nr.	Mutterteere, I. Ca-Formiat-Generation		Würfe, II. Ca-Formiat-Generation			Remarks Bemerkungen
	Gewicht in g	Alter in Monaten	Zahl	Gewicht in g	Länge in cm	
a) 0,2% Calciumformiat in Trinkwasser						
1	240	7	11	4,2 ± 0,24	3,7 ± 0,10	Würfe erfolgten in der Zeit vom 17.–18. 11. 1965
2	210	6	6	5,7 ± 0,[illegible]	4,1 ± 0,21	
3	220	7	8	4,8 ± 0,20	3,9 ± 0,16	
4	240	7	12	4,9 ± 0,[illegible]7	4,1 ± 0,26	
5	250	10	7	6,9 ± 0,51	4,7 ± 0,25	2 Tage alt
b) Unbehandelte Kontrollratten:						
1	270	8	10	4,5 ± 0,39	3,9 ± 0,18	
2	240	8	8	5,1 ± 0,43	4,2 ± 0,24	

Mother animals; Litters; Weight; Age in months; No.; Weight; Length; in drinking water; Litters resulting in the time from; days old; Untreated controls

Tab. 4. Chronic feeding tests on Wistar rats. Daily addition of approximately 200 mg/kg calcium formate to the drinking water. Data regarding the mean body weights with standard deviation.

Tab. 4. Chronische Fütterungsversuche an Wistar-Ratten. Tägliche Gabe von rd. 200 mg/kg Calciumformiat im Trinkwasser. Angabe der mittleren Körpergewichte mit Standardabweichung

	I. Generation n = 11	I. Generation n = 10	II. Generation n = 9	II. Generation n = 8	III. Gen. n = 10	Kontrolle n = 10
Geburtsgewicht	5,0 ± 0,46	5,8 ± 0,61	5,5 ± 0,33	4,9 ± 0,55	5,6 ± 0,58	5,2 ± 0,52
1. Woche	16,0	19,5	18,2	15,9	14,8	14,5
2. Woche	34,0	32,5	31,7	28,0	29,1	30,0
3. Woche	49,0	55,0	48,0	48,0	52,0	50,0
4. Woche	79,0 ± 3,8	84,0 ± 3,7	78,0 ± 4,0	85,0 ± 3,2	76,0 ± 3,[illegible]	88,0 ± 4,2
5. Woche	108	118	112	109		113
6. Woche	128	138	136	134		145
7. Woche	160	159	155	153		165
8. Woche	179 ± 3,9	174 ± 4,8	177 ± 6,0	180 ± 4,3		182 ± 5,1
10. Woche	216	220	217	224		221
14. Woche	291	297	301	295		293
18. Woche	297	309	—	—		—
20. Woche	309 ± 5,0	318 ± 6,2	310 ± 6,8	313 ± 5,2		320 ± 5,4

Weight at birth; Weet; control

[1]The test is not pursued further.

A year and a half ago we began a new series of tests with a still higher dosage of formate with the same number of animals at the start (drinking water with a content of 1% natrium formate). A short time ago we did research ~~on six rats with a daily intake of formic acid~~ on the daily formic acid intake and excretion in six rats (see Tab . 5). It is known that 185.4 mg of formic acid are taken in per animal and that 26.2 mg of formic acid are excreted. Thus 13.8% of the total formic acid taken in is eliminated through the urine.

Tab. 5. Daily intake and excretion of formic acid in a chronic test on rats (no. = 6). Mean body weight 375 g. Fed in the drinking water as 1% natrium formate solution. Data regarding the mean values with standard deviation.

a) Natrium formate intake and

Tab. 5. Tägliche Ameisensäureaufnahme und -ausscheidung im chronischen Versuch an Ratten (n = 6). Mittleres Körpergewicht: 375 g. Zufuhr im Trinkwasser als 1% Natriumformiatlösung. Angabe der Mittelwerte mit Standardabweichungen

a) Aufgenommenes Natriumformiat

Quantity in drinking water → Trinkmenge in ml	Na-Formiat mg	Calculated as Berechnet als AS mg
27,4 ± 4,45	274,0 ± 44,5	185,4 ± 30,3

b) Excretion of formic acid

b) Ameisensäure-Ausscheidung

Quantity of urine Urinmenge ml	AS mg %	total AS insges. mg	AS in % der Aufnahme of the intake
12,1 ± 3,46	223,9 ± 43,6	26,2 ± 9,6	13,8 ± 3,1

For purposes of comparison, in other tests with an approximately 10-fold lower feeding of natrium formate, only 3% of the formate fed was excreted in the 24-hour urine.

In the chronic toxicity Studies on the dog--four dogs received 5 g of natrium formate in chopped meat daily--it is shown that

evidentally in the carnivorous animal the oxidation of formic acid is much slower than in the rat. Within 24 hours, about 38-40% of the formate fed is excreted in the urine. In one dog, the test could be carried out for 12 days' time (with 2 empty days); the other dogs refused the pieces of chopped meat after 3 or 4 days.

Professor Lindner of the Pathological Institute of the University of Hamburg carried out the pathological-anatomical examinations. These examinations covered more than 250 Wistar rats. There were included old animals and adult male and female rats from three new generations, which had received over a period of 2 years a daily dose of 200 mg/kg calcium formate in the drinking water.

The macroscopic and histological examination of all the rats treated with calcium formate yielded no findings that aroused suspicions, only slight phagocytosis processes occasionally in moderately proliferated reticulo-endothelial and reticulo-histiocytic elements especially of the lung and spleen, partially also in the lymphatic tissue in so far as it was dissected, thus abdominal lymph nodes etc., however not in the liver. In the liver itself no peculiarities were observed. Apart from 2 benign spontaneous tumors in old animals, there were neither in the digestive tract, including the liver and kidney, nor in the other tissues, any malignant growths to be observed. Thus no signs of a chronic poisoning are to be observed on any of the organs examined, but there are only findings which belong in the range of the normal.

7/

Furthermore the course of fertility and pregnancy as well as the development of the embryos, the number in litters and the growing up of the new generation were observed. It was shown that mother animals of the first, second, and third calcium formate generations which had received calcium formate from their birth on, had brought into the world a normal number of well formed fetuses. The fetuses were distinguished ~~neight~~ neither in body weight nor in body length from the fetuses of the controls. One part of the fetuses (76) were killed and dissected immediately after birth.

Malformations were not observable, pathologically-anatomically, either on the embryos or on the placentas, whithered implants, so-called deciduomata, were not to be found, mutation-causing or teratogenic effects were not identifiable. The capabilities and development of organs were regular.

3. The tests for toxicity and teratogenicity on the chicken embryo.

Natrium formate leads to no increased dying off of the embryos after injection into the air bladder of the hatched chicken egg (48 or 96 hours after the beginning of hatching) in doses of 5 mg, 10 mg or 20 mg per egg. The rate of survival remains on the same level as the survival rate of the controls. The final embryo weights of the eggs treated with natrium formate do not show any deviations. Natrium formate, which is completely eliminated after 10-12 days of hatching time, and to be sure chiefly through oxidation, shows itself as teratogenically not remarkable in the hatched chicken egg.

Vis-a-vis the untreated controls (no. = 1051), no change in the frequency of malformation either quantitatively or qualitatively is observed. On the other hand, with the same kind of test, a highly significant increase in the malformation rate is shown with hydrocortisone (0.025 mg per egg).

The decrease in ~~reduction of~~ formic acid, which had been applied on the fourth hatching day in the form of a natrium salt and which could act upon the embryo continually during the first embryonic period, is clearly to be seen from the adjoining illustration.

AS in the whole egg after injection of 10 mg Na-formate (6.9 mg AS) on the fourth hatching day (total number = 74)

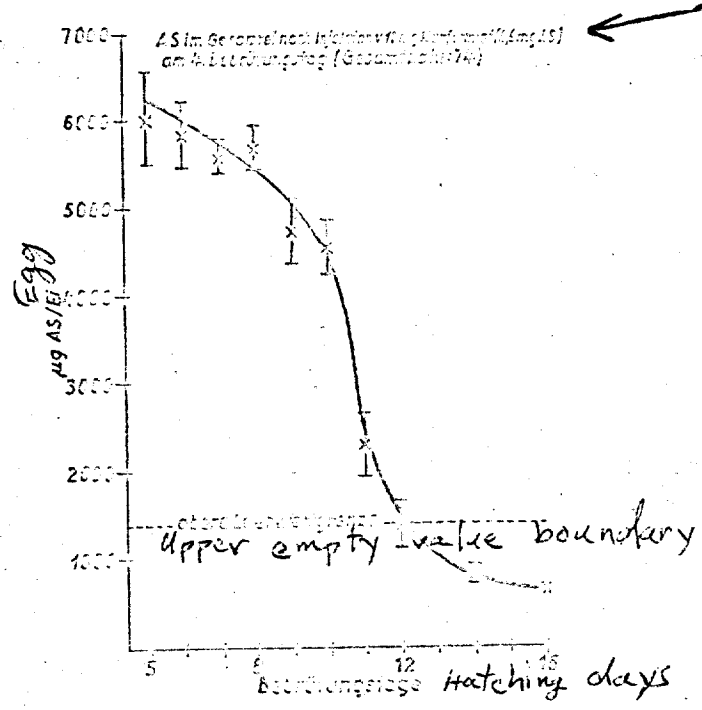

Abb. 1. Ameisensäuregehalt des Gesamteies während der Embryonalentwicklung. Injektion von 10 mg Na-formiat (6,9 mg AS) am 4. Bebrütungstag (n = 74). [Übernommen aus LANGE, G.: Inaug. Diss. Hamburg 1968]

Illustration 1. The formic acid content of the whole egg during the embryonic development. The injection of 10 mg Na-formate (6.9 mg AS) on the fourth hatching day (no. = 74). [taken from Lange, G.: Inaug. Diss. Hamburg 1968).

4. Biochemical-Pharmacological Effects.

After infusion of a 0.2 M Ringer's solution of formic acid (0.918% AS) in a total dose of 1.17 mM/kg, a strong metabolic acidosis is formed in the dog, which is reversable after 4 hours (see Tab. 6). No methaemoglobin arises. The concentration of formic acid reaches a value in the blood of 18.25 mg%[sic] and ~~falls off to~~ declines continually, following an exponential function (curve), in 4 hours to the ~~final value~~ starting value. Similar results--with the exception of the strong pH-decrease in the blood--are obtained with the infusion of a 0.2 M natrium formate solution (1.36%). The biological half-life for formic acid in the dog amounts to 77 minutes. The maximum of the formate oxidation--measureable on the CO_2 excretion--is found one hour after stopping the infusion.

The cat shows a half-life of 67 minutes ~~after~~ with infusion of the same quantity of formate per kilogram of body weight. The half-life in rodents (the rabbit 32 min., the guinea pig 22 min., and the rat 12 min.) is considerably lower. The more rapid oxidation of the formate in herbivorous animals can be connected with the ~~higher~~ larger supply of formic acid and especially of folic acid. According to our research ~~from~~ on the cat, an ~~increase in~~ addition of folic acid leads to an acceleration of the oxidation of formic acid. Correspondingly the antagonists of folic acid, for example methotrexate, inhibit the oxidation of formic acid.

Tab. 6. The infusion of equimolecular solutions of natrium formate and formic acid in the dog. Data for the mean values and standard deviations

Taken from Malorny, G., N. Rietbrock and M. Schneider: Naunyn-Schmiedebergs Arch. exp. Path. u. Pharmak. 250, 419 (1965.

D_0=empty value, D_{50}=50% of the quantity of infusion, D_{100}=100% of the quantity of infusion

Tab. 6. Infusion äquimolarer Lösungen von Natriumformiat und Ameisensäure beim Hund. Angabe der Mittelwerte und Standardabweichungen [Entnommen aus MALORNY, G., N. RIETBROCK und M. SCHNEIDER: Naunyn-Schmiedebergs Arch. exp. Path. u. Pharmak. 250, 419 (1965)].
D_0 = Leerwert, D_{50} = 50% der Infusionsmenge, D_{100} = 100% der Infusionsmenge

			Infusion			Zeit nach Infusion (Time after infusion)							
n	kg		D_0	D_{50}	D_{100}	2	9	29	49	60	120	180	240
a) Infusion einer 0,2 M Natriumformiat-Ringerlösung (pH 6,89), 8 ml/min													
		pH	7,44 ± 0,03	7,41 ± 0,03	7,40 ± 0,03	7,40 ± 0,03	7,39 ± 0,03	7,39 ± 0,01	7,39 ± 0,02	7,41 ± 0,01	7,41 ± 0,02	7,42 ± 0,03	7,42 ± 0,03
6	12,1 ± 2,9	AS mg %	0,70 ± 0,31	11,90 ± 3,48	18,25 ± 4,09	14,51 ± 2,98	12,23 ± 2,00	10,51 ± 2,14	8,33 ± 1,32	7,60 ± 1,74	4,17 ± 2,02	2,35 ± 2,06	0,98 ± 0,89
b) Infusion einer 0,2 M Ameisensäure-Ringerlösung (pH 2,35), 8 ml/min													
		pH	7,45 ± 0,03	7,33 ± 0,07	7,30 ± 0,06	7,31 ± 0,06	7,33 ± 0,04	7,35 ± 0,05	7,37 ± 0,05	7,38 ± 0,05	7,38 ± 0,03	7,41 ± 0,05	7,42 ± 0,03
6	13,0 ± 4,4	AS mg %	1,15 ± 1,18	12,58 ± 4,53	20,28 ± 3,67	15,20 ± 1,84	11,85 ± 1,62	10,65 ± 3,18	8,65 ± 1,17	7,61 ± 1,59	4,57 ± 2,06	2,31 ± 1,70	0,90 ± 0,70

In the metabolism of formic acid, the liver has special importance. If a rabbit, from which the liver has been removed in the most sparing manner possible, is administered a dosage of 0.2 M natrium formate Ringer's solution (1.17 mM/kg), then the formic acid half-life, which amounts to only 35 minutes in the controlled tests, is extended to 130 minutes. The oxidation of formic acid is thus inhibited, the elimination delayed. ~~In vitro also~~ The central position of the liver is recognized as well in experiments in vitro. The rat liver washed free of remains of the blood and homogenized, yields considerable ~~formate~~ quantities of formate with the incubation of the homogenate in the ~~blood chest (?)~~ incubator. After two hours, only 57% of the added formate is ~~are~~ still found.

SUMMARY

According to the results of our research, formic acid and its salts, according to my opinion, up to the determined boundary quantities as they are given for example in List 2 of the revised and completed law concerning foodstuffs of 21 December 1958 (highest daily quantity 1 g, highest concentration 1 g/kg) are not to be regarded as dangerous to health. As the reasons therefore let it be stated:

Formic acid is a normal ~~ingredients~~ constituent of human blood and tissue; it plays a highly important role in the intermediary metabolism at the transfer of (transmission) of C_1 bodies. ¶ Formic acid occurs in certain foodstuffs if only in small quantities, as for example in the musculature of sea fish, in honey, in roasted products, and in some kinds of fruit.

Added formic acid is completely withdrawn and excreted; no danger of cumulation exists.

The chronic feeding of 200 mg/kg calcium formate in the rat in a experiment lasting two years produces no damage attributable to a particular substance with regard to the organic functions and does not inhibit the growth and development of the new generation.

Formic acid in the form of its natrium salt (5 mg, 10 mg, and 20 mg per egg) has no toxic or teratogenic effect in the hatched chicken egg upon the hatched chicken embryo.

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Die akute und chronische Toxizität der Ameisensäure und ihrer Formiate*)

Von G. MALORNY

Mit 1 Abbildung und 6 Tabellen

(Eingegangen am 19. Februar 1969)

Ameisensäure und Formiate wurden verschiedentlich schon als Konservierungsstoffe für bestimmte Lebensmittel verwendet. Über ihre akute und chronische Toxizität sowie ihr Verhalten im Stoffwechsel war aber bis jetzt nur wenig Sicheres bekannt.

Im "Eight Report of the Joint FAO/WHO Expert Committee on Food Additives, Genf 1965" (1) finden sich über die Wirkungen der Ameisensäure nur spärliche Angaben. Es heißt dort: "Exact LD_{50} values are not available." Und an einer anderen Stelle: "Since long-term toxicity studies are lacking, it is not possible to give guidance on an unconditional acceptable daily intake level in man."

*) Bericht an die Fremdstoffkommission der Deutschen Forschungsgemeinschaft.

1. Akute Toxizität

Freie Ameisensäure übt auf alle Gewebe, insbesondere auch auf Schleimhäute, einen starken Reiz aus. In der Konservierungstechnik gelangt sie deswegen meist in Form eines ihrer Salze zur Anwendung. Um den Toxizitätsanteil, der auf die freien Wasserstoffionen entfällt, erfassen zu können, haben wir an der weißen Maus die akute Toxizität (LD_{50}) sowohl für die reine Ameisensäure als auch für ihre Salze bestimmt. In Tab. 1 sind die LD_{50}-Angaben nach oraler Applikation und nach intravenöser Injektion zusammengefaßt.

Tab. 1. LD_{50} für Ameisensäure und ihre Salze (Maus)

Substanz	LD_{50} mg/kg	LD_{50} Range mg/kg	LD_{50} berechnet auf Formiat	Anzahl der Tiere
per os				
Ameisensäure	1,100	1,000– 1,200	1,076	55
Na-Formiat	11,200	9,600–12,800	7,410	45
K-Formiat	5,500	5,000– 6,000	2,950	50
NH_4-Formiat	2,250	2,050– 2,460	1,610	50
Ca-Formiat	1,920	1,280– 2,560	1,330	45
intravenös				
Ameisensäure	145	138–151	142	50
Na-Formiat	807	800–813	534	50
K-Formiat	95	93– 97	51	55
NH_4-Formiat	410	408–412	293	50
Ca-Formiat	154	150–158	107	55

Die höchste Toxizität bei intravenöser Zufuhr besitzt Kaliumformiat, es folgt die freie Ameisensäure und danach Calciumformiat. Am indifferentesten wirkt Natriumformiat; seine Toxizität ist achtmal geringer als die des Kalziumformiat. Als Ursache für diese Unterschiede ist die Eigentoxizität der Kationen anzusehen. Es ist bekannt, daß K^+ und Ca^{++} z. B. bei höherer Dosierung herztoxisch wirken.

Ähnliche Beziehungen findet man bei den oralen LD_{50}-Werten. Die höchste Toxizität zeigt die freie Ameisensäure (1,1 g/kg). Ihre Giftigkeit ist in erster Linie durch die freien H-Ionen bedingt, weniger durch die Formiationen. Dies erhellt aus dem Vergleich mit Natriumformiat. Hier liegt die LD_{50} bei 11,2 g/kg, bzw. auf Formiat bezogen, bei 7,41 g/kg. Vergleichsweise beträgt die LD_{50} für Kochsalz 3,096 g/kg bei intraperitonealer Applikation.

2. Chronische Toxizität

Seit mehr als 3 Jahren laufen chronische Fütterungsversuche an Wistar-Ratten mit Calciumformiat (0,2% im Trinkwasser). In Versuch genommen wurden zu Anfang 8 männliche und 24 weibliche Tiere mit einem Körpergewicht von 160–210 g, dazu 8 Kontrollen.

Nach unseren Messungen nehmen die Tiere 150–200 mg Calciumformiat pro Kilogramm täglich zu sich. Wir stehen gegenwärtig in der 5. Generation, ohne daß sich hier Auffälligkeiten hinsichtlich Wachstum und Fertilität ge-

zeigt hätten. Auch die Organfunktionen waren nicht gestört. Insbesondere wurden keine Augenhintergrundstörungen beobachtet, weder in vivo noch bei der späteren neuropathologischen Untersuchung. In Tab. 2 ist der Wachstumsverlauf in den ersten Versuchswochen wiedergegeben; in Tab. 3 die Fertilität der Muttertiere aus der I. Calciumformiatgeneration; in Tab. 4 der Wachstumsverlauf der I. bis III. Generation.

Tab. 2. Gewichtskurven bei 4 Versuchs- und 2 Kontrollgruppen mit je 4 Wistar-Ratten (160–210 g). Angabe der Mittelwerte und Standardabweichungen

	0,2% $Ca(HCOO)_2$ im Trinkwasser				Kontrollen	
Wochen	I ♀	II ♀	III ♀	IV ♂	V ♀	VI ♂
1	175 ± 8,9	170 ± 12,5	170 ± 11,0	195 ± 9,4	170 ± 11,2	200 ± 9,8
2	205	190	185	230	195	235
3	220	190	195	250	210	260
4	230	200	200	270	225	275
5	240	205	210	285	235	288
6	240	215	220	300	238	290
7	240	220	225	310	240	305
8	255	220	230	320	260	318
10	262	228	235	340	268	340
12	275 ± 10,6	240 ± 9,8	238 ± 9,5	353 ± 13,4	270 ± 9,0	360 ± 14,2
15	283 ± 9,5	245 ± 10,5	240 ± 13,0	355 ± 11,8	290 ± 10,6	370 ± 12,7

Seit 2 Jahren läuft bei gleichem Versuchsansatz eine weitere Versuchsserie mit der doppelten Calciumformiat-Dosis (0,4% im Trinkwasser). Unsere Versuchstiere stehen in der 2. Generation. Auch hier sind keine Störungen im Vergleich zu den Kontrollgruppen aufgetreten, wie die pathologisch-histologischen Untersuchungen – durchgeführt von Herrn Prof. LINDNER (Pathologisches Institut der Universität Hamburg) – ergeben haben.

Tab. 3. Fertilität von Ratten, die täglich – ebenso wie ihre Elterntiere – 200 mg/kg Calciumformiat erhalten hatten. Körpergewicht und Länge der Feten mit Angabe der Standardabweichung

Nr.	Muttertiere, I. Ca-Formiat-Generation: Gewicht in g	Alter in Monaten	Würfe, II. Ca-Formiat-Generation: Zahl	Gewicht in g	Länge in cm	Bemerkungen
a) 0,2% Calciumformiat im Trinkwasser:						
1	240	7	11	4,2 ± 0,24	3,7 ± 0,18	Würfe erfolgten
2	210	6	6	5,7 ± 0,56	4,1 ± 0,21	in der Zeit vom
3	220	7	8	4,8 ± 0,23	3,9 ± 0,16	17.–19. 11. 1965
4	240	7	12	4,9 ± 0,47	4,1 ± 0,26	
5	280	10	7	6,9 ± 0,51	4,7 ± 0,26	2 Tage alt
b) Unbehandelte Kontrollratten:						
1	270	8	10	4,5 ± 0,39	3,9 ± 0,18	
2	240	8	8	5,1 ± 0,43	4,2 ± 0,24	

Tab. 4. Chronische Fütterungsversuche an Wistar-Ratten. Tägliche Gabe von rd. 200 mg/kg Calciumformiat im Trinkwasser. Angabe der mittleren Körpergewichte mit Standardabweichung

	I. Generation n = 11	I. Generation n = 10	II. Generation n = 9	II. Generation n = 8	III. Gen. n = 10	Kontrolle n = 10
Geburtsgewicht	5,0 ± 0,46	5,8 ± 0,61	5,5 ± 0,33	4,9 ± 0,55	5,6 ± 0,58	5,2 ± 0,52
1. Woche	16,0	19,5	18,2	15,9	14,8	14,5
2. Woche	34,0	32,5	31,7	28,0	29,1	33,0
3. Woche	49,0	55,0	48,0	48,0	52,0	53,0
4. Woche	79,0 ± 3,8	84,0 ± 3,7	78,0 ± 4,0	85,0 ± 3,2	76,0 ± 3,6 [1]	83,0 ± 4,2
5. Woche	108	118	112	109		113
6. Woche	128	138	136	134		145
7. Woche	160	159	155	153		165
8. Woche	179 ± 3,9	174 ± 4,8	177 ± 6,0	180 ± 4,3		182 ± 5,1
10. Woche	216	220	217	224		221
14. Woche	291	297	301	295		293
18. Woche	297	309	—	—		—
20. Woche	309 ± 5,0	318 ± 6,2	310 ± 6,8	313 ± 5,2		320 ± 5,8

Vor $1^1/_2$ Jahren setzten wir eine neue Versuchsserie mit noch höherer Formiatdosis bei gleicher Ausgangstierzahl an (Trinkwasser mit einem Gehalt von 1% Natriumformiat). Vor kurzem untersuchten wir bei 6 Ratten die tägliche Ameisensäureaufnahme und -ausscheidung (s. Tab. 5). Man erkennt, daß pro Tier 185,4 mg Ameisensäure aufgenommen und 26,2 mg Ameisensäure ausgeschieden werden. Somit werden 13,8% der insgesamt aufgenommenen Ameisensäure durch den Urin eliminiert.

Tab. 5. Tägliche Ameisensäureaufnahme und -ausscheidung im chronischen Versuch an Ratten (n = 6). Mittleres Körpergewicht 375 g. Zufuhr im Trinkwasser als 1% Natriumformiatlösung. Angabe der Mittelwerte mit Standardabweichungen

a) Aufgenommenes Natriumformiat

Trinkmenge in ml	Na-Formiat mg	Berechnet als AS mg
27,4 ± 4,45	274,0 ± 44,5	185,4 ± 30,3

b) Ameisensäure-Ausscheidung

Urinmenge ml	AS mg %	AS insges. mg	AS in % der Aufnahme
12,1 ± 3,46	223,9 ± 43,6	26,2 ± 9,6	13,8 ± 3,1

[1]) Versuch wurde nicht weiter verfolgt.

Vergleichsweise wurden in anderen Versuchen bei etwa 10fach niedrigerer Natriumformiatzufuhr nur 3% des zugeführten Formiats, im 24-Stunden-Urin ausgeschieden.

Bei chronischen Toxizitätsstudien am Hund – vier Hunde erhielten 5 g Natriumformiat im Fleischhack täglich – läßt sich zeigen, daß offenbar beim Fleischfresser die Oxydation der Ameisensäure viel langsamer verläuft als bei der Ratte. Es werden innerhalb von 24 Stunden etwa 38–40% des zugeführten Formiats im Urin ausgeschieden. Bei einem Hund konnte der Versuch 12 Tage durchgeführt werden (mit 2 Leertagen); die anderen Hunde verweigerten nach 3–4 Tagen die Annahme der Fleischhappen.

Die pathologisch-anatomischen Untersuchungen nahm Herr Prof. LINDNER vom Pathologischen Institut der Universität Hamburg vor. Sie erstreckten sich auf mehr als 250 Wistar-Ratten. Es handelte sich um Alttiere und um erwachsene männliche und weibliche Ratten aus 3 Nachwuchsgenerationen, die über einen Zeitraum von 2 Jahren eine tägliche Dosis von 200 mg/kg Calciumformiat im Trinkwasser erhalten hatten.

Die makroskopische und histologische Untersuchung aller mit Calciumformiat behandelten Ratten ergab keine verdachterregenden Befunde, nur stellenweise geringe Phagozytosevorgänge in mäßig proliferierten retikuloendothelialen und retikulo-histiozytären Elementen speziell von Lunge und Milz, teilweise auch im lymphatischen Gewebe, soweit es mitgeschnitten wurde, also Bauchlymphknoten etc., jedoch nicht in der Leber. An der Leber selbst waren keinerlei Besonderheiten festzustellen. Abgesehen von 2 gutartigen Spontantumoren bei Alttieren waren weder im Digestionstrakt, einschließlich Leber und Niere, noch in den anderen Geweben bösartige Geschwülste festzustellen. Somit sind Zeichen einer chronischen Intoxikation an keinem der untersuchten Organe festzustellen, sondern lediglich Befunde, die in den Bereich der Norm gehören.

Es wurden ferner Fertilität und Schwangerschaftsverlauf, sowie Entwicklung der Embryonen, Wurfzahl und Heranwachsen der neuen Generation beobachtet. Es zeigte sich, daß Muttertiere der 1., 2. und 3. Calciumformiat-Generation, die von ihrem Lebensbeginn an Calciumformiat erhalten hatten, eine normale Zahl wohl ausgebildeter Feten zur Welt gebracht hatten. Die Feten unterschieden sich weder im Körpergewicht noch in der Körperlänge von den Feten der Kontrollen. Ein Teil der Feten (76) wurde gleich nach der Geburt getötet und seziert.

Pathologisch-anatomisch waren Verbildungen an den Embryonen oder an der Placenta nicht feststellbar, abgestorbene Implantate, sogen. Deciduomata, waren nicht auffindbar, mutationsauslösende oder teratogene Wirkungen nicht nachweisbar, Organanlagen und -entwicklungen regelrecht.

3. Toxizitäts- und Teratogenitätsprüfungen am Hühnerembryo

Natriumformiat führt bei Injektion in die Luftblase des bebrüteten Hühnereies (48 bzw. 96 h nach Bebrütugsbeginn) in Dosen von 5 mg, 10 mg oder 20 mg/Ei zu keinem vermehrten Absterben der Embryonen. Die Überlebensrate liegt auf der gleichen Höhe wie die der Kontrollen. Die Embryonen-Endgewichte der mit Natriumformiat behandelten Eier lassen keine Abweichungen erkennen. Natriumformiat, das nach 10–12 Tagen Bebrütungsdauer vollständig eliminiert wird, und zwar vorzugsweise durch Oxydation, erweist sich am bebrüteten Hühnerei als teratogen unauffällig. Gegenüber den unbehandelten Kontrollen (n = 1051) wird keine Änderung der Mißbildungshäufigkeit weder in quantitativer noch qualitativer Hinsicht beobachtet. Am gleichen Versuchsmodell läßt sich hingegen mit Hydrocortison (0,025 mg/Ei) eine hochsignifikante Zunahme der Mißbildungsrate beweisen.

Der Abbau der Ameisensäure, die am 4. Brütungstag in Form ihres Natriumsalzes appliziert worden war und während der 1. Embryonalperiode kontinuierlich auf den Embryo einwirken konnte, ist aus der beigefügten Abbildung deutlich zu erkennen.

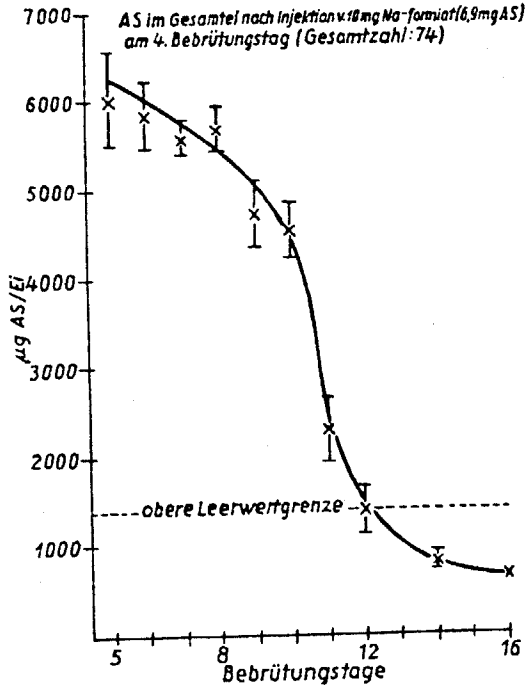

Abb. 1. Ameisensäuregehalt des Gesamtei während der Embryonalentwicklung. Injektion von 10 mg Na-formiat (6,9 mg AS) am 4. Bebrütungstag (n = 74). [Entnommen aus LANGE, G.: Inaug. Diss. Hamburg 1968]

4. Biochemisch-pharmakologische Wirkungen

Nach Infusion einer 0,2 M Ameisensäure-Ringer-Lösung (0,918% AS) in einer Gesamtdosis von 1,17 mM/kg bildet sich beim Hund eine starke metabolische Acidose aus, die nach 4 h reversibel ist (s. Tab. 6). Es entsteht kein Methaemoglobin. Die Ameisensäure-Konzentration erreicht im Blut einen Wert von 18,25 mg% und fällt stetig, einer Exponentialfunktion folgend, in 4 h zum Ausgangswert ab. Ähnliche Ergebnisse – mit Ausnahme des starken pH-Abfalls im Blut – erhält man bei Infusion einer 0,2 M Natriumformiat-

Tab. 6. Infusion äquimolarer Lösungen von Natriumformiat und Ameisensäure beim Hund. Angabe der Mittelwerte und Standardabweichungen

[Entnommen aus MALORNY, G., N. RIETBROCK und M. SCHNEIDER: Naunyn-Schmiedebergs Arch. exp. Path. u. Pharmak. **250**, 419 (1965)].

D_0 = Leerwert, D_{50} = 50% der Infusionsmenge, D_{100} = 100% der Infusionsmenge

n	kg		Infusion			Zeit nach Infusion								
			D_0	D_{50}	D_{100}	2	9	20	40	60	120	180	240
a) Infusion einer 0,2 M Natriumformiat-Ringerlösung (pH 6,89), 8 ml/min													
		pH	7,44 ± 0,03	7,41 ± 0,03	7,40 ± 0,03	7,40 ± 0,03	7,39 ± 0,03	7,39 ± 0,01	7,39 ± 0,02	7,41 ± 0,01	7,41 ± 0,02	7,42 ± 0,03	7,42 ± 0,03
6	12,1 ± 2,9	AS mg %	0,70 ± 0,31	11,90 ± 3,48	18,25 ± 4,09	14,51 ± 2,08	12,23 ± 2,00	10,51 ± 2,14	8,33 ± 1,82	7,60 ± 1,74	4,17 ± 2,02	2,35 ± 2,06	0,98 ± 0,89
b) Infusion einer 0,2 M Ameisensäure-Ringerlösung (pH 2,35), 8 ml/min													
		pH	7,45 ± 0,03	7,33 ± 0,07	7,30 ± 0,06	7,31 ± 0,06	7,33 ± 0,04	7,35 ± 0,05	7,37 ± 0,05	7,38 ± 0,05	7,38 ± 0,03	7,41 ± 0,05	7,42 ± 0,03
6	13,0 ± 4,4	AS mg %	1,15 ± 1,18	12,58 ± 4,53	20,28 ± 3,57	15,30 ± 1,84	11,85 ± 1,52	10,65 ± 3,18	8,65 ± 1,17	7,61 ± 1,59	4,57 ± 2,06	2,31 ± 1,70	0,90 ± 0,70

lösung (1,36%) Die biologische Halbwertszeit (HWZ) für Ameisensäure beim Hund beträgt 77 min. Das Maximum der Formiatoxydation – meßbar an der CO_2-Ausscheidung – findet man 1 h nach Absetzen der Infusion.

Die Katze zeigt eine HWZ von 67 min bei Infusion der gleichen Formiatmenge pro Kilogramm Körpergewicht. Erheblich niedriger liegt die HWZ bei den Nagern (Kaninchen 32 min, Meerschweinchen 22 min und Ratte 12 min). Die raschere Formiatoxydation bei Pflanzenfressern mag mit dem höheren Angebot an Ameisensäure und vor allem an Folsäure zusammenhängen. Nach unseren Untersuchungen an der Katze führt Zulage von Folsäure zu einer Beschleunigung der Ameisensäure-Oxydation. Dementsprechend hemmen Folsäure-Antagonisten, z. B. Methotrexat, die Ameisensäure-Oxydation.

Im Stoffwechsel der Ameisensäure kommt der Leber eine besondere Bedeutung zu. Belastet man Kaninchen, bei denen die Leber in möglichst schonender Weise ausgeschaltet wurde, mit einer 0,2 M Natriumformiat-Ringer-Lösung (1,17 mM/kg), so wird die Ameisensäure-Halbwertszeit, die in den Kontrollversuchen nur 35 min beträgt, auf 130 min verlängert. Die Oxydation der Ameisensäure ist also gehemmt, die Elimination verzögert. Auch bei Versuchen in vitro läßt sich die zentrale Stellung der Leber erkennen. Die von Blutresten freigespülte und homogenisierte Rattenleber setzt bei Inkubierung des Homogenats im Brutschrank erhebliche Formiatmengen um. Nach 2 h findet man nur noch 57% des zugesetzten Formiats wieder.

Zusammenfassung

Gemäß unserer Untersuchungsergebnisse sind Ameisensäure und ihre Salze meines Erachtens bis zu bestimmten Grenzmengen, wie sie beispielsweise in der Liste 2 des geänderten und ergänzten Lebensmittelgesetzes vom 21. 12. 1958 angegeben sind (Tageshöchstmenge 1 g, Höchstkonzentration 1 g/kg), als nicht gesundheitsschädlich anzusehen. Als Gründe seien angeführt:

Ameisensäure ist ein normaler Bestandteil des menschlichen Blutes und der Gewebe; sie spielt im intermediären Stoffwechsel eine hochwichtige Rolle bei der Übertragung von C_1-Körpern.

Ameisensäure kommt in bestimmten Lebensmitteln, wenn auch nur in geringen Mengen, vor, wie z. B. in der Seefischmuskulatur, im Honig, in Röstprodukten und in einigen Früchten.

Zugeführte Ameisensäure wird vollständig abgebaut und ausgeschieden; es besteht keine Cumulationsgefahr.

Die chronische Zufuhr von 200 mg/kg Calciumformiat bei der Ratte im Zweijahres-Versuch führt keine stoffspezifischen Schädigungen der Organfunktionen herbei und hemmt nicht Wachstum und Entwicklung neuer Generationen.

Am bebrüteten Hühnerei wirkt Ameisensäure in Form ihres Natriumsalzes (5 mg, 10 mg und 20 mg/Ei) nicht toxisch und nicht teratogen auf den bebrüteten Hühnerembryo.

Literatur

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Journal for Nutrition Science, Vol. 9, brochure 4

METABOLISM TESTS WITH SODIUM FORMATE AND FORMIC ACID IN MAN. *)

By G. MALORNY

With 2 illustrations and 7 tables.

(Received on February 19,1969).

To complement our animal experiment work concerning the acute and chronic toxicity of formic acid (AS) and of its salts we also undertook examinations regarding the metabolism of said substances in man. Since we found that the biological half-time (HWZ) of the AS varies considerably in single animal species, we considered it desirable to determine the HWZ in man as well. We furthermore reviewed the effect of AS on the acid base metabolism of the blood as well as on the AS elimination velocity in the urine.

Sixteen persons made themselves voluntarily available for the examinations, some of them on several occasions. The following questions had to be clarified:

1. Absorption, tolerability and excretion of limiting quantities for AS, as indicated in List II of the modified and supplemented LMG of December 21, 1958 (maximum daily quantity 1 g, maximum concentration 1 g/kg). This quantity, as well as the double and triple quantity, was administered in the form of sodium salt, and for some tests, in the form of pure AS as well.

2. Verification of the biological HWZ in the blood plasma after oral administration of 2 & 3 g of AS respectively, as Na-salts. The excretion velocity was to be determined on the basis of hourly taken urine samples.

3. Investigation of the acid base metabolism in the blood when loaded with 2 or 3 g respectively, of AS in the form of Na formate, and in some cases also when loaded with 1 or 2 g respectively, of pure AS.

*) Report submitted to the Foreign Substance Commission of the German Research Association (June 1958).

Applied methods.

1. Quantitative determination of AS in the blood and in the urine according to a method developed in our Institute by RIETBROCK and HINRICHS (1964). The method is based on the reduction of AS into formaldehyde by nascent hydrogen in the presence of metallic magnesium and 25% HCl in ice bath. The developing formaldehyde is determined with chromotropic acid according to BREMANIS (1949). The isolation of AS from blood and urine is performed by means of water vapor distillation of the acidified samples.

2. Micro-procedure of ASTRUP and others for the determination of pH, p CO_2, of the standard bicarbonate and of the excess base in the blood.

Results

1. Formic acid excretion in man.

The AS consumed in food is for the most part oxidized in the course of metabolism and only a minor part of the same is excreted in the urine. The daily excretion is indicated as amounting to approximately 25 mg. In our tests normal excretion amounts to somewhat less, i.e. it is 13 mg in 24 hours (see Table 1).

When 1,48 g Na-formate (1 g AS) is orally administered, a total of 34 ± 4,5 mg AS is excreted in the 24-hour urine. After substraction of the void value (Leerwert), this will amount to 21 ± 3,5 mg AS in 24 hours. When said volume is correlated with the administered 1000 mg, it results that 2,1% of the additionally assimilated AS is excreted in the urine.

TABLE 1. Formic acid excretion in the urine in man after the oral intake of 1,48 g sodium formate (~ 1 g formic acid).

3 Nr.	4 Vpn.,	5 Alter	6 Urin-Leerwerte Vol.	pH	mg AS insges. 7	Vol. 8	Nach Formiatgabe mg AS insges. 9	mg AS abzügl. Leerwert 10	Ausscheidung in % abzügl. Leerwert 11
1	H. B.	27 ♀	730	5,78	13,30	600	57,42	44,12	4,41
2	E. F.	53 ♀	710	5,33	11,01	1255	33,20	22,19	2,22
3	V. G.	48 ♂	1200	6,04	30,25	1365	68,40	38,15	3,82
4	G. G.	26 ♂	840	5,62	11,23	1454	42,50	31,27	3,13
5	R. M.	28 ♂	1350	5,92	9,48	870	34,70	25,22	2,52
6	G. T.	42 ♀	660	5,34	8,97	1035	21,31	12,34	1,23
7	B. W.	26 ♂	1100	5,05	13,61	1580	24,23	10,62	1,06
8	G. G.	27 ♂	645	6,05	12,21	735	37,52	25,31	2,53
9	G. T.	42 ♀	610	5,20	6,84	795	20,92	14,08	1,41
10	P. S.	29 ♂	1400	6,34	12,62	1525	26,89	14,27	1,43
11	St. B.	27 ♂	1200	5,34	13,91	1920	17,92	4,01	0,40
12	B. J.	25 ♀	560	5,70	13,08	710	23,85	10,78	1,09
12	Mittelwerte $\bar{x}$		917	5,63	13,04	1154	34,08	21,64	2,16
13	(deren mittl. Fehler) $S\bar{x}$		± 90	±0,10	± 1,68	± 119	± 4,48	± 3,52	± 0,35

1. Urine - void values 2. After formate administration
3. No. 4. Test subject 5. Age 6. Volume 7. mg AS total
8. Volume 9. mg AS total 10. mg AS less void value
11. in %, less void value 12. Average values 13. (their average errors).

The question is raised here concerning the velocity of the AS excretion in the urine. Table 2 contains the answer. When 1,48 g Na formate is taken (Group 2 on the Table), the major part of the AS is excreted during the first 6 hours. This effect is especially conspicuous in Group 3 of the Table, in which case--after administration of 2,96 g Na formate (2 g AS)-- 64,3 mg AS was already eliminated within 6 hours, while the total daily excretion was 77,8 mg AS. Starting from the 12th hour, the control values are reached again in all tests.

The results for Group 3 on Table 2 are of interest from several viewpoints. When the void value is substracted, a total of 66,11 mg AS is excreted in 24 hours, i.e .3,3% of the supplied 2000 mg AS.

TABLE 2. Oral doses of sodium formate, distributed in ground meat. Group 1 = control with ground meat only; Group 2 = 1,48 g Na formate in ground meat; Group 3 = 2,96 g Na formate in ground meat.

Tab. 2. Orale Gaben von Natriumformiat in Hackfleisch verteilt. Gruppe 1 = Kontrolle mit Hackfleisch allein Gruppe; 2 = 1,48 g Na-formiat im Hackfleisch; Gruppe 3 = 2,96 g Na-formiat im Hackfleisch

3' Gruppe	n	h	1 Ameisensäure mg/100 ml	4 mg insgesamt	2 AS-Ausscheid. in % d. Zufuhr 5	6 Urin pH	Vol. ml	7 Bemerkungen
1	5	0- 6	1,42 ± 0,17	3,53 ± 0,74	—	6,01 ± 0,14	272 ± 62	
		6-12	1,72 ± 0,30	2,97 ± 0,60	—	5,87 ± 0,21	185 ± 31	
		12-24	1,99 ± 0,30	5,25 ± 0,98	—	5,60 ± 0,09	269 ± 58	
		0-24		11,73 ± 1,25			726 ± 82	
2	12	0- 6	8,34 ± 1,52	21,97 ± 2,59	2,20 ± 0,26	6,52 ± 0,20	326 ± 57	Keine Störungen 9
		6-12	2,71 ± 0,62	6,74 ± 1,93	0,67 ± 0,19	6,30 ± 0,14	283 ± 43	1 Inder erbrach 10
		12-24	1,49 ± 0,28	5,37 ± 1,22	0,54 ± 0,12	5,52 ± 0,10	413 ± 67	(Versuch wurde nicht 11
		0-24 8		34,08 ± 4,48	3,41 ± 0,45		1022 ± 118	gewertet)
		abzüglich Leerwert		22,35	2,24			
3	7	0- 6	14,72 ± 2,83	64,33 ± 5,07	3,21 ± 0,42	7,08 ± 0,11	438 ± 103	1 Vp gab vorübergehende
		6-12	2,53 ± 0,32	6,80 ± 0,71	0,34 ± 0,04	6,59 ± 0,28	276 ± 20	13 Kopfschmerzen an; bei
		12-24	2,13 ± 0,33	6,71 ± 0,92	0,34 ± 0,05	5,88 ± 0,16	369 ± 76	fast allen Durstgefühl
		0-24		77,84 ± 8,98	3,89 ± 0,45		1033 ± 125	14 (Hack gesalzen)
		abzüglich Leerwert 12		63,11	3,20			

1. Formic acid 2. AS-excretion 3. Group 4. mg total
5. in % of the supply 6. Urine pH 7. Remarks
8. less void value 9. no disorders 10. 1 Inder vomited
11. (Test was not evaluated) 12. Less void value
13. 1 test subject complained of temporary headaches; all experienced thirst sensation. 14. (Ground meat unsalted).

The percentage is only slightly higher after the administration of 1000 mg AS, in which case 2,1% was verified. Accordingly, no accumulation of AS occurs in the organism.

The survey of the urine pH shows that during the time interval of the maximal AS-elimination the urine pH shifts into the alkaline zone. The explanation is simple. Base equivalents are released following the oxidative elimination of the major part of the AS; said base equivalents are excreted and thereby act diuretically (see below).

2. Determination of the biological half-time (HWZ) and of the elimination velocity for formic acid.

The biological HWZ for AS has, to the best of my knowledge, not been measured to date in man; it is higher for carnivores as compared to herbivores. Conclusions concerning man are not unconditionally admissible.

The most precise values for HWZ are obtained when the substance to be examined is intravenously administered. We could however demonstrate with tests in dogs that the AS-level in the plasma speedily reaches high values without oral AS administration as well, because the resorption velocity is high (see below). Therefore identical half-times are obtained by means of plasma-concentration verification.

Illustration 1 represents a test during which test subject Ju. had taken 2,96 g Na formate dissolved in well water, in the morning while fasting. Blood samples were taken from the arm vein in intervals of 30 min. During this test the plasma-HWZ amounted to 45'.

The AS content of the hourly collected urine samples declined speedily. As shown on Table 3, 84 mg AS was already eliminated during the 1st hour, which amounts to 52% of the total quantity, i.e. 161 mg AS.

In the 2nd hour, this amounted to as much as 73,2% and to 84,5% in the 3rd hour. The speedy elimination is conspicuous.

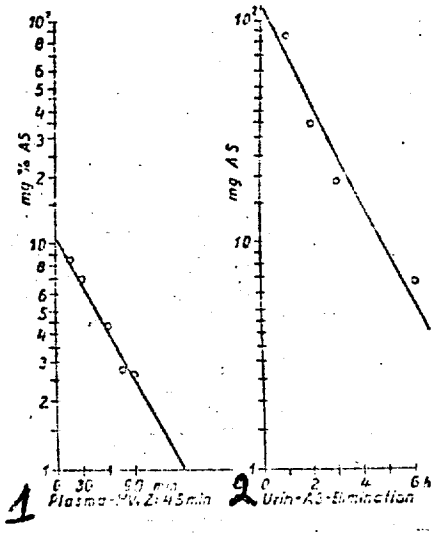

ILLUSTRATION 1. 2,96 g Na formate per os (~ 2 g formic acid) Test subject Ju ♀, 57,5 kg.

1. Plasma - HwZ : 45 min. 2. Urine AS-elimination.

TABLE 3. Formic acid concentration and excretion in the urine after oral administration of 2,96 g sodium formate (2 g AS). Test subject B.J., age 27 years, ♀ 38,5 mg AS/kg.

1 AS-Gehalt des Plasmas min	mg/100 ml	2 h	AS-Ausscheidung im Urin mg/100 ml	3 Gesamtausscheidung in mg	4 in % d. Einnahme	pH	Urin Vol/ml 5
6 Leerwert	1,32	6 Leerwert 0-24		13,08		5,70	560
15	8,50	0- 1	140,00	84,00	4,20	6,54	60
30	6,95	1- 2	77,80	34,20	1,71	7,44	44
45	4,94	2- 3	5,45	18,30	0,92	6,68	335
60	4,32	3- 6	1,67	6,00	0,30	7,38	395
75	2,78	6-12	2,24	5,26	0,26	6,70	235
90	2,63	12-24	3,59	12,58	0,63	6.12	350
				160,94	8,05		1410
		7 abzüglich Leerwert		147,86	7,40		

1. AS content of the plasma 2. AS excretion in the urine
3. Total excretion in mg 4. in % of consumption
5. Urine vol/ml 6. Void value 7. less void value.

The behavior of the urine pH is noteworthy as well. It unequivocally shifts into the alkaline zone and slowly drops to standard after 12 hours only.

The urine volume is $1\frac{1}{2}$ times increased as compared to the control value. The Na formate acts as diuretic.

Findings identical with those for test subject Ju. result also with test subject Gr. Illustration 2 shows a test performed with a large dose Na formate, i.e. 4,44 g (corresponding to 3 g AS). The plasma HWZ for AS amounted in this test to 46 minutes. It is remarkable here as well that the AS is eliminated by the kidneys with unusual speed.

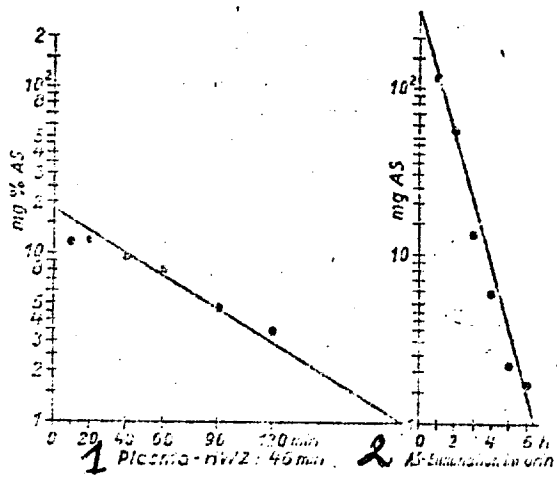

ILLUSTRATION 2. 4,44 g Na formate per os (~3 g formic acid); test subject Gr. ♂ 74 kg.
1. Plasma . . 2. AS elimination in the urine

Table 4 offers more specific information. Here the urine values for 2 doses of Na formate are recorded. After Dose I (2,96 g Na formate) 84 mg of a total of 107 mg AS is excreted during the first two hours, i.e. 75,5%. After Dose II (4,44 g Na formate) the test subject excreted 56% within 1 hour, and an additional 27% during the 2nd hour from the total intake volume, which is 83% in the first two hours. A transient alkalization is observed here in the urine as well, simultaneously with increased urine production.

TABLE 4. Formic acid excretion in the urine after oral administration of 2 doses sodium formate. I) 2,96 g sodium formate; II) 4,44 g sodium formate. Test subject Gr., 25 years old, 74 kg.

Tab. 4. Ameisensäureausscheidung im Urin nach oraler Gabe von 2 Dosen Natriumformiat. I 2,96 g Natriumformiat; II 4,44 g Natriumformiat. Vp. Gr. 25 J. 74 kg

	1 Harnprobe h	2 Menge ml	pH	3 AS-Ausscheidung im Urin mg/100 ml	mg ges.	4 Gesamtausscheidung in % der aufgenommenen Menge	5 Bemerkungen
6	I Leerwert (0–24)	645	5,87	1,93	12,45	—	
	0– 2	100	7,55	84,00	84,00	4,20	
	2– 3	30	7,66	17,90	5,37	0,27	
	3– 6	235	6,63	2,71	6,38	0,32	
	6–12	400	6,99	1,87	7,48	0,37	
	12–24	200	5,63	1,87	3,74	0,19	
	0–24	965			107,00	5,35	
		7 abzüglich Leerwert			94,55	4,72	
II	0– 1	52	6,31	227,00	118,00	3,94	30 min nach Einnahme
	1– 2	46	6,85	122,50	56,40	1,88	8 Müdigkeit
	2– 3	60	6,30	22,30	13,38	0,45	45 min nach Einnahme
	3– 4	120	6,50	4,94	5,94	0,20	9 sehr angeregt
	4– 5	75	6,74	2,96	2,22	0,07	Danach normales
	5– 6	100	6,68	1,72	1,72	0,06	Befinden
	6–12	430	7,14	1,92	8,25	0,28	
	12–24	255	7,00	1,72	4,38	0,15	
	0–24	1138			216,8	7,03	
		7 abzüglich Leerwert			197,85	6,38	

1. Urine sample 2. Volume, ml 3. AS excretion in the urine mg/ 100 ml mg total
4. Total excretion in % of the quantity taken
5. Remarks 6. Void value 7. less void value
8. 30 min after ingestion, lassitude
9. 45 minutes after ingestion, strongly stimulated; condition then returns to normal.

TABLE 5 . Formic acid excretion in the urine after oral ingestion of 2 g formic acid. Test subject: Vp.

Tab. 5. Ameisensäureausscheidung im Urin nach oraler Einnahme von 2 g Ameisensäure Vp Gr.

1 Harnprobe h	2 Menge ml	pH	3 AS-Ausscheidung im Urin mg/100 ml	mg insgesamt	in % der Einnahme
4 Leerwert (0–24)	645	5,87	1,93	12,45	
0– 1	35	5,77	29,00	10,15	
1– 2	48	5,94	57,76	27,70	
2– 3	44	8,01	32,80	14,42	
3– 4	35	6,37	9,07	3,16	
4– 5	63	7,54	4,52	2,98	
5– 6	59	7,78	2,65	1,56	
6–24	1170	6,73	2,44	28,66	
0–24	1157			88,57	
5 abzügl. Leerwert:				76,12	3,81

1. Urine sample. 2. Volume.
3. AS excretion in the urine
   mg/100 ml mg total in % of ingestion
4. Void value. 5. Less void value.

Three self-tests were likewise performed with pure AS. The results coincide largely with findings obtained with Na formate. The ingestion of 2 g AS as a watery solution while fasting caused transient nausea and minor apathy. The test subject felt comfortable after 10 to 15 minutes; the hourly AS excretion values in the urine are shown on Table 5. The excretion total in % of ingestion amounts to 3,81% after 24 hours. Accordingly, the value is within the order of magnitude of the comparable Na formate value (see Table 2, Group 3).

3. Effect on the acid base metabolism

We were able to determine in the course of earlier tests with dogs that a transient metabolic acidosis occurs after the infusion of 0,2 M formic acid Ringer solution (MALORNY, RIETBROCK and SCHNEIDER, 1965). It begins to decline after 3 hours (see aforementioned study). Following the infusion of Na formate, as in case of the dog, a blood pH drop was observed, which , however, was minor.

Our tests in man are shown on Table 6. The blood values at issue are those of test subject Gr, whose values in urine (Table 4) were discussed earlier. No conspicuous symptoms are evident regarding the behavior of the actual blood pH either after Dose I (2,96 g Na formate) or after Dose II (4,44 g Na formate). Slight fluctuations are conditioned by respiration. The standard bicarbonate remains constant.

During the examinations of the acid base metabolism in man 2 phenomena are observed which require discussion:

1. The absence of a metabolic acidosis in the blood.
2. The transient alkalinization of the urine.

An explanation can be found for both phenomena when the fate of the Na formate in the body is followed.

The de-ionization of Na formate takes place in the stomach. The free lipoid-soluble acid is resorbed at the site, i.e. in the stomach. This process evidently requires little time. As Table 4 shows, 10 minutes after oral administration of 4,4 g Na formate, 11,8 mg% of AS can already be verified in the plasma.

The pK value of AS is 3,7, i.e. with a pH of 3,7, 50% of AS is dissociated. The comparable pK value of lactic acid is 3,86; that is: it is approximately at the same level as the pK value of AS. As known, lactic acid is likewise resorbed in the stomach.

With a pH of 7,36, the resorbed AS is immediately transformed in the blood into the completely dissociated Na formate.

Since--as shown on Table 6--only 4,72% of the total AS intake is excreted in the urine after Dose I and 6,58% is excreted after Dose II, and therefore the major part of AS is oxidized in the course of metabolism, the conclusion can be reached that a base excess forms thereby in the blood. The hydrocarbonate ion replaces the oxidized formate anion. More bases are excreted and the urine becomes alkaline. A similar phenomenon is evident [illegible] (end of sentence illegible).

TABLE 6. Formic acid content of the plasma and acid base metabolism of the blood after oral administration of 2 doses of sodium formate. Test subject 25 years old, ♂, 74 kg. Dose I: 2,96 g sodium formate, corresponding to 2 g formic acid (27,0 mg/kg); Dose II : 4,44 g sodium formate, corresponding to 3 g formic acid (40,6 mg/kg).

1 Blutprobenentnahme	2 Plasma-AS mg/100 ml	3 Aktuelles Blut-pH	pCO_2 mmHg	4 Standard-Bic. mäqu/l Plasma	5 Puffer-basen mäqu/l Blut	6 Gesamt-CO_2 mäqu/l Plasma
I. Leerwert 7	0,46	7,35	44,1	22,7	40,6	24,7
30'	4,32	7,33	49,5	23,4	40,9	26,5
60'	1,93	7,38	40,0	23,4	40,9	24,6
120'	0,98	7,35	46,2	23,4	40,9	25,[illegible]
240'	0,[illegible]	7,[illegible]	42,0	22,8	40,6	25,[illegible]
II. Leerwert 7	0,46	7,34	43,5	23,1	49,3	27,1
10'	11,80	7,33	51,0	23,4	49,3	27,3
20'	12,05	7,33	51,0	23,4	49,3	27,3
40'	9,58	7,34	49,0	23,4	49,3	2[illegible]
60'	8,18	7,[illegible]	51,0	23,4	49,3	27,[illegible]
90'	4,78	7,33	51,0	23,4	49,3	27,[illegible]
120'	3,55	7,32	53,0	23,4	49,3	27,[illegible]

1. Blood sample taken
2. Plasma-AS mg/100 ml
3. Actual blood pH
4. Standard bicarbonate mäqu/l plasma
5. Buffer bases mäqu /l blood
6. Total CO_2 mäqu/plasma
7. Void value

Table 7 represents the behavior of the acid base metabolism and of the plasma-AS after oral administration of 1 and 2 g AS respectively, as a 0,2 and 0,4% watery solution respectively. It is evident that the actual blood pH is not affected by the smaller dose except for a drop of short duration; nor is the standard bicarbonate affected. With the larger dose these effects are somewhat more clearly defined and of longer duration.

TABLE 7. Formic acid content of the plasma and acid base metabolism of the blood after oral administration of 1 g and 2 g formic acid respectively. Test subject: Ma., 27 years old, 80 kg, 1 g AS per os (12,5 mg/kg); Test subject: Gr., 25 years old, 75 kg; 2 g AS per os (26,7 mg/kg).

1 Blut-Vp probe	2 Pl. AS mg/100 ml	3 Aktuelle Blut-pH	4 Standard-Bicarbonat mÄqu/l Plasma	5 Puffer-basen mÄqu/l Blut	6 Total CO_2 mÄqu/l Plasma
Ma. Leerwert 7	—	7,87	41,7	24,0	51,0	27,7
15'	0,51	7,85	41,4	24,5	51,0	25,[illegible]
30'	—	7,87	40,8	24,2	50,0	26,5
45'	0,51	7,80	42,5	24,5	51,0	26,[illegible]
60'	0,31	—	—	24,5	51,0	26,5
90'	0,31	7,[illegible]	42,0	24,5	51,0	26,[illegible]
Gr. Leerwert 7	0,[illegible]	7,87	41,4	22,8	47,0	24,2
15'	1,24	7,82	46,4	21,7	46,0	24,4
30'	1,70	7,[illegible]	42,8	21,7	46,0	23,5
45'	2,01	7,85	41,2	21,7	46,0	23,0
60'	2,01	7,85	41,2	21,7	46,0	23,0
90'	1,70	7,85	41,2	21,7	46,0	23,0

1. Blood Vp samples
2. Plasma-AS mg/100 ml
3. Actual blood pH
4. Standard bicarbonate mÄqu/l plasma
5. Buffer bases mÄqu/l blood
6. Total CO_2 mÄqu/l plasma
7. Ma. Void value
   Gr. " "

Summary

1. The biological half-time for formic acid in the blood plasma in man is 45 minutes and 46 minutes in another case. It is measured after the oral administration of 2 and 3 g AS respectively, in the form of its sodium salt.

2. The resorption of AS occurs already in the stomach. When AS is offered in the form of a salt, de-ionization will likewise take place , due to its pK value of 3.7; resorption will occur thereby. As early as 10 minutes after the oral administration of 4,44 g sodium formate, AS can be verified in the blood.

3. The resorbed AS is transformed in the blood into dissociated Na formate. The major part of the formate is oxidized during metabolism. The base equivalents released thereby hinder the occurrence of a metabolic acidosis. Since excess bases are excreted in the urine, the pH of the urine increases temporarily up to 7; an increased volume of water is simultaneously excreted.

4. After 1,48 g Na formate (corresponding to 1 g AS), 2,1% of the additionally absorbed AS excretes in urine within 24 hours; after 2,96 g Na formate (~ 2 g AS),(the excretion) will amount to 3,3%, and after 4,4 g Na formate (~3 g AS) the excreted amount will be 6,58%. More than half of the total excretion occurs already 1 hour after oral intake. Therefore no accumulation of AS will result in the body.

5. Pure AS--1 and 2 g respectively was administered as watery solution--is subject to the same fate in human metabolism as Na formate.The acid base metabolism of the blood is not noticeably affected by these doses.

6. The general condition of the test subjects was upset in quite sporadic cases only after Na formate administration. Even the intake of thoroughly thinned AS solutions resulted merely in stomach mucous membrane irritation of short duration.

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Stoffwechselversuche mit Natrium-formiat und Ameisensäure beim Menschen*)

Von G. Malorny

Mit 2 Abbildungen und 7 Tabellen

(Eingegangen am 19. Februar 1969)

In Ergänzung unserer tierexperimentellen Arbeiten über die akute und chronische Toxizität der Ameisensäure (AS) und ihrer Salze nahmen wir auch Untersuchungen über den Stoffwechsel dieser Substanzen beim Menschen vor. Da wir gefunden hatten, daß die biologische Halbwertszeit (HWZ) der AS bei den einzelnen Tierspezies recht unterschiedlich ist, erschien es uns wünschenswert, die HWZ auch beim Menschen zu bestimmen. Weiterhin überprüften wir die Wirkung der AS auf den Säure-Basen-Haushalt des Blutes sowie auf die Eliminationsgeschwindigkeit der AS im Harn.

Für die Untersuchungen stellten sich 16 freiwillige Versuchspersonen zur Verfügung, davon einige mehrmals. Die folgenden Fragen sollten geklärt werden:

1. Aufnahme, Verträglichkeit und Ausscheidung der Grenzmengen für AS, wie sie in Liste II des geänderten und ergänzten LMG vom 21. 12. 1958 angegeben sind (Tageshöchstmenge 1 g, Höchstkonzentration 1 g/kg). Diese Menge, wie auch die doppelte und dreifache Menge wurde in Form des Natriumsalzes verabfolgt, in einigen Versuchen auch in Form der reinen AS.
2. Ermittelung der biologischen HWZ im Blutplasma nach oraler Aufnahme von 2 bzw. 3 g AS als Na-Salze. Anhand stündlich entnommener Urinproben sollte die Ausscheidungsgeschwindigkeit bestimmt werden.
3. Untersuchung des Säure-Basen-Haushalts des Blutes bei Belastung mit 2 bzw. 3 g AS als Na-formiat und in einigen Fällen auch bei Belastung mit 1 bzw. 2 g reiner AS.

Verwendete Methoden

1. Quantitative Bestimmung der AS im Blut und Harn nach einer in unserem Institut von Rietbrock und Hinrichs (1964) entwickelten Methode. Die Methode beruht auf der Reduktion der AS zu Formaldehyd durch nascierenden Wasserstoff in Anwesenheit von metallischem Magnesium und 25% HCl im Eisbad. Der entstehende Formaldehyd wird mit Chromotropsäure nach Bremanis (1949) bestimmt. Die Isolierung der AS aus Blut und Harn erfolgt durch Wasserdampfdestillation der angesäuerten Proben.
2. Mikroverfahren von Astrup et al. zur Bestimmung des pH, pCO_2, des Standardbikarbonats und des Basenüberschusses des Blutes.

*) Bericht an die Fremdstoffkommission der Deutschen Forschungsgemeinschaft (Juni 1968).

Ergebnisse

1. Die Ameisensäureausscheidung beim Menschen

Die mit der Nahrung aufgenommene AS wird zum größten Teil im Stoffwechsel verbrannt und nur zu einem kleinen Teil im Harn ausgeschieden. Die tägliche Ausscheidung wird mit etwa 25 mg angegeben. In unseren Versuchen liegt die Normalausscheidung niedriger, nämlich bei 13 mg in 24 h (s. Tab. 1).

Tab. 1. Ameisensäure-Ausscheidung im Urin beim Menschen nach oraler Einnahme von 1,48 g Natriumformiat (~1 g Ameisensäure)

Nr.	Vpn.,	Alter	Urin-Leerwerte Vol.	pH	mg AS insges.	Nach Formiatgabe Vol.	mg AS insges.	mg AS abzügl. Leerwert	Ausscheidung in % abzügl. Leerwert
1	H. B.	27 ♀	730	5,78	13.30	660	57,42	44,12	4,41
2	E. F.	53 ♀	710	5,33	11,01	1255	33,20	22,19	2,22
3	V. G.	48 ♂	1200	6,04	30,25	1365	68,40	38,15	3,82
4	G. G.	26 ♂	840	5,62	11,23	1454	42,50	31,27	3,13
5	R. M.	28 ♂	1350	5,92	9,48	870	34,70	25,22	2,52
6	G. T.	42 ♀	660	5,34	8,97	1030	21,31	12,34	1,23
7	B. W.	28 ♂	1100	5,65	13,61	1530	24,23	10,62	1,06
8	G. G.	27 ♂	645	6,05	12,21	735	37,52	25,31	2,53
9	G. T.	42 ♀	610	5,20	6,84	795	20,92	14,08	1,41
10	P. S.	29 ♂	1400	6,34	12,62	1525	26,89	14,27	1,43
11	St. B.	27 ♂	1200	5,34	13,91	1920	17,92	4,01	0,40
12	B. J.	25 ♀	560	5,70	13,08	710	23,95	10,78	1,09
Mittelwerte $\bar{x}$			917	5,69	13,04	1154	34,08	21,04	2,10
(deren mittl. Fehler) $S_{\bar{x}}$			± 90	±0,10	± 1,68	± 119	± 4,48	± 3,52	± 0,35

Bei oraler Zufuhr von 1,48 g Na-formiat (1 g AS) werden im 24-Stunden-Urin 34 ± 4,5 mg AS insgesamt ausgeschieden. Bei Abzug des Leerwerts sind es dann 21 ± 3,5 mg AS in 24 h. Bezieht man diese Menge auf die zugeführten 1000 mg, so werden somit 2,1% der zusätzlich aufgenommenen AS durch den Harn eliminiert.

Es erhebt sich nun die Frage nach der Geschwindigkeit der AS-Ausscheidung im Urin. Aufschluß hierüber gibt Tab. 2. Bei Einnahme von 1,48 g Na-formiat (Gruppe 2 der Tabelle) wird die Hauptmenge an AS in den ersten 6 h ausgeschieden. Dieser Effekt ist besonders ausgeprägt bei Gruppe 3 der Tabelle, wo nach Zufuhr von 2,96 g Na-formiat (2 g AS) in 6 h bereits [illegible] mg AS eliminiert werden, bei einer Tagesgesamtausscheidung von 77,8 mg AS. Von der 12. Stunde ab sind in allen Versuchen die Kontrollwerte wieder erreicht.

Die Ergebnisse der Gruppe 3 in Tab. 2 sind in mehrfacher Hinsicht interessant. Insgesamt werden bei Abzug des Leerwerts 66,11 mg AS in 24 h ausgeschieden, das sind 3,3% der zugeführten AS von 2000 mg. Der Prozentsatz liegt nur wenig höher als nach Zufuhr von 1000 mg AS, wo 2,1% ermittelt wurden. Es kommt demnach nicht zur Anstauung von AS im Organismus.

Tab. 2. Orale Gaben von Natriumformiat in Hackfleisch verteilt. Gruppe 1 = Kontrolle mit Hackfleisch allein Gruppe; 2 = 1,48 g Na-formiat im Hackfleisch; Gruppe 3 = 2,96 g Na-formiat im Hackfleisch

Gruppe	n	h	Ameisensäure mg/100 ml	Ameisensäure mg insgesamt	AS-Ausscheid. in % d. Zufuhr	Urin pH	Vol. ml	Bemerkungen
1	5	0–6	1,42 ± 0,17	3,53 ± 0,74	—	6,01 ± 0,14	272 ± 62	
		6–12	1,72 ± 0,30	2,97 ± 0,60	—	5,87 ± 0,21	185 ± 31	
		12–24	1,99 ± 0,30	5,25 ± 0,98	—	5,60 ± 0,09	269 ± 58	
		0–24		11,73 ± 1,25			726 ± 82	
2	12	0–6	8,34 ± 1,52	21,97 ± 2,59	2,20 ± 0,26	6,52 ± 0,20	326 ± 57	Keine Störungen
		6–12	2,71 ± 0,62	6,74 ± 1,93	0,67 ± 0,19	6,30 ± 0,14	283 ± 43	1 Inder erbrach
		12–24	1,49 ± 0,28	5,37 ± 1,22	0,54 ± 0,12	5,52 ± 0,10	413 ± 67	(Versuch wurde nicht gewertet)
		0–24		34,08 ± 4,48	3,41 ± 0,45		1022 ± 118	
		abzüglich Leerwert		22,35	2,24			
3	7	0–6	14,72 ± 2,83	64,33 ± 5,07	3,21 ± 0,42	7,08 ± 0,11	438 ± 103	1 Vp gab vorübergehende Kopfschmerzen an; bei fast allen Durstgefühl (Hack gesalzen)
		6–12	2,53 ± 0,32	6,80 ± 0,71	0,34 ± 0,04	6,59 ± 0,28	276 ± 20	
		12–24	2,13 ± 0,33	6,71 ± 0,92	0,34 ± 0,05	5,88 ± 0,16	369 ± 76	
		0–24		77,84 ± 8,88	3,89 ± 0,45		1083 ± 125	
		abzüglich Leerwert		66,11	3,30			

Ein Blick auf das Verhalten des Urin-pH zeigt, daß in der Zeitspanne der höchsten AS-Elimination das Urin-pH in den alkalischen Bereich verschoben wird. Die Erklärung ist einfach. Durch oxydative Beseitigung des größten Teils der AS werden Basenäquivalente frei, die ausgeschieden werden und dabei diuretisch wirken (s. weiter unten).

2. Bestimmung der biologischen Halbwertzeit (HWZ) und der Eliminationsgeschwindigkeit für Ameisensäure

Die biologische HWZ für AS ist meines Wissens beim Menschen bisher nicht gemessen worden. Sie liegt bei Carnivoren vergleichsweise höher als bei Herbivoren. Rückschlüsse auf den Menschen sind nicht ohne weiteres statthaft.

Die exaktesten Werte für die HWZ erhält man durch intravenöse Zufuhr des zu untersuchenden Stoffes. In Versuchen an Hunden konnten wir indessen zeigen, daß auch bei der oralen Zufuhr von AS – wegen der hohen Resorptionsgeschwindigkeit (s. weiter unten) – der AS-Spiegel im Plasma rasch hohe Werte erreicht, so daß durch Ermittelung der Plasma-Konzentrationen identische Halbwertszeiten erhalten werden.

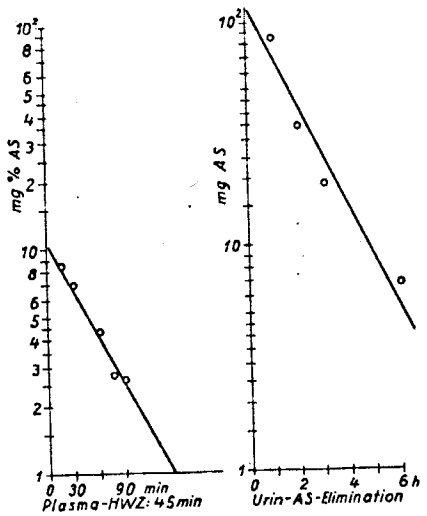

Abb. 1. 2,96 g Na-formiat per os (~ 2 g Ameisensäure) Vp. Ju. ♀, 57,5 kg

In Abb. 1 ist ein Versuch dargestellt, wo die Vp Ju. morgens nüchtern 2,96 g Na-formiat, in Brunnenwasser gelöst, eingenommen hatte. Im Abstand von 30 min wurden bei ihr aus der Armvene Blutproben entnommen. Die Plasma-HWZ betrug in diesem Versuch 45′.

In den stündlich gesammelten Urinproben fiel der AS-Gehalt rasch ab. Wie aus Tab. 3 zu erkennen ist, wurden in der 1. Stunde bereits 84 mg AS eliminiert, das sind 52% der Gesamtmenge von 161 mg AS. In der 2. Stunde waren es bereits 73,2% und in der 3. Stunde 84,5%. Die rasche Elimination ist auffallend.

Tab. 3. Ameisensäurekonzentration im Plasma und Ausscheidung im Urin nach oraler Gabe 2,96 von g Natriumformiat (2 g AS). Vp B. J. 27 J. ♀ 52 kg 38,5 mg AS/kg

AS-Gehalt des Plasmas min	mg/100 ml	h	AS-Ausscheidung im Urin mg/100 ml	Gesamtausscheidung in mg	in % d. Einnahme	pH	Urin Vol/ml
Leerwert	1,82	Leerwert 0–24		13.08		5,70	560
15	8,50	0– 1	140,00	84.00	4,20	6,54	60
30	6,95	1– 2	77,80	34,20	1,71	7,44	44
45	4,94	2– 3	5,45	18,30	0.92	6,68	335
60	4,32	3– 6	1,67	6,60	0,33	7,38	395
75	2,78	6–12	2,24	5,26	0,26	6,70	235
90	2,63	12–24	3,59	12,58	0,63	6,12	350
				160,94	8,05		1419
		abzüglich Leerwert		147,86	7,40		

Bemerkenswert ist auch das Verhalten des Urin-pH. Es verschiebt sich eindeutig in den alkalischen Bereich, um erst nach 12 h langsam zur Norm abzusinken.

Die Urinmenge ist gegenüber dem Kontrollwert um das Anderthalbfache vermehrt. Na-formiat verhält sich wie ein Diureticum.

Die gleichen Beobachtungen wie bei der Vp Ju. erhebt man auch bei der Vp Gr. In Abb. 2 ist ein Versuch mit einer großen Dosis Na-formiat, nämlich 4,44 g (entsprechend 3 g AS), dargestellt. Die Plasma-HWZ für AS betrug in diesem Versuch 46 min. Auch hier fällt auf, daß die AS außerordentlich rasch durch die Nieren eliminiert wird.

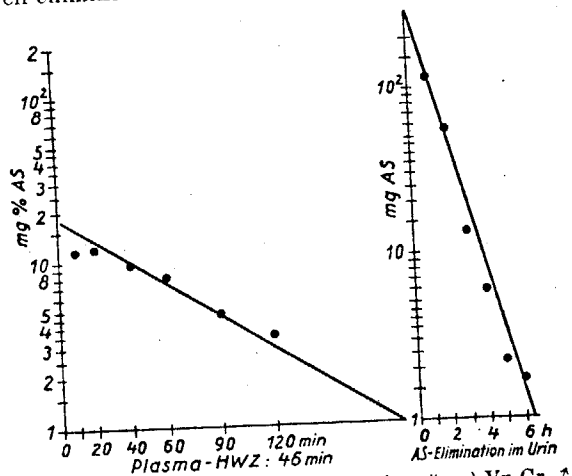

Abb. 2. 4,44 g Na-formiat per os (~ 3 g Ameisensäure) Vp Gr. ♂ 74 kg

Näheren Aufschluß gewährt die Tab. 4. Hier sind die Harnwerte für 2 Dosen Na-formiat wiedergegeben. Nach Dosis I (2,96 g Na-formiat) werden in den ersten beiden Stunden 84 mg von insgesamt 107 mg AS ausgeschieden, das sind

78,5%. Nach Dosis II (4,44 g Na-formiat) schied die Vp in der 1. Stunde 56% und in der 2. Stunde weitere 27% der Gesamtmenge aus, das sind in den ersten beiden Stunden 83%. Auch hier beobachtet man im Urin eine vorübergehende Alkalisierung, begleitet von einer vermehrten Harnproduktion.

Tab. 4. Ameisensäureausscheidung im Urin nach oraler Gabe von 2 Dosen Natriumformiat. I 2,96 g Natriumformiat; II 4,44 g Natriumformiat. Vp. Gr. 25 J. 74 kg

	Harnprobe h	Menge ml	pH	AS-Ausscheidung im Urin mg/100 ml	mg inges.	Gesamtausscheidung in % der aufgenommenen Menge	Bemerkungen
I	Leerwert (0-24)	645	5,87	1,93	12,45	—	
	0– 2	100	7,55	84,00	84,00	4,20	
	2– 3	30	7,66	17,90	5,37	0,27	
	3– 6	235	6,68	2,71	6,38	0,32	
	6–12	400	6,99	1,87	7,48	0,37	
	12–24	200	5,63	1,87	3,74	0,19	
	0–24	965			107.00	5,35	
		abzüglich Leerwert			94,55	4,72	
II	0– 1	52	6,31	227,00	118.00	3,94	30 min nach Einnahme
	1– 2	46	6,85	122,50	56,40	1,88	Müdigkeit
	2– 3	60	6,30	22,30	13,38	0,45	45 min nach Einnahme
	3– 4	120	6,50	4,94	5,94	0,20	sehr angeregt
	4– 5	75	6,74	2,96	2,22	0,07	Danach normales
	5– 6	100	6,68	1,72	1,72	0,06	Befinden
	6–12	430	7,14	1,92	8,25	0,28	
	12–24	255	7,00	1,72	4,38	0,15	
	0–24	1138			210,3	7,03	
		abzüglich Leerwert			197,85	6,58	

Tab. 5. Ameisensäureausscheidung im Urin nach oraler Einnahme von 2 g Ameisensäure. Vp Gr.

Harnprobe h	Menge ml	pH	AS-Ausscheidung im Urin mg/100 ml	mg insgesamt	in % der Einnahme
Leerwert (0–24)	645	5.87	1,93	12,45	
0– 1	35	5,77	29,00	10,15	
1– 2	48	5,94	57,76	27,70	
2– 3	44	8,01	32,80	14,42	
3– 4	35	6,37	9,07	3,16	
4– 5	66	7,54	4,52	2,98	
5– 6	59	7,78	2,65	1,56	
6–24	1170	6,73	2,44	28,60	
0–24	1157			88,57	
			abzügl. Leerwert:	76,12	3,81

Es wurden auch 3 Selbstversuche mit reiner AS durchgeführt. Die Ergebnisse entsprachen weitgehend den mit Na-formiat erzielten Befunden. Die Einnahme von 2 g AS als 0,4% wäßrige Lösung auf nüchternen Magen führte zu vorübergehendem Übelkeitsgefühl und leichter Apathie. Nach 10–15 min trat Wohlbefinden auf. Die stündlichen AS-Ausscheidungswerte im Urin sind in Tab. 5 wiedergegeben. Die Gesamtausscheidung in % der Einnahme beträgt nach 24 h 3,81%. Damit liegt dieser Wert in der Größenordnung des vergleichbaren Na-formiat-Wertes (vgl. Tab. 2, Gruppe 3).

3. *Beeinflussung des Säure-Basen-Haushalts*

In früheren Versuchen am Hund konnten wir feststellen, daß nach Infusion einer 0,2 M Ameisensäure-Ringer-Lösung eine vorübergehende metabolische Acidose auftritt (Malorny, Rietbrock und Schneider, 1965). Sie beginnt nach 3 h abzuklingen (vgl. voranstehende Arbeit). Nach Na-formiat-Infusion wird beim Hund zwar auch ein Absinken des Blut-pH beobachtet; es ist aber geringgradiger.

Unsere Versuche am Menschen sind aus Tab. 6 zu ersehen. Es handelt sich um die Blutwerte der Vp Gr., deren Werte im Urin (Tab. 4) bereits besprochen wurden. Sowohl nach Dosis I (2,96 g Na-formiat) als auch nach Dosis II (4,44 g Na-formiat) beobachtet man keine Auffälligkeiten im Verhalten des aktuellen Blut-pH. Geringgradige Schwankungen sind respiratorisch bedingt. Das Standardbikarbonat bleibt konstant.

Bei den Untersuchungen des Säure-Basen-Haushalts beim Menschen werden 2 Phänomene beobachtet, die der Erörterung bedürfen:

1. Das Fehlen einer metabolischen Acidose im Blut
2. Die vorübergehende Alkalisierung des Urins.

Für beide Phänomene läßt sich eine Erklärung finden, wenn man das Schicksal des Na-formiats im Körper verfolgt.

Na-formiat wird im Magen entionisiert. Die freie lipoidlösliche Säure wird bereits an Ort und Stelle, also im Magen, resorbiert. Dieser Vorgang verbraucht offenbar wenig Zeit. Wie Tab. 4 erweist, sind bereits 10 min nach oraler Einnahme von 4,4 g Na-formiat im Plasma 11,8 mg% AS nachzuweisen.

Der pK-Wert der AS beträgt 3,7; d. h. bei einem pH von 3,7 sind 50% der AS dissoziiert. Der pK-Wert der Milchsäure beträgt vergleichsweise 3,86; er liegt also etwa auf der gleichen Höhe, wie der pK-Wert der AS. Es ist bekannt, daß auch Milchsäure bereits im Magen resorbiert wird.

Die resorbierte AS wird im Blut bei einem pH von 7,36 sofort in das vollständige dissoziierte Na-formiat umgewandelt.

Da, wie aus Tab. 6 ersichtlich ist, nach Dosis I nur 4,72% und nach Dosis II 6,58% der insgesamt aufgenommenen AS im Urin ausgeschieden werden und somit der größte Teil der AS im Stoffwechsel verbrannt wird, ist zu folgern, daß auf diese Weise ein Basenüberschuß im Blut entsteht. Anstelle des verbrannten Formiat-Anions tritt das Hydrocarbonat-Ion. Es werden mehr Basen ausgeschieden, und der Urin wird alkalisch. Das gleiche Phänomen sieht man übrigens auch nach Na-laktat.

Tab. 6. Ameisensäuregehalt des Plasmas und Säure-Basen-Haushalt des Blutes nach oraler Gabe von 2 Dosen Natriumformiat. Vp 25 J. ♂ 74 kg. Dosis I: 2,96 g Natriumformiat, entsprechend 2 g Ameisensäure (27,0 mg/kg); Dosis II; 4,44 g Natriumformiat, entsprechend 3 g Ameisensäure (40,6 mg/kg)

Blutproben-entnahme	Plasma-AS mg/100 ml	Aktuelles Blut-pH	pCO_2 mmHg	Standard-Bic. mäqu/l Plasma	Pufferbasen mäqu/l Blut	Gesamt-CO_2 mäqu/l Plasma
I. Leerwert	0,46	7,35	44,1	22,7	46,6	24,7
30′	4,32	7,33	49,5	23,4	46,8	26,5
60′	1,86	7,39	40,0	23,4	46,8	24,6
120′	0,93	7,35	46,2	23,4	46,8	25,9
240′	0,93	7,33	48,0	22,8	46,6	25,6
II. Leerwert	0,46	7,34	49,5	23,1	49,3	27,1
10′	11,80	7,33	51,0	23,4	49,3	27,3
20′	12,05	7,33	51,0	23,4	49,3	27,3
40′	9,58	7,34	49,0	23,4	49,3	26,8
60′	8,18	7,33	51,0	23,4	49,3	27,3
90′	4,78	7,33	51,0	23,4	49,3	27,3
120′	3,55	7,32	53,0	23,4	49,3	27,8

In Tab. 7 ist das Verhalten des Säure-Basen-Haushalts und der Plasma-AS nach oraler Gabe von 1 bzw. 2 g AS als 0,2 bzw. 0,4% wäßrige Lösung wiedergegeben. Man erkennt, daß das aktuelle Blut-pH bei der niedrigeren Dosis außer einer schnell vorübergehenden Senkung unbeeinflußt bleibt, ebenso das Standardbicarbonat. Bei der höheren Dosis sind diese Effekte etwas deutlicher ausgeprägt und länger anhaltend.

Tab. 7. Ameisensäure-Gehalt des Plasmas und Säure-Basen-Haushalts des Blutes nach oraler Gabe von 1 g bzw. 2 g Ameisensäure. Vp Ma. 27 J. 80 kg 1 g AS per os (12,5 mg/kg); Vp Gr. 25 J. 75 kg 2 g AS per os (26,7 mg/kg)

Vp	Blut-proben-	Plasma-AS mg/100 ml	Aktuelles Blut-pH	pCO_2 mmHg	Standard-Bic. mäqu/l Plasma	Pufferbasen mäqu/l Blut	Gesamt-CO_2 mäqu/l Plasma
Ma.	Leerwert	—	7,37	47,5	24,8	51,0	27,7
	15′	0,31	7,35	51,3	24,8	51,0	28,6
	30′	—	7,37	45,8	24,2	50,0	26,5
	45′	0,51	7,40	42,3	24,8	51,0	26,4
	60′	0,31	—	—	24,8	51,0	26,5
	90′	0,31	7,39	44,0	24,8	51,0	26,9
Gr.	Leerwert	0,46	7,37	41,4	22,8	47,9	24,2
	15′	1,24	7,32	46,4	21,7	46,0	24,4
	30′	1,70	7,34	42,8	21,7	46,0	23,5
	45′	2,01	7,35	41,2	21,7	46,0	23,0
	60′	2,01	7,35	41,2	21,7	46,0	23,0
	90′	1,70	7,35	41,2	21,7	46,0	23,0

Zusammenfassung

1. Die biologische Halbwertszeit für Ameisensäure im Blutplasma beträgt beim Menschen 45 Minuten, in einem anderen Fall 46 Minuten. Sie wird gemessen nach oraler Verabfolgung von 2 g bzw. 3 g AS in Form ihres Natriumsalzes.
2. Die Resorption der AS erfolgt bereits im Magen. Wird sie in Form eines Salzes angeboten, so kommt es wegen ihres pK-Wertes von 3,7 ebenfalls zur Entionisierung und damit zur Resorption. AS läßt sich bereits 10 min nach oraler Gabe von 4,44 g Na-formiat im Blut nachweisen.
3. Die resorbierte AS wird im Blut in dissoziiertes Na-formiat umgewandelt. Der größte Teil des Formiats wird im Stoffwechsel verbrannt. Die dabei freiwerdenden Basenäquivalente verhindern das Auftreten einer metabolischen Acidose. Da die überschüssigen Basen im Urin ausgeschieden werden, steigt das Urin-pH vorübergehend bis auf 7 und darüber an; gleichzeitig wird vermehrt Wasser ausgeschieden.
4. Im Urin werden nach 1,48 g Na-formiat (entsprechend 1 g AS) innerhalb von 24 h 2,1% der zusätzlich aufgenommenen AS ausgeschieden; nach 2,96 g Na-formiat (~ 2 g AS) sind es 3,3% und nach 4,4 g Na-formiat (~ 3 g AS) sind es 6,58%. Mehr als die Hälfte der Gesamtausscheidung vollzieht sich bereits in der 1. Stunde nach der oralen Einnahme. Es kommt demnach nicht zu einer Kumulation der AS im Körper.
5. Reine AS – verabfolgt wurden 1 g bzw. 2 g oral als wäßrige Lösung – erfährt im Stoffwechsel des Menschen das gleiche Schicksal wie Na-formiat. Der Säure-Basen-Haushalt des Blutes wird durch diese Dosen nicht nennenswert belastet.
6. Störungen des Allgemeinbefindens nach Na-formiat traten bei den Vpn nur ganz vereinzelt auf. Selbst die Einnahme stark verdünnter AS-Lösungen führten nur zu rasch vorübergehender Reizung der Magenschleimhaut.

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Formate Metabolism in the Vitamin B_{12}-Deficient Rat

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(*Received* 19 *May* 1959)

It was reported earlier (Fatterpaker, Marfatia & Sreenivasan, 1955; Lavate & Sreenivasan, 1956; Kasbekar, Lavate, Rege & Sreenivasan, 1959) that the protective action exerted by vitamin B_{12} in experimentally induced hyperthyroidism in the rat is also observable in terms of a restoration of the uncoupling of oxidative phosphorylation caused by thyroxine. These studies were extended to ascertain whether the incorporation of formate into the purines of liver acid-soluble nucleotides is impaired in the thyrotoxic rat and whether vitamin B_{12} protection is reflected in altered purine synthesis from administered formate.

METHODS

Animals. The basal diet employed was a modification of the corn-soya diet of Ericson, Harper, Williams & Elvehjem (1956) known to cause vitamin B_{12} deficiency, and consisted of (percentages): corn meal 46·2, defatted pea-nut meal 46·2, salts (Ericson *et al.* 1956) 2, L-cystine 0·3, sesame oil 5·0, and vitamin mixture (Ericson *et al.* 1956) 0·3. Rats of either sex (Wistar strain) weighing 70–80 g. were divided into four groups and fed *ad lib.* on the basal diet either as such or with supplements of iodinated casein (Protomone: Cerophyll Laboratories, Kansas City, Mo., U.S.A.) or vitamin B_{12} or both. Iodinated casein known to enhance vitamin B_{12} deficiency (Lewis, Tappan, Register & Elvehjem, 1950) was added at 0·1 g./100 g. and vitamin B_{12} at 20 μg./100 g. Feeding was continued for 7 weeks, during which period the animals on the basal diet with iodinated casein showed some 10% loss in weight when they were considered hyperthyroid (Du Toit, 1952).

Incorporation of formate into adenine and guanine of liver acid-soluble nucleotides. To animals from each group were administered intraperitoneally 0·5 ml. of a solution of 0·1 M-sodium [^{14}C]formate ($2·25 \times 10^6$ counts/min.). The animals were operated on under ether anaesthesia 2 hr later and their livers perfused with cold aq. 0·09% NaCl, excised, weighed and extracted with 40 ml. of ice-cold 6% (w/v) trichloroacetic acid solution for 30 min. The protein precipitate was separated and washed by centrifuging in the cold at 5000 rev./min. The supernatant and washings were combined, neutralized and treated with 5 ml. of 20% (w/v) mercuric acetate solution to precipitate the acid soluble nucleotides (Kerr, 1942). After separation and washing of the precipitate with 0·5% (w/v) mercuric acetate solution, the nucleotides were freed from Hg by H_2S and the excess of H_2S was removed by aeration. The nucleotides were then hydrolysed in N-H_2SO_4 for 1 hr. over a boiling-water bath. The liberated purines were precipitated at pH 1 with 1 ml. of 5M-$AgNO_3$ solution (Hayder, Chernigov, Khatchadourian & Kerr, 1957) and extracted from their silver salts with hot N-HCl. The purine hydrochlorides were freeze-dried and dissolved in 0·2 ml. of N-HCl. The solutions were then chromatographed on Eaton–Dikeman (Mt. Holly Springs, Pa., U.S.A.) no. 613 paper strips, with 65% (v/v) propan-2-ol in 2N-HCl (Hayder *et al.* 1957) as the developing solvent. The separated purines were then identified as their mercury complexes made visible by ammonium sulphide (Vischer & Chargaff, 1948). Segments from the air-dried strips corresponding to the positions of the separated purines were directly eluted in 0·1 N-HCl for adenine and N-HCl for guanine. They were estimated according to the procedure recommended by Vischer & Chargaff (1948) with a Beckman DUV spectrophotometer. Measured portions were evaporated to dryness on 25 mm. diameter Pyrex-glass planchets by heat lamps. Their activities were determined at infinite thinness in a helium-filled SC-1 Flow gas counter with an SC-51 Autoscaler (Tracerlab Inc., Waltham, Mass., U.S.A.) operating at 40–50% efficiency.

Folic acid and vitamin B_{12}. Other animals from each group were killed for determinations in liver of total folic

acid, vitamin B_{12} and formate oxidation. For folic acid assays, 10% (w/v) liver homogenates were prepared in ice-cold water and autolysed overnight in 0·1M-phosphate buffer, pH 7·2, at 37° under toluene. The samples were then steamed, appropriately diluted, filtered and assayed microbiologically with *Streptococcus faecalis* (Mitbander & Sreenivasan, 1954).

Portions of the liver homogenates were incubated at 37° for 12 hr. under toluene with papain (25 mg./g. of fresh liver) in 0·1M-acetate buffer, pH 4·6. These samples were then analysed for vitamin B_{12} with *Euglena gracilis* as the test organism according to the method of Hoff-Jørgensen (1954).

Formate oxidation. This was studied at 37° in Warburg vessels. Each flask contained 1·0 ml. of a 20% (w/v) aq. liver homogenate, 1·0 ml. of Krebs–Ringer phosphate buffer, pH 7·2, and 0·066M-formate in a total volume of 3·0 ml. When used, [^{14}C]formate corresponded to a total activity of $1·25 \times 10^4$ counts/min./flask. The centre well contained 0·2 ml. of 10% (w/v) KOH solution. Oxygen uptake was measured for 1 hr. and the CO_2 evolved was trapped in the alkali and later precipitated as $BaCO_3$. The precipitate was washed, plated and counted at infinite thinness in the helium flow-gas counter.

Liver catalase. This was determined by a microcolorimetric method (S. P. Manjrekar, unpublished work). Aq. liver homogenate (1:2000) (2 ml.) was added to 10 ml. of buffered (5·0 mM phosphate buffer, pH 6·8) hydrogen peroxide solution (1·25 mM) at 0°. Portions (2 ml.) of the reaction mixture were pipetted out at different time intervals into 5 ml. of titanium sulphate reagent (Feigl, 1954). The colour developed for residual H_2O_2 was read at 420 mμ in a Klett–Summerson photoelectric colorimeter. Catalase activity was determined in terms of Kat.f. units (Euler & Josephson, 1927), the pseudo-first-order reaction constant K being equal to $D(\log x_0 - \log x_t)/t$, where x_0 and x_t are Klett–Summerson readings (corrected for proper blanks) at 0 and t min. and D is the dilution of tissue preparation used for assay.

All results reported are averages for determinations from at least four independent samples ± S.E.M.

RESULTS AND DISCUSSION

The protection by vitamin B_{12} against weight loss caused by iodoprotein feeding, as well as the increased depletion of the vitamin under this condition (Table 1), are in conformity with earlier observations (Fatterpaker *et al.* 1955; Kasbekar *et al.* 1959). There is also a decrease in liver stores of folic acid in experimental thyrotoxicosis. Vitamin B_{12} protection was not, however, observable in terms of a correction of this effect. 'Active formate', as it functions in the exchange and synthesis of C-2 and C-8 of the purine skeleton, is a reduced and formylated derivative of folic acid (Greenberg, Daenicke & Silverman, 1955). It was therefore of interest to examine whether the decreased levels of folic acid in thyrotoxicosis are reflected in the lowering of [^{14}C]formate incorporation into adenine and guanine of the acid-soluble nucleotides.

Table 1. *Folic acid and vitamin B_{12} levels, and incorporation of [^{14}C]formate into acid-soluble nucleotides in livers of thyrotoxic and vitamin B_{12}-protected rats*

Young adult rats were reared on a vitamin B_{12}-deficient basal diet with or without additions of iodinated casein and vitamin B_{12}. The animals were killed at the end of 7 weeks. The incorporation of formate into the adenine and guanine of liver acid-soluble nucleotides was studied 2 hr. after [^{14}C]formate administration *in vivo*. For details and methods see text.

Diet group	Wt. gain in 7 weeks (g.)	Vitamin B_{12} (μμg./g.)	Folic acid (μg./g.)	Adenine (μμ-moles/g.)	Guanine (μμ-moles/g.)	[^{14}C]Formate incorporation: Adenine (counts/min./μmole)	[^{14}C]Formate incorporation: Guanine (counts/min./μmole)	[^{14}C]Formate incorporation: Adenine (counts/min./g. of liver)	[^{14}C]Formate incorporation: Guanine (counts/min./g. of liver)
Basal	116 ± 5	33 ± 4	3·74 ± 0·05	604 ± 27	43 ± 4	674 ± 10	750 ± 52	428 ± 22	31 ± 4
Thyrotoxic	95 ± 11	24 ± 4	2·96 ± 0·19	725 ± 36	54 ± 7	608 ± 12	660 ± 27	442 ± 27	38 ± 4
Basal + vitamin B_{12}	124 ± 6	162 ± 8	3·70 ± 0·37	721 ± 35	63 ± 8	151 ± 10	139 ± 8	110 ± 12	9 ± 2
Thyrotoxic + vitamin B_{12}	114 ± 5	103 ± 6	2·98 ± 0·15	798 ± 20	66 ± 9	211 ± 14	271 ± 25	166 ± 13	18 ± 3

The observed decrease (Table 1) in specific activity (counts/min./μmole) of liver acid-soluble nucleotide adenine and guanine in the thyrotoxic rat is mostly a dilution effect, explainable in terms of increased endogenous synthesis of these nucleotides in thyrotoxicosis. When the total incorporation/g. of liver is considered, this difference levels off. Guggenheim & Halevy (1957) and Halevy & Guggenheim (1958) have shown that hepatic nucleic acid levels are not affected by various conditions adversely affecting folic acid metabolism, e.g. protein deficiency and incorporation of either succinylsulphathiazole or 4-aminopterin in the diet. Dinning, Cosgrove & Day (1957) have also shown in guinea-pig-liver homogenates that ascorbic acid deficiency depresses the utilization of formate in the synthesis of serine by 78% and in that of methionine by 85%. However, under identical conditions of folic acid metabolism, the incorporation of formate into acid-soluble nucleotides and nucleic acid purines remains remarkably unaffected. It would seem, therefore, that there may be differences in the requirements for 1-C transfer reactions and that, in mild folic acid-deficiency states, systems other than those involved in nucleic acid-purine synthesis are more susceptible.

The incorporation of vitamin B_{12} into the basal or the iodocasein-supplemented rations resulted in decreased incorporation of [^{14}C]formate into acid-soluble nucleotides (Table 1). This decreased incorporation cannot be attributed to dilution of activity due to increased synthesis of acid-soluble nucleotides on vitamin B_{12} administration.

From Table 2 it would seem that oxidation of formate to CO_2 is significantly increased ($P < 0.05$) on vitamin B_{12} administration, partially explaining the observed (Table 1) decrease in formate incorporation into acid-soluble nucleotides. This would suggest a role for vitamin B_{12} in formate metabolism, directly or indirectly linked to formate oxidation. In a recent note Arnstein (1958) has proposed a similar role for vitamin B_{12}.

Table 2. *Oxidation of formate by liver enzymes in thyrotoxic and vitamin B_{12}-protected rats*

For details of grouping, see Table 1 and text. Oxidation of formate by liver enzymes was followed manometrically in conventional Warburg flasks with sodium [^{14}C]formate, the $^{14}CO_2$ being recovered as carbonate in the centre well.

Diet group	O_2 uptake (μl./hr./flask)	Activity of total $^{14}CO_2$ (counts/min.)
Basal	16·0 ± 1·1	8170 ± 318
Thyrotoxic	18·4 ± 2·9	8750 ± 120
Basal + vitamin B_{12}	25·5 ± 4·0	9530 ± 380
Thyrotoxic + vitamin B_{12}	23·6 ± 1·2	9410 ± 320

Since formate oxidation has been linked to catalase activity in rat liver (Nakada & Weinhouse, 1953), it is possible that the decreased formate oxidation in vitamin B_{12} deficiency may be due to an effect of the vitamin on haem-protein synthesis. Catalase activity did not show any appreciable increase in the vitamin B_{12}-supplemented group, the Kat.f. values being 14 220 ± 1730 units for the basal group and 14 940 ± 1460 units for the vitamin B_{12}-supplemented group (C. Bhuvaneswaran, unpublished work).

Other work from this Laboratory would also point to the possibility that the oxidation of liver formate is not entirely linked to catalase activity. It was not enhanced when xanthine was included in the reacting system. Again it was inhibited to a less extent than was catalase by cyanide. For example, 3 mM-cyanide completely inhibits catalase activity, whereas a tenfold concentration of cyanide inhibited formate oxidation only to a negligible extent. Kruhoffer (1951) has shown formate oxidation in rat liver to be diphosphopyridine nucleotide-linked.

Formate oxidation is lowered, but to a less extent in the thyrotoxic rat (Table 2), even though vitamin B_{12} deficiency is augmented. Similarly, the increase in formate oxidation on vitamin B_{12} supplementation is less marked ($0.1 > P > 0.05$) in the iodoprotein-fed group. This would suggest that although administration of iodinated casein intensifies the severity of vitamin B_{12} deficiency it is also accompanied by the development of other unknown metabolic complications. Adverse effects of prolonged iodocasein feeding have been demonstrated in other studies (Fatterpaker *et al.* 1959) with rats and mice, on blood haemoglobin and serum proteins, on the retention of vitamins and soluble sulphydryl compounds in liver, as well as on the ability of liver enzymes to effect conjugation *in vitro* of *p*-aminobenzoic acid and glycine.

SUMMARY

1. An observed decrease in liver folic acid levels in the hyperthyroid rat is not corrected by vitamin B_{12} administration.

2. It is also not accompanied by any clear-cut decrease in the incorporation of [^{14}C]formate *in vivo* into the purines of liver acid-soluble nucleotides.

3. It is concluded that incorporation of formate into the purines of rat liver acid-soluble nucleotides is independent of mild disturbances in folic acid metabolism.

4. Vitamin B_{12} administration to a vitamin B_{12}-deficient rat markedly decreased the incorporation of [^{14}C]formate *in vivo* into liver acid-soluble nucleotides. This could be partly explained in terms of the

accompanying increase in the oxidation of formate studied *in vitro*.

5. Vitamin B_{12} has a role, directly or indirectly, in the oxidation of formate.

6. In rat liver, oxidation of formate may not necessarily be catalase-linked.

We are indebted to the Department of Atomic Energy, Government of India, for a grant from which the expenses of this work were defrayed. Our thanks are due to Mr D. G. Padval for technical assistance and to Mr S. P. Manjrekar for allowing us to give details of his method for determination of catalase activity in advance of its publication.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 231, No. 7, July 1959
Printed in U.S.A.

Formate Metabolism by Animal Tissues*†

II. THE MECHANISM OF FORMATE OXIDATION

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(Received for publication, February 11, 1959)

Many studies on the metabolism of formic acid by a variety of animal tissues have shown that an appreciable portion of this substrate is oxidized to carbon dioxide (6–13). Since the initial observation of Battelli (6) and Battelli and Stern (7) on the oxidation *in vitro* of formate, various uncorrelated attempts have been made to identify the responsible enzymes to explain the mechanism of this reaction.

Battelli and Stern (7) first established that the oxidation of formate by animal tissues in presence of H_2O_2 was an enzyme-catalyzed reaction. Plaut *et al.* (14) were the first to demonstrate that injected formate-C^{14} was oxidized to $C^{14}O_2$ and that a major portion of the formate was incorporated into serine. These results led Plaut *et al.* to postulate that formate oxidation occurred by incorporation into serine, followed by oxidation of serine to pyruvate, and finally the degradation of pyruvate to CO_2. Later Plaut and Lardy (15) and Kruhoffer (16) demonstrated the direct oxidation of formate to CO_2 without the intermediate formation of serine. Mathews and Vennesland (17) demonstrated that in pea extracts a DPN-formic dehydrogenase was responsible for the oxidation, and AMP did not influence this oxidation. However, supernatants from rat liver and kidney oxidized formic acid in presence of either AMP, ATP, or ATP and DPN, respectively, thus differing from the pea extracts.

Nakada and Weinhouse (18) presented evidence that in animal tissues the peroxidative activity of catalase, in the form of a catalase peroxide complex, is responsible for the oxidation of formic acid. In 1955, Weinhouse (19) described a model system with the use of xanthine oxidase and catalase to indicate that hypoxanthine oxidation by xanthine oxidase furnishes hydrogen peroxide which, in combination with catalase, oxidized formate. He summarized the findings from his laboratory as follows: "We believe this may be a coupled oxidation phenomenon."

During the studies on the metabolism of formate by animal tissues (20), it was desirable to find the exact mechanism of formate oxidation, hence the present investigations were undertaken. These studies establish that in the rat liver extracts catalase forms a complex with hydrogen peroxide derived from other enzymic systems, and it is this complex which is responsible for the oxidation of formic acid to carbon dioxide.

* A preliminary report of these findings has been presented (1). Since then, several papers have been published (2–5) confirming essentially our results.

† Supported by a research grant from the American Cancer Society.

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EXPERIMENTAL

Three- to 5-months old Sprague-Dawley rats were used in these experiments. They were fasted 24 hours before they were killed by cervical fracture. Liver and small intestine were removed and placed in cold isotonic sodium chloride adjusted to pH 7.4. The tissues were homogenized in two parts of 0.1 M phosphate buffer, pH 7.4, by means of a jacketed Waring Blendor maintained at 5° with ice. The resulting homogenates were centrifuged at 20,000 × *g* for 30 minutes and the clear supernatants were separated and dialyzed against 0.1 M phosphate buffer, pH 7.4, for 15 hours at 5–7°.

Dialyzed extracts were incubated in 0.1 M phosphate buffer, pH 7.4, with 15 μmoles of formate-C^{14} and in presence of various additives as described under each table of experimental results (Tables I to VI). With the exceptions of the experiments in Thunberg tubes, all incubations were carried out in an atmosphere of oxygen. Arrangements to collect labeled CO_2 were those described in a previous paper (20).

RESULTS AND DISCUSSION

Effect of Purine Derivatives—Previous reports showed that addition of either DPN (16), ATP, or AMP (17) to rat liver extracts enhanced oxidation of C^{14}-formate. In order to ascertain the nature of this enhancement, a comparative study was made by incubating DPN, ATP, and other purine derivatives with rat liver extracts. Tables I and II show the results of these experiments. Equimolar quantities of DPN, ATP, or hypoxanthine enhanced the oxidation of formic acid in rat liver extracts to the same degree. Data in Table I show that there was an additive effect when both ATP and DPN are present, dependent only on the concentration of the adenine derivative. Table I shows that both hydrolyzed and intact DPN enhance formate oxidation to approximately the same degree.

Effect of Specific Inhibitors of Xanthine Oxidase—Commercial folic acid contains 1.6 per cent of diazotizable amine, AHPA[1] (22). Since AHPA is known as a specific inhibitor of xanthine oxidase (23), 1.6×10^{-5} M AHPA, contained in 0.001 M PGA, was added to rat liver extracts to test the effect of AHPA on formate oxidation in presence of ATP, hypoxanthine or DPN, respectively. The results are shown in Table II. In the presence of ATP, hypoxanthine, or DPN, the over-all oxidation of formate was inhibited by the addition of folic acid containing AHPA.

In order to test the effect of higher concentrations of pure

[1] The abbreviations used are: AHPA, 2-amino-4-hydroxy-6-pteridyl aldehyde; PGA, pteroylglutamic acid.

TABLE I

Effect of purine derivatives on $HC^{14}OOH$ oxidation by rat liver extracts

3 ml. of rat liver extract in 0.1 M phosphate buffer, pH 7.4, incubated for 2.5 hours at 37° in oxygen. C^{14}-formate 0.005 M, 730 × 10^3 c.p.m. per flask. All additives were 0.002 M.

Additions	$C^{14}O_2$ activity
	c.p.m. × 10^{-3}
None	58
DPN, 0.002 M	151
DPN, 0.001 M + ATP, 0.001 M	147
ATP, 0.002 M + nicotinamide, 0.002 M	143
Nicotinamide, 0.002 M	60
None	25
DPN	104
Hydrolyzed DPN*	96
Hypoxanthine	90
Inosine	79
AMP	78

* DPN hydrolyzed with warm 0.2 N NaOH (21).

AHPA,[2] the manometric technique (25) was used. Fig. 1 shows the uptake of oxygen during formate oxidation by rat jejunum extracts in the presence of DPN with and without the addition of pure AHPA (3 × 10^{-3} M). Data from Fig. 1 establish that 3 × 10^{-3} M AHPA completely inhibited oxygen uptake by rat jejunum extracts in the presence of 0.002 M DPN and 0.005 M formate; lower concentrations of AHPA (6 × 10^{-4} M, Table II) inhibited formate oxidation only 45 per cent in presence of DPN. This indicated that the enhancing effect of DPN on formate oxidation was indirect and mediated by the presence of xanthine oxidase in the tissue extracts.

If the formation of hydrogen peroxide from the oxidation of DPN were dependent on the reaction of oxygen with reduced xanthine oxidase, any oxidizing agent which could compete with oxygen for reduced xanthine oxidase would decrease the oxidation for formic acid to CO_2 to an equivalent extent. It is known that reduced xanthine oxidase will transfer the electrons to cytochrome *c* or nitrate if the reaction is carried out in the presence of phosphate ions (26). Table II shows the effect of added cytochrome *c* and nitrate, respectively, on the oxidation of formate by rat liver extracts in the presence of DPN. It can be seen from this table that the addition of cytochrome *c* decreased the oxidation of formic acid to the endogenous level, thus nullifying the enhancing influence of DPN. If the responsible enzyme were a DPN-dehydrogenase, the addition of cytochrome *c* would have enhanced the oxidation of formate.

Methylene blue is known to act as a hydrogen acceptor for reduced xanthine oxidase and for reduced dehydrogenases. If a DPN-dehydrogenase were responsible for the oxidation of formic acid, it would proceed in the absence of oxygen but in the presence of methylene blue. However, if this oxidation were dependent on the formation of peroxide by xanthine oxidase, no appreciable oxidation should occur under anaerobic conditions. With the Thunberg technique (25), hypoxanthine and DPN were incubated anaerobically with rat liver extracts in the pres-

[2] AHPA was a gift from Lederle Laboratory Division of the American Cyanamid Company through the kindness of Dr. B. L. Hutchings.

TABLE II

Effect of additives on formate oxidation enhanced by purine derivatives in rat liver extracts

Additions	$C^{14}O_2$ activity
	c.p.m. × 10^{-3}
Experiment I	
None*	58
ATP, 0.002 M	113
ATP, 0.002 M + PGA,† 0.001 M	8.3
Experiment II	
None*	18
Hypoxanthine, 0.002 M	113
Hypoxanthine, 0.002 M + PGA,† 6 × 10^{-4} M	2.5
DPN, 0.002 M	118
DPN, 0.002 M + PGA,† 6 × 10^{-4} M	65
DPN, 0.002 M + PGA,† 6 × 10^{-4} M + cytochrome *c*, 1 × 10^{-4} M	5.6
Experiment III	
None*	18
DPN, 0.002 M	116
DPN, 0.002 M + cytochrome *c*, 1 × 10^{-4} M	21
Experiment IV	
None‡	4.6
DPN, 0.001 M	40.2
DPN, 0.001 M + $NaNO_3$, 0.020 M	21.5

* Conditions of incubation the same as described in Table I. Formate-C^{14} concentration, 0.005 M (310 × 10^3 c.p.m. per flask).

† Commercial sample of folic acid (PGA) contained 1.6 per cent diazotizable amine (AHPA) as determined by the method of Bratton and Marshall (21).

‡ 3 ml. of rat liver extract in 0.1 M phosphate buffer, pH 7.2, incubated for 2 hours at 37° in O_2. C^{14}-formate, 0.005 M, 175 × 10^3 c.p.m. per flask.

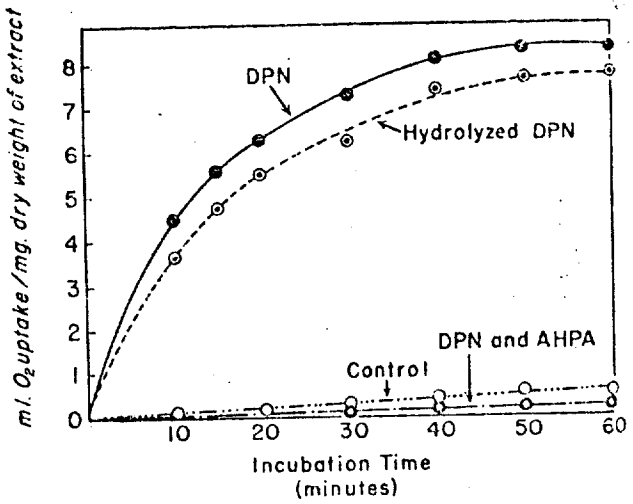

FIG. 1. Effect of AHPA on the oxygen uptake of rat jejunum extracts incubated with DPN and formate. Incubated 2 ml. of dialyzed rat jejunum extract in 0.1 M phosphate buffer, pH 7.4, with 0.002 M DPN or hydrolyzed DPN (21), 0.005 M formate, with or without 0.003 M AHPA in an oxygen atmosphere with a Warburg apparatus. Total volume of incubation mixture was 3 ml.

ence of C^{14}-formate and methylene blue. Table III shows that practically no formate was oxidized under these conditions in the presence of DPN and methylene blue or with methylene blue alone. These results indicate that a dehydrogenase is not responsible for formate oxidation.

Effect of Other Peroxide-forming Systems—Since xanthine oxidase forms hydrogen peroxide, it was of interest to test whether other enzymes in rat liver extracts known to produce hydrogen peroxide would have any influence on formate oxidation. Monoamine oxidase (27) uricase (28), and D-amino acid oxidase (29) are present in rat liver and are known to produce hydrogen peroxide in the oxidation of their respective substrates. The participation of these enzymes in formate oxidation was tested and the results are given in Table IV.

The addition of tyramine, uric acid, and DL-methionine, respectively, enhanced formate oxidation to an appreciable extent. Since the oxidation of uric acid by uricase is not inhibited by AHPA (23), it was of considerable interest to test the effect of AHPA on the oxidation of formate by rat liver extracts in the presence of added uric acid. Addition of AHPA, which completely inhibits formate oxidation in the presence of hypoxanthine (Fig. 1 and Table II), did not inhibit this oxidation in the presence of uric acid (Table IV). This established that AHPA affected formate oxidation by inhibition of xanthine oxidase and that it does not prevent peroxide formation from the action of uricase on uric acid.

TABLE III

Effect of methylene blue and anaerobiosis on oxidation of formic acid enhanced by DPN or hypoxanthine in rat liver extracts

3 ml. of rat liver extract incubated in 0.1 M phosphate buffer, pH 7.4, for 2 hours at 37°; C^{14}-formate 0.005 M, 230 × 10^3 c.p.m. per flask. All other additions 0.002 M.

Additions	$C^{14}O_2$-activity
	c.p.m. × 10^{-3}
Methylene blue alone*	5.1
Hypoxanthine + methylene blue*	5.8
DPN + methylene blue*	6.7
Hypoxanthine†	41.2
DPN†	47.8

* Evacuated Thunberg tubes.

† Flasks were gassed with oxygen.

TABLE IV

Effect of hydrogen peroxide-forming enzymes on oxidation of formic acid by rat liver extracts

Additions	$C^{14}O_2$-activity
	c.p.m. × 10^{-3}
Experiment I*	
None	58
Tyramine, 0.002 M	106
Experiment II†	
None	19
Uric acid, 0.006 M	52
Uric acid, 0.006 M + PCA, 1 × 10^{-4} M	48
DL-Methionine, 0.012 M	69

* Conditions as in Table I.

† Conditions as in Table III.

TABLE V

Effect of hydrogen donors on oxidation of formic acid by rat liver extracts

Additions	$C^{14}O_2$-activity
	c.p.m. × 10^{-3}
Experiment I	
None*	12.0
Hypoxanthine, 0.002 M	39.2
Hypoxanthine, 0.002 M + nitrite, 0.004 M	15.1
DPN, 0.002 M	46.3
DPN, 0.002 M + nitrite, 0.004 M	10.2
Experiment II (9-day rats)	
None†	89
Ethanol, 0.04 M	8.9
Experiment III (mature rats)	
None‡	1.0
Ethanol, 0.08 M	0.35
Sodium nitrite, 0.04 M	0.30

* 3 ml. of rat liver extract in 0.1 M phosphate buffer, pH 7.4; incubated for 2 hours at 37° in oxygen; DPN, 0.002 M; C^{14}-formate, 0.003 M (120 × 10^3 c.p.m.).

† 2.7 ml. of rat liver extract (2 parts tissue and 9 parts Krebs-Ringer buffer, pH 7.4). Incubated 1 hour in oxygen at 37°. Contained formate-C^{14}, 3.7 × 10^{-6} M (4.8 × 10^6 c.p.m.); DL-homocysteine, 3.7 × 10^{-6} M; and glycine, 3.7 × 10^{-6} M.

‡ 4 ml. of rat liver extract (1 part tissue and 2 parts 0.1 M phosphate buffer, pH 7.4). Incubated 2 hours at 38° in oxygen containing 5 × 10^{-3} M formate-C^{14} (9.8 × 10^4 c.p.m.).

Effect of Hydrogen Donors on Catalase-Hydrogen Peroxide Complex—The previous results have demonstrated that the oxidation of formic acid in animal tissues is induced by enzymatic reactions which produce hydrogen peroxide. Chance established (30) that catalase forms a hydrogen peroxide complex which is capable of oxidizing formic acid, among other substrates. Experiments were then designed to ascertain the nature of the enzyme that is responsible for the oxidation of formic acid in the presence of hydrogen peroxide. Since Weinhouse (19) has suggested that a catalase peroxide complex is also responsible for the oxidation of formic acid in animal tissues, the presence of other substrates which can interact with this complex and thus compete with formic acid, should result in a decreased oxidation of the latter. Nitrous acid and ethanol are also oxidized by catalase hydroperoxide (31, 32). Table V shows the effect of sodium nitrite on the oxidation of C^{14}-formate in the presence of either hypoxanthine or DPN. The addition of sodium nitrite inhibited the C^{14}-formate oxidation 61 per cent in presence of hypoxanthine and 78 per cent in presence of DPN.

The effect of added ethanol and nitrite, respectively, on formate oxidation, in the absence of added purines, was tested with rat liver extracts from 9-day-old rats and mature rats. Table V shows the results of these tests. Appreciable inhibition of formate oxidation was noted in presence of these additives.

Effect of Added DPN—The enhancing effect of DPN on the oxidation of formic acid is probably due to the initial degradation of DPN by liver DPN nucleotidases and the resulting purine is

then oxidized by xanthine oxidase producing the H_2O_2. Since nicotinamide inhibits the action of DPN nucleotidases, experiments were designed to test the enhancing influence of DPN on formate oxidation in the presence of nicotinamide. In the presence of 2×10^{-2} M nicotinamide, the oxidation of formic acid induced by DPN was significantly inhibited (30 per cent, Table VI). Nicotinamide alone had no affect on this oxidation. These results indicate that DPN is hydrolyzed by DPN nucleotidases of rat liver extracts to a purine derivative, which is then oxidized to uric acid by xanthine oxidase.

TABLE VI

Effect of nicotinamide on oxidation of formic acid in rat liver extracts enhanced by DPN

3 ml. of rat liver extract in phosphate buffer, pH 7.4, incubated for 2 hours at 37° in oxygen. C^{14}-formate, 0.005 M, 175×10^3 c.p.m. per flask.

Additions	$C^{14}O_2$-activity
	c.p.m. $\times 10^{-3}$
None	4.6
DPN, 0.002 M	40.2
DPN, 0.002 M + nicotinamide, 0.020 M	28.0
Nicotinamide, 0.020 M	5.1

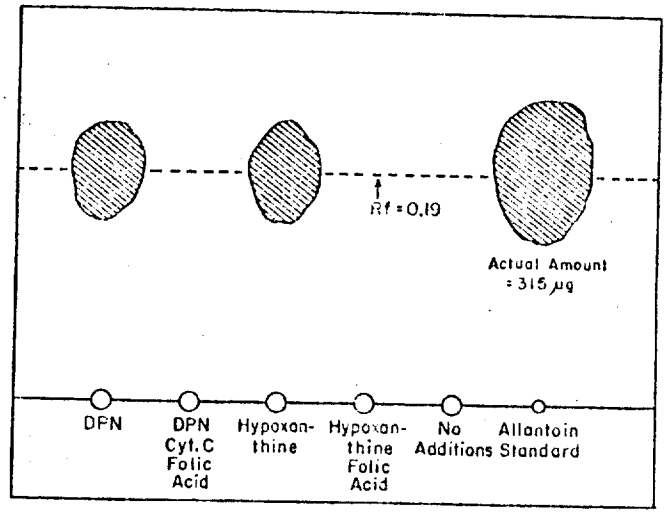

FIG. 2. Diagramatic drawing of a chromatogram showing allantoin formation from DPN incubated with rat liver extracts, with additives as shown.

It seemed likely that uric acid was then further oxidized to allantoin by the action of uricase which is known to be present in rat liver extracts (28). Investigations were carried out with rat liver extracts to establish whether allantoin was formed when DPN was used to enhance formate oxidation. The incubation mixtures (see heading in Table VI) were inactivated with perchloric acid to a final concentration of 3 per cent, the protein precipitate was centrifuged off, washed several times with 3 per cent perchloric acid, and the supernatant was subsequently neutralized with KOH and made up to 10 ml. Aliquots of 4 ml. were evaporated to dryness under vacuum at room temperature. To the residues 0.2 ml. of distilled water was added, and 60 μl. aliquots of these solutions were placed on Whatman No. 1 paper and descending chromatographs were developed with a mixture of *n*-propanol, 90 per cent formic acid, and water (8:1:1). A sample of 60 μl. of a saturated solution of allantoin was used as a standard. After chromatography the paper was dried and sprayed with *p*-dimethylaminobenzaldehyde in 1.2 N HCl solution (33) and the resultant yellow spots indicated the presence of allantoin (Fig. 2).

Mechanism of Formate Oxidation—This investigation has shown that the necessary components responsible for formate oxidation by animal tissue extracts are catalase and a source of hydrogen peroxide. Since catalase has been shown to be widely distributed in various tissues including liver and intestine (31), it was only necessary to establish that the source of hydrogen peroxide was the determinant factor in this oxidation. The possibility that oxidation may occur through the mediation of DPN dehydrogenase was eliminated when it was demonstrated that DPN after alkaline hydrolysis was as effective as DPN itself (Table I). Also, the presence of nicotinamide, which inhibited the degradation of DPN to purine by DPN nucleotidase in the tissue extract, obviated the enhancing effect of DPN on formate oxidation (Table VI). Thus the oxidation can be attributed to the liberation of free purine which subsequently was oxidized by xanthine oxidase and uricase to form hydrogen peroxide and allantoin (Table I and Fig. 2).

Additional evidence that xanthine oxidase is involved in this oxidation is demonstrated by the inhibitor influence of AHPA on formate oxidation (Table II and Fig. 1) where AHPA is a specific inhibitor of xanthine oxidase (22). In addition, the presence of cytochrome *c* and nitrate as acceptors of electrons from reduced xanthine oxidase eliminated the enhancing influence of purine derivatives on formate oxidation under aerobic conditions (Table II) by preventing hydrogen peroxide formation. In the absence of oxygen, methylene blue, whether alone or in

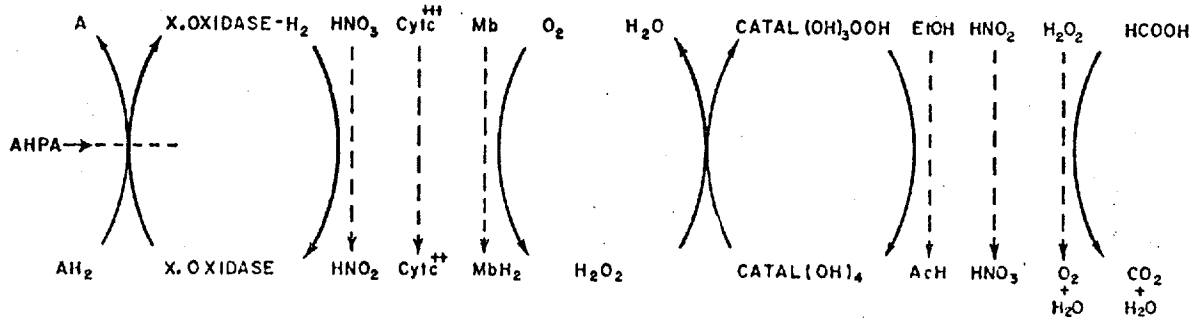

FIG. 3. Scheme representing the coupling of catalase with various H_2O_2-producing systems in the oxidation of formic acid. AH_2 is the substrate for xanthine oxidase; CATAL-$(OH)_3OOH$ is the catalase-peroxide complex; vertical broken arrows are competitive or noncompetitive inhibitors.

presence of hypoxanthine or DPN, did not facilitate formate oxidation (Table III). Thus the presence of oxygen was essential for the formation of hydrogen peroxide from xanthine oxidase activity. A schematic summary of these interrelated reactions is illustrated in Fig. 3. Other peroxide-forming systems such as monoamine oxidase (27), uricase (28), and D-amino acid oxidase (29) can serve as sources of peroxide for formate oxidation (Table IV).

Availability of hydrogen peroxide and the presence of catalase implicated the participation of a catalase-peroxide complex (30), first indicated by the model experiments of Weinhouse (19), as the primary agent responsible for oxidation of formate to CO_2. Ethanol, nitrite, and formate, respectively, are known to be substrates for the catalase peroxide complex system (30). Thus the addition of either ethanol or nitrite to an incubation mixture containing formic acid prevented oxidation of formic acid by interaction with the catalase peroxide complex (Table V).

SUMMARY

Formate is oxidized to carbon dioxide in rat liver and jejunum extracts by a catalase-hydrogen peroxide complex. Dehydrogenases do not participate in this oxidation. Enzymes responsible for the formation of hydrogen peroxide are xanthine oxidase, uricase, monoamine oxidase, and D-amino acid oxidase. Thus, the oxidation of formate is the result of coupled reactions between the enzymes capable of yielding hydrogen peroxide for the formation of a catalase peroxide complex and the subsequent oxidation of formate by this complex.

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Synthesis of proteins, lipids and nucleic acids from formate in young rats[1]

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RAPPOPORT, DONALD A., BETTIE W. SEWELL AND FERRIN B. MORELAND. *Synthesis of proteins, lipids and nucleic acids from formate in young rats.* Am. J. Physiol. 197(5): 1045–1047. 1959.—Formate-C^{14} was injected into a 21-day pregnant rat and into 1-, 3- and 10-day-old rats. Whole fetal and 1-day-old rats and liver, heart, brain and intestine from 1-, 3- and 10-day-old rats were blended with trichloroacetic acid. The brei's were fractionated into acid-soluble, protein, DNA, RNA and lipid fractions and the radioactivity of each fraction measured. Most of the activity was always in the acid-soluble fraction. Protein activity was high in all 1-day rat tissues and decreased progressively with age; it was higher 1 day after birth than before. Activity of the lipids was increased on the 1st and 3rd days and then decreased. RNA showed high activity in 1-day rats, then dropped markedly. DNA was always of low activity. It may be concluded that the rate of formation of protein, lipid and RNA from formate reflects the activity of tissues during development.

IN AN ATTEMPT to correlate normal mammalian growth with changes in protein and nucleic acid synthesis, it was of considerable interest to determine the rate of formate-C^{14} incorporation *in vivo* by young rats. Since formate is a precursor of serine (1), methionine (2), cysteine (3) and purines (4, 5), it is ultimately incorporated in the form of these precursors into proteins and nucleic acids (6, 7).

Studies were made to determine whether the rate of formation of proteins, nucleic acids and lipids *in vivo* from formate-C^{14} can be correlated with the growth of the rat. This report presents the results of such studies in *a*) 21-day fetal and 1-day old rats, and *b*) in liver, heart, brain and intestine from rats aged 1, 3 and 10 days.

PROCEDURES

Fetal metabolism in utero. A 368-gm rat, 21 days pregnant, was anesthetized with ether; the fetuses (12, average 6.5 gm) were then exposed by abdominal surgery, and 0.2 ml of a saline solution containing C^{14}-formate (7.5×10^6 cpm) was injected into the jugular vein. After 5 minutes the first three fetuses in the right uterine horn were isolated by means of a suture from the remaining fetuses and from the connecting blood vessels. Each fetus was thus removed, weighed and homogenized in an ice-jacketed micro Waring Blendor with cold 10% trichloroacetic acid (TCA). At intervals of 5, 17, 30 and 47 minutes following the injection, 3 of the 12 fetuses were treated in this manner. Subsequently the tissue components were separated and analyzed for C^{14} distribution (for details see section on METHODS).

Metabolism by newborn rats. Ten weighed, newborn Sprague-Dawley rats from two litters (approximately 20 hr. after birth) were injected with 0.2 ml of a saline C^{14}-formate solution (containing 3×10^6 cpm) intraperitoneally. They were kept in a box filled with absorbent cotton until used; laboratory temperature was 26°. Pairs of animals were homogenized separately in a Blendor with cold 10% TCA at 2, 5, 10, 25 and 60 minutes after injection. The tissue components were separated and analyzed for isotope distribution as described in METHODS.

In vivo tissue metabolism. For these experiments a litter of six to eight rats was used at one time. The rats, 1, 3 or 10 days of age, were weighed, injected intraperitoneally with 0.05 ml saline solution of C^{14}-formate (containing 1.15×10^6 cpm), and kept at room temperature. At intervals of 30, 60 and 90 minutes animals were decapitated and the liver, heart, brain and intestine were removed, weighed and homogenized in cold 10% TCA. The tissue components were separated and the amount of C^{14} in each fraction determined.

METHODS

Tissue fractionation was carried out by the following procedure. The cold tissue mixture was centrifuged and the supernatant solution separated from the precipitate. The precipitate was washed with cold 5% TCA and centrifuged. This was repeated once more. The washings and the first supernatant solution were combined. To remove unchanged formate-C^{14}, aliquots of this solution were transferred to stainless steel planchets, 0.4 ml of

Received for publication June 15, 1959.
[1] This investigation was supported by a Research Grant (4432-MG) from the National Institutes of Health.

50% formic acid was added, and the planchets were dried at 60° in a stream of air for one-half hour. This treatment was repeated until C^{14} activity in the planchet remained constant as determined with a thin window Geiger-Mueller tube. The remaining C^{14} compounds were designated as the 'acid-soluble' fraction.

The TCA-insoluble residue was extracted twice with 95% ethyl alcohol. It was then treated three times by being suspended in a solution of ether-alcohol (1:1 by vol.), heated at 60° for 4 minutes and centrifuged. The alcohol and the ether-alcohol extracts were combined and designated as the lipids. Aliquots were transferred to planchets and air dried at 60° for determination of C^{14} activity.

The residue left after extraction of the lipids was suspended in 1 N KOH and allowed to stand overnight at room temperature. The solution thus obtained was chilled and acidified with 6 N HCl to pH 3; the resultant precipitate (DNA and protein) was separated by centrifugation. The supernatant contained RNA nucleotides. Aliquots were transferred to planchets and dried; C^{14} determinations were made with the GM tube.

The residue of DNA and protein was washed three times with cold 5% TCA, suspended in 5 ml of 5% TCA, and heated for 20 minutes at 90°C. The residue (protein) was separated from the supernatant (DNA-hydrolysate) by centrifugation. The protein residue was washed three times with 5% TCA, then dissolved in 1 ml of 90% formic acid in a waterbath at 70°. Aliquots of the DNA solutions and the protein solutions, respectively, were transferred to planchets and dried, and C^{14} activity determined with the GM counter. When the combined nucleic acids were used, the alkali treatment following lipid extraction was omitted and both RNA and DNA were hydrolyzed and extracted with hot 5% TCA as described above.

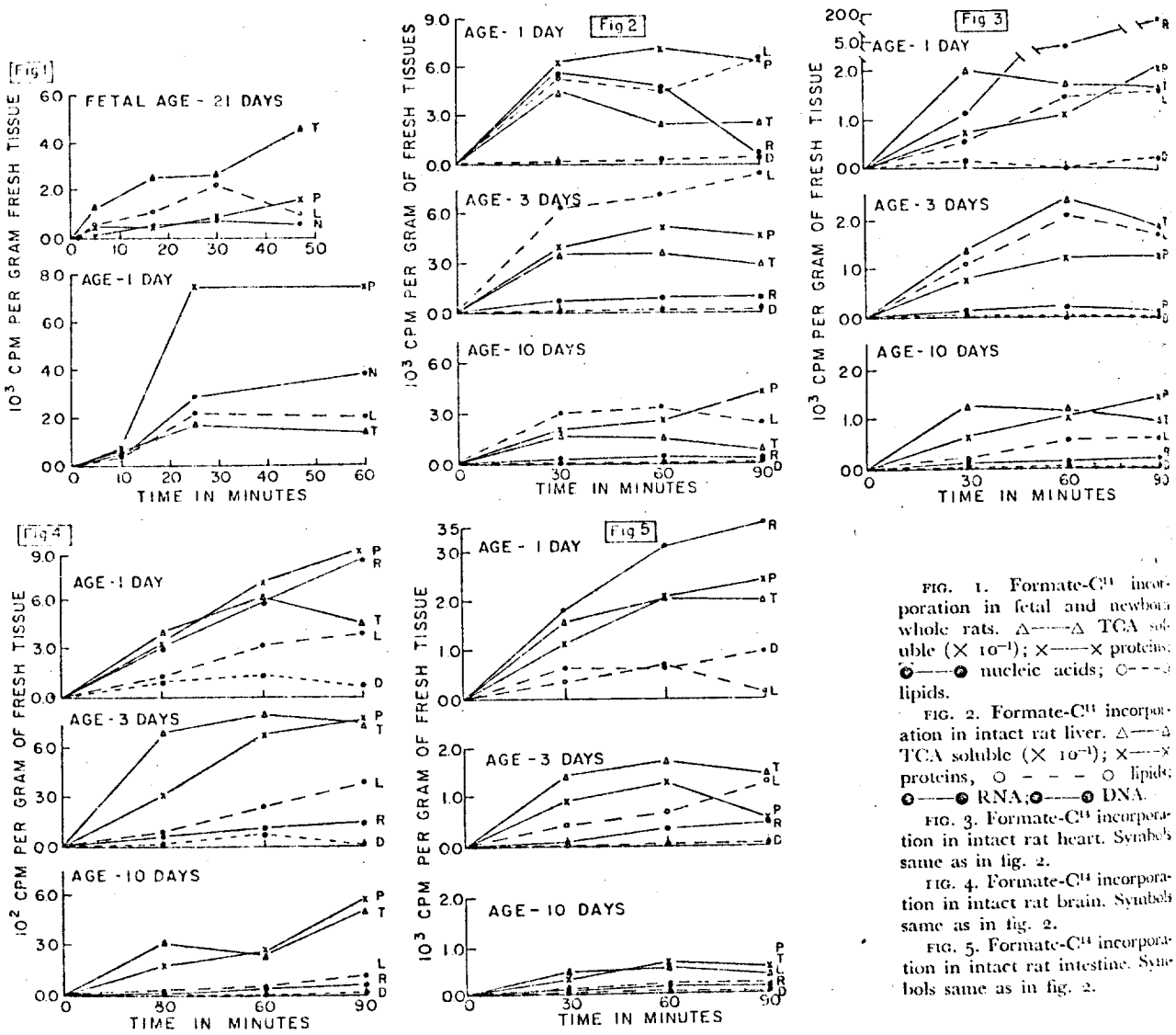

FIG. 1. Formate-C^{14} incorporation in fetal and newborn whole rats. △——△ TCA soluble (× 10^{-1}); ×——× proteins; ●——● nucleic acids; ○- - -○ lipids.

FIG. 2. Formate-C^{14} incorporation in intact rat liver. △——△ TCA soluble (× 10^{-1}); ×——× proteins, ○ - - - ○ lipids; ●——● RNA; ●——● DNA.

FIG. 3. Formate-C^{14} incorporation in intact rat heart. Symbols same as in fig. 2.

FIG. 4. Formate-C^{14} incorporation in intact rat brain. Symbols same as in fig. 2.

FIG. 5. Formate-C^{14} incorporation in intact rat intestine. Symbols same as in fig. 2.

Radioactive products in the acid-soluble fraction were parated and identified by descending paper chromatography. For two-dimensional chromatography the llowing solvents were used: *n*-butanol, acetic acid and ater (16:4:9 vols.); and *n*-butanol, acetone, water and thylamine (10:10:5:2 vols.) with Whatman 3MM aper. For unidimensional chromatography either solent was used. Radioactive components were detected scanning the chromatograms with a thin window GM be, and also by autoradiography on x-ray film.

ESULTS

Fetal rats. As is shown in the upper part of figure 1, the ost rapid and the greatest incorporation of C^{14}-formate y the fetal rats was into the acid-soluble fraction. This as also true in all tissues and at all ages examined in is study (note that in all the graphs the TCA-soluble action is plotted at 1/10 the scale of the other fractions). aper chromatography established that of the amino ids in the TCA-soluble fraction, the most radioactive ere serine, methionine and cystathionine. No further alysis of the remaining tissue fractions was made.

Newborn rats. The lower portion of figure 1 illustrates e results from time studies on the uptake of formate-C^{14} whole newborn rats. The maximum incorporation of C^{14} into all components was attained within 25 minutes. f the insoluble fractions, protein showed the maximum C^{14} incorporation.

Young rats. As in the fetal and newborn rats, the acid-oluble fraction in all the tissues studied contained the aximum C^{14} activity (figs. 2, 3, 4 and 5). In rat liver ig. 2) the protein and the acid soluble fractions showed progressive drop in C^{14} activity from the rats aged 1, 3 nd 10 days, respectively. The radioactivity of the lipids all tissues was as high or higher on the third day as on e first, although dropping in the 10-day rats (figs. 2–5). he RNA in the 1-day rats showed relatively high C^{14} ctivity but was very low in the 3- and 10-day rats. The ctivity of DNA, in all the tissues studied from all the age roups, was extremely low.

Paper chromatography of the acid soluble fractions howed that the main radioactive amino acids from all ssues were serine, cystathionine and cysteine, and, to a ser extent, methionine. Some unknown radioactive ompounds were also noted in this fraction.

ISCUSSION

Formate-C^{14} injected into newborn rats was rapidly tilized in the formation of proteins, lipids, and nucleic acids (fig. 1). The simultaneous increase of C^{14} in the acid-soluble fraction is in accord with the fact that prior to incorporation of formate into proteins and nucleic acids, it is first converted into amino acids and purine precursors (1–5). Thus the data in figure 1 indicate that the rate of formate incorporation can be a useful approach towards correlation of such biochemical observations with various phases of growth.

For specific information on the relationship between the development of the rat and formate metabolism, the liver, heart, brain and intestine from rats, aged 1, 3 and 10 days were studied. Examination of figures 2, 3, 4 and 5 indicates that the most consistent change was the progressive decrease in the rate of formate-C^{14} uptake into proteins in 1-, 3- and 10-day rat tissues, respectively.

Deoxyribonucleic acids (DNA) did not incorporate significant amounts of C^{14} in the time intervals used in these experiments, thus indicating that the turnover or synthesis of DNA was insignificant compared to protein (figs. 2–5) in the tissues studied. Since these tissues are actively growing, the very low rate of DNA formation was unexpected, nor is the reason for this observation clear.

The rate of ribonucleic acid (RNA) formation was very high in liver, heart, brain and intestine from 1-day-old rats (figs. 2–5); however in the 3- and 10-day rat tissues this rate decreased to an extremely low value, almost to the level of DNA. This sudden decrease in rate of RNA formation indicates a profound change in the metabolic behavior of these organs. It is probable that the newborn rat in the neonatal period goes through metabolic changes caused by transition from placental dependence *in utero* to the postnatal independence, and this change may be reflected in the above observations concerning RNA. The changes in the neonatal period are also shown in figure 1. It is difficult to make accurate comparisons between the fetal and 1-day whole animals since in one case the mother was injected intravenously and in the other the young were directly injected intraperitoneally. The dose of formate in counts per minute per gram of body weight was 2.3 times as large for the 1-day rats as for the fetuses. Allowing for this, it is seen that the activity of the protein is up and that of the acid-soluble fraction is down on the first day of life, implying a stimulus to protein synthesis in the neonatal period with a resultant depletion of soluble protein precursors.

From the data presented in figures 2–5, only the rate of incorporation of formate into protein showed a definite correlation with the age of the young rats, i.e. a progressive decrease in protein synthesis with increased age of the postnatal rat.

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Reversible Inactivation of Trypsin by Anhydrous Formic Acid

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(Received for publication, October 16, 1958)

The inactivation of lysozyme and ribonuclease by anhydrous formic acid has been shown by Josefsson and Edman (1, 2) to be reversed by adjustment of an aqueous solution of the inactivated enzyme to pH 6.0 to 8.5. Reactivation was accompanied by an uptake of base, approximately equivalent to the number of serine and threonine residues in the proteins. With both proteins an increase in the positive electrophoretic mobility was observed, and it was postulated that the action of anhydrous formic acid was to bring about an *N*- to *O*-acyl migration at the hydroxyamino acid residues of the proteins. Such a migration should result in the generation of primary amine groups equivalent to the number of residues of serine and threonine involved in the shift. In the case of lysozyme there was an increase in amino nitrogen content as measured by the Van Slyke method, but the yields were poor and no dinitrophenylserine could be detected after reaction at pH 5.0 with fluorodinitrobenzene. In the case of ribonuclease, attempts to demonstrate an increase in the amino nitrogen content of the protein failed completely. Other observations (3) included an increase in the sedimentation coefficient and viscosity of ribonuclease but no change in the optical rotatory properties of the enzyme.

Because of the high content of serine and threonine in trypsin (10 and 11 residues, respectively (4)), it was of considerable interest to extend the investigation of the action of anhydrous formic acid to this enzyme. As with lysozyme and ribonuclease, trypsin was inactivated and could be regenerated by incubation at alkaline pH, and the reactivation was accompanied by an uptake of base. However, the physical and chemical properties of the inactivated enzyme do not support the hypothesis of an *N*- to *O*-acyl shift produced by formic acid.

EXPERIMENTAL

Materials and Methods—Trypsin was prepared from trypsinogen by the rapid activation procedure of Pechère and Neurath (5). Trypsinogen (Lot No. TG 522) was a product, crystallized once, and containing 50 per cent $MgSO_4$, prepared by the Worthington Biochemical Corporation, Freehold, New Jersey. The protein was dissolved in 0.01 M HCl, dialyzed extensively in the cold against 0.001 M HCl, pH 3.0, and lyophilized. After activation to trypsin, the activation mixture was dialyzed against 0.001 M HCl and lyophilized.

Anhydrous formic acid was prepared by drying the Baker and Adamson (98 to 100 per cent) product over boric anhydride for at least 24 hours, followed by distillation at reduced pressure (1, 6).

Urea was a reagent grade product and was used without further purification for the electrophoresis experiments. For all other experiments, it was recrystallized once from 30 per cent ethanol.

Tryptic activities were determined by the esterase method, with BAE[1] as substrate (7). The reagents were combined to give a final concentration of 0.001 M Tris buffer, 0.05 M $CaCl_2$, 0.1 M KCl, and 0.01 M BAE. The assays were carried out at pH 8.0 and 25 ± 0.2° with a Jacobsen-Léonis autotitrator (8), manufactured by Ole Dich, Instrument Maker, Copenhagen, Denmark, in conjunction with a titrator type TTT-1 from the Radiometer Corporation, Copenhagen, Denmark. The assembly was used with a temperature-controlled reaction vessel designed for small volumes (9).

Protein and protein nitrogen concentrations were calculated by measuring the absorption at 280 mμ in a Hilger Uvispek spectrophotometer. An extinction coefficient, $E^{1\%}_{1cm.} = 14.4$, and a nitrogen content of 15 per cent were used for trypsin (10). The same values were assumed for formic acid-inactivated trypsin. For the calculation of molar concentrations, the molecular weight of trypsin was taken as 23,800 (11).

pH measurements in the presence of urea were recorded as measured with the glass electrode and a Cambridge model R pH meter.

Moving boundary electrophoresis was carried out in the Spinco model H electrophoresis apparatus.

Optical rotation was measured at room temperature in 10 ml.-cells with the standard Keston model D polarimeter attachment for the Beckman model DU spectrophotometer. The instrument was calibrated with sucrose solutions of known concentration.

RESULTS

Inactivation of Trypsin with Formic Acid and Reactivation at pH 8—In preliminary experiments trypsin was dissolved in anhydrous formic acid and kept in a closed vessel at 25° for 15 to 18 hours. The dry powder, after removal of the formic acid by lyophilization, was insoluble in aqueous solutions from pH 1 to 12, in 0.5 per cent sodium dodecyl sulfate, and in 66 per cent dimethylformamide. It was soluble, however, in 4 to 6 M guanidine hydrochloride and in 7 to 8 M urea solutions. Since trypsin is reversibly inactivated by concentrated urea solutions (12), the following procedure was adopted for a study of the time dependence of the inactivation reaction.

* Recipient of a Donner Fellowship for Medical Research awarded by the Division of Medical Sciences, National Academy of Sciences, National Research Council.

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[1] The abbreviations used are: BAE, benzoylarginine ethyl ester; DNP, dinitrophenyl; FDB, fluorodinitrobenzene; Tris, tris-(hydroxymethyl)aminomethane.

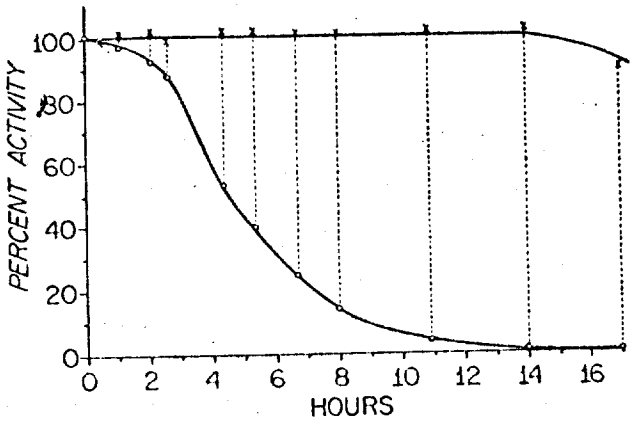


FIG. 1. Inactivation of trypsin in anhydrous formic acid (O——O) and reactivation at pH 8 (X——X).

200 mg. of trypsin were dissolved in 20 ml. of freshly distilled anhydrous formic acid and kept at 25° in a closed vessel for 17 hours. At suitable intervals 1-ml. samples were pipetted into 10-ml. Erlenmeyer flasks, immediately frozen, and the formic acid was removed by lyophilization. Each sample of dry protein was dissolved in 1 ml. of 8 M urea previously adjusted to pH 3.0 with HCl. Of this solution, 0.200 ml. was diluted 25-fold with 8 M urea, pH 3.0, and 25- or 50-μl. aliquots of the diluted sample were taken for assay with the use of 2.5 ml. of BAE substrate. The remainder of each sample was diluted with 4 ml. of 8 M urea in 0.1 M Tris buffer, pH 8.45, whereupon the pH of this solution dropped to 8.1. The samples were stored at 7° for 24 hours to permit complete reactivation when the pH had dropped to 7.95. For assay of esterase activity 25-μl. samples were taken.

Inactivation of the enzyme was complete in 14 to 17 hours as shown in Fig. 1, and full activity[2] was restored by subsequent incubation at pH 8.

Correlation of Enzymic Activity with Base Consumption during Reactivation—Josefsson and Edman (1, 2) have shown that the rate of restoration of enzyme activity of lysozyme and ribonuclease was closely related to the rate of consumption of alkali. It was of interest, therefore, to follow the alkali consumption of formic acid-treated trypsin during reactivation. This was done in the following manner, at pH 8.00 and pH 9.00.

15 mg. of trypsin, treated for 14 hours with formic acid, were dissolved in 6 ml. of 8 M urea, previously adjusted to pH 8.0 or 9.0 with 0.1 N NaOH. 5 ml. of this solution were pipetted into the reaction chamber of the autotitrator and the pH readjusted to pH 8.0 or 9.0 with 0.1 N NaOH. The temperature was maintained at 25°, and the consumption of base required to maintain the pH at these values was recorded automatically. For assay purposes 100 μl. samples of the reaction mixture were diluted with 2 ml. of 8 M urea, pH 3.00, and stored at 7° until assayed. The results plotted in Fig. 2 indicate an initially rapid uptake of base followed by a slow, continuous base consumption which reached a constant rate after several hours.

[2] When 25- or 50-μl. aliquots of trypsin dissolved in 8 M urea were assayed in 2.5 ml. of BAE substrate solutions, the activity was only 80 per cent of similar aliquots of trypsin dissolved in 0.001 M HCl. However, the activity did not decrease over many hours of storage at 7°. The activities indicated in Fig. 1 are expressed as the percentage activity of aliquots of trypsin dissolved in 8 M urea.

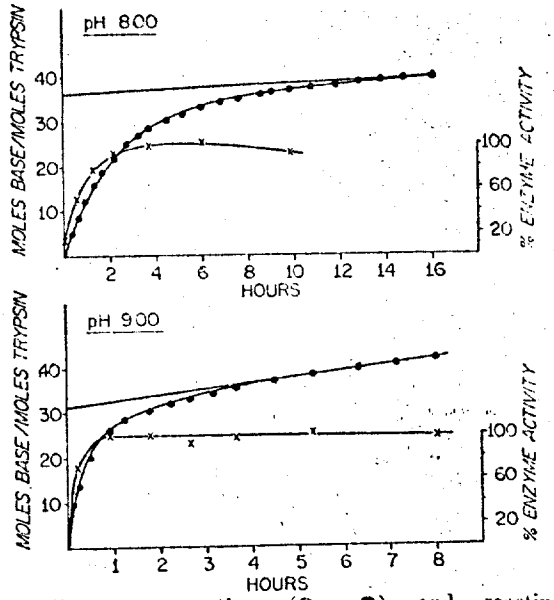


FIG. 2. Base consumption (●——●) and reactivation (X——X) of inactivated trypsin at 25°.

It is possible that the slow reaction was caused by the uptake of atmospheric carbon dioxide, even though every attempt was made to minimize this effect by protecting the solution by a layer of paraffin oil or by flushing with nitrogen gas. For calculation of the base consumed during reversal of the inactivation process, the reaction was allowed to continue until the base consumption had reached a constant rate and an extrapolation to zero time could be made, as indicated in Fig. 2.

Both at pH 8.0 and 9.0, the rate of restoration of enzymic activity was faster than the rate of alkali consumption, and the reaction reached completion earlier. Thus at pH 8.0 trypsin was fully reactivated in less than 4 hours, whereas the base consumption did not reach a constant rate until 10 hours had elapsed. Similarly, at pH 9.0, the corresponding time intervals were 1 hour and 4 hours. It seems safe to conclude that the activity of trypsin is not dependent on all of the chemical groups affected by the formic acid treatment. Thus at pH 8.0 full activity was restored when only 25 of the 32 groups had been restored to their original state, as calculated from the alkali consumption, whereas at pH 9.0, the corresponding values were 27 out of 34.

"Immediate" and "Time-dependent" Uptake of Base at Different pH Values from 7.0 to 11.0—If the action of anhydrous formic acid is to produce an *N*- to *O*-acyl shift, there should be a liberation of serine and threonine amino groups equivalent to the number of peptide bonds involved in the acyl shift. Depending on the pK of the amino groups, a certain proportion of them would be titrated by adjusting the solution with alkali to a particular pH ("immediate" base consumption). The remaining groups would release their hydrogen ions only upon reversal of the acyl shift ("time-dependent" alkali consumption). By measuring the "immediate" and "time-dependent" alkali consumption at a series of pH values, the pK of the amino groups could be determined. For this purpose the following procedure was adopted at pH 7.0, 8.0, 9.0, 10.0, and 11.0, respectively. Trypsin, 15 mg., previously treated for 17 hours with anhydrous formic acid, was dissolved in 6 ml. of 8 M urea adjusted to the pH at which the experiment was to be done. There was no

immediate drop of pH to between 4.5 and 4.9. Five ml. of this solution were pipetted into the reaction chamber of the autotitrator and immediately titrated to the required pH with 0.1 N NaOH. This procedure took only 0.6 minute at pH 7.0, but at pH 10.0 and 11.0 it required 3.9 and 5.7 minutes, respectively. The amount of base so consumed has been designated as the "immediate" alkali consumption. The reactivation mixture was maintained at the required pH by the automatic addition of 0.1 N NaOH. The contents of the reaction chamber were covered with paraffin oil, and the reaction was allowed to continue until the base consumption had reached a constant rate. The temperature of the reaction mixture was maintained at 25° throughout. The results were plotted and the "time-dependent" alkali consumption was computed by extrapolation to zero time, as shown in Fig. 2.

The results of these experiments are shown in Table I. The "time-dependent" alkali consumption is, within experimental error, a constant value at pH 7.0, 8.0, and 9.0; the value shows a small decrease at pH 10.0 and a large decrease at pH 11.0. The "immediate" base consumption, on the other hand, shows a large increase between pH 10.0 and 11.0. These data are consistent with a pK of about 10.5 for the postulated amino groups. However, at pH 11.0 the "time-dependent" alkali consumption is very rapid and is complete in less than 20 minutes. Undoubtedly, much of the true "time-dependent" alkali consumption is included in the "immediate" alkali consumption, since 5.7 minutes were required for adjustment of the pH to 11.0. A pK of 10.5 for these groups is therefore a minimal value. It should be noted that these data are also consistent with any base-catalyzed reaction involving the release of a hydrogen ion *e.g.* hydrolysis of an ester bond.

Ninhydrin Color Yield during Inactivation—The ninhydrin color yield of trypsin during inactivation with formic acid was measured in the following manner. 15 mg. of trypsin were dissolved in 3 ml. of anhydrous formic acid at zero time and incubated in a water bath at 25°. At suitable intervals duplicate 100-μl. samples were pipetted into calibrated tubes and immediately lyophilized. After storage at 7° overnight, the samples were analyzed by the ninhydrin method, with the modified reagent described by Dixon *et al.* (13). The results in Table II show that there was no change in ninhydrin color yield during 14 hours of inactivation.

N-Terminal Group Analysis—An attempt was made to demonstrate the presence of extra serine amino groups in formic acid-treated trypsin by means of reaction with FDB. Elliott (14) has shown that the dinitrophenylation reaction can be carried out at pH 5.0 and has used this reaction as one means of demonstrating the *N*- to *O*-acyl shift of silk fibroin occurring under the influence of concentrated sulfuric acid. However, Josefsson and Edman (1) were unable to demonstrate by this method serine amino groups in lysozyme treated with formic acid.

Since the reaction with FDB proceeds more readily at alkaline pH, the reaction with formic acid-treated trypsin was carried out at pH 7.0 for 2 hours at 25°. It was calculated from previous experiments that during this time reactivation would have proceeded to only 14 per cent completion. Guanidine hydrochloride (6M) was chosen as the solvent for the insoluble inactivated trypsin because of the success achieved by Phillips (15) with this reagent for disaggregation in his studies on the *N*-terminal groups of histones. As a control, a sample of native trypsin was carried through the entire end group analysis.

TABLE I

"Immediate" and "time dependent" base consumption during reactivation of inactivated trypsin at several pH values

pH	Time to titrate to pH	Moles of base per mole of trypsin		
		"Immediate"	"Time dependent"	Total
	min.			
7.0	0.6	31.2	28.5*	59.7*
8.0	2.4	32.9	33.0	65.9
9.0	3.0	43.0	35.4	78.4
10.0	3.9	46.9	28.8	75.7
11.0	5.7	78.5	6.3	84.8

* The base consumption at pH 7.0 had not reached a constant rate even after 48 hours and, as a result, this value is probably low.

TABLE II

Ninhydrin color yield of trypsin during inactivation with formic acid

Time of inactivation	Absorbancy at 570 mμ*
min.	
2	0.405
110	0.380
283	0.395
457	0.406
857	0.397

* Average of duplicate samples.

Details of the procedure are as follows. 2 μmoles of trypsin or inactivated trypsin in 3 ml. of 6 M guanidine hydrochloride (pH 7.0) were treated with 0.1 ml. of pure FDB in the autotitrator at pH 7.0 and 25° for 2 hours, when the reaction was terminated by lowering the pH to 3.0. The solution was transferred to a 50-ml. centrifuge tube and diluted to 20 ml. with 0.001 M HCl and extracted once with ether. The yellow precipitate was washed repeatedly with 0.001 M HCl, followed by acetone and ether, and finally dried, first under a stream of air and then in a vacuum over P_2O_5. Approximately 0.3 μmole of dried DNP-protein was suspended in 0.2 ml. of 5.7 N HCl in a small test tube, and after evacuation and sealing, heated for 16 hours at 105° (butyl phthalate bath). The ether phases of the hydrolysates were examined by one-dimensional paper chromatography with *tert*-amyl alcohol-pH 6.0 phthalate buffer according to Blackburn and Lowther (16). The DNP-amino acid spots were cut out, eluted, and their yield was estimated as described by Fraenkel-Conrat *et al.* (17). The results, uncorrected for losses during hydrolysis and chromatography, are presented in Table III. It is evident that the FDB reaction at pH 7.0 had proceeded to a satisfactory extent with both proteins, as judged by the yield of DNP-isoleucine. It is also evident that no significant difference was found between trypsin and inactivated trypsin.

Electrophoresis of Trypsin and Inactivated Trypsin in 8 M Urea at pH 5.1—Josefsson and Edman (1, 2) have shown both with lysozyme and ribonuclease that an increase of electrophoretic mobility occurs upon inactivation with formic acid. Because of the insoluble nature of the inactivated trypsin, it was necessary to carry out the electrophoresis in 8 M urea

TABLE III

Yields of DNP-amino acids from reaction of FDB with trypsin and inactivated trypsin

	Trypsin	Inactivated trypsin
	moles/mole protein	*moles/mole*
DNP-serine	0.02	0.03
DNP-isoleucine	0.50	0.61
DNP-aspartic and/or DNP-glutamic acid	trace	trace
DNP-valine	trace	trace

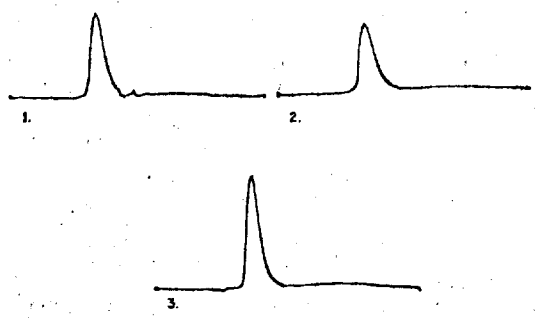


FIG. 3. Ascending electrophoresis patterns of (1) 0.7 per cent trypsin, (2) 0.7 per cent inactivated trypsin, (3) mixture of 0.5 per cent trypsin and 0.5 per cent inactivated trypsin; after 1123 minutes in 8 M urea, acetate buffer, pH 5.1, ionic strength 0.1, voltage gradient 1.28 volts per cm. at 4 ma.

TABLE IV

Electrophoretic mobilities of trypsin and inactivated trypsin in 8 M urea, acetate buffer, pH 5.1

	Mobility	
	Ascending	Descending
	cm.² sec⁻¹ volt⁻¹ × 10⁵	
Trypsin	+3.5	+2.9
Inactivated trypsin	+3.6	+3.0
Trypsin plus inactivated trypsin	+3.7	+2.7

This was successfully achieved by running the experiment at 20° and at a low current to avoid the development of convection anomalies usually experienced under such conditions.

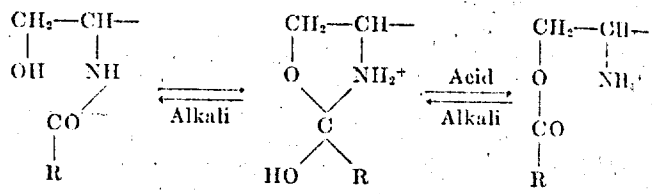
The patterns of the ascending boundaries of trypsin, of inactivated trypsin, and of a trypsin-inactivated trypsin mixture are shown in Fig. 3. The similarities of the patterns and of the mobilities of the ascending and descending boundaries (Table IV) indicate no significant difference in charge between trypsin and inactivated trypsin.

Optical Rotation of Trypsin in Anhydrous Formic Acid—In view of the dependence of optical rotation on the helical configuration of proteins, the introduction of an additional atom into the backbone of the polypeptide chain, arising from an *N*- to *O*-acyl shift, would be expected to cause a significant change in the optical rotatory power of the protein. Accordingly, a 0.75 per cent solution of trypsin in anhydrous formic acid was prepared and the optical rotation followed over a 15-hour period at room temperature. Immediately after mixing, the specific optical rotation at 5890 A was −56° and had changed only to −59° at the end of the experiment. Although these measurements were made under different conditions, the results are in agreement with those of Josefsson (3), who found no differences in the optical rotatory properties of native and formic acid-inactivated ribonuclease.

DISCUSSION

The hypothesis of an *N*- to *O*-peptidyl shift in the inactivation of enzymes by anhydrous formic acid is not substantiated by the results of this investigation. Attempts to demonstrate the presence of additional amino groups by the ninhydrin method or by reaction with FDB gave negative results. The titration data indicate that if these groups exist they cannot have a pK value of less than 10.5, which seems unlikely for a primary amino group. The electrophoretic experiments have failed to show a charge increase, and there was no change in the optical rotatory properties upon inactivation.

The participation of the hydroxyamino acids in the inactivation by formic acid is indicated by the fact that with all three enzymes the amount of hydrogen ion liberated is of the same order of magnitude as the number of serine and threonine residues in the proteins. The difficulties encountered in demonstrating the presence of free amino groups in inactivated lysozyme and ribonuclease have been attributed to the nonavailability, for steric reasons, of these groups in the *O*-peptidyl molecules (1, 2). It is difficult, in the case of trypsin, to conceive of any mechanism by which over 30 of these groups could be completely masked. An alternative explanation could be that the reaction had proceeded only to a hydroxyoxazolidine structure, the intermediate considered by Elliott as the most probable in the *N*- to *O*-acyl shift (14).



The nitrogen of this structure would be expected to possess strong basic properties and this would be consistent with a pK value of not less than 10.5, as indicated by the results of this investigation. However, such a structure would increase the positive charge on the protein in solutions of pH less than 10.5, resulting in an increase in the electrophoretic mobility of the inactivated enzyme. This is consistent with the findings with lysozyme and ribonuclease (1, 2), but not with trypsin.

A third possibility is that the inactivation with formic acid involves an acylation of the hydroxyl groups of serine and threonine. Such an ester bond would be labile at alkaline pH values, and the resulting uptake of base would be consistent with the titration data as previously indicated in this communication. This mechanism would also be consistent with all the experimental evidence presented above for trypsin, but would not account for the increase in amino nitrogen observed by Josefsson and Edman (1) during the inactivation of lysozyme or for the increased electrophoretic mobility of inactivated lysozyme and ribonuclease. It is conceivable that in the latter case the higher mobility could result from the marked aggregation observed with this inactivated enzyme. These authors have also shown that neither serine nor threonine in the free state reacts with formic acid. The formylation of these amino

acids would thus require that they be more reactive in a polypeptide chain than in the free state.

Until more definite experimental evidence is forthcoming, we believe it is unwise to conclude that the inactivation of trypsin by formic acid is attributable to an *N*- to *O*-acyl shift. A decision about other possible mechanisms must await further investigation.[3]

SUMMARY

Trypsin is inactivated by treatment for 14 to 17 hours with anhydrous formic acid. The inactivated enzyme is insoluble in aqueous solutions at pH values from 1 to 12 but is soluble in concentrated urea and guanidine hydrochloride solutions. By incubation in 8 M urea, at pH 8, the enzyme was reactivated, and the reactivation was accompanied by an uptake of 30 to 35 moles of base per mole of trypsin. Attempts to demonstrate the presence of additional amino groups in the inactivated enzyme by the ninhydrin method or by reaction with dinitrofluorobenzene gave negative results. Titration data indicated that if these groups exist, they cannot have a pK of less than 10.5. Electrophoretic experiments failed to show an increase in mobility, and there was no significant change in optical rotation upon inactivation. These results do not support the hypothesis of an *N*- to *O*-acyl shift in trypsin produced by anhydrous formic acid, and other possible mechanisms of inactivation are considered.

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[3] After completion of these experiments, we have learned that Dr. J. Rabinowitz, in Professor Linderstrøm-Lang's laboratory, has obtained direct evidence that treatment of histidyl-L-serine and poly-L-serine with C^{14}-labeled, anhydrous formic acid, under the present conditions, leads to the formylation of the serine side chains.

RANGE-FINDING TOXICITY DATA

List V

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SINCE the appearance of our previous communications on the range-finding test,* additional data from our work have become available for presentation. They are listed in the Table. All materials named either are being handled in commercial quantities or have been tentatively evaluated for specific commercial applications within the past few years. A few are known by trade names not suggested by the chemical names given here. In particular, Cellosolve compounds are tabulated as oxyethanols; Carbitol compounds appear as oxyethoxyethanols, ethanolamines as substituted 2-aminoethanols, and diethanolamines as substituted 2,2'-iminodiethanols.

It should be emphasized that the range-finding test is relied upon only to allow predictions of the comparative hazards of handling new chemicals. Acute toxicity studies, no matter how carefully planned, yield no more than indications of the degree of care necessary to protect exposed workmen and indications that certain technically feasible applications of a chemical may or may not eventually be proved safe.

It appears inevitable that minor changes in details will take place in any method which is employed for a period of years. For that reason, as well as to save reference to the earlier papers in this series,† the methods used for obtaining the data of the Table are briefly described again.

Single dose oral toxicity for rats is estimated by intubation of dosages in a logarithmic series to groups of five male rats or at times to female rats. The animals are Carworth-Wistar rats, raised in our own colony, fed Rockland rat diet complete, weighing 90 to 120 gm., and not fasted before dosing. Chemicals are diluted with water, corn oil, or a 1% solution of sodium 3,9-diethyl-6-tridecanol sulfate (Tergitol Penetrant 7) when necessary to bring the volume given one rat to between 1 and 10 ml. Fourteen days after dosing, mortality is considered complete. The most

From the Mellon Institute of Industrial Research, University of Pittsburgh.

* References 1 to 4.

† References 1 to 4.

Range-Finding Toxicity Data

Material Studied	Single-Dose Oral Toxicity for Rats (R. F. L. D_{50} After 14 Days), Gm./Kg.*	Penetration of Rabbit Skin (R. F. L. D_{50} After 14 Days), Ml./Kg.*	Concentrated Vapor Inhalation by Rats: Maximum for No Death	Inhalation of Known Vapor Concentration by Rats: Concentration, Ppm	Time, Hr.	Mortality in 14 Days	Primary Skin Irritation on Rabbits	Eye Injury in Rabbits from Fluid
Hydroxy Compounds								
Propanol-1	1.87 (1.31-2.60)	5.04 (3.40-7.46)	2 hr.	4,000	4	2/6	1	5
Butanol-2	6.48 (5.73-7.32)	.....	.....	16,000	4	5/6	1	4
Isobutyl alcohol	2.46 (1.60-3.78)	4.24 (2.52-7.12)	2 hr.	8,000	4	2/6	1	7
Pentanol-3	1.87 (1.34-2.60)	2.52 (1.33-4.78)	2 hr.	.....	..	...	2	5
2-Methylpentanol-1	1.41 (0.96-2.08)	3.56	8 hr.	.....	..	...	1	[illegible]
2-Methyl-2-pentene-1-ol	1.92 (3.75-6.46)	3.00 (2.13-4.21)	4 hr.	.....	..	...	2	5
2-Ethylbutanol-1	1.85 (1.52-2.24) †	1.26 (0.85-1.87)	8 hr.	.....	..	...	2	4
Heptanol-2	2.58 (2.29-2.91) †	1.78 (1.10-2.88)	8 hr.	.....	..	...	[illegible]	[illegible]
2,6,8-Trimethylnonanol-4	17.0 (12.2-23.8)	11.22 (5.10-24.73)	8 hr.	.....	..	...	3	1
2,8-Dimethyl-6-isobutylnonanol-4	16.3 (14.5-18.3) †	.....	8 hr.	.....	..	...	3	1
Pentanediol-2,4	6.86 (6.29-7.48)	14.1	8 hr.	.....	..	...	1	1
2,2-Diethylpropanediol-1,3	0.85 (0.78-0.93)	4.21 (3.01-5.95)	4 hr.	.....	..	...	1	3
2,4-Dimethyl-2-ethoxymethylpentane-2,5-diol	5.65	.....	8 hr.	.....	..	...	2	4
1,2,6-Hexanetriol	17.0 (12.2-23.8)	> 20	4 hr.	.....	..	...	1	1
2-Methyloltetrahydro-1,4-pyran	3.73 (2.68-5.21)	4.00 (2.18-6.45)	8 hr.	.....	..	...	1	7
3,4-Dimethylpyrrolidine-ethanol	1.23 (0.94-1.62)	0.85 (0.60-1.19)	8 hr.	.....	..	...	[illegible]	6
2-Ethylhexylphenol	2.46 (1.88-3.23)	2.52 (1.70-3.73)	8 hr.	.....	..	...	6	10
Pyrocatechol, 4-*tert*-butyl-	2.82 (2.23-3.58)	0.63 (0.43-0.94)	8 hr.	.....	..	...	[illegible]	10
Hydroxy Ethers								
2-(Hexyloxy)ethanol	1.48 (1.30-1.60)	0.89 (0.55-1.44)	8 hr.	.....	..	...	1	[illegible]
2-(2-ethylbutoxy)ethanol	1.91 (1.60-2.28)	0.32 (0.17-0.60)	8 hr.	.....	..	...	2	9
2-(1-ethylamyloxy)ethanol	2.28 (1.92-2.72) †	.....	8 hr.	.....	..	...	4	[illegible]
2-(2-ethylhexyloxy)ethanol	3.08 (2.49-3.81)	2.12 (1.26-3.56)	8 hr.	.....	..	...	5	9
2-(Isobutyl-3-methylbutoxy)ethanol	5.41 (4.79-6.11)	.....	8 hr.	.....	..	...	3	5
2-(Methoxy-methoxy)ethanol	6.50 (4.84-8.52)	4.23 (2.52-7.12)	.....	.....	..	...	2	2
2-(2-[1-ethylamyloxy]ethoxy)ethanol	2.94 (2.41-3.53) †	.....	8 hr.	.....	..	...	4	9
2-(2-[1-isobutyl-3-methylbutoxy]ethoxy)ethanol	8.68 (5.53-13.63)	3.00 (1.78-5.04)	4 hr.	.....	..	...	4	5
2-(2-phenoxyethoxy)ethanol	2.14 (1.54-2.99)	2.12 (1.26-3.56)	8 hr.	.....	..	...	1	7
3-(2-ethoxyethoxy)propanol	7.06	.....	8 hr.	.....	..	...	1	3
3-(2-butoxyethoxy)propanol	5.16 (3.53-9.06)	3.00 (2.13-4.21)	8 hr.	.....	..	...	4	5
Ethers								
Butyl ether	7.40 (6.41-8.53)	10.08 (4.41-23.04)	30 min.	4,000	4	2/6	4	1
Hexyl ether	30.9 (27.8-34.4) †	6.9 (3.6-10.0)	8 hr.	.....	..	...	5	1
Di-(2-ethylhexyl) ether	33.9 (22.0-52.0)	.....	4 hr.	.....	..	...	3	1
Vinyl butyl ether	10.30 (8.40-12.63)	1.24 (3.02-5.95)	5 min.	8,000‡	4	0/6	3	2

Vinyl 2-ethylhexyl ether	1.35 (1.17-1.56)	3.56 (2.20-5.76)	4 hr.	.....	..	...	3	1
Vinyl 2,6,8-trimethylnonyl ether	1.22 (0.98-1.52)	5.0	4 hr.	.....	..	...	5	1
Vinyl 2-methoxyethyl ether	3.90 (3.49-4.30)	7.13 (4.41-11.52)	1 hr.	8,000	4	4/6	1	2
2-Ethoxy-4-methyl-2,3-dihydro-4H-pyran	3.40 (3.11-3.72)	1.06 (0.63-1.79)	4 hr.	.....	..	...	3	2
1,2-Dibutoxyethane	3.25 (2.82-3.74)	3.56 (2.20-5.76)	8 hr.	.....	..	...	4	2
2,2'-Dibutoxyethyl ether	3.90 (3.35-4.50)	4.04 (3.41-7.46)	8 hr.	.....	..	...	3	2
1-Butoxy-2-ethoxyethane	2.83 (1.93-4.15)	2.12 (1.26-3.56)	2 hr.	.....	..	...	2	4
2-Butoxyethyl vinyl ether	3.10 (2.77-3.46)	3.00 (2.13-4.21)	4 hr.	2,000	8	2/6	2	3
1,1-Dibutoxyethane	8.79 (7.96-9.70) †	.....	8 hr.	.....	..	...	5	1
1,1-Di(2-methoxyethoxy)ethane	3.26 (2.73-3.90)	4.24 (2.52-7.12)	8 hr.	.....	..	...	1	1
1,1,3-Trimethoxybutane	1.48 (1.35-1.62)	.....	2 hr.	2,000	1	1/6	2	2
Epoxy Compounds								
1,2,3,4-Diepoxybutane	0.078 (0.059-0.102)	0.089 (0.055-0.144)	15 min.§	.....	..	...	6	9
Diisobutylene oxide	4.92 (3.75-6.46)	14.1 (8.7-22.9)	10 min.	4,000	4	2/6	1	2
Phenyl glycidyl ether	4.26 (3.90-4.60)	1.50 (1.07-2.10)	8 hr.	.....	..	...	5	2
Epoxyethylbenzene	4.29 (3.07-5.98)	1.06 (0.63-1.79)	1 hr.	500	4	2/6	2	2
Ketones								
Pentanone-3	2.14 (1.54-2.99)	20	15 min.	8,000	1	1/6	2	1
Hexanone-2	2.59 (2.11-3.18)	5.99 (4.27-8.42)	30 min.	4,000‡	4	0/6	1	3
5-Ethyl-5-nonen-2-one	8.12 (7.27-9.06)	8.48 (5.61-11.21)	8 hr.	.....	..	...	2	1
Aldehydes								
Isobutyraldehyde	3.73 (2.68-5.21) †	7.13	15 min.	8,000	1	1/6	1	5
Hexanal	4.89 (4.52-5.61)	.....	1 hr.	2,000	1	1/6	2	2
Hexa-2,4-dienal	0.73 (0.61-0.86)	0.27 (0.16-0.45)	15 min.	2,000	4	1/6	6	8
2-Methyl-2-pentene-1-al	4.29 (3.07-5.98)	4.5 (2.0-9.8)	1 hr.	2,000	1	2/6	2	5
3-Methylglutaraldehyde	0.78 (0.46-1.31)	0.30 (0.14-0.67)	8 hr.	.....	..	...	6	9
α-Hydroxyadipaldehyde	17.0 (12.2-23.8)	> 20	8 hr.	.....	..	...	2	1
2,3,5-Trimethylcyclohexanecarboxaldehyde	4.14 (3.18-5.38)	15.8	8 hr.	.....	.	...	1	2
Acids, Anhydrides, Lactones								
Propionic anhydride	2.36 (2.06-2.71) †	10.0 (5.3-19.0)	1 hr.	.....	..	...	2	8
Butyric acid	8.79 (8.06-9.58) †	6.35 (3.91-10.28)	.....	.....	..	...	5	9
α-Methoxybutyric acid	3.03 (2.44-3.75)	.....	8 hr.	.....	..	...	4	8
2-Ethylbutyric acid	2.20 (1.97-2.46) †	0.52 (0.38-0.75)	8 hr.	.....	..	...	2	9
Hexanoic acid	6.44 (5.77-7.19)	0.63 (0.33-1.20)	8 hr.	.....	..	...	6	8
2-Methylpentanoic acid	2.01 (1.82-2.27)	.....	8 hr.	.....	..	...	4	9
3-Butoxypropionic acid	5.19 (4.20-6.41)	0.63 (0.43-0.91)	8 hr.	.....	..	...	4	9
Hexanoic acid, γ-lactone	4.29 (3.07-5.98)	5.99 (4.27-8.42)	8 hr.	.....	..	...	1	8
3(2-Ethylhexoxy)propionic acid	3.73 (2.43-5.71)	0.53 (0.38-0.75)	4 hr.	.....	..	...	6	8
3,5-Dimethyl-3-hydroxy-4-hexanoic acid, β-lactone	2.70 (2.31-3.41)	.....	4 hr.	.....	..	...	1	5
3(2-ethylhexyloxy)propionic acid	3.73 (2.68-5.21)	0.75 (0.54-1.06)	8 hr.	.....	..	...	6	7
2-Cyclohexene-1-carboxylic acid	1.26 (3.65-1.98)	1.00 (0.68-1.18)	4 hr.	.....	..	...	6	9

Range-Finding Toxicity Data—Continued

Material Studied	Single-Dose Oral Toxicity for Rats (R. F. L. D_{50} After 14 Days), Gm./Kg.*	Penetration of Rabbit Skin (R. F. L. D_{50} After 14 Days), Ml./Kg.*	Concentrated Vapor Inhalation by Rats: Maximum for No Death	Inhalation of Known Vapor Concentration by Rats: Concentration, Ppm	Time, Hr.	Mortality in 14 Days	Primary Skin Irritation on Rabbits	Eye Injury in Rabbits from Fluid
Esters								
Carbonic acid, cyclic ethylene ester	10.40 (7.94-13.62)	> 20	8 hr.	.....	..	...	1	1
Carbonic acid, cyclic propylene ester	29.1 (23.8-35.5)	> 20	8 hr.	.....	..	...	1	1
Formic acid, ethyl ester	4.29 (3.07-5.98)	> 20	5 min.	8,000	1	5/6	1	1
Acetic acid, isopropyl ester	6.75 (6.16-7.38)	> 20	30 min.	32,000	1	5/6	1	2
Acetic acid, butyl ester	14.13 (11.84-16.85)	> 20	4 hr.	.....	..	...	1	5
Acetic acid, 3-methoxybutyl ester	4.21 (3.68-4.82)	.....	8 hr.	.....	..	...	2	1
Acetic acid, 1,3-dimethylbutyl ester	6.16 (5.52-6.88)	> 20	2 hr.	4,000	4	2/6	1	2
Acetic acid, 2-methylamyl ester	7.40 (6.41-8.53)	> 20	8 hr.	.....	..	...	2	2
Acetic acid, 3-heptanol ester	8.35 (7.40-9.40) †	.....	8 hr.	.....	..	...	3	1
Acetic acid, 2[2-(2-methoxyethoxy)ethoxy]ethyl ester	11.26 (7.60-16.53)	8.00 (3.60-17.78)	8 hr.	.....	..	...	1	2
Acrylic acid, 2-ethoxyethanol ester	1.07 (0.77-1.49)	1.01 (0.68-1.49)	1 hr.	500	4	5/6	2	5
Methacrylic acid, 2-butyloctyl ester	25.8 (16.8-39.7)	.....	8 hr.	.....	..	...	2	1
Acetoacetic acid, butyl ester	11.26 (7.60-16.53) †	.....	8 hr.	.....	..	...	1	2
2-Ethylhexoic acid, vinyl ester	4.29 (3.07-5.98)	.....	8 hr.	.....	..	...	5	1
Succinic acid, dipropyl ester	6.49 (4.95-8.53)	.....	8 hr.	.....	..	...	1	1
Succinic acid, di-2-hexyloxyethyl ester	4.28 (3.08-5.98)	12.31 (8.01-18.92)	8 hr.	.....	..	...	2	1
Crotonic acid, vinyl ester	6.50 (4.95-8.53)	.....	1 hr.	2,000	4	0/6	1	2
Fumaric acid, diisopropyl ester	3.25 (2.48-4.26)	10	8 hr.	.....	..	...	4	2
Fumaric acid, di-(2-ethylhexyl) ester	29.2 (15.8-53.9)	> 20	8 hr.	.....	..	...	5	1
Maleic acid, diisopropyl ester	2.14 (1.54-2.99)	.....	8 hr.	.....	..	...	2	1
Maleic acid, dibutyl ester	3.73 (2.68-5.21)	10.1 (6.8-14.9)	.....	.....	..	...	2	1
Maleic acid, di(1,3-dimethylbutyl) ester	7.46 (4.68-11.47)	11.9 (8.5-16.7)	8 hr.	.....	..	...	1	1
Maleic acid, mono(hydroxyethyl) ester	2.46 (1.88-3.23)	.....	8 hr.	.....	..	...	1	7
Maleic acid, mono(hydroxyethoxyethyl) ester	2.83	7.13	8 hr.	.....	..	...	1	5
Maleic acid, mono(2-hydroxypropyl) ester	3.73 (2.68-5.21)	8.48 (6.04-11.90)	8 hr.	.....	..	...	2	7
Adipic acid, di(2-ethylbutyl) ester	5.62 (4.36-7.25)	16.8 (12.0-23.6)	8 hr.	.....	..	..	1	1
Adipic acid, di(2-hexyloxyethyl) ester	4.29 (3.07-5.98)	12.31 (8.01-18.92)	.....	.....	..	...	2	1
Adipic acid, di(2[2-ethylbutoxy]ethyl) ester	3.25 (2.48-4.26)	4.24 (2.52-7.12)	8 hr.	.....	..	...	4	1
Benzoic acid, methyl ester	3.43 (2.83-4.15)	.....	8 hr.	.....	..	...	3	1
Benzoic acid, ethyl ester	6.48 (5.66-7.42) †	.....	8 hr.	.....	..	...	4	1
Benzoic acid, vinyl ester	3.25 (2.48-4.26)	.....	8 hr.	.....	..	...	5	2
Benzoic acid, butyl ester	5.14 (4.70-5.62) †	.....	8 hr.	.....	..	...	5	1
Phthalic acid, dihexyl ester	29.6 (27.0-32.4)	> 20	8 hr.	.....	..	...	1	1
1,2-Carbo(2-ethyl)hexyloxycyclohexane	> 60	> 20	8 hr.	.....	..	...	1	1

Halogen Compounds								
1,1-Dichloropropane	6.50 (4.95-8.53)	14.1 (8.8-22.6)	2 min.	4,000	4	4/6	1	2
1,1,2-Trichloropropane	1.23 (0.94-1.62)	14.1 (8.8-22.9)	30 min.	2,000	4	3/6	4	5
1-Chloropropene	1.95 (1.40-2.72)	20	2 min.§	32,000	4	1/6	1	2
1-Chlorobutane	2.67 (2.32-3.06) †	> 20	15 min.	8,000	4	2/6	3	2
1,4-Dibromo-2-butene	0.075 (0.057-0.097)	.....	10 min.	.....	..	...	6	10
Nitriles								
3-Butoxypropionitrile	7.46 (5.35-10.41)	8.98 (4.10-19.68)	8 hr.	.....	..	...	1	1
3-(2-ethylbutoxy)-propionitrile	2.46 (1.88-3.23)	10	8 hr.	.....	..	...	1	1
3-(2-ethylhexyloxy)-propionitrile	4.92 (3.75-6.46)	5.09 (1.27-8.42)	4 hr.	.....	..	...	1	1
3,3'-Oxydipropionitrile	2.83	.....	8 hr.	.....	..	...	1	1
3,3'-Iminodipropionitrile	4.92 (3.75-6.46) †	2.52 (1.70-3.73)	8 hr.	.....	..	...	1	1
N-Methyl-3,3'-iminodipropionitrile	0.89 (0.61-1.31)	0.80 (0.39-1.62)	8 hr.	.....	..	...	1	2
2-Hydroxy-3-butenenitrile	0.065 (0.041-0.081)	0.0075 (0.0051-0.0106)	.....	16	4	3/6	7	10
2-Cyclohexene-1-carbonitrile	0.46 (0.38-0.55)	9.46 (6.76-13.29)	30 min.	62‡	4	0/6	1	2
Amino Compounds								
Ethylamine	0.40 (0.29-0.56)	0.39 (0.28-0.55)	2 min.§	8,000	4	2/6	1	9
Diisopropylamine	0.77 (0.61-0.94)	.....	5 min.§	1,000	4	2/6	1	8
Dibutylamine	0.55 (0.48-0.62)	1.01 (0.68-1.49)	.....	250‡	4	0/6	5	9
Hexylamine	0.67 (0.62-0.74)	0.42 (0.30-0.60)	1 hr.	500	4	2/6	6	8
2-Ethylbutylamine	0.39 (0.34-0.45)	2.00 (1.35-2.96)	15 min.	500‡	4	0/6	6	9
1-Methyl-4-ethylbutylamine	0.73 (0.64-0.82)	0.38 (0.27-0.53)	8 hr.	.....	..	...	6	10
N,N-Diethylethylenediamine	2.83	0.82 (0.53-1.26)	8 hr.	.....	..	...	6	10
N,N'-Di(α-methylbenzyl)ethylenediamine	1.29 (1.12-1.49)	0.53 (0.38-0.75)	8 hr.	.....	..	..	4	5
1,3-Diaminopropane	2.23 (1.90-2.62) †	0.50	4 hr.	.....	..	...	6	8
N,N-Dimethyl-α-methylbenzylamine	0.42 (0.27-0.66)	0.89	30 min.	125	4	2/6	6	4
2-Methylaminoethanol	2.34 (2.13-2.56)	.....	8 hr.	.....	..	...	3	9
2-Ethylaminoethanol	1.48 (1.35-1.62)	0.36	8 hr.	.....	..	...	2	9
2-Butylaminoethanol	1.15 (1.04-1.27) †	.....	8 hr.	.....	..	...	6	9
2-Phenylethylaminoethanol	1.87 (1.34-2.60)	3.00 (2.13-4.21)	8 hr.	.....	..	...	2	8
2-Diisopropylaminoethanol	1.07 (0.77-1.50)	0.45 (0.28-0.72)	8 hr.	.....	..	...	1	8
2-Dibutylaminoethanol	1.07 (0.98-1.17)	1.68 (1.20-2.36)	8 hr.	.....	..	...	6	5
2-N-(2-ethylhexyl)aminoethanol	4.92 (3.75-6.46)	2.52 (1.33-4.78)	8 hr.	.....	..	...	3	1
N-Methyl-2,2'-iminodiethanol	4.78 (4.46-5.12) †	5.09 (3.57-10.07)	8 hr.	.....	..	...	2	4
N-Ethyl-2,2'-iminodiethanol	4.57 (4.11-5.07)	.....	8 hr.	.....	..	...	2	8
N-Butyl-2,2'-iminodiethanol	4.25 (3.60-5.00) †	.....	8 hr.	.....	..	...	2	8
1,1-Dimethylaminopropanol-2	1.89 (1.59-2.18)	.....	2 hr.	.....	..	...	3	9
N-Butylaniline	1.62 (1.24-2.13)	5.09 (3.57-10.07)	8 hr.	.....	..	...	5	2
3'-Aminoacetophenone	1.87 (1.31-2.60)	1.31 (3.01-5.95)	4 hr.	.....	..	...	1	2
3-Amino-α-methylbenzyl alcohol	3.10 (2.77-3.46)	3.56 (2.20-5.76)	8 hr.	.....	..	...	1	2

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Range-Finding Toxicity Data—Continued

Material Studied	Single-Dose Oral Toxicity for Rats (R. F. L. D_{50} After 14 Days), Gm./Kg.	Penetration of Rabbit Skin (R. F. L. D_{50} After 14 Days), Ml./Kg.*	Concentrated Vapor Inhalation by Rats; Maximum for No Death	Inhalation of Known Vapor Concentration by Rats: Concentration, Ppm	Time, Hr.	Mortality in 14 Days	Primary Skin Irritation on Rabbits	Eye Injury in Rabbits from Fluid
Heterocyclic Nitrogen Compounds								
5-Ethyl-2-methylpiperidine	0.54 (0.38-0.75)	0.63 (0.43-0.91)	30 min.	250	4	5/6	6	9
5-Ethyl-2-vinylpyridine	1.23 (0.94-1.62)	0.89	4 hr.	8,000	4	2/6	6	4
4-Picoline	1.29 (1.12-1.50)	0.27 (0.19-0.38)	30 min.	1,000	4	1/6	5	8
Morpholine	1.05 (0.95-1.16) †	0.50 (0.31-0.81)	1 hr.	8,000	8	0/6	6	7
4-Ethylmorpholine	1.78 (1.49-2.12)	.....	2 hr.	2,000	4	1/6	1	7
4-Acetylmorpholine	6.13 (5.00-7.51)	7.5	8 hr.	.....	..	...	1	2
Silanes								
Vinyltriethoxysilane	22.5	10	.....	4,000	4	2/6	1	1
Vinyltrichlorosilane	1.28 (1.05-1.59)	0.68 (0.48-0.94)	15 min.	2,000ƨ	4	0/6	6	10
Amyltriethoxysilane	19.6 (14.9-25.5)	7.13 (4.45-11.41)	8 hr.	.....	..	...	3	1
Tris(2-chloroethoxy)silane	0.19 (0.16-0.22)	0.089	8 hr.	.....	..	...	3	5
Phenyltrichlorosilane	2.39 (2.19-2.61)	0.89 (0.61-1.31)	8 hr.	.....	..	...	6	9
Miscellaneous								
Cyclopentadiene dimer	0.82 (0.53-1.26)	6.72 (3.15-14.36)	2 hr.§	2,000	4	4/6	5	2
3,4-Dihydro-2H-pyran-2-carboxaldehyde	4.92 (3.75-6.46)	.....	8 hr.	.....	..	...	1	8
2,3-Dihydro-2,5-dimethyl-2-formyl-1,4-pyran	2.46 (1.88-3.23)	.....	2 hr.	.....	..	...	2	4
Azoxybenzene	0.62 (0.47-0.81)	1.09 (0.59-2.01)	.....	.....	..	...	3	1
3'-Nitroacetophenone	3.25 (2.11-4.99)	3.0 (1.8-5.0)	8 hr.	.....	..	...	1	1

* Figures in parentheses show limits of ± 1.96 standard deviations calculated by method of Thompson; absence of parentheses marks L_{50} obtained graphically.
† Female rats.
§ Inhalation time shown killed all six rats.
ƨ Twice concentration shown killed all six rats.

probable L. D_{50} value and its fiducial range are estimated by the method of Thompson[5] using the tables of Weil.[6] When fractional mortality is not observed, the L. D_{50} value is recorded without a fiducial range.

Penetration of rabbit skin is estimated by a technique essentially the one-day cuff method of Draize and associates,[7] using groups of four male New Zealand giant albino rabbits weighing 2.5 to 3.5 kg. The fur is closely clipped over the entire trunk, and the dose, retained beneath an impervious plastic film, contacts about 1/10 of the body surface. Dosages greater than 20 ml. per kilogram of body weight cannot be retained in contact with the skin. After 24 hours' contact the film is removed, and mortality is considered complete after 14 additional days.

Earlier papers in this series ‡ have spoken of saturated vapor inhalation. Recent reexamination of our methods indicates that a less definite term is desirable. Accordingly, we now speak of concentrated vapor inhalation. This consists in exposing groups of six male albino rats to a flowing stream of air approaching saturation with vapors. The stream is prepared by passing dried air through a fritted disc gas washing bottle initially at room temperature. When carbon dioxide will react with the sample, the air stream is freed of that gas. Inhalations are continued for periods of time in an essentially logarithmic series with a ratio of two, extending to eight hours, until the period killing half the rats within 14 days of inhalation is defined. The Table records the longest period which allowed all rats to survive 14 days or, in a few instances marked by a symbol, the shortest period tried when this killed all rats.

Inhalation of known vapor concentrations by rats is conducted with flowing streams of vapors prepared by various styles of proportioning pumps. Nominal concentrations are recorded, not confirmed by analytical methods. Exposures are four hours long, although rarely an eight-hour period is used. Concentrations are in an essentially logarithmic series with a factor of two, and the Table records the concentration yielding fractional mortality among six rats within 14 days. Where no fractional mortality was observed, both the concentration yielding no mortality and that yielding complete mortality are indicated.

Primary skin irritation on rabbits records in a 10-grade ordinal series the severest reaction on the clipped skin of any of five albino rabbits within 24 hours of application of 0.01 ml. of undiluted sample or of dilutions in water, propylene glycol, or kerosene. Grade 1 in the Table indicates the least visible capillary injection from undiluted chemical. Grade 6 indicates necrosis when undiluted, and Grade 10 indicates necrosis from a 0.01% solution.

Eye injury in rabbits records the degree of corneal necrosis from various volumes and concentrations of chemical, as detailed by Carpenter and Smyth.[8] Grade 1 in the Table indicates at most a very small area of necrosis resulting from 0.5 ml. of undiluted chemical in the eye; Grade 5 indicates a so-called severe burn from 0.005 ml., and Grade 10 indicates a severe burn from 0.5 ml. of a 1% solution in water or propylene glycol.

‡ References 1 to 4.

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STUDIES OF CHRONIC INTOXICATIONS ON ALBINO RATS

III. ACETIC AND FORMIC ACIDS

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Received for publication September 25, 1920

INTRODUCTION

The immediate occasion for investigating these acids was the question of the safety of the use of formic acid for the preservation of foods. The supposed safety of acetic acid for similar purposes made this an admirable measure of comparison; so that parallel experiments were carried out with both acids. However, the investigation was planned with a view to possible wider applications.

The effects of continued use of acetic acid have not been experimentally studied. It seemed of interest to learn whether the continued ingestion of considerable quantities of acid influenced digestion and metabolism, for instance by direct effects on the digestive tract, which is so largely controlled by the acid and alkaline reactions.

Acetic acid is so readily oxidized that it would probably not affect the acid-base equilibrium of the tissues. The oxidation of formic acid is less complete (Fleig), and might therefore conceivably alter the equilibrium, and this would probably be reflected in growth.

Formic acid has a further interest as an oxidation product of methyl alcohol (Pohl). Chronic or subacute poisoning with formic acid, if it exists, might therefore throw some light on chronic or subacute methyl alcohol poisoning.

Finally, some fifteen or eighteen years ago, formic acid and formates were loudly proclaimed as a sort of elixir of youth, destined to redeem the world, and to confer on men the indefatigability of insects. This action, if it existed, should be reflected in the nutrition and growth.

The interesting history of this fantastic propaganda as well as its scientific refutation, are given in the comprehensive paper of Fleig, 1907. He showed the remarkable tonic effects to be merely imaginary. The descriptions of his experiences are worth reading; for they apply equally to more modish tonics and rejuvenants; especially interesting is the description of the asthenic patient (page 215) who recovered his vigor on sodium chlorid, that he mistook for the more advertised formate.

Fleig's paper also contains the scientific data as to formates, of which his own are the most important. He reviews the literature so thoroughly that it is not necessary to enter further into that subject; except to abstract very briefly the data that apply to the present investigation. There seems to have been very little scientific work with formates since Fleig.

METHODS

The experiments were conducted as described in the first and second papers of this series; the acids being added to the drinking water, so that they were consumed continuously.

Dosage. This is determined by the concentration of the drug and by the thirst of the animals. The mean doses are shown in table 1. The weekly variations appear unimportant. They could be calculated from the curve of fluid consumption.

For the preservation of food, 0.1 to 0.15 per cent of free formic acid is said to suffice (Lebbin). This falls within the limit of the concentration (0.01 to 0.5 per cent) of the present experiments.

Lebbin administered to men for four weeks, daily doses of 0.5 gram of formic acid per kilogram of body weight, without any effects. This would correspond to about 8 mgm. per kilogram, which is our smallest dosage. Our largest dosage is 45 times as great. Lebbin also tried doses larger than 0.5 gram. They produced only local actions, analogous to acetic acid. In rabbits, continued administration of either formic or acetic acid damaged the kidneys.

Of sodium formate, Fleig found the acute toxic dose for dogs to be 3 grams per kilogram by vein, 4 grams per kilogram by mouth; this would correspond to 250 grams for man; i.e., the toxicity of the formate is of the same order as that of sodium chlorid. Men consumed daily doses of 10 grams of sodium formate, equivalent to 150 mgm. per kilogram, for some time without any harm. Eppinger observed hematuria in one out of three patients taking 3 to 4 grams per day; but this looks like an accidental coincidence.

TABLE 1

Dosage of acetic and formic acids

CONCENTRATION OF ACID	EXPERIMENT NUMBER	NUMBER OF ANIMALS IN EXPERIMENT	DURATION	MEAN DOSAGE, MILLIGRAMS OF ACID PER KILOGRAM OF RAT PER DAY	
Acetic acid					
per cent			*weeks*		
0.01	56	6	11	8.2	(5.7–11.3)
0.01	107	3	9	10.9	(9.6–12.9)
0.1	63	6	15	81.0	(63.0–95.0)
0.25 (after 10 weeks of 0.01)	5695	4	15	210.0	(79.0–390.0)
0.5 (after 16 weeks of 0.1)	6305	3	9	360.0	(310.0–710.0)
Formic acid					
0.01	57	6	11	8.2	(5.6–12.1)
0.01	108	3	14	10.25	(8.4–14.3)
0.1	64	6	15	90.0	(80.0–131.0)
0.25 (after 12 weeks of 0.01)	5796	4	15	160.0	(120.0–210.0)
0.5 (after 17 weeks of 0.1)	6406	3	9	360.0	(290.0–670.0)

CONSUMPTION OF FLUID

Table 2 gives the mean daily consumption of fluid in each experiment; as well as the mean for the first, middle and last third of each experiment, so as to indicate the influence of the continuance of the experiment.

Table 2 shows that there are no distinctive differences in the fluid consumption as between acetic or formic acids, or the different concentrations, or the different periods. It may there-

fore be concluded that neither acetic nor formic acid affect the consumption of fluid when added to the drink in concentrations up to at least 0.5 per cent.

TABLE 2

Mean daily consumption of fluid

ACIDS	EXPERIMENT NUMBER	DURATION	FLUID CONSUMPTION (CUBIC CENTIMETERS PER KILOGRAM OF RAT)			
			Entire period	First third	Middle third	Last third
		weeks				
Acetic acid, 0.01 per cent	56	11	82.0 (57-113)	104	80	80
Acetic acid, 0.01 per cent	107	9	109.0 (96-129)	106	112	107
Acetic acid, 0.1 per cent	63	15	81.0 (63-95)	83.5	69	80
Acetic acid, 0.25 per cent	5695	15	90.0 (74-155)	106	88	90
Acetic acid, 0.5 per cent	6305	9	71.0 (63-142)	71	67	86
Formic acid, 0.01 per cent	57	11	82.0 (56-121)	101	75	62
Formic acid, 0.01 per cent	108	14	102.5 (84-125)	122	89	99
Formic acid, 0.1 per cent	64	15	90.0 (80-109)	86	89	92
Formic acid, 0.25 per cent	5796	15	64.0 (49-85)	64	62	66
Formic acid, 0.5 per cent	6406	9	72.0 (58-135)	73	58	82
Formic acid, 0.75 per cent	138	2	127.0 (116-138)			

DIURETIC ACTION

The fact that formic acid does not increase the consumption of fluid implies that it does not act as a diuretic. According to Fleig, formates had considerable reputation as diuretics; and he quoted from Huchard several cases of marked diuresis following the administration of formates to cardiac and renal patients. If these were not accidental coincidences, they must have been due to the alkaline element of the formates. Fleig himself attributes the supposed diuresis to renal vasodilation that he observed on intravenous injection of sodium formate in animals; but this renal effect was evidently due to hydremic plethora from the injection of the solution.

EFFECTS ON GROWTH

The data are shown in table 3 and in figures 1, 2 and 3. It may be recalled that in non-medicated groups of rats, the variation from the standard growth do not exceed −1 to +1.8 per cent per week. The last column of table 3 shows that both

TABLE 3

Effects of acetic and formic acid on growth

DRUG AND CONCENTRATION	DOSAGE, CUBIC CENTIMETERS PER KILOGRAM OF RAT PER DAY	EXPERIMENT NUMBER	DURATION	OBSERVED WEIGHT	NORMAL WEIGHT	DIFFERENCE IN GRAMS	DIFFERENCE IN PER CENT OF NORMAL WEIGHT	DIFFERENCE PER CENT PER WEEK
			weeks					
Acetic acid, 0.01 per cent	8.2	56	11	175	250	−75	−30.0	−2.7
Acetic acid, 0.01 per cent	10.9	107	9	232	230	+2	+0.43	+0.04
Acetic acid, 0.1 per cent	81.0	63	15	304	295	+9	+3.2	+0.21
Acetic acid, 0.25 per cent (after 10 weeks of 0.01 per cent)	210.0	5695	5	209	212	−3	−1.4	−0.28
			15	215	258	−43	−16.0	−1.06
Acetic acid, 0.5 per cent (after 16 weeks of 0.1 per cent)	360.0	6305	1	264	312	−48	−15.0	−15.0
			5	270	315	−45	−14.0	−2.8
			9	240	319	−79	−24.0	−2.6
Formic acid, 0.01 per cent	8.2	57	10	199	190	+9	+4.6	−0.46
			11	162	192	−30	−15.0	−1.3
Formic acid, 0.01 per cent	10.25	108	14	160	177	−17	−9.0	−0.63
Formic acid, 0.1 per cent	90.0	64	15	275	282	−7	−2.0	−0.13
Formic acid, 0.25 per cent (after 12 weeks of 0.01 per cent)	160.0	5796	2	165	168	−3	−1.8	−0.9
			4	222	175	+47	+26.0	+6.4
			10	235	191	+44	+23.0	+2.3
			15	216	197	+19	+9.0	+0.6
Formic acid, 0.5 per cent (after 17 weeks of 0.1 per cent)	360.0	6406	9	180	295	−115	−56.0	−6.7

acetic and formic acids, in concentrations of 0.1 and 0.25 per cent are generally within these limits, and therefore had no effect on growth. With each acid, one experiment with 0.01 per cent also comes within the limits; and one experiment exceeds the

limits. In view of the negative results with 25 times this concentration, this experiment was evidently a coincidence.

By doubling the concentration, that is, with 0.5 per cent acids, and a dosage of 0.36 cc. per kilogram, both acids produced immediate and progressive check of growth, averaging 2.6 per cent per week for acetic acid, and 6.7 for formic acid or total

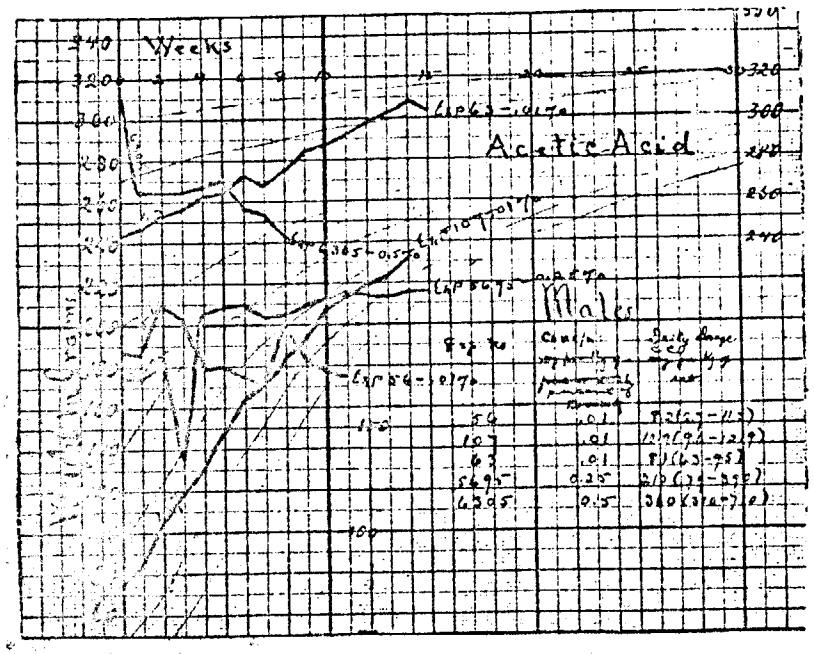

FIG. 1. EFFECT OF ACETIC ACID ON GROWTH

The heavy lines represent the growth of the alcohol rats; the light line represents the standard normal growth curves.

deficits, in the weight, of 24 per cent for acetic acid and 58 per cent for formic acid. The difference between the two acids is evidently accidental; for similar differences, but in the opposite direction, occur with the lower concentrations.

It may therefore be concluded that formic and acetic acid are quantitatively alike in their influence on growth. Neither has any effect in fifteen weeks when the concentration in the drink

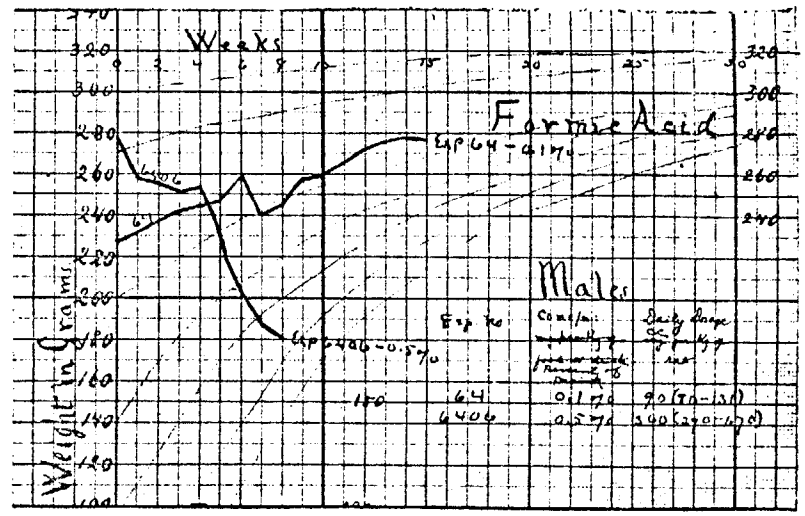

FIG. 2. FORMIC ACID ON GROWTH OF MALE RATS

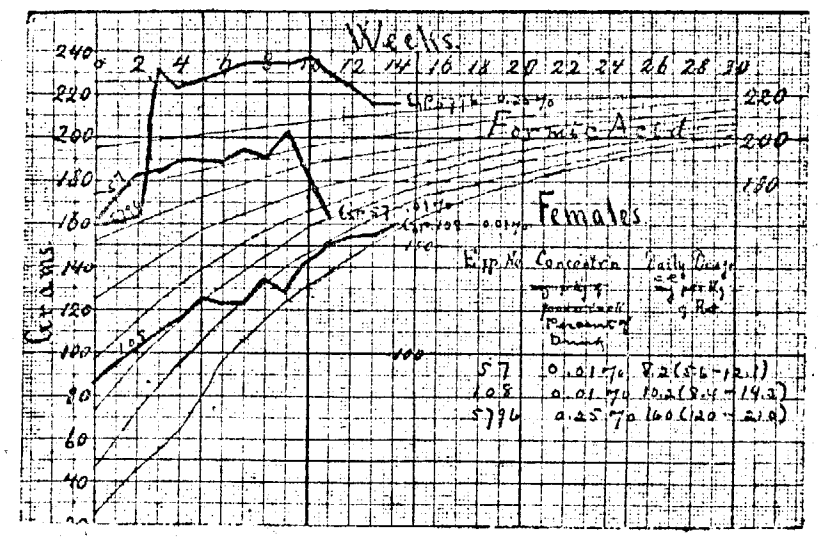

FIG. 3. FORMIC ACID ON GROWTH OF FEMALE RATS

does not exceed 0.25 per cent, with the daily dosage 0.2 cc. per kilogram of rat.

With a concentration of 0.5 per cent and daily dosage of 0.36 cc. per kilogram, growth is definitely checked from the start of the administration.

This does not depend on any change of the total fluid consumption, since this is not materially altered.

EFFECTS ON FOOD CONSUMPTION

These are shown in table 4 and figures 4 and 5.

In interpreting the data of table 4, it must be remembered that the extreme variation of unpoisoned rats from the standard

TABLE 4

Food consumption

ACIDS	EXPERIMENT NUMBER	DURATION OF EXPERIMENT	GROWTH, MEAN DIFFERENCE FROM NORMAL STANDARD PER CENT PER WEEK	FOOD CONSUMPTION, MEAN DIFFERENCE FROM NORMAL STANDARD GRAMS		MEAN DIFFERENCE (PER CENT) IN FOOD FOR SERIES
				Grams per rat per day	Per cent	
		weeks				
Acetic acid, 0.01 per cent	56	11	−2.7	−1.6	−15	−5.5
Acetic acid, 0.01 per cent	107	9	+0.04	+0.45	+4	
Acetic acid, 0.1 per cent	63	15	+0.21	+0.2	+1	+1.0
Acetic acid, 0.25 per cent (after 10 weeks of 0.01 per cent)	5695	15	−1.06	−0.8	−7	+7.0
Acetic acid 0.5 per cent (after 16 weeks of 0.1 per cent)	6305	9	−2.6	−3.8	−27	−27.0
Formic acid, 0.01 per cent	57	11	−1.3	−0.4	−4	−1.0
Formic acid, 0.01 per cent	108	14	−0.63	+0.2	+2	
Formic acid, 0.1 per cent	64	15	−0.13	+1.0	+8	+8.0
Formic acid, 0.25 per cent (after 12 weeks of 0.01 per cent)	5796	15	+0.6	+0.6	+6	+6.0
Formic acid, 0.5 per cent (after 17 weeks of 0.1 per cent)	6406	9	−0.66	−3.8	−29	−29.0

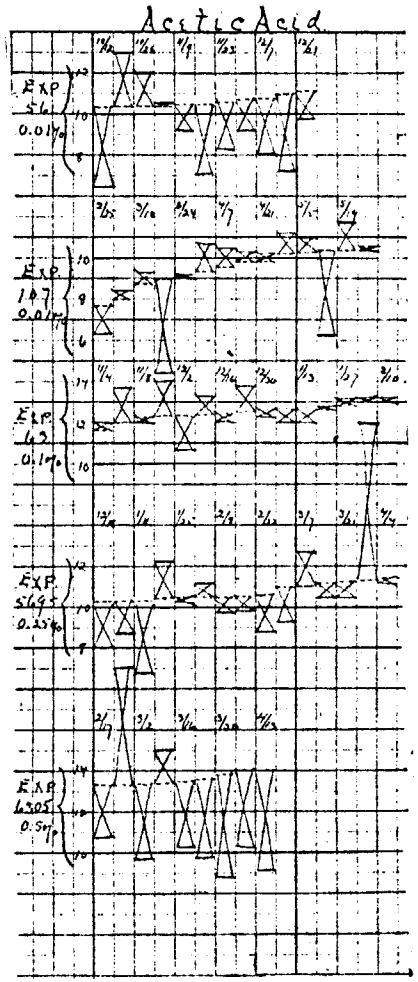

FIG. 4. ACETIC ACID ON FOOD CONSUMPTION

The numbers to the left represent grams of food consumed daily per rat. The numbers above each experiment are the dates of the observations. The dotted horizontal lines represent the standard food consumption; and the solid horizontal lines, joined to the dotted lines by crossed lines represent the actual food consumption.

food averages lies between −0.01 and +1.4 grams per rat per day, or −0.08 to +19 per cent.

The experiments with 0.1 per cent and 0.25 per cent acetic and formic acids arrange themselves in close proximity to both extremes; that is, the food consumption is normal; if anything, formic acid seems to stimulate appetite somewhat; but this is

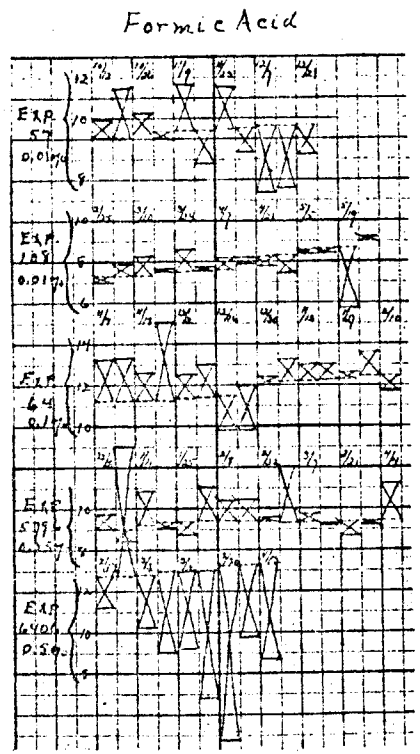

FIG. 5. FORMIC ACID ON FOOD CONSUMPTION

doubtless an accidental coincidence. With the 0.01 per cent acids, one experiment for each acid gave normal results, and the other a deficit; doubtless accidental, as explained under growth.

With the 0.5 per cent acids, there is serious interference with appetite; the food-consumption being reduced to 27 per cent for each acid.

The food and growth curves are in general very closely parallel. It is therefore impossible to say whether the failure of growth is the consequence of the failure of appetite, or vice versa.

MORTALITY

The data are shown in table 5. Briefly, no fatality occurred with either acid for concentrations of 0.5 per cent, equivalent to 0.36 cc. per kilogram of rat per day, for nine weeks, following after sixteen weeks of 0.1 per cent. In view of this, the occasional deaths from lower concentrations must be considered as accidental coincidences.

TABLE 5

Mortality

DRUG AND CONCENTRATION	DOSAGE	EXPERIMENT NUMBER	NUMBER OF ANIMALS	WEEKS OF FATALITIES	TOTAL DURATION	PER CENT OF FATALITIES
					weeks	
Acetic acid, 0.01 per cent	8.2	56	6	8	11	16
Acetic acid, 0.01 per cent	10.9	107	3	0	9	0
Acetic acid, 0.1 per cent	81.0	63	6	0	15	0
Acetic acid, 0.25 per cent (after 10 weeks of 0.01 per cent)	210.0	5695	4	2, 4, 11	15	75
Acetic acid, 0.5 per cent (after 16 weeks of 0.1 per cent)	360.0	6305	3	0	9	0
Formic acid, 0.01 per cent	8.2	57	6	11	11	16
Formic acid, 0.01 per cent	10.25	108	3	0	14	0
Formic acid, 0.1 per cent	90.0	64	6	0	15	0
Formic acid, 0.25 per cent (after 12 weeks of 0.01 per cent)	160.0	5796	4	2	15	25
Formic acid, 0.5 per cent (after 17 weeks of 0.1 per cent)	360.0	6406	3	0	9	0

CONCLUSIONS

Acetic and formic acids behave approximately quantitatively alike, when added to the drinking water of rats, in concentrations up to 0.5 per cent and daily doses up to 0.36 cc. of absolute acid per kilogram of body weight, for two to four months; this being the sole source of fluid for the animals.

Concentrations of 0.01 per cent to 0.25 per cent, corresponding to daily dosage of 0.2 cc. of acid per kilogram of body weight, produced no effect on growth, appetite, or consumption of fluid. They are therefore quite harmless.

The diuresis described in the literature for formates and acetates must be due to the sodium, potassium or lithium, and not to the formic radicle.

With concentrations of 0.5 per cent and daily dosage of 0.36 cc. of absolute acid per kilogram of rat, the appetite and growth but not the fluid consumption are materially, immediately and progressively diminished with both acids. This is evidently due to their acidity; but the experiments do not throw any light as to whether this is due to local action on digestion, or whether to some more profound disturbance of the acid-base equilibrium.

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Comparative Excretion and Distribution of C^{14}-Labeled Carbonate and Formate in Large Albino Rats

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THE FATE, excretion and retention of C^{14} incorporated in sodium carbonate has aroused much interest since a preliminary report on this question was published by Bloom, Curtis and McLean (1). It was expected that C^{14} incorporated in other simple molecules would have a different fate and for this reason a comparative study was undertaken of the behavior of sodium carbonate and sodium formate under strictly comparable conditions. In addition it was hoped that such study of the formate might throw some light on its metabolism, especially its rate of oxidation, which has given rise to much speculation in connection with the mechanism of the toxicity of methanol and other monosubstituted methane derivatives. For this reason the studies were made with single intraperitoneal injections and the animals observed for a period of 8 days. Armstrong, Schubert and Lindenbaum (2) had developed suitable methods which were followed in the present study.

Since the experiments to be reported below were undertaken, several papers have been published on the distribution of C^{14} after the administration of labeled carbonate, by various means, in mice (3, 4) and rats (5) and on its presence in glycogen, blood glucose and lactate (6), in amino acids (7) and in nucleic acid (8). The excretory rates by various routes have been reported in rats (2, 5, 6). Similarly certain phases of the fate and pulmonary excretion of C^{14} from labeled formate have been reported in rats (9–11). None of these latter studies considered the excretion and distribution of formate and none have direct bearing on the problem discussed below.

MATERIALS AND METHODS

Eleven male Osborne-Mendel rats, 12–18 months of age, weighing 400–537 gm, were used, thus eliminating variants due to estrus, growth and senility. The animals were fed a diet of 10% dextrose by weight in whole milk, which was introduced into the cage through a funnel with a stopcock, thus excluding atmospheric CO_2 and preventing the escape of respiratory CO_2. Each rat was placed on this diet exclusively for 10 days – 2 weeks prior to injection.

Each rat was injected intraperitoneally with a single dose of a solution of either 0.03–0.05 mc of sodium carbonate or 0.07–0.10 mc of sodium formate. Six rats received 1 cc/100 gm body wt. of a solution containing 1.1×10^{-5} M/cc sodium carbonate. Four rats received similar doses in similar volumes of sodium formate. One rat received 40% of this dose of formate.

Immediately after injection each rat was placed in a glass cage of 12-liters capacity, similar to that used by Armstrong *et al.* (2), the cage sealed, and the rat kept there for 8 days. The air, which was drawn through the cage, was first filtered through soda lime to remove CO_2 and then through a solution of barium hydroxide, which served to indicate the complete absorption of atmospheric CO_2 by the soda lime. Air-flow rate was measured by means of a 'rotameter,' and was kept at about 1 l/min. All metal and rubber surfaces in the cage were coated with 'Glyptal' to prevent reaction and leakage of CO_2. Respiratory CO_2 in the air passing through the cage was absorbed in 500 cc of a 20% solution of sodium hydroxide in a glass tower. The air was then passed through a wash bottle containing barium hydroxide solution.

Urine and feces were separately collected as described by Armstrong *et al.* (2). Those fecal pellets which stuck to the sides of the cage were collected at the end of the experiment and separately analyzed. The hair in the cage, which the animals had shed because of self-grooming, was also removed and analyzed.

The towers were changed at the end of 1, 2, 3, 6 and 24 hours and then daily, for the determination of CO_2. Urine was collected at 7 hours, at 24 hours and then daily. Feces were collected daily when possible. Occasionally the amount of feces passed during a 24-hour period was inadequate for analysis so that two-day samples had to be used.

The plan of analysis was similar to that of Armstrong *et al.* (2). Aliquots of all materials, with the exception of exhaled CO_2, underwent wet combustion by the method of Van Slyke and Folch (12). The products of combustion were passed through granulated zinc and then into a solution of normal, CO_2-free sodium hydroxide. Formates, when oxidized by this method, yield both CO and CO_2. To obtain complete recovery

Received for publication May 1, 1953.

the combustion gases were passed over hot copper oxide before entering the absorption vessel, in order to convert CO to CO_2. The contents of the absorption vessels were then added to a hot aqueous solution of M/4 barium chloride-M ammonium chloride and the precipitated barium carbonate was filtered off in sintered crucibles which were then dried and weighed.

For the determination of respiratory CO_2, aliquots were taken from the tower contents and added directly to the barium-ammonium chloride solution. Fat was extracted from the feces by the method of Hawk and Bergheim (13). This and the fat-free residue were separately oxidized. Urine and the hair from the cage were dried and weighed before oxidation. Aliquots of the injected materials were converted to barium carbonate by the same technique, in order to determine their activities, and, for direct comparison with the samples from the rats.

At the end of 8 days, the rat was removed from the cage and injected intraperitoneally with 4 mg of pentothal sodium/100 gm body wt. When anesthetized, 7–10 cc of heart blood were withdrawn. The abdomen of the living animal was then opened and a portion of the liver removed, blotted, weighed and placed in hot potassium hydroxide solution for glycogen extraction by the method of Stetten and Boxer (14). Similarly, samples of the abdominal and thigh muscles were removed immediately and treated in the same way. The other organs were then removed, blotted, weighed and the fats and proteins extracted (2, 15). These tissue constituents and the residues remaining from the various extractions, were then oxidized and converted to barium carbonate.

The barium carbonate was ground in alcohol and the slurry poured into planchets to make infinitely thick plates, which were dried and counted in a gas-flow proportional counter which registered about 35 background counts/min. Each sample received two consecutive counts. If 10,000 counts/sample could not be recorded in a reasonable time, then two consecutive 1-hour counts were made. The average of the two counts was used in the calculations.

All calculations were made in terms of percentage of injected dose and will hereafter be noted as percent. Tissue constituents were further calculated in terms of percentage of injected C^{14}/mg dry wt. of each fraction. Many of the tissue constituents in the carbonate-injected group produced very low counts. Those which counted at a rate of 10 or less/min. over background were arbitrarily considered to have no activity worthy of calculation. Since this was the case, and since significance tests would be required for over a thousand separate determinations, a method (16) of ranking was employed to compare concentrations of C^{14} among the tissue constituents and between the two groups of animals.

Each tissue constituent from each animal was ranked according to the percent of injected dose found in that tissue constituent. The sum of the numerical positions in rank for each constituent from each rat in the group was calculated and the constituents regrouped according to this sum. The rankings between the two groups were then compared.

TABLE 1. C^{14} RECOVERED AT THE END OF 8 DAYS IN PERCENTAGE OF INJECTED DOSE

	Carbonates						Formates				
Animal No.	1	2	3	4	5	6	7*	8	9	10	11§
Total CO_2 exhaled	94.1	93.9	98.9	96.5	95.6	95.5	74.1	82.1	78.4	89.1	84.5
Total urine	2.3	1.1	1.0	2.7	0.6	0.7	5.7	3.9	3.0	7.0	6.9
Total feces residue	0.1†	0.9	0.9	0.1	0.1	0.3	0.7	0.7	1.1	1.6	1.8
Total feces fat	0.0†	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.3	0.1	0.3
Total excreted	96.5	96.0	100.8	99.3	96.3	96.5	80.6	86.8	82.8	97.8	93.5
Total all fats							1.4	2.3	2.3	2.0	2.7
Total all proteins	0.2	0.3	0.2		0.2	0.3	6.7	3.9	7.9	5.8	8.2
Blood							‡	‡	1.4	0.5	0.9
Glycogen							‡	‡	0.1	0.1	0.1
Residues							3.7	1.6	2.6	3.9	3.2
Total recovered	96.7	96.3	101.0		96.5	96.8	92.4	94.6	97.1	110.1	108.6

* 5 days only. † Cage feces lost. ‡ Lost. § Received 40% of dose.

RESULTS

The sum of the C^{14} recovered from all avenues of excretion and from all tissues ranged from 96–101% among the carbonate-injected rats, and from about 92–110% among the formate-injected rats. All data from the formate-injected rat which received 40% of the dose matched those of the other animals in the group (table 1).

The rate of pulmonary excretion of $C^{14}O_2$ in both groups of rats is illustrated in figure 1. The mean CO_2 excretion during the first hour by the carbonate-injected rats was about 72%; by the formate-injected rats, 42%. At 24 hours the hourly rates had fallen to 0.14 and 0.34% respectively and at the end of 8 days these had dropped to about 0.002 and 0.025%. At the end of 24 hours, the carbonate-

injected rats had excreted 94.8% and the formate-injected rats, 75.1%. For the remainder of the 8 days the $C^{14}O_2$ excretion by the carbonate-injected rats totaled about 1%, while that of the formate-injected group totaled about 9%. It should be noted that although the carbonate-injected rats excreted a larger total quantity of $C^{14}O_2$ than did the formate-injected rats, the excretion rate patterns in both groups were similar.

Urinary excretion of C^{14} in the two groups of rats followed a pattern similar to that for pulmonary $C^{14}O_2$; rapid excretion during the first 24 hours after injection was followed by a slower rate for the remainder of the experiment. The carbonate-injected rats excreted in the urine an average of 0.7% in 7 hours and a total of 1.2% in 8 days. The formate-injected rats excreted 1.6% during the first 7 hours, and at the end of 8 days had excreted 5%.

Most of the C^{14} in the feces of the carbonate-injected rats was found in the fat-free residue, from which 0.05–0.9% was recovered in 8 days. On the other hand little C^{14} was found in the feces fat. In 2 of the 6 rats the C^{14} in the feces fat was insufficient for calculation, while in the other 4 the average recovered after 8 days was 0.03%. Similarly the formate-injected rats also excreted less C^{14} in the feces fat than in the fat-free residue. An average total of 0.13% was recovered from the fat and between 1.1 and 1.8% from the residue. Although the C^{14} content in both feces fat and feces residue fluctuated daily in both groups, it showed a slow, steady decline.

The total C^{14} excreted by the lungs, kidney and bowel during the 8 days of the experiment (table 1), by the carbonate-injected group, was about 96% and about 90% by the formate-injected rats. Of these totals, the former excreted 95% during the first 24 hours after injection, and the latter, 78%. In both groups, the greatest proportion was excreted during the first 7 hours after injection. The biological half-life of the remainder was calculated to be 14 days in the carbonate group and 7 days in the formate group.

The radioactive carbon retained by both groups of rats was distributed in all tissues of each animal. In the carbonate-injected rats each tissue or tissue constituent exhibited a low order of radioactivity. In the formate-injected rats, these exhibited relatively high counting rates. An end-window Geiger tube passed over the surface of the carbonate-injected rats, just prior to sacrifice, gave no indication of radioactivity. When the tube was passed over the formate-injected rats, it registered several hundred counts per minute over background.

The amount of C^{14} in the fat extracted from the various tissues of the carbonate-injected rats was, with the exception of the testes fat, too low to be counted. Aliquots of the

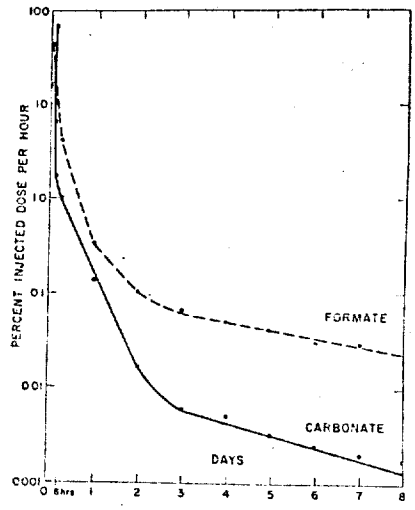

FIG. 1. Rate of $C^{14}O_2$ excretion (percentage of injected dose).

testes fats gave between 13 and 20 counts/min. over background. It was calculated that the fats from each pair of testes contained approximately 0.001%. In contrast, all the fats for the tissues of the formate-injected rats exhibited appreciable radioactivity. The aliquot counts ranged from 150 to as much as 1500/min. and the fats from each pair of testes contained between 0.002 and 0.17%. The testes fats in this group showed a greater concentration of C^{14} than all other fats except that from the kidney. In addition, the fats of the heart, spleen, and lungs showed relatively high concentrations of C^{14}, while the spinal cord and depot fat had the lowest concentrations. The other tissue fats were intermediate between these two extremes (table 2). The total fats, in each rat of this group, contained about 2%.

Aliquots of the proteins from the tissues of both groups of rats gave acceptable counts. Samples from the carbonate group gave counts which ranged between 20 and 60 over background. The total C^{14} in the proteins in each rat was about 0.2%. The proteins from the formate group gave counts which ranged from 200–2000/min. over background. The total C^{14} in the proteins ranged between 4 and 8%.

TABLE 2. SUM OF RANKING BASED ON TISSUE FAT CONTENT OF C^{14} FORMATE-INJECTED ANIMALS*

Organ	Sum	Rank
Kidneys	62	1
Testes	53.5	2
Heart	53	3
Spleen	50.5	4
Lungs	49.5	5
Lg. int.	41.5	6
Sm. int.	40.5	7
Bone	39	8
Liver	38	9
Brain	36	10
Stomach	34	11
Muscle	30	12
Pelt	27	13
Depot fat	11	14
Spinal cord	8	15

* Only testes fat gave acceptable counts, of all the fats from the carbonate-injected rats.

When the tissues were ranked with regard to percentage concentration of C^{14}/mg of proteins (table 3) it was noted, in both groups, that spinal cord had the lowest concentration, and that stomach, spleen, kidney, testes and liver had the highest. The correlation between the two groups of rats was 0.86, where a perfect correlation would be 1.0.

During the extraction of the fats from the tissues, a water-soluble product remained after the alcohol-extract was reextracted with petroleum ether. These residues weighed a few milligrams each. They exhibited no activity which could be calculated in the carbonate group, but they did exhibit considerable activity in the formate-injected group, and in the latter accounted for about 2% in each rat. Similarly, products soluble in trichloracetic acid and in the various washes which were removed during protein extraction from the tissues of the carbonate group, showed insufficient activity for calculation; those from the formate group accounted for about 1.5% in each rat. Calculations of the concentrations of percent of C^{14} per mg of these products were not made. The fat residues were too small for analysis unless those from several tissues were pooled. The protein residues remaining dissolved in trichloracetic acid and in the various washes used in the purification of the proteins, could not be separated from each other because of technical difficulties.

The total amount of C^{14} in the defatted bone of the carbonate group was about 0.3%, and in the formate group, about 1.3%. However, the concentrations, in both groups, were low, and were but little higher than that found in the spinal cord protein. The blood, both cells and plasma, from the carbonate-injected rats had very low counting rates, and calculations were not made. That from the formate group had good counting rates, and calculations showed that about 1% was present in the blood 8 days after injection; three-fourths of the activity was in the cells, and the remainder in the plasma.

Similarly, because of very low counting rates, calculations could not be made of the activity in the glycogen obtained from liver and muscle in the carbonate group, but the glycogen from the formate group, both from liver and muscle, contained about 0.03% each. The liver glycogen, however, had about five times the concentration of muscle glycogen. In both groups of rats, of all tissue fractions studied, proteins had the greatest concentration of C^{14} and fats the lowest.

DISCUSSION

The initial rapid excretion of C^{14} through the lungs and in the urine in both groups may have reflected the direct chemical, excretory and metabolic response of the rats to the carbonate and formate, even though the mechanism might differ. The later, slower excretion of pulmonary-, urinary-, and fecal-C^{14} may have represented the excretion by these routes of both stored and converted carbonate and formate. The early, rapid excretion following the administration of carbonate has been reported (1, 3, 8 and others), but the few reports on C^{14} excretion after formate administration have no agreement with each other and each was incidental to the experiment reported (17–19).

It can be seen (fig. 1) that the carbonate

group excreted larger amounts of $C^{14}O_2$ than the formate group, and smaller amounts of urinary-C^{14}, but it should be noted that the rate pattern was similar in both groups. This quantitative difference and similarity of rate pattern was also apparent in the fecal excretion of C^{14}. In both groups, the fat-free portion of the feces contained considerably more C^{14} than did the fat. In both groups the rates of fecal-C^{14} excretion were similar, but more C^{14}, in both fractions, was excreted by the formate group than by the carbonate group.

The findings reported here are in general agreement with other reports dealing with the incorporation and retention of carbonate-C^{14}, except that calculations were not made where very low activity was present, as in glycogen, fat and blood (1–6). The data presented in tables 2 and 3 indicate that proteins and fats from testes were among those tissue constituents which had the greatest concentration of C^{14}. Of those tissues and tissue constituents for which calculations were made, defatted bone, spinal cord and muscle had the lowest concentration.

The pattern of retention in the formate group was similar to that in the carbonate group, but the quantities retained were greater. Here again the testes showed a greater concentration, both in the fat fraction and in the protein fraction, than most other tissues, and defatted bone, spinal cord and muscle were among the tissues with low concentrations of C^{14}. Several reports (8, 17, 18, 20) showed that various amino acids contained C^{14} following formate administration, but there have been no reports dealing with the general distribution of formates in rats. The present experiments indicate the incorporation into glycogen and fats, as well. In addition, formate-C^{14} was found in blood cells, blood serum and in products remaining after the extraction of fats and the purification of proteins.

Calculations of the biological half-life, as indicated earlier, showed that carbonate-C^{14} was retained for longer periods of time than formate-C^{14}, even though a smaller percentage of the former was present at the end of 8 days due to the difference in rates of excretion. Both these substances appear to be readily eliminated by the means noted above. However the larger amount of formate retained may be suggestive of its delayed oxidation with regard to methanol intoxication. It should be remembered that the doses used in these experiments were very small as compared to toxic doses of methanol.

In general then, the pattern of distribution and excretion of C^{14} from carbonates and formates was quite similar. C^{14} was found in all tissues and tissue constituents analyzed. Those tissues and tissue constituents which exhibited a higher concentration of C^{14} following the administration of one of these compounds, showed a higher concentration following the other. Both groups showed a similar pattern of pulmonary, urinary, fecal and total excretion. The carbonate group differed from the formate group in having greater $C^{14}O_2$ excretion, and a lower urinary and fecal excretion, and in retaining lesser amounts in the tissues and tissue fractions. The differences between the two groups were of degree rather than kind.

TABLE 3. SUM OF RANKING BASED ON TISSUE PROTEIN CONTENT OF C^{14} INJECTED ANIMALS

Carbonates	Rank	Organ	Rank	Formates
87	1	Liver	1	71
80	2	Testes	3	56
73.5	3	Kidney	2	66
61	4	Spleen	4.5	50.5
51.5	5	Stomach	4.5	50.5
47	6	Sm. int.	9	36.5
42.5	7	Lg. int.	8	39
41.5	8	Pelt	12	20
36	9	Lungs	6	43
34.5	10	Brain	11	21.5
29	11	Heart	7	40
22	12	Muscle	10	32.5
19	13	Bone	14	6
8	14	Spinal cord	13	13

Correlation:

$$R = 1 - \frac{6\Sigma d^2}{n^2(n-1)} = 0.80$$

CONCLUSIONS

Carbonates and formates have the same general pattern of excretion and distribution following intraperitoneal injection into large, fully mature albino rats.

More than 90% of the injected dose is excreted in 24 hours by the carbonate-injected rats. The formate-injected rats excreted about 80%.

Carbonate-injected rats excrete about 95% of the injected dose in 8 days. Formate-injected rats excrete about 90%.

Carbonate-injected rats excreted, in the urine, somewhat more than 1% of the dose in 8 days; most of this during the first 7 hours. Formate-injected rats excreted almost 2% in the first 7 hours, and more than 5% during the 8 days.

Fecal excretion for both groups of animals was similar in rate, but greater by the formate-injected group. In both groups, the feces fat contained far less C^{14} than did the fat-free portion.

The biological half-life of the retained C^{14}, 24 hours after injection, was calculated to be about 14 days for the carbonate group and about 7 days for the formate group.

The retained C^{14} was distributed throughout all tissues in both groups of rats, but the formate group retained a greater percentage at the end of 8 days.

In both groups, testes fat had a higher concentration of C^{14} than the fat from most other tissues. The fats from the lungs, spleen, heart, kidneys and testes in the formate group, had the highest concentration of C^{14}, and that from the spinal cord and depot fat had the lowest.

The pattern of concentration of C^{14} in the proteins, in both groups of rats, was similar. Stomach, spleen, kidney, testes and liver had the highest concentration, while spinal cord had the lowest.

In both groups, defatted bone had a low concentration of C^{14}, but the formate group had a higher concentration than the carbonate group.

We would like to express our appreciation to Robert McLaughlin and especially to Walter S. Cool for technical assistance, and to Marvin Schneiderman of the Section on Biometrics of the National Institutes of Health for his advice on the statistical evaluation of our data.

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IGIENA
vol. XI, nr. 6, anul 1962

RESEARCH REGARDING THE TOXICITY OF FORMIC ACID

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Formic acid is used for the preservation of syrups, fruit juices, and of preserved fruits in ~~doses~~ amounts that vary between 0.6 and 1 g ‰. The situation with regard to the use of formic acid in the preservation of foodstuffs ~~and~~ or beverages varies from country to country. While some countries permit its use (the Socialist Republic of Czechoslovakia, Switzerland, the Federal Republic of Germany) (2), other countries prohibit it--the USSR (10), France (3), the USA (4). This is due to the fact that the data from the literature are not sufficient to enable us to evaluate the harmfulness of formic acid in the ~~doses~~ amounts used for preservation. From the data in the literature, it can be seen that formic acid in certain amounts has an irritating effect on the mucous membranes and the skin, that it influences the activity of the central nervous system producing, in small ~~amounts~~ doses, a nervous depression with decrease in the reflexes and in large doses convulsions, that it has ~~have~~ a methemoglobinizing ~~action~~ effect, that it ~~synthe~~ inhibits the synthesis of pantothenic acid and that it decreases the activity of the blood catalase (6, 7, 11).

The data regarding the toxicity of formic acid are based in the great majority of cases on pharmacological experiments, in which the ways of administration and the doses used do not allow us to draw valid conclusions for the ~~elementary~~ alimentary sector. For this reason, in all the international conferences that have taken place in recent years formic acid has been placed on the list of insufficiently known substances from the toxicological point of view (1, 9).

Taking this into consideration, we have considered it necessary to carry out some systematic studies regarding acute, sub-acute, and chronic toxicity of formic acid.

EXPERIMENTAL METHOD

The acute toxicity of the substance was determined on young rats, by the administration per os of various doses of the substance to white mice, by the intraperitoneal injection of the substance, ~~into~~ and on daphnias, ~~as well~~ through the inclusion of formic acid in the nutritive medium.

The sub-acute toxicity was tested with the aid of a test for restoring the hepatic proteins after hunger and with a test of growth.

The test for restoring the hepatic proteins after hunger was carried out on 21 adult white rats divided into three groups of which one group received 0.5 ‰ of formic acid, whereas the other received 1 ‰ in the drinking water. The substance was added to the water of the experimental groups both in the hunger period and in the period of restoration (7 days). Along with the body weight, the weight of the liver, and the nitrogen content of the liver, the weight of the suprarenal glands, their

ascorbic acid content, the values of the hemoglobin [? because only semilegible] and their Catalasic coefficient were followed.

The test of growth was carried out on 48 young white rats of approximately equal weight, originating from the same generation. The animals were divided into six groups, the experiments being carried out on the two levels of protein from the ration--11.8% and 18.2%--and in both concentrations of the substance tested--0.5 and 1 ‰ . The use of two protein rations was determined from the fact that in our previous research, it was shown that under these conditions, ~~it becomes more practicable to modify the~~ the modifications of the protein metabolism become more easily known. The test for growth was carried out during a six weeks period with a hypoprotein ration and for five weeks with a normo-protein ration. In the course of the experiments, the weight of the animal, the weekly gain in weight, the ingestion of food, and the protein efficiency were followed.

At the end of the experiment the animals were killed, and measurements were made of the nitrogen and lipid content of the liver, the weight of the liver, the weight of the suprarenal glands, of the spleen, and of the kidney, the ~~content~~ ascorbic acid content of the suprarenal glands, the Catalasic coefficient and the peroxidasic index.

RESULTS OBTAINED
(Acute Toxicity)

The LD_{50}, at the administration per os of the substance to young white rats, was 1830 mg/kilo of body weight. ~~[decimal point added by translator].~~ The animals die with symptoms of

dyspnea and cyanosis. In the case of the survivors, diarrhea, weakness, and a slow restoration appear.

In the intraperitoneal injection of the substance into white mice, the LD50 was 940 mg/kilo of body weight.

* In daphnias , the LD_{50} after 48 hours, was 70 mg per litre.

SUB-ACUTE TOXICITY

The restoration of hepatic proteins after hunger. It is seen from the analysis of the results obtained (Tab. I) that the administration of formic acid did not influence the loss and restoration of weight in the animals. The nitrogen content of the liver is somewhat smaller in the group which received 1‰ formic acid and significantly lower in the group which received 0.5 ‰ (t is 2.79, greater than 2.18). The lowest values obtained for the group that received 0.5 ‰ formic acid are correlated with the smaller amounts ingested recorded for this group.

Tab. 1. The influence of formic acid on the restoration of the hepatic proteins after hunger, on the ~~content~~ ascorbic acid content of the suprarenal glands and ~~unde~~ on the ~~catalytic coefficient~~ catalasic coefficient.

The Mean Data Per Group

Tabelul I

Influența acidului formic asupra refacerii proteinelor hepatice după foame, a conținutului în acid ascorbic al glandelor suprarenale și coeficientului catalazic

Date medii pe lot

Group Lotul	Greutatea Inițială	Greutatea După foame	Greutatea După refacere	Ingestie	mg N ficat	Greutatea suprarenalei	Acid ascorbic în suprarenală	Coeficient catalasic
					In % greutatea corporală			
Control Martor	177,8	150,4	170,6	44,4	137,7	0,157	136,0	16,4
Formic acid Acid formic 1‰	182,7	156,0	177,2	49,4	129,2	0,146	128,4	13,7
Acid formic 0,5‰	182,1	155,0	180,1	38,9	122,3	0,132	129,4	18,9

The weight of the suprarenal gland is lower in the group that received 1 ‰ formic acid and particularly in the group that received 0.5 ‰. The ascorbic acid content is approximately the same in all the experimental groups, the differences among the groups being statistically insignificant. The catalasic coefficient is slightly greater in the group that received 1‰ formic acid and slightly lower in the group that received 0.5 ‰.

The growth test. The results obtained are presented in Tab. II.

Tab. II. The influence of the administration of formic acid on the growth of the white rats.

Mean Data Per Group

Tabelul II

Influenţa administrării acidului formic asupra creşterii şobolanului alb

Date medii pe lot

Group Lotul	Initial intake Indicatorul iniţial	Week Săptămîna I	II	III	IV	V	VI	Total medie mean Totals
I. Martor Control Caseină Casein 18,2%	Greutatea 40,1 Weight	56,8	79,0	96,3	116,9	131,9		
	Ingestia	49,1	63,6	77,8	85,4	85,0		360,8
	Eficienţa proteică Protein efficiency	1,86	1,91	1,22	1,32	0,96		1,39
II. Acid formic 0,5 ‰	Greutatea 40,5 "	58,6	77,6	90,3	105,3	120,0		
	Ingestia "	46,0	63,9	69,9	80,0	83,1		342,9
	Eficienţa proteică "	2,16	1,63	0,99	1,33	1,03		1,28
III. Acid formic 1‰	Greutatea 40,6 "	60,3	80,7	100,6	117,6	131,0		
	Ingestia "	48,9	69,0	80,1	83,3	86,6		367,9
	Eficienţa proteică "	2,20	1,62	1,36	1,12	0,90		1,36
Casein Control IV. Martor caseină 11,8 %	Greutatea 40,1 "	53,5	64,1	75,3	93,5	107,0	115,8	
	Ingestia "	53,9	62,0	71,6	81,0	76,4	71,3	416,2
	Eficienţa proteică "	2,10	1,58	1,32	1,99	1,49	1,04	1,54
V. Acid formic 0,5‰	Greutatea 40,1 "	51,3	63,1	73,1	88,2	98,8	107,1	
	Ingestia "	51,6	64,8	65,4	74,5	70,8	66,0	392,6
	Eficienţa proteică "	1,83	1,54	1,29	1,71	1,27	1,56	1,44
VI. Acid formic 1‰	Greutatea 40,1 "	51,1	63,8	75,9	88,8	100,0	108,3	
	Ingestia "	51,6	65,5	71,4	74,4	69,0	64,1	396,0
	Eficienţa proteică "	1,82	1,64	1,43	1,46	1,37	1,09	1,45

The analysis of the results obtained shows that the administration of formic acid in a concentration of 1‰ did not influence the growth of the group which received a ration of 18.2 % casein, producing a slight decrease in the growth of the group that received 11.8% casein. The administration of formic acid in a concentration of 0.5 ‰ produced a more powerful inhibition of growth with both food rations. The differences vis-à-vis the control groups are not however significant from the statistical point of view.

The ingestion of food varies in correspondence with growth, being less in the groups which receive 0.5‰ formic acid, close to that of the control group in the group which received 1‰ formic acid, with the ration of 18.2% casein.

The protein efficiency is lower in all the groups that received formic acid especially in the groups that received 0.5‰. In the group which received a ration with 18.2% casein, with all the indicators there is registered a phasicity in the effect of the substance with a slight stimulation of growth and protein efficiency in the first week and an inhibition of them in the later weeks.

The weight of the suprarenal gland, of the liver, of the spleen, and of the kidney is reduced in the group which received 0.5‰ formic acid and the food ration with 18.2% casein. With 1‰ formic acid, there is noticed a slight increase in the weight of the suprarenal gland and of the spleen and a slight decrease in the weight of the liver and of the kidney (Tab. III).

Tab. III. The influence of formic acid on the weight of the liver, of the kidney, of the suprarenal, and of the spleen.

Mean Data Per Group.

Tabelul III

Influ[illegible]a acidului formic asupra greutăţii ficatului, rinichiului, suprarenalei şi splinei

Date medii pe lot

Group Lotul	Liver Ficat	Kidneys Rinichi	Suprarenale	Spleen Splină
Control casein				
I. Martor 18,2% cazeină	5,443	1,302	0,2 494	0,424
II. Acid formic 0,5‰	5,143	1,191	0,2 394	0,355
III. Acid formic 1‰	5,090	1,241	0,2 745	0,443
IV. Martor 11,8% cazeină	4,318	1,030	0,247	0,310
V. Acid formic 0,5‰	3,995	1,015	0,226	0,261
VI. Acid formic 1‰	4,092	1,025	0,226	0,241

In the groups which received 11.8% casein in the ration, formic acid produced a slight decrease in the weight of the liver, of the suprarenal gland, and of the spleen in the group with 0.5‰ formic acid and an increase in the weight of the spleen in the group with 1‰.

The nitrogen content of the liver is slightly lower in the ~~group~~ second group and significantly lower in the third group (the test [result] of 4.72 is greater than 2.18). In the fifth and sixth groups, no differences in the nitrogen content of the liver were registered vis-à-vis the control group (Tab. IV).

Tab.IV. The influence of formic acid on the nitrogen and lipid content of the liver, on the ascorbic acid content of the suprarenal glands and on the catalasic coefficient.

Mean Data Per Group.

Tabelul IV

Influenţa acidului formic asupra conţinutului în azot şi lipide al ficatului, conţinutului în acid ascorbic al glandelor suprarenale şi a coeficientului catalazic

Date medii pe lot

Group Lotul	mg N ficat % greutate corporală	Lipide ficat	Acid ascorbic suprarenală % greutate corporală	Coeficient catalazic
Control casein				
I. Martor 18,2% cazeină	131,0	0,296	118,8	16,7
II. Acid formic 0,5‰	128,0	0,228	125,5	19,5
III. Acid formic 1‰	118,0	0,226	117,5	18,8
IV. Martor 11,8% cazeină	115,0	0,214	126,3	17,[illegible]
V. Acid formic 0,5‰	117,0	0,214	135,0	17,5
VI. Acid formic 1‰	117,0	0,204	132,8	18,8

The lipid content of the liver is lower vis-à-vis the control groups, ~~respectively~~ in the second and third groups respectively and unchanged in the fifth and sixth groups.

The ascorbic acid content of the suprarenal glands is approximately the same as in the control group for the second and third group and somewhat higher in the fifth group.

The catalasic coefficient is either approximately the same vis-à-vis the control groups or slightly higher (the second and third groups).

The peroxidasic index is ~~high~~ increased in the groups which receive formic acid especially in the groups which receive a ration with 18.2% casein (Fig. 1).

DISCUSSION

The analysis of the results obtained in the acute experiments shows that formic acid can be placed among the substances with a medium toxicity, smaller than that of salicylic ~~than~~ or boric acid but greater than that of benzoic acid. In the experiments on daphnias , formic acid was however more toxic that salicylic or boric acid (8).

The results obtained from the sub-acute experiments allow the conclusion to be drawn that, under the experimental conditions used by us, the acute and sub-acute toxicity of the substance was low ~~decreased~~. With regard to this, the data show ~~with regard to~~ the lack of any ~~same~~ significant modifications in the majority of the tests used. It might be presupposed that the lack of modifications is due to the fact that in our

experiments, we have used concentrations similar to those used in practice, therefore too small to assure a coefficient of certainty. This presupposition is, however, erroneous because, while in practice only a small part of the liquids ingested contain formic acid, in our experiments, using a dry synthetic ration, the whole quantity of liquid consumed contained formic acid. In this way we consider that a corresponding coefficient of certainty has been assured, the increase in concentrations administered representing an aggravating condition which exceeds the limits of the coefficient of certainty.

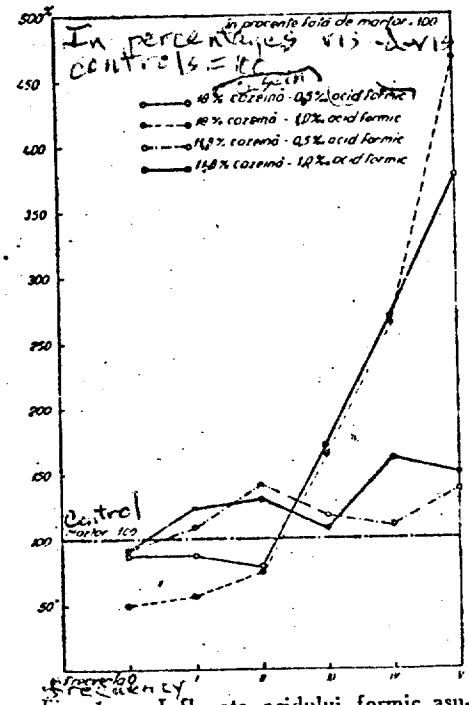

Fig. 1. — Influența acidului formic asupra indicelui peroxidazic.

Fig. 1. The influence of formic acid on the peroxidasic index.

It would be mistaken to drawn the conclusion that formic acid is indifferent in its effect on the organism. With regard to this, the data show, with regard to the acute toxicity of the substance, modifications in the nitrogen content of the liver upon restoration after hunger as well as at the end of the test of growth, a decrease in weight of the suprarenal glands, of the liver, of the spleen, and of the kidney, a tendency to inhibit the growth and the protein efficiency, a phasicity in the effect of the substance on the normo-proteic feeding, and a direct inhibition of growth in the groups with hypo-proteic feeding, and an important increase in the peroxidasic index. But the data obtained are insufficient for prohibiting the use of formic acid in the preservation of juices, syrups or fruits. In order to solve this problem, we have carried out long-term research.

CHRONIC TOXICITY

Experimental Method

The experiments were carried out on 60 adult white mice (50 females and 10 males), with observation of the influence of prolonged administration of the substance upon reproduction, the blood picture, the nitrogen content of the liver and the ascorbic acid content of the suprarenal glands. The animals were divided into five groups as follows:

-- group I, the control group;

-- group II, females, received formic acid during the whole experimental period;

-- group III, males, received formic acid during the whole experimental period;

-- group IV, both males and females, received formic acid during the whole experimental period;

-- group V, females, received formic acid during lactation.

In this way, the separate testing of the effects of the substance was assured on the reproductive capacities of the females and males as well as the possibility of the passage of some of the toxic components through the milk.

In order to test the effects obtained at different periods of administering the substance as well as in order to verify the results obtained, three repetitions (reproductions) were carried out: first one month from the beginning of the administration of the substance, the second three months ~~later~~ after the beginning, ~~the~~ and the third seven months after the beginning. In all, the substance was administered for eight months' time. ¶ In the experimental period, the animals received the accustomed food from the ~~incubator~~ foster-mother, the experimental groups received formic acid in the concentration of 1 g ‰, ~~placed~~ in relation to the water consumed.

In the test on reproduction the following indicators were studied:

-- the number of females that became pregnant;

-- the number of offspring born, both ~~living~~ alive and dead;

-- the weight of offspring at birth;

-- the number of living offspring and their weight at seven days;

-- the number of living offspring and their weight at 21 days.

The offspring of the first generation were subject to a test for growth after four weeks, without the administration of the substance, with the study of their weight, the weekly gain in weight, the ingestion of food and the protein efficiency.

At the end of the experiment, the adult animals that received the substance for 8 months as well as the animals of the control group were examined with regard to the blood picture, the nitrogen content of the liver, and the ascorbic acid content of the suprarenal glands.

RESULTS OBTAINED

I. The influence of prolonged administration of the substance on reproduction and on the development of offspring

The number of females that were pregnant, at the first repetition, varies between 5 and 6 in one single group (in which the males received formic acid) there being recorded 8 pregnant females. No differences are noted among the groups which received formic acid vis-à-vis the control group (Tab. V).

At the second repetition, the number of females that were pregnant varies between 5 and 7 in the group in which both females and males received formic acid, the number of pregnancies being lower (3).

In the third repetition, there were recorded a small number of pregnancies (2--4) in all the groups, which does not permit an interpretation of the results obtained.

The number of offspring born, in the first repetition ~~reproduction~~ is ~~in the mean~~ on the average 6.6 per female for the control group, varying between 5.5 and 7.3 for the other groups (Tab. V).

In the second repetition ~~reproduction~~, the number of offspring born ~~varyi~~ varies between 6 and 6.3 for the control group and for ~~groups~~ the third and fifth groups. In the groups in which the females received formic acid during the whole ~~experiem~~ experimental period (II and IV), the number of offspring born on the average per female is 4.1 and 4 respectively.

The number of offspring born alive in the first repetition ~~reproduction~~ varies between 5 and 7.0, without there being ~~regester~~ recorded significant differences among the groups.

At the second repetition ~~reproduction~~, the number of offspring born alive is 4 for the control group, 5.7 for the group in which the females received formic acid during the period of lactation, 6 for the group in which the males received formic acid, and 2.8 and 1 respectively for the groups in which the females received formic acid during the whole experimental period (Tab. V).

Tab. V. The influence of prolonged administration of formic acid on the reproduction of white rats

Mean Data Per Group

Tabelul V

Influența administrării îndelungate a acidului formic asupra reproducerii șobolanilor albi

Date medii pe lot.

Lotul	Femele gravide	Pui născuți	Pui vii	Greutatea la naștere	Pui vii 7 zile	Greutatea 7 zile	Pui vii 21 de zile	Greutatea 21 de zile
			Reproducerea I					
I	6	6,6	6,3	5,2	5,0	10,6	5	34,3
II	6	5,5	5,0	5,1	0,5	—	0,5	—
III	8	6,3	6,1	5,9	3,8	12,5	3,8	34,[illegible]
IV	5	6,0	5,4	4,9	2,2	9,6	2,2	34,[illegible]
V	6	7,3	7,0	5,2	5,5	10,2	5,5	23,5
			Reproducerea a II-a					
I	6,0	6,0	4,0	5,1	4	11,5	4	5[illegible]
II	6,0	4,1	2,8	5,1	2,1	11,2	2,1	5[illegible]
III	6,0	6,3	6,0	5,8	6,0	13,1	6,0	2[illegible]
IV	3,0	1,0	1,0	5,0	0	—	—	—
V	7,0	6,3	5,7	5,2	4,7	13,6	4,7	23,6

The weight at birth of the offspring in the first repetition ~~reproduction~~ varies among the groups between 4.9 g and 5.9 g on the average per offspring, without any significant differences being recorded among the groups. Nor at the second reproduction are there recorded significant differences among the groups, the weight of the offspring at birth varying between 5 and 5.8 g (Tab. V).

The number of offspring remaining alive after seven days varies significantly among the groups (Tab. V).

It can be seen from Tab. V that at the first repetition ~~reproduction~~, while in the control group after 7 days an average of 5 offspring remain alive whereas in the group that received formic acid in the period of lactation, 5.5 offspring remained alive after 7 days, in the groups in which the females received formic acid, the number of offspring remaining alive after 7 days is only 0.5 and 2.2 respectively. At the second repetition ~~reproduction~~ the phenomenon is repeated, the number of offspring remaining alive for the groups in which the females took formic acid being 0 and 2.1 respectively.

The weight of the offspring after 7 days does not present significant differences among the groups both at the first and at the second repetition ~~reproduction~~. The figures vary ~~value varies~~ at the first repetition ~~reproduction~~ between 9.6 and 12.5 g, but at the second repetition ~~reproduction~~ between 11.2 and 13.6 g.

In the period between the seventh and twenty-first day, not even 1 (one) offspring dies in any of the groups (Tab. V).

The weight of the offspring after 21 days does not present significant differences among the groups in either the first or the second repetition. It varies at the first repetition between 28.5 and 34.4 g but at the second repetition between 29.6 and 34.4 g (Tab. V).

The test for growth of the offspring likewise does not indicate the existence of significant differences among the groups which received formic acid vis-à-vis the control group or among the groups which received formic acid.

Thus, for example, in the second repetition, while the animals of the control group reach 60.4 g after 4 weeks, the animals of the groups which received formic acid reach on the average 59.8-62.8 g.

II. The effect of prolonged administration of formic acid on the nitrogen content of the liver, on the ascorbic acid in the suprarenal gland, and on the blood picture.

The nitrogen content of the liver was determined for the 16 control animals and the 15 animals which received formic acid over a period of 8 months. The results obtained indicate a lack of any significant modifications in both groups, the figures being 103 mg per 100 g of body weight for the control group and 96 mg % for the group which received formic acid.

The ascorbic content of the suprarenal glands likewise does not indicate the existence of any significant differences in both groups, the figures being 76.2 mg% for the control group and 74.8 mg% respectively for the group which received formic acid.

The blood picture indicates the existence of a certain degree of hyperchromic anemia in the group which received formic acid, likewise a leukocytosis, a basophile neutropenia, and a slight lymphocytosis (Tab. VI).

Tab. VI. The effect of ~~the pro~~ prolonged administration of formic acid on the blood picture. Mean Data ~~of the~~ per Group

Tabelul VI

Influenţa administrării îndelungate a acidului formic asupra tabloului sanguin

Date medii pe lot

Group Lotul	Hematii/mm³	Hb %	Valoarea globulară	Leucocite/mm³	Neutrofile (%)	Limfocite (%)	Eozinofile (%)	Bazofile (%)	Mononucleare (%)	Celule tinere (%)
Control Martor	6,140	94	0,77	10 300	30,4	58,8	3,4	0,9	3,8	2,9
Acid formic	5,310	106	1,00	26 800	17,8	68,0	3,6	5,7	2,3	2,7

DISCUSSION

It is seen from the analysis of the data obtained that the prolonged administration of formic acid certainly influenced ~~certainly~~ some of the indicators in the reproduction tests. Thus the number of females that became pregnant, the weight at birth, after 7 days, and after 21 days do not show significant differences among the groups which received formic acid vis-à-vis the control group.

With regard to the number of offspring born on the average per female as well as the number of offspring born alive, the differences appear only at the second repetition ~~reproduction~~. This fact could be attributed to modifications which appear following prolonged administration of formic acid. Taking into account the multitude of factors which can affect the number of offspring born, the variability which is registered

normally in this indicator, the fact that differences among the groups are relatively small, we consider that such an interpretation of the results is not sufficiently justified.

The important and significant differences between the groups in which the females received formic acid and the control group are registered in the viability of the offspring in the first 7 days. Taking into account the fact that the experiments were carried out under the same conditions and in the same length of time for all the groups, the fact that the phenomenon is confirmed at both repetitions and that the differences are very obvious, we can draw the conclusion that the administration of formic acid has a negative influence on the viability of the offspring. The fact that the offspring originating from the females that received formic acid in the lactation period show a viability similar to that of the control group and the fact that after giving birth, the females have received no further formic acid indicates that the effect of formic acid on the viability of the offspring is due to its action before birth.

The harmful action of prolonged administration of formic acid also results from the modifications of the blood picture which appear in adult animals that received the substance for a more prolonged period.

Our research does not provide data which might permit determining the action mechanism of formic acid, but it shows its negative effect on the organism.

Taking into account the results obtained and the fact that relatively small concentrations of formic acid were used in the experiment, we consider that its use in the preservation of foodstuffs is not indicated.

CONCLUSIONS

1. The acute toxicity of formic acid has been established on ~~for~~ white mice through the administration of the substance per os and ~~intrap~~ by intraperitoneal injection, and on daphnias. The results obtained indicate that formic acid shows a ~~smaller~~ lesser acute toxicity than does salicylic acid or boric acid but greater than the toxicity of benzoic acid.

2. In the sub-acute experiment, it was established that formic acid has a low toxicity without being without effect on the organism. Thus, formic acid produces a ~~lowering~~ decrease in the restoration of hepatic nitrogen after hunger, a decrease in the weight of the organs, a tendency to reduce ~~growth~~ the growth rate and to reduce the protein effeciency.

3. In the experiments of long duration (8 months), it was established that formic acid influences ~~a~~ negatively the viability of offspring in the first 7 days of life as well as the blood picture.

4. On the basis of the results obtained our opinion is against the use of formic acid in the preservation of foodstuffs.

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IGIENA
vol. XI, nr. 6, anul 1962

Cercetări cu privire la toxicitatea acidului formic

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Lucrare efectuată în Secţia de igiena alimentaţiei a Institutului de igienă şi sănătate publică al R.P.R.

Acidul formic se utilizează pentru conservarea siropurilor, a sucurilor de fructe şi a fructelor zaharate în doze ce variază între 0,6 şi 1 g ‰. Poziţia faţă de utilizarea acidului formic în conservarea alimentelor sau băuturilor diferă de la ţară la ţară. În timp ce unele ţări permit utilizarea sa (R.S. Cehoslovacă, Elveţia, R.F. Germană) (2), altele o interzic — U.R.S.S. (10). Franţa (3), S.U.A. (4). Aceasta se datoreşte faptului că datele din literatură nu sînt suficiente pentru a se putea aprecia nocivitatea acidului formic în dozele utilizate pentru conservare.

Din datele din literatură rezultă că acidul formic în anumite doze are o acţiune iritativă asupra mucoaselor şi a pielii, că influenţează activitatea sistemului nervos central producînd, în doze mici, o depresiune nervoasă cu scăderea reflexelor şi, în doze mari, convulsii, că are o acţiune methemoglobinizantă, inhibează sinteza acidului pantotenic, scade activitatea catalazei sanguine (6, 7, 11).

Datele privitoare la toxicitatea acidului formic se bazează în marea lor majoritate pe experienţe farmacologice, în care căile de administrare şi dozele utilizate nu permit să se tragă concluzii valabile pentru sectorul alimentar. Din această cauză, la toate conferinţele internaţionale care au avut loc în ultimii ani, acidul formic a fost trecut pe lista substanţelor insuficient cunoscute din punct de vedere toxicologic (1, 9).

Ţinînd seama de acest considerent, am considerat necesară efectuarea unor studii sistematice cu privire la toxicitatea acută, subacută şi cronică a acidului formic.

METODA EXPERIMENTALĂ

Toxicitatea acută a substanţei s-a determinat pe şobolani tineri, prin administrarea *per os* a diferitelor doze de substanţă la şoareci albi, prin injectarea intraperitoneală a substanţei şi pe dafnii, prin includerea acidului formic în mediul nutritiv.

Toxicitatea subacută s-a testat cu ajutorul probei de refacere a proteinelor hepatice după foame şi a probei de creştere.

Proba de refacere a proteinelor hepatice după foame s-a efectuat pe 21 de şobolani albi adulţi împărţiţi în 3 loturi din care un lot primea 0,5‰ acid formic, iar altul 1‰ în apa de băut. Substanţa s-a adăugat în apa loturilor experimentale atît în perioada de foame, cît şi în perioada de refacere (7 zile). Pe lîngă greutatea corporală, greutatea ficatului şi conţinutul în azot al ficatului, s-a urmărit şi greutatea glandelor suprarenale, conţinutul lor în acid ascorbic, valorile hemoglobinei şi coeficientul catalazic.

Proba de creştere s-a efectuat pe 48 de şobolani albi tineri de greutate apropiată, provenind din aceeaşi generaţie. Animalele au fost împărţite în 6 loturi, experienţele efectuîndu-se la două nivele de proteine din raţie — 11,8% şi 18,2% — şi la ambele concentraţii de substanţă testate — 0,5 şi 1‰. Utilizarea a două raţii proteice a fost determinată de faptul că în cercetările noastre anterioare s-a demonstrat că în aceste condiţii devin mai sesizabile modificările metabolismului proteic. Proba de creştere s-a efectuat timp de 6 săptămîni la raţia hipoproteică şi 5 săptămîni la raţia normoproteică. În cursul experienţelor s-a urmărit greutatea animalelor, cîştigul săptămînal în greutate, ingestia de alimente şi eficienţa proteică.

La sfîrşitul experienţei animalele au fost sacrificate, determinîndu-se conţinutul în azot şi în lipide al ficatului, greutatea ficatului, a glandelor suprarenale, splinei şi rinichiului, conţinutul în acid ascorbic al glandelor suprarenale, coeficientul catalazic şi indicele peroxidazic.

REZULTATELE OBŢINUTE

Toxicitatea acută

DL_{50}, la administrarea *per os* a substanţei la şobolanii albi tineri, a fost de 1 830 mg/kilocorp. Animalele mor cu semne de dispnee şi cianoză. În cazul supravieţuirii apar diaree, slăbire şi o refacere lentă.

La injectarea intraperitoneală a substanţei la şoareci albi, DL_{50} a fost de 940 mg/kilocorp.

Pe dafnii, DL_{50}, la 48 de ore, a fost de 70 mg la litru.

Toxicitatea subacută

Refacerea proteinelor hepatice după foame. Din analiza rezultatelor obţinute (tabelul I) reiese că administrarea acidului formic nu a influenţat pierderea şi refacerea în greutate a animalelor. Conţinutul în azot al ficatului este ceva mai mic la lotul care a primit 1‰ acid formic şi semnificativ mai scăzut la lotul care a primit 0,5‰ (t este 2,79, mai mare decît 2,18). Valorile mai scăzute obţinute la lotul care a primit 0,5‰ acid formic se corelează cu ingestia mai mică înregistrată la acest lot.

Tabelul I

Influenţa acidului formic asupra refacerii proteinelor hepatice după foame, a conţinutului în acid ascorbic al glandelor suprarenale şi coeficientului catalazic

Date medii pe lot

Lotul	Greutatea			Ingestia	mg N ficat	Greutatea suprarenalei	Acid ascorbic în suprarenală	Coeficient catalazic
	Iniţială	După foame	După refacere		In % greutatea corporală			
Martor	177,8	150,4	170,6	44,4	137,7	0,157	136,0	16,4
Acid formic 1‰	182,7	156,0	177,2	49,4	129,2	0,146	128,4	13,7
Acid formic 0,5‰	182,1	155,0	180,1	38,9	122,3	0,132	129,4	18,9

Greutatea glandei suprarenale este mai scăzută la lotul care a primit 1‰ acid formic şi mai ales la lotul care a primit 0,5‰. Conţinutul în acid ascorbic este apropiat la toate loturile experimentale, diferenţele între loturi fiind nesemnificative statistic. Coeficientul catalazic este uşor crescut la lotul care a primit 1‰ acid formic şi uşor scăzut la lotul cu 0,5‰.

Proba de creştere. Rezultatele obţinute sînt prezentate în tabelul II.

Tabelul II

Influenţa administrării acidului formic asupra creşterii şobolanului alb
Date medii pe lot

Lotul	Indicatorul iniţial	Săptămîna I	II	III	IV	V	VI	Total medie
I. Martor Caseină 18,2%	Greutatea 40,1	56,8	79,0	96,3	116,9	131,9		360,8
	Ingestia	49,1	63,6	77,8	85,4	85,0		
	Eficienţa proteică	1,86	1,91	1,22	1,32	0,96		1,39
II. Acid formic 0,5%	Greutatea 40,5	58,6	77,6	90,3	105,3	120,9		342,9
	Ingestia	46,0	63,9	69,9	80,0	83,1		
	Eficienţa proteică	2,16	1,63	0,99	1,33	1,03		1,28
III. Acid formic 1‰	Greutatea 40,6	60,3	80,7	100,6	117,6	131,9		367,9
	Ingestia	48,9	69,0	80,1	83,3	86,6		
	Eficienţa proteică	2,20	1,62	1,36	1,12	0,90		1,36
IV. Martor caseină 11,8%	Greutatea 40,1	53,5	64,1	75,3	93,5	107,0	115,8	416,2
	Ingestia	53,9	62,0	71,6	81,0	76,4	71,3	
	Eficienţa proteică	2,10	1,58	1,32	1,90	1,49	1,04	1,54
V. Acid formic 0,5‰	Greutatea 40,1	51,3	63,1	73,1	88,2	98,8	107,1	392,6
	Ingestia	51,6	64,8	65,4	74,5	70,3	66,0	
	Eficienţa proteică	1,83	1,54	1,29	1,71	1,27	1,56	1,44
VI. Acid formic 1‰	Greutatea 40,1	51,1	63,8	75,9	88,8	100,0	108,3	396,0
	Ingestia	51,6	65,5	71,4	74,4	69,0	64,1	
	Eficienţa proteică	1,82	1,64	1,43	1,46	1,37	1,09	1,45

Din analiza rezultatelor obţinute reiese că administrarea acidului formic în concentraţie de 1‰ nu a influenţat creşterea lotului care a primit o raţie de 18,2% cazeină, producînd o uşoară reducere a creşterii la lotul care a primit 11,8% cazeină. Administrarea acidului formic în concentraţia de 0,5‰ a produs o inhibiţie mai puternică a creşterii la ambele raţii alimentare. Diferenţele faţă de loturile martor nu sînt însă semnificative din punct de vedere statistic.

Ingestia de alimente variază corespunzător cu creşterea, fiind mai mică în loturile care au primit 0,5‰ acid formic, apropiată de lotul martor la lotul care a primit 1‰ acid formic, la raţia cu 18,2% cazeină.

Eficienţa proteică este mai scăzută la toate loturile care au primit acid formic, mai ales la loturile cu 0,5‰.

La lotul care a primit o raţie cu 18,2% cazeină, la toţi indicatorii se înregistrează o fazicitate în acţiunea substanţei, cu o uşoară stimulare a creşterii şi eficienţei proteice în prima săptămînă şi inhibarea ei ulterioară.

Greutatea glandei suprarenale, a ficatului, splinei şi rinichiului este scăzută la lotul care a primit 0,5‰ acid formic şi raţia alimentară cu 18,2% cazeină.

La 1‰ acid formic se remarcă o uşoară creştere a greutăţii glandei suprarenale şi splinei şi o uşoară scădere a greutăţii ficatului şi rinichiului (tabelul III).

Tabelul III

Influenţa acidului formic asupra greutăţii ficatului, rinichiului, suprarenalei şi splinei
Date medii pe lot

Lotul	Ficat	Rinichi	Suprarenală	Splină
I. Martor 18,2% cazeină	5,443	1,302	0,2 494	0,424
II. Acid formic 0,5‰	5,143	1,191	0,2 394	0,355
III. Acid formic 1‰	5,090	1,241	0,2 745	0,443
IV. Martor 11,8% cazeină	4,318	1,030	0,247	0,310
V. Acid formic 0,5‰	3,995	1,015	0,226	0,261
VI. Acid formic 1‰	4,092	1,025	0,226	0,341

La loturile care au primit 11,8% cazeină în raţie, acidul formic a produs o uşoară scădere a greutăţii ficatului, glandei suprarenale şi splinei la lotul cu 0,5‰ acid formic şi o creştere a greutăţii splinei la lotul cu 1‰.

Conţinutul în azot al ficatului este uşor scăzut la lotul al II-lea şi semnificativ scăzut la lotul al III-lea (testul de 4,72 este mai mare decît 2,18). La loturile al V-lea şi al VI-lea nu s-au înregistrat diferenţe în conţinutul în azot al ficatului faţă de lotul martor (tabelul IV).

Tabelul IV

Influenţa acidului formic asupra conţinutului în azot şi lipide al ficatului, conţinutului în acid ascorbic al glandelor suprarenale şi a coeficientului catalazic
Date medii pe lot

Lotul	mg N ficat % greutate corporală	Lipide ficat	Acid ascorbic suprarenală % greutate corporală	Coeficient catalazic
I. Martor 18,2% cazeină	131,0	0,296	118,8	16,7
II. Acid formic 0,5‰	123,0	0,228	125,5	19,5
III. Acid formic 1‰	118,0	0,226	117,5	18,8
IV. Martor 11,8% cazeină	115,0	0,214	126,3	17,4
V. Acid formic 0,5‰	117,0	0,214	135,9	17,5
VI. Acid formic 1‰	117,0	0,204	132,8	18,8

Conţinutul în lipide al ficatului este mai scăzut faţă de loturile martor, respectiv la loturile al II-lea şi al III-lea, şi nemodificat la loturile ale V-lea şi al VI-lea.

Conţinutul în acid ascorbic al glandelor suprarenale este apropiat de cel al lotului martor la loturile al II-lea şi al III-lea şi ceva mai crescut la lotul al V-lea.

Coeficientul catalazic este fie apropiat faţă de loturile martor, fie uşor crescut (lotul al II-lea şi al III-lea).

Indicele peroxidazic este crescut la loturile care au primit acid formic, mai ales la loturile care au primit o raţie cu 18,2% cazeină (fig. 1).

Discuţii

Din analiza rezultatelor obţinute în experienţele acute rezultă că acidul formic poate fi încadrat printre substanţele cu o toxicitate medie, mai mică ca aceea a acidului salicilic sau boric, dar mai mare ca aceea a acidului benzoic.

în experiențele pe dafnii, acidul formic a fost însă mai toxic decît acidul salicilic sau boric (8).

Rezultatele obținute în experiențele subacute permit să se tragă concluzia că, în condițiile experimentale utilizate de noi, toxicitatea acută și subacută a substanței a fost redusă. Despre aceasta vorbesc datele cu privire la lipsa unor modificări semnificative la majoritatea testelor utilizate. S-ar putea presupune că lipsa modificărilor se datorește faptului că în experiențele noastre am folosit concentrații asemănătoare cu cele utilizate în practică, deci prea mici pentru a asigura un coeficient de siguranță. Această presupunere este însă greșită, deoarece, în timp ce în practică numai o mică parte din lichidele ingerate conțin acid formic, în experiențele noastre, utilizîndu-se o rație sintetică uscată, toată cantitatea de lichid consumată a conținut acid formic. În acest fel considerăm că s-a asigurat un coeficient de siguranță corespunzătoare, creșterea concentrațiilor administrate reprezentînd o condiție agravantă care depășește limitele coeficientului de siguranță.

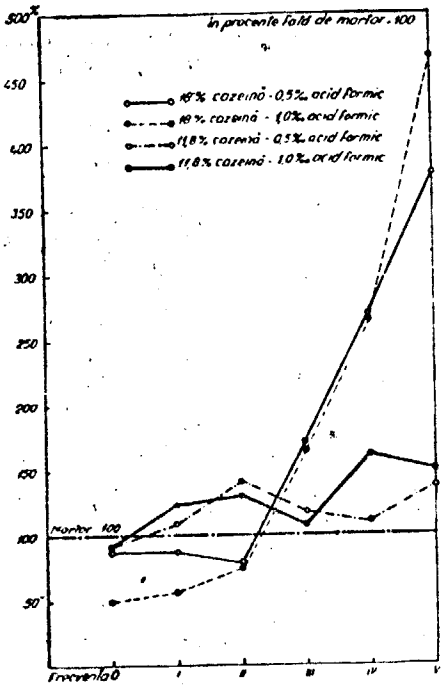

Fig. 1. — Influența acidului formic asupra indicelui peroxidazic.

Ar fi greșit să tragă concluzia că acidul formic este indiferent pentru organism. Despre aceasta vorbesc datele cu privire la toxicitatea acută a substanței, modificările în conținutul în azot al ficatului la refacerea după foame, ca și la sfîrșitul probei de creștere, scăderea în greutate a glandelor suprarenale, ficatului, splinei și rinichiului, tendința de inhibare a creșterii și a eficienței proteice, fazicitatea în acțiunea substanței la alimentația normoproteică și inhibiția directă a creșterii la loturile cu alimentație hipoproteică, creșterea importantă a indicelui peroxidazic. Datele obținute sînt însă insuficiente pentru a interzice utilizarea acidului formic în conservarea sucurilor, siropurilor sau fructelor. Pentru a rezolva această problemă, am efectuat cercetări de lungă durată.

TOXICITATEA CRONICĂ

Metoda experimentală

Experiențele s-au efectuat pe 60 de șoareci albi adulți (50 femele și 10 masculi), urmărindu-se influența administrării îndelungate a substanței asupra reproducerii, tabloului sanguin, conținutului în azot al ficatului și conținutului în acid ascorbic al glandelor suprarenale. Animalele au fost împărțite în 5 loturi după cum urmează:

— lotul I martor;

— lotul al II-lea, femele, a primit în toată perioada experimentală acid formic;

— lotul al III-lea, masculi, a primit în toată perioada experimentală acid formic;

— lotul al IV-lea, atît masculii cît și femelele, a primit în toată perioada experimentală acid formic;

— lotul al V-lea, femele, a primit acid formic în perioada alăptării.

În acest fel s-a asigurat testarea separată a acțiunii substanței asupra capacității de reproducere a femelelor și masculilor, ca și posibilitatea trecerii unor substanțe toxice prin lapte.

Pentru a testa efectul obținut la diferite perioade de administrare a substanței, ca și pentru a verifica rezultatele obținute, s-au efectuat 3 reproduceri; prima la o lună de la începutul administrării substanței, a doua la 3 luni și a treia la șapte luni. În total, substanța s-a administrat timp de 8 luni.

În perioada experimentală animalele au primit hrana obișnuită de creșcătorie, loturile experimentale au primit acidul formic în concentrație de 1 g‰, raportat la apa consumată.

În proba de reproducere s-au urmărit următorii indicatori:

— numărul de femele rămase gravide;

— numărul de pui născuți, cîți vii și cîți morți;

— greutatea puilor la naștere;

— numărul de pui vii și greutatea lor la 7 zile;

— numărul de pui vii și greutatea lor la 21 de zile.

Puii din prima generație au fost supuși probei de creștere timp de 4 săptămîni, fără a se administra substanța, urmărindu-se greutatea lor, cîștigul săptămînal în greutate, ingestia de alimente și eficiența proteică.

La sfîrșitul experienței la animalele adulte care au primit timp de 8 luni substanța, ca și la animalele din lotul martor s-au cercetat tabloul sanguin, conținutul în azot al ficatului și conținutul în acid ascorbic al glandelor suprarenale.

REZULTATELE OBȚINUTE

I. *Influența administrării îndelungate a substanței asupra reproducerii și dezvoltării puilor*

Numărul de femele rămase gravide, la prima reproducere, variază între 5 și 6 la un singur lot (la care masculii au primit acid formic), înregistrîndu-se 8 gravide. Nu se remarcă diferențe între loturile care au primit acid formic, față de lotul martor (tabelul V).

La a doua reproducere, numărul de femele rămase gravide oscilează între 5 și 7 la lotul la care atît femelele, cît și masculii au primit acid formic, numărul de gravide fiind mai redus (3).

La a treia reproducere, la toate loturile se înregistrează un număr mic de gravide (2—4), ceea ce nu permite o interpretare a rezultatelor obținute.

Numărul de pui născuți la prima reproducere este în medie, de femelă, de 6,6 pentru lotul martor, oscilînd între 5,5 și 7,3 la celelalte loturi (tabelul V).

La a doua reproducere numărul de pui născuți oscilează între 6 și 6,3 pentru lotul martor și loturile al III-lea și al V-lea. La loturile în care femelele au primit acid formic în toată perioada experimentală (II și IV), numărul puilor născuți în medie de o femelă este de 4,1, respectiv 4.

Numărul de pui născuți vii la prima reproducere oscilează între 5 și 7,0, fără a se înregistra diferențe semnificative între loturi.

La a doua reproducere, numărul de pui născuţi vii este de 4 pentru lotul martor, de 5,7 pentru lotul la care femelele au primit acid formic în perioada alăptării, de 6 pentru lotul la care masculii au primit acid formic şi de 2,8, respectiv 1, pentru loturile la care femelele au primit acid formic în toată perioada experimentală (tabelul V).

Tabelul V

Influenţa administrării îndelungate a acidului formic asupra reproducerii şobolanilor albi

Date medii pe lot

Lotul	Femele gravide	Pui născuţi	Pui vii	Greutatea la naştere	Pui vii 7 zile	Greutatea 7 zile	Pui vii 21 de zile	Greutatea 21 de zile
				Reproducerea I				
I	6	6,6	6,3	5,2	5,0	10,6	5	34,3
II	6	5,5	5,0	5,1	0,5	—	0,5	—
III	8	6,3	6,1	5,9	3,8	12,5	3,8	34,8
IV	5	6,0	5,4	4,9	2,2	9,6	2,2	34,4
V	6	7,3	7,0	5,2	5,5	10,2	5,5	28,5
				Reproducerea a II-a				
I	6,0	6,0	4,0	5,1	4	11,5	4	31,4
II	6,0	4,1	2,8	5,4	2,1	11,2	2,1	32,2
III	6,0	6,3	6,0	5,8	6,0	13,1	6,0	33,5
IV	3,0	4,0	1,0	5,0	0	—	—	—
V	7,0	6,3	5,7	5,2	4,7	13,6	4,7	29,6

Greutatea la naştere a puilor la prima reproducere oscilează între loturi între 4,9 g şi 5,9 g în medie de pui, fără a se înregistra diferenţe semnificative între loturi. Nici la a doua reproducere nu se înregistrează diferenţe semnificative între loturi, greutatea puilor la naştere oscilînd între 5 şi 5,8 g (tabelul V).

Numărul de pui rămaşi în viaţă la 7 zile variază semnificativ între loturi (tabelul V).

Din tabelul V rezultă că la prima reproducere, în timp ce la lotul martor la 7 zile rămîn în viaţă în medie 5 pui, iar la lotul care primeşte acid formic în perioada alăptării 5,5 pui, la loturile la care femelele primesc acid formic, numărul de pui care rămîn în viaţă la 7 zile este de numai 0,5, respectiv 2,2. La a doua reproducere fenomenul se repetă, numărul de pui rămaşi în viaţă pentru loturile la care femelele au primit acid formic fiind de 0 respectiv 2,1.

Greutatea puilor la 7 zile nu prezintă diferenţe semnificative între loturi atît la prima, cît şi la a doua reproducere. Valorile oscilează la prima reproducere între 9,6 şi 12,5 g, iar la a doua reproducere între 11,2 şi 13,6 g.

În perioada între 7 şi 21 de zile nu moare nici un pui la nici unul din loturi (tabelul V).

Greutatea puilor la 21 de zile nu prezintă diferenţe semnificative între loturi atît la prima, cît şi la a doua reproducere. Ea oscilează la prima reproducere între 28,5 şi 34,4 g, iar la a doua reproducere între 29,6 şi 34,4 g (tabelul V).

Proba de creştere a puilor nu indică, de asemenea, existenţa unor diferenţe semnificative între loturile care au primit acid formic faţă de lotul martor sau între loturile care au primit acid formic.

Astfel, de exemplu, în reproducerea a doua, în timp ce animalele din lotul martor ajung la 4 săptămîni la 60,4 g, animalele din loturile care au primit acid formic ajung în medie la 59,8—62,8 g.

II. *Acţiunea administrării îndelungate a acidului formic asupra conţinutului în azot al ficatului, a acidului ascorbic în glanda suprarenală şi a tabloului sanguin.*

Conţinutul în azot al ficatului s-a determinat pe 16 animale martor şi 15 animale care au primit acid formic, timp de 8 luni. Rezultatele obţinute indică lipsa unor modificări semnificative între ambele loturi, valorile fiind de 103 mg la 100 g greutate corporală pentru lotul martor şi 96 mg % pentru lotul care a primit acid formic.

Conţinutul în acid ascorbic al glandelor suprarenale de asemenea nu indică existenţa unor diferenţe semnificative între ambele loturi, valorile fiind de 76,2 mg % pentru lotul martor, respectiv 74,8 mg % pentru lotul care a primit acid formic.

Tabloul sanguin indică existenţa unui oarecare grad de anemie hipercromă la lotul care a primit acid formic, de asemenea o leucocitoză, neutropenie bazofilă şi o uşoară limfocitoză (tabelul VI).

Tabelul VI

Influenţa administrării îndelungate a acidului formic asupra tabloului sanguin

Date medii pe lot

Lotul	Hematii/mm³	Hb %	Valoarea globulară	Leucocite/mm³	Neutrofile (%)	Limfocite (%)	Eozinofile (%)	Bazofile (%)	Mononucleare (%)	Celule tinere (%)
Martor	6,140	94	0,77	10 300	30,4	58,8	3,4	0,9	3,8	2,9
Acid formic	5,310	106	1,00	26 800	17,8	68,0	3,6	5,7	2,3	2,7

Discuţii

Din analiza datelor obţinute rezultă că administrarea îndelungată a acidului formic a influenţat doar unii indicatori ai testului de reproducere. Astfel, numărul de femele rămase gravide, greutatea la naştere la 7 zile şi la 21 de zile nu înregistrează diferenţe semnificative la loturile care au primit acid formic faţă de lotul martor.

În ceea ce priveşte numărul de pui născuţi în medie de o femelă, ca şi numărul de pui vii născuţi, diferenţele apar numai la a doua reproducere. Acest fapt ar putea fi atribuit modificărilor care apar în urma administrării îndelungate a acidului formic. Ţinînd seama de multitudinea factorilor de care poate depinde numărul de pui născuţi, de variabilitatea care se înregistrează în mod normal la acest indicator, de faptul că diferenţele între loturi sînt relativ mici, considerăm că o asemenea interpretare a rezultatelor nu este suficient de justificată.

Diferenţe importante şi semnificative între loturile în care femelele au primit acid formic şi lotul martor se înregistrează în viabilitatea puilor în primele 7 zile. Ţinînd seama de faptul că experienţele s-au efectuat în aceleaşi condiţii şi în aceeaşi perioadă pentru toate loturile, că fenomenul se confirmă la ambele reproduceri, că diferenţele sînt foarte evidente, se poate trage concluzia că administrarea acidului formic influenţează negativ viabilitatea puilor. Faptul că puii care provin de la femelele care au primit acid formic în perioada alăptării prezintă o viabilitate asemănătoare cu cea a lotului martor, că după naştere femelele

nu au mai primit acid formic arată că efectul acidului formic asupra viabilității puilor se datorește acțiunii sale înainte de naștere.

Acțiunea nocivă a administrării îndelungate a acidului formic rezultă și din modificările tabloului sanguin care apar la animalele adulte care au primit substanța pe o perioadă mai îndelungată.

Cercetările noastre nu furnizează date care să permită stabilirea mecanismului de acțiune a acidului formic, ele demonstrează însă efectul său negativ asupra organismului.

Ținînd seama de rezultatele obținute, de faptul că în experiment s-au folosit concentrații relativ mici de acid formic, considerăm că utilizarea sa în conservarea alimentelor nu este indicată.

Concluzii

1. S-a stabilit toxicitatea acută a acidului formic pe șoareci albi prin administrarea substanței *per os* și intraperitoneal, și pe dafnii. Rezultatele obținute arată că acidul formic prezintă o toxicitate acută mai mică decît aceea a acidului salicilic sau boric, dar mai mare decît aceea a acidului benzoic.

2. În experiența subacută s-a stabilit că acidul formic are o toxicitate redusă, fără a fi indiferent pentru organism. Astfel, acidul formic produce o scădere a refacerii azotului hepatic după foame, o scădere în greutatea organelor, o tendință de scădere a creșterii și eficienței proteice.

3. În experiențele de lungă durată (8 luni) s-a stabilit că acidul formic influențează negativ viabilitatea puilor în primele 7 zile de viață, cît și tabloul sanguin.

4. Pe baza rezultatelor obținute se opiniază împotriva utilizării acidului formic în conservarea alimentelor.

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Articol intrat în redacție la 10.V.1962.

Indicele de clasificare: 613.2 : 615.734.46—099

SIXTEEN YEARS' EXPERIENCE WITH FORMIC ACID AS A THERAPEUTIC AGENT.*

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My attention was first drawn to the medicinal qualities of formic acid by a semi-secret preparation styled by its manufacturer, Hensel, "Ferrum formicarum oxidatum oxydulatum." This was in 1888. The same or a similar preparation is now sold by a homeopathic drug house under the name of "Hensel's tonicum."

HISTORICAL REVIEW.

There is no doubt in my mind that Hensel's formic acid compound is a latter-day transformation of Hoffmann's "Vinegar of Magnanimity," in which a preparation of ants played the leading rôle. In the seventeenth century this famous vinegar had found entrance in all European courts and was employed by men and women as a general tonic, stomachic and diuretic, and probably also as an aphrodisiac. For formic acid seems to possess certain qualities stimulating sexual appetite (in South America a species of large ants are consumed), and the proprietary tonics demanding a good sale, as a general rule, are those which increase sexual desire.

Otto Braunfels, a German botanist, and physician (1488-1534), to whom Linnæus refers to as "the father of botany," already drew attention to the fact that an acid fume reddening blue vegetable colors emanated from ant hills. Formic acid, however, was first isolated and discovered by J. Wray or Rey in 1670 by distilling red ants with water; others ascribe the first isolation of this substance to Samuel Fischer (1670). Marggraf, in 1749, experimentally proved the dissimilarity of formic and acetic acids. Döbereiner, in 1822, produced formic acid artificially by the oxidation of tartaric acid with manganese dioxid and sulphuric acid thus:

$$\begin{matrix} CH(OH).CO.OH \\ | \\ CH(OH).CO.OH \end{matrix} + MnO_2 + H_2SO_4 = 2H.CO.OH + 2H_2O + 2CO_2.$$

Tartaric acid + Manganese dioxid + Sulphuric acid = Formic acid + Water + Carbon dioxid.

Berzelius and Liebig devoted much time to the minute study of formic acid.

* Read in the Section on Pharmacology and Therapeutics of the American Medical Association, at the Fifty-sixth Annual Session, July, 1905.

PREPARATION OF FORMIC ACID.

Formic acid is prepared in different ways. The best mode of obtaining it is by heating equal weights of oxalic acid and glycerin to a temperature of 100 to 110 C. for about fifteen hours, when carbon dioxid is given off and dilute formic acid distils over. The dilute acid may be concentrated by saturating it with lead carbonate or lead oxid, after which the mixture is filtered and evaporated to a small bulk (complete dryness). The dry lead formate, reduced to a fine powder, is then heated in a glass tube connected with a condensing apparatus, through which a current of sulphureted hydrogen is passed. The glycerin apparently exerts no chemical action, but seems to bring about decomposition of oxalic acid at a lower temperature than would otherwise be necessary. At a temperature above 110 C. decomposition of the formic acid would ensue.

The name-formic acid is an appropriate one, for, as is well known, the ant, especially the red ant, *Formica rufa,* ejects, when irritated, a secretion, the greater part of which is composed of formic acid. Ants, however, are not the only insects which produce the acid. This substance is produced or accumulated in a special gland from which, by means of the sting, it finds its way under the skin, where it gives rise to a local inflammatory process. Bees impart some of their formic acid to the honey. The acidity is particularly noticeable in fresh honey of wild bees. In domestic medicine, honey at one time was considered to be a good preventive of diphtheria. The formic acid contained in it is said to inhibit the growth of the Klebs-Loeffler bacillus. The diuretic qualities of certain isopoda, like *Armadillo officinalis* and *Oniscus asellus,* have also been ascribed to the formic acid which they contain. Free formic acid is also contained in the leaves of the stinging nettle, *Urtica urens,* and in other plants, and it has been demonstrated in unripe grapes. Being a decomposition product of vegetable débris, it is very likely the potent medicinal constituent of mud baths. The mud bath acts as a local rubefacient.

REVIEW OF LITERATURE.

The effect of formic acid or the formates on the animal organism has not been studied exhaustively. Mitscherlich was the first[1] to bestow more than casual attention on this question. He reported the following:

Rabbits were poisoned with lethal doses of free formic acid. The autopsy revealed pronounced thickening of the outer layer of the gastric mucosa without marked cellular changes; the capillary vessels and veins were engorged and showed extravasation of blood; in short, the lining of the stomach and the upper part of the small intestines was violently inflamed. The dilute acid produced the same effect but, of course, in a less pronounced degree. That the acid was absorbed was evinced by the acid reaction of the urine which in rabbits is normally alkaline. The blood appeared to be little altered. The kidneys were discolored dark red brown, and contained a large amount of blood. The urine exhibited blood and numerous cylindrical bodies. This evinced that formic acid like other acid substances exerted a specific influence on the kidneys. In this respect they acted in a similar manner to cantharides.

The toxic state due to formic acid, according to Mitscherlich, is different from that due to any of the other acids he had experimented with. The experiment-animals become restless and excited. Asthenia supervenes only after some hours. Death ensues after a period of mild convulsions and difficult respiration.

According to the same author, formic acid is a very violent poison, as half an ounce of a liquid containing in 100 parts 7 parts of the acid caused death after twenty-four hours, and two drams even called forth marked poisonous symptoms. The acid in the same dilution applied on the skin of various individuals caused but a slight burning sensation; the pure acid, on the other hand, gave rise to a severe inflammatory process which only subsided after a number of weeks.

Rabuteau,[2] in experimenting with formic acid and sodium formate, came to different results. He showed that the acid is again eliminated by the urine as a carbonate (combined with alkali), and that it has no deleterious effect on the organism if administered as free diluted acid or if combined with sodium. He took 3.5 gm. of sodium formate without experiencing any undue effects.

Arloing[3] observed the action of sodium formate in dogs and horses, and found that it diminishes cardiac activity and lowers the blood pressure and the temperature. Massive doses, as one gram per kilogram body weight, cause death by cardiac paralysis.

Hoffmann[4] studied the influence of formic acid on the lower organisms. Infusoria became immediately paralyzed in dilutions of the acid down to 0.1 per cent. Their body-contents were rendered dark and granulated and finally the enveloping membrane burst in most organisms. Bursting of the infusoria could not be observed in a 0.01 per cent. solution of the acid, although all other phenomena were still well marked.

A leech put into a beaker containing formic acid in 0.25 per cent. dilution immediately contracts its body and relaxes it again and makes vain attempts to escape from the vessel. Its movements, at first lively, gradually become weaker and after twenty-five minutes the leech is dead. The same phenomena occur in the 0.1 per cent. solution of the acid, but death ensues only after fifty minutes. Placed in a 0.05 per cent. solution of formic acid, the leech immediately fastens itself on the glass and performs moderate movements with the free end of his body. After five minutes it tries to get out of the fluid; again placed in the solution, the animal fastens itself again, renewing at the same time its movements, and attempts to escape. The leech becomes gradually weaker, and after fifty minutes the sucking discs no longer work. The movements cease temporarily while the animal lies on its side contracting its body vehemently. After two hours neither spontaneous nor reactive movements are perceptible. In a 0.01 per cent. solution of the acid the leech apparently was not disturbed, but twenty-four hours after it was found dead at the bottom of the vessel. A leech put for control purposes into a 0.05 per cent. solution of acetic acid fastened itself at once on the wall of the vessel and remained in a quiescent state. The animal was found in the position after twenty-four hours, although means to escape were offered it. In the same dilution formic acid caused the death of the leech in two hours.

Frogs placed in a beaker containing 100 c.c. of a 0.5 per cent. solution of formic acid at once make attempts

1. Mitscherlich: "De acidi acetici, oxalici, tartarici, citrici, formicici et boracici effectu in animalibus observatio," 1845. See also Hoffmann: "Experimentelle Untersuchungen ueber die Wirkung der Ameisensaure, 1884."

2. Abstract from Gaz. med. de Paris, 1871, in Jahresber. ueber die Fortschr. d. Pharmakognosie, Pharmacologie und Toxikologie, 1872.

3. Abstract from Bull. gen. de therapie, 1879, in Jahresber. ueber d. Fortschr. d. Pharmakognosie, etc., 1879. Both quotations after Hoffmann.

4. Hoffmann: Experimentelle Untersuchungen ueber die Wirkung der Ameisensaure, 1884.

to escape; after fifteen minutes the animal suddenly falls on its abdomen, holding its head above the fluid and making violent motions with its hind legs. Soon it becomes unable to rise and its head droops down. The extremities are moderately rigid after twenty-five minutes. This rigidity slowly increases and becomes more general, so that in fifty minutes the animal gives the impression as if it were frozen; an hour and a half after starting the experiment it is lifeless. The blood spectroscopically examined exhibits the bands of oxyhemoglobin. In a 0.5 per cent. solution of acetic acid a frog lived for two hours and a half. In this frog muscular rigidity did not ensue.

Hoffmann also inquired into the antiseptic and antifermentative properties of formic acid. He found that free formic acid in a solution of 1 per cent. prevented even the slightest degree of putrefaction; that a 0.5 per cent. solution averted putrefaction of Buchholtz's nutrient (distilled water 100 c.c., commercial rock candy 10 gm., ammonium tartrate 1 gm., and potassium phosphate 0.5 gm), and that a 0.25 per cent. solution exerted a definite but limited preventive influence on putrefaction. He showed that the conversion of albumin by pepsin and hydrochloric acid is not prevented if as much as 1 per cent. of formic acid is contained in the digestive fluid, and that formic acid can not replace hydrochloric acid in proteid digestion.

Sodium formate inhibits proteid digestion by combining with the hydrochloric acid to form sodium chlorid, setting free thereby formic acid. Fermentation, of course, is also inhibited when the liquid to be fermented contains formic acid in rather large proportion; in greater dilution, however, as demonstrated by the same observer, formic acid exerts a stimulating influence on the process of fermentation. It appears that the activity of the yeast cell is enhanced when high dilutions of formic acid are added to the glucose solution.

The latter observation I am able to corroborate to a certain extent. I found that while in dilution of 1 to 3000 formic acid suppresses the development of bacteria in weakly acid media like beer, and while in dilutions of 1:7000 and 1:10000 the bacteria are rendered more or less inactive; culture, as well as wild yeasts, thrive very well and often times become even more active in these dilutions.[5]

During the last few years a number of investigators (notably French) have studied the antiseptic, antifermentative and clinical possibilities of formic acid. Concerning its antiputrefactive and antifermentative qualities, they have, in substance, not gone far beyond Hoffmann's experiments; concerning its clinical value they have said nothing. We owe thanks, however, to the French clinicians for having resuscitated from oblivion this very potent remedial agent.

The effect of formic acid on the healthy human organism as studied by me is as follows: In doses of two drops, well diluted, it gives rise to no symptoms besides an occasional irritation in the throat and a feeling of warmth and emptiness in the stomach. In doses of five drops it causes a slight irritation in the pharynx and larynx; in 10-drop doses it exerts mild diuretic and diaphoretic influence; in 20-drop doses it produces the same phenomenon, but in a more pronounced degree and gives rise to contracting sensations in the pyloric region when the stomach is empty. Administered to the amount of from 3 to 4 c.c., very well diluted, and when the stomach is empty, it causes dryness in the throat, ringing in the ears, slight vertigo, burning sensation in the gastric organ and sometimes mild pyloric spasms, lassitude, slight motor disturbances and elevation of temperature of from one-half to two degrees, which latter is followed after half an hour or an hour by marked perspiration and increased micturition. In doses of 0.5 to 1 c.c., repeated three or four times a day for some weeks, it acts as a muscular excitant in some instances. Clement[6] states that formic acid augments muscular force, activity and resistance. By its action on the muscular structure of the bladder it facilitates emission of the urine and enhances the contractility of the muscles of the larynx.

There is no authentic case of fatal formic-acid poisoning on record. In very massive doses as recommended by Clement (2 to 3 gm. a few times during the day), we find not infrequently toxic symptoms, as vertigo, nausea and vomiting, albuminuria, hematuria, vesical tenesmus, dyspnea and lowered temperature. According to Lewin,[7] 1 gr. of sodium formate per kilogram of animal weight is a lethal dose.

THERAPEUTICS OF FORMIC ACID.

The therapeutics of formic acid are manifold, but indefinite. It has been employed, as we have already seen, in a similar way as cantharides to increase sexual desire. At present the people of Paris, as I have learned from a trustworthy source, are again beginning to utilize it for the same purpose. Formic acid has been turned to practical account—in years gone-by especially—in the treatment of atonic conditions of the urinary organs, vesical weakness, dropsy, paralysis and gouty and rheumatic affections, in general when a stimulant of the mucous membranes or a diaphoretic and diuretic agent was indicated.

It was often used as an external remedy, especially in the form of the *Spiritus formicarum*. This is prepared by mixing four parts of formic acid, seventy parts of alcohol and twenty-six parts of water. Topically this was employed alone or with other rubefacients, as compound spirit of angelica, spirits of camphor, etc. Internally it was prescribed in doses of from 0.5 to 2.0 c.c. several times a day. Popular medicine made use of the ants and of formic acid. Placed in little linen bags and crushed therein, the ants were pressed against the portion of the body affected with paralysis, neuralgia, gout or rheumatism, or their juice was employed by rubbing it directly into the diseased joint or muscle. At other times the ants were used for local or general baths or for inhalation (diseases of the respiratory organs) or finally in the form of fomentations. The Russian peasant prepares his ant bath in the following way: Finding an ant's nest, he puts into a linen bag the ants, their eggs and necessarily considerable dirt. Returning to his home, he plunges his bag of ants, which he has previously fastened tightly at the mouth, into the hot water of the bath. After several minutes the water gives off a very strong penetrating odor of formic acid. The bath is now ready and the invalid is put

5. Formaldehyd is partially oxidized to formic acid in the organism and excreted as such by the urine. I have repeatedly observed that the urine of diabetic patients, when these had taken urinary antiseptics (so called) which liberate formaldehyd and consequently formic acid, had no inhibiting influence on distiller's yeast which was added in order to perform the fermentation test for urinary glucose. Again, Effront, of Brussels, has lately demonstrated that yeast which has become accustomed to gradually increased amount of formol, is capable of decomposing the latter even before it attacks maltose or glucose contained in the liquid.

6. Lyon Medical, Feb. 26, 1905.

7. Lehrbuch der Toxikologie, 1897.

into it. In certain diseases of the respiratory organs the fumes emanating from the decoction are inhaled by the patient.

Looking over the field of formic-acid therapeutics we find that it was and still is chiefly employed as an epispastic. It has been generally utilized on account of its vesicatory, rubefacient and irritating qualities and not on account of a well-defined chemical or constitutional action. Its employment even as an aphrodisiac or as a diuretic must be ascribed to its irritating influence. Applied to a paralyzed limb or to a rheumatic joint, its beneficial influence is solely due to its power of exciting the circulation and setting up a somewhat enduring counter-irritation. Hypodermic injection of the dilute acid into limbs affected with rheumatic conditions as practiced by Couch[8] may effect beneficial results by reason of its antiseptic qualities if a septic state underlies the local manifestation. In the majority of instances, however, it is the epispastic property of this substance which brings about an amelioration of the painful condition.

My experience with formic acid as a therapeutic agent has been obtained by its internal administration mostly, or at least in conditions in which its rubefacient qualities seemed to play an unimportant or secondary rôle. Besides using this remedy in a variety of other affections, I have tested its therapeutic efficacy in a comparatively large number of cases of gout, acute articular rheumatism, muscular pain, cancer, diphtheria, intestinal toxicoses, syphilis and phthisis.

Gout.—Internally administered in doses of from two to ten drops, formic acid does not seem to reduce the gouty state. Employed hypodermically (not in the lesion itself) in from 0.5 to 1.5 c.c. of a 2 per cent. solution, it proved equally ineffective even when it was injected three times daily for from three to five consecutive days.

Acute Articular Rheumatism.—Administered alone internally, formic acid in my hands proved of no avail in ameliorating rheumatic fever. The same must be said of its hypodermic introduction when injected into non-affected parts for eventual constitutional effects. Combined with citric acid, however, as in the following formula, I have employed it with a certain degree of satisfaction, although its efficacy can not be compared with that of sodium salicylate:

℞. Acidi formici
Acidi citrici, āā ..... 5 parts
Glycerini ..... 40 parts
Tinct. cardamoni ..... 1 part
Aquæ ..... 49 parts
M. Sig.: Teaspoonful doses every two to four hours.

Muscular Pain.—Muscular pain of vague character which ceases when an ointment of salicylic acid and methyl salicylate or dry hot air is applied is not relieved by the internal continued administration of formic acid in the usual doses.

Cancer.—The internal administration of formic acid has certainly averted a quick fatal issue in a number of inoperable cases of cancer. In such cases I have usually given it in essence of pepsin which makes an excellent vehicle for its administration. A saturated formic-acid-iodin solution (about 0.5 gram iodin in 60 c.c. formic acid) has given similar results, either when administered by the mouth or hypodermically. Injected in and around the edges of the cancerous tumor (epithelioma, etc.), the iodin-formic-acid solution has given very gratifying results to some of my surgical colleagues in New York (Dr. Daniel Lewis, the late Dr. Thomas H. Manley). The advent of Roentgen-ray therapy by which similarly located cancerous growths are successfully treated prompted us to discontinue our experiments in this particular field.

Diphtheria.—Before the days of the diphtheria antitoxin I used nothing but formic acid, both locally and internally, in the treatment of diphtheritic affections. I do not remember having lost a single case of diphtheria so treated. Internally in this condition I usually gave formic acid in drop doses well diluted in water, with the occasional addition of some whisky. This dose was repeated every one, two or three hours according to the severity of the attack. When the disease was well under control the medication was continued three times a day for from four to eight days. Locally, that is to the faucial mucosa or to any other affected area of the mucous membrane, a 5 per cent. formic-acid-glycerin solution was at once applied and this was followed by swabbing with a 1 or 2 per cent. glycerin solution every few hours. To prevent the spreading of diphtheria in a family I employed locally the 1 per cent. glycerin solution, which was applied once daily to the nares and three times a day to the fauces of the healthy members.

Intestinal Toxicoses.—In toxicoses of supposedly intestinal origin I have made use of formic acid very frequently. I have already dwelt on this point.[9] Formic acid exerts a distinct antidotal value in certain forms of enterotoxicosis and even autotoxicosis. It may be taken before or after removal of the gastric and colonic contents in doses of from two to five drops, well diluted, every hour. Besides its pronounced antiseptic qualities, which it has no occasion to exhibit in genuine intrabiontic toxicosis, formic acid undoubtedly exerts a specific influence on the enteric secretion, a property which sodium formate and the other formates, ingested as such, do not seem to possess.

Syphilis.—In the secondary and tertiary stages of syphilis the iodin-formic acid solution, internally administered, has been well borne in most of the instances in which I employed it, and has been productive of splendid results in almost every case. It does not, as a rule, call forth the same degree of depression of the nervous system as potassium iodid, neither does it occasion muscular asthenia or lowered tone of the heart, which will invariably follow the prolonged administration of iodin or any of its therapeutically active salts.

The iodin-formic acid solution, which some of my chemical and medical colleagues have styled "Stern's solution," for the treatment of syphilitic manifestations is best prescribed in gradually increasing doses and in a menstruum like essence of pepsin. I had a patient who took as much as 3 c.c. of the solution three times a day. Such large doses, however, are hardly called for and may prove injurious to the gastrointestinal mucosa. Applied locally in syphilitic (and gonorrheal) rheumatism, so-called, some of the iodin, probably on account of the vesicatory action of the formic acid, penetrates into the deeper structures, frequently affording relief of the symptoms. The local application, undiluted, is rather a painful expedient and the diluted solution is of but little, if any, therapeutic value. Hypodermically administered, it will improve the symptoms in a comparatively short time. Still, for obvious reasons, I would

[illegible] Amer. Medical Record, June 24, 1905.

9. Medical Standard, September, 1904.

prefer the internal administration of this remedy. The solution is especially indicated in the treatment of the syphilitic manifestations of the nervous system, preeminently in those conditions which are the result of syphilitic nodes or gummata.

Pulmonary Phthisis.—Formic acid, and more particularly the iodin-formic acid solution, which must be prepared without heating, as the latter would cause decomposition of the formic acid, in my hands has proved the most valuable medicinal agent thus far advanced for the treatment and cure of—let me call it by the old names—chronic ulcerative and chronic fibrous phthisis. It would lead me too far to state on this occasion my experience with these medicinal agents in detail. A more thorough study of the subject will be published in a separate article in the near future. I only wish to state that I have employed it in the treatment of pulmonary phthisis ever since the monumental tuberculin failure, both in private and hospital practice, in more than 800 cases.

Of course, neither formic acid nor the iodin solution, which latter I have used exclusively in phthisis in the last ten years, is an infallible remedy in this affection. If this were the case I would have given to the world my experience with the drug long ago. I am sorry to say that the solution did not cure all of these 800 patients, neither do I wish to maintain at the present moment that it is even a rational remedial agent in the treatment of phthisis, but I wish to state that thus far it is the only drug which has given me positive results, that is, in improving or curing the condition in over 70 per cent. of all of my cases.

It is certainly a much more rational agent than creosote and its preparations and all other symptomatic makeshifts, including rest cure, open air and dietary treatment, devised to this day for the amelioration of this, the most common of all the chronic pathologic conditions.

DISCUSSION.

Dr. W. C. Abbott, Chicago, asked: Taking formic acid as a remedial agent, what does it do physiologically? Clinically, he said, the result has been demonstrated, but he asked how the result is obtained.

Dr. W. F. Waugh, Chicago, called attention to the fact that formic acid has been said to stimulate the growth of plants, so much so that it has been alleged that the Hindoo fakir who shows a plant growing before one's eyes employs an earth that contains formic acid. If this be true, Dr. Waugh said that it would be exceedingly interesting to know whether or not formic acid stimulates any of the tissues or organs of the body, or its cells, so as to increase their power of resisting noxious agents such as tubercle bacilli, or the possible germ of cancer.

Dr. H. Stern, New York, said that he had tried to give all the real pharmacodynamics of formic acid and that he did not succeed because formic acid is produced constantly in the human body, as it is one of the members of the fatty acid series found in every organism, healthy or diseased. When formic acid as such is taken by the mouth it combines to form sodium formate in the stomach, but it is again excreted as formic acid, provided it is taken in medicinal doses. Therefore, there is a double conversion, into sodium formate and then back into formic acid. Dr. Stern said he does not know how it acts. Whether or not it exerts a specific action on the tubercle he does not know, but in combination with iodin, he said, it has healing virtues and facilitates the production of fibrous tissue. In other words, in most of the patients who have taken the iodin preparation a chronic fibrosis has been produced.

A PRACTICAL METHOD OF ABOLISHING THE CAUSE OF ONE-QUARTER OF THE UNNECESSARY BLINDNESS IN THE UNITED STATES.

F. PARK LEWIS, M.D.

President New York State Commission for Improving the Condition of the Blind, 1903-1904.

BUFFALO, N. Y.

When an enlightened, civilized and progressive nation quietly and passively, year after year, permits a multitude of its people unnecessarily to become blind, and more especially when one-quarter of these are infants, the reason for such a startling condition of affairs demands explanation. That such is the fact practically all reliable ophthalmologists agree.

From a summary of carefully tabulated statistics it has been demonstrated that at least four-tenths of all existing blindness might have been avoided had proper preventative or curative measures been employed, while one-quarter of this, or one-tenth of the whole, is due to ophthalmia neonatorum, an infectious, preventable and almost absolutely curable disease. Perhaps this statement will take on a new meaning when it is added that there are in the State of New York alone more than six thousand and in the United States more than fifty thousand blind people; of these, six hundred in the one state and five thousand in the country would have been saved from lives of darkness and unhappiness, in having lost all the joys that come through sight, and of more or less complete dependence, for no individual can be as self-sufficient without as with eyes—if a simple, safe and easily applied precautionary measure had been taken at the right time and in the right way to prevent this affliction. The following three vital facts are not questioned, but are universally accepted by those qualified to know:

1. The ophthalmia of infancy is an infectious germ disease.

2. By the instillation of a silver salt in the eyes of a new-born infant the disease is prevented from developing in all but an exceedingly small number of the cases in which it would otherwise have appeared.

3. In practically all those few exceptional cases the disease is absolutely curable if like treatment is employed at a sufficiently early period.

Since these facts are no longer subjects of discussion, but are universally accepted by all educated medical men, the natural inquiry follows: Why, as a commonsense proposition, are not these simple, harmless, preventive measures invariably employed, and why, in consequence of this neglect, does a nation sit quietly and indifferently by, making no attempt to prevent this enormous and needless waste of human eyes?

The reasons are threefold and lie, first, with the medical profession; second, with the lay public; third, with the state.

The medical profession, great as have been its advances during recent years and strenuous as have been the efforts of its teachers and leaders to promulgate the fundamental importance of germs in disease, is by no means yet universally familiar with the facts concerning infantile ophthalmia, as to its prevalence, its dangers, its prevention and the measures that may be successfully instituted for its treatment. While the total number of cases is large, the disease may occur very rarely in the experience of any individual physician, even though he may have had an extensive general practice. When it does occur, unless the physician is fully informed, he

Theoretical and Applied Genetics 39, 330–334 (1969)

Formaldehyde-Induced Mutations in *Drosophila melanogaster* in Dependence of the Presence of Acids

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Summary. The mutagenic activity of various combinations of formaldehyde, formic acid, acetic acid and hydrochloric acid was investigated by a sex-linked lethal test. All combinations were mutagenic and showed a mutation pattern from which it is concluded that in feeding experiments spermatocytes I are especially sensitive to the pairs of chemicals tested. In vapour experiments all germ cell stages were found to be susceptible.

The presence of volatile acids was found to be necessary for the mutagenic activity of formaldehyde in the vapour state. Mutagenic effects were also observed in larval feeding experiments, in which only these acids were added to the medium. Experiments with stabilized pH at 7.5 did not show a significant mutagenic effect of formaldehyde.

It is postulated that the tested agents are catalase inhibitors, which promote the formation of peroxides or free radicals which interfere with DNA replication, thus producing mutations.

Introduction

The sensitivity of *Drosophila* germ cells to the mutagenic action of formaldehyde was described by RAPOPORT (1946) and confirmed by AUERBACH (1949, 1951, 1953, 1967), AUERBACH and MOSER (1953), KAPLAN (1948) and several other investigators (ALDERSON, 1960a, 1960b, 1961, 1964; KHISHIN, 1964; NAFEI and AUERBACH, 1964; SOBELS, 1956, 1958, 1962, 1963).

As briefly published earlier (STUMM-TEGETHOFF, 1964) we found that any mutagenic activity of formaldehyde depends on pH values below 7. Detailed experiments which include also the effect of impurities of commercial formaline are presented which support the earlier findings, and give information on the possible mechanisms of mutation induction.

Material and Methods

A standard strain of *Drosophila melanogaster*, Oregon-K (inbred for about 10 years) was used. This strain has a spontaneous mutation rate of 0.15% sex-linked lethals in males.

For *treatments with the vapour phase* of an agent, males were exposed for 24 hrs in specially designed bottles. The flies themselves were kept in small glass tubes (⌀: 2.5 cm), which were closed with gauze on the lower end and with cotton wool on the upper end (see figure 1). Samples of each of the agents to be tested (5 ml) were applied to the cotton wool in the bottom of the bottle (volume: 125 ml).

During *larval feeding experiments*, we used a nutrient medium consisting of 12 g of agar, 35 g of killed brewers yeast, 150 g of corn meal and 100 g of sucrose per 1000 ml distilled water. This medium was autoclaved, cooled, and supplemented with the chemicals to be tested. These were formaldehyde, formic acid, acetic acid and hydrochloric acid. The pH of the media varied from 4.2 to 5.6 depending on the added reagent. A 37% solution of formaldehyde of pH 7 was obtained by neutralization with calcium carbonate. To distinguish this formaldehyde from the commercial formaline (which is known to contain formic acid), it will be referred to as neutral "n"-formaldehyde, and the commercial formaldehyde as acid "a"-formaldehyde. Females were allowed to lay eggs for 2 to 43 hrs on the prepared medium, and were then discarded. Males, which spent their whole larval life on the medium, were collected 24 hrs after emergence (BRIDGES, 1952) and mated individually to two M-5 virgins.

To *test for sex-linked lethals* we employed the MULLER-5 technique (DEMEREC, 1948; SPENCER and STERN, 1948). About 30 treated males were individually crossed with one or two virgins of the M-5 strain (also inbred for about 10 years). Every third day the males were transferred to two fresh virgins in order to produce three successive broods.

All experiments were performed at a temperature of 24 °C (± 1 °C). 60–80% of the flies survive treatment with the tested agents.

In order to test the results statistically, we used the χ^2-test, the rank correlation method of KENDALL (1955), or a special test for mutation frequency of STEVENS (1942).

1. *Vapour Experiments*

The data obtained from vapour experiments are summarized in table 1. When males were treated 24 hrs after emergence with "a"-formaldehyde, formic acid, acetic acid, hydrochloric acid, or the combination of "n"-formaldehyde and the acids, all experiments produced mutations. The data were found to be significantly different ($p \leq 0.001$) from the spontaneous mutation rate of 0.15%.

For each chemical tested, no significant deviation in mutation frequency for sex-linked lethals could be found between the results obtained with each of the three broods (STEVENS, 1942). This result allows us to consider the mutation frequencies for the total of the three broods. If we compare these data, we find significant differences ($p \leq 0.05$) when comparing "a"-formaldehyde with acetic acid; "a"-formaldehyde with hydrochloric acid; "a"-formaldehyde with "n"-formaldehyde + formic acid; and "a"-formaldehyde with "n"-formaldehyde + hydrochloric

Table 1. *Vapour experiments*

treatment		brood							
substance	concentration	1st		2nd		3rd		total	
	%	n[1]	%[2]	n	%	n	%	n	%
nF	7.0	—	—	—	—	—	—	3803	0.16
aF	7.0	436	0.69	712	1.97	423	1.18	1571	1.49
FA	0.1	1068	1.12	1130	1.42	850	1.41	3048	1.31
AA	0.1	1703	0.82	1359	0.66	1279	1.10	4332	0.85
HA	0.01	1047	0.67	977	0.61	396	0.50	2420	0.62
nF + FA	7.0 0.1	1491	0.80	2198	0.82	1809	0.99	5498	0.87
nF + AA	7.0 0.1	786	1.02	1004	1.29	1020	1.18	2810	1.17
nF + HA	7.0 0.01	1263	1.03	1466	0.68	852	0.70	3680	0.79
mutation control	—	—	—	—	—	—	—	2584	0.15

[1] number of chromosomes tested. — [2] percentage of sex-linked lethals found.

abbreviations:

aF = acid formaldehyde
nF = neutral formaldehyde
FA = formic acid
AA = acetic acid
HA = hydrochloric acid.

All mutation frequencies are significant at the $p < 0.001$ level when compared with the non-treated control.

ric acid. However, no significant differences were found in the comparison of "a"-formaldehyde with "n"-formaldehyde + acetic acid; "a"-formaldehyde with formic acid; and acetic acid with hydrochloric acid. It may be concluded that all germ cell stages can be influenced by "a"-formaldehyde and all the tested acids. It has been shown already in earlier investigations (STUMM-TEGETHOFF, 1964) that "n"-formaldehyde causes no mutation in vapour experiments.

2. *Larval Feeding Experiments*

It could be argued that the acid vapour treatment may interfere with the normal ventilation of the tracheal system. Therefore experiments were carried out with the mutagenic substances in the feeding substrate. As seen from table 2 all tested substances have a significant increased mutagenic effect compared with the control.

In contrast to the results of the vapour experiments the highest mutation rate of sex-linked lethals in most of the larval feeding experiments were found in the second brood. The significance of these results was determined by the rank correlation method. With 0.05% "n"-formaldehyde and 0.05% "n"-formaldehyde + formic acid, however, the highest frequency of mutation was found in the first brood. Within individual experiments there were no significant differences between the broods, as shown by the test of STEVENS (1913), except in the cases of 0.05% "n"-formaldehyde and 0.05% "n"-formaldehyde + formic acid. Therefore, totals were compared as in the vapour experiments, revealing no

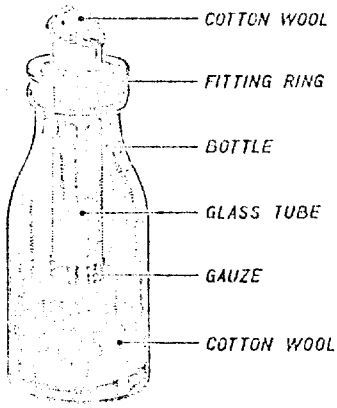

Fig. 1. Bottle for vapour experiments with *Drosophila*

differences between the tested agents except for acetic acid and hydrochloric acid, which were less effective ($p < 0.05$).

The finding that "n"-formaldehyde is as mutagenic as "a"-formaldehyde for larvae, contrasts with the results of the vapour experiments. The pH of "n"-formaldehyde is 7. An explanation could be given by the following experiments.

When hatched larvae were transferred every day to fresh nutrient medium which was adjusted to pH 7.5 with glycine-NaOH buffer (table 3), no mutation was found in the second brood for 0.025% formaldehyde, and one mutation (0.27%) was found for 0.05% formaldehyde.

In another experiment with 0.1% formic acid in the food, but stabilized on pH 7.5 a mutation rate

Table 2. *Larval feeding experiments A*

treatment		brood							
		1st		2nd		3rd		total	
substance	concentration %	n[1]	%[2]	n	%	n	%	n	%
nF	0.025	910	0.77	717	1.40	737	0.68	2364	0.93
nF	0.05	1266	1.26	1503	0.33	1601	0.31	4370	0.59
aF	0.025	1287	0.70	1213	0.91	1089	0.73	3589	0.78
aF	0.05	754	1.06	802	1.75	901	0.78	2457	1.18
FA	0.1	786	1.15	522	1.34	571	0.88	1879	1.11
AA	0.1	981	0.61	1007	0.60	1157	0.43	3145	0.54
HA	0.01	757	0.40	668	0.75	800	0.50	2225	0.55
nF + FA	0.025 0.1	839	0.95	857	1.17	587	1.19	2283	1.09
nF + FA	0.05 0.1	981	1.33	1352	0.96	1574	0.76	3907	0.97
nF + AA	0.025 0.1	716	0.98	782	1.28	652	0.77	2150	1.02
nF + AA	0.05 0.1	770	0.91	840	1.79	1092	1.10	2702	1.25
nF + HA	0.025 0.01	478	1.05	594	1.18	548	0.91	1620	1.05
nF + HA	0.05 0.01	446	2.02	514	1.95	447	1.12	1407	1.71
Mutation control	—	—	—	—	—	—	—	2584	0.15

[1] number of chromosomes tested. — [2] percentage of sex-linked lethals found.

Abbreviations:
aF = acid formaldehyde
nF = neutral formaldehyde
FA = formic acid
AA = acetic acid
HA = hydrochloric acid.

All mutation frequencies are significant at the $p < 0.001$ level when compared with the non-treated control.

Table 3. *Larval feeding experiments B*

substance	concentration	pH of the medium	tested chromosomes	mutations found	mutation frequency
nF	0.025%	7.5	398	0	0.00%
nF	0.05%	7.5	366	1	0.27%
FA	0.1%	7.5	544	2	0.38%
Mutation control	—	7.0	2584	4	0.15%

For these experiments a special prepared medium of pH > 7 was used (see "Materials and Methods"). Only the 2nd brood was analyzed. Concentration of the glycine-NaOH buffer: 0.1 M. The mutation frequency shows no significant deviation from the (untreated) control (0.15%) in contrast to the results obtained by testing the same substances in an acid medium.

of 0.38% was observed in the second brood. These data are not significantly different from the spontaneous mutation frequency.

Comparing the results of the feeding experiments one can conclude that there is a positive correlation between the low pH of the nutrient medium and the mutation frequency over all broods.

Discussion

AUERBACH (1951), KAUFMANN and GAY (1963) and KHISHIN (1964) proved that during larval development spermatogenesis stops with spermatocytes I. Therefore, only this type of germ cells (plus spermatogonia) is capable of reacting with mutagenic agents present in the larval food. These findings are in agreement with our results from larval feeding experiments. No clusters of identical lethals from the same male have been found. Therefore, with great probability, only spermatocytes I are influenced (AUERBACH, 1953; SOBELS, 1954). In comparison with the controls, all broods showed significant higher mutation frequency.

The highest mutation rate occurred in the larval feeding experiments (table 2) in the second brood, which is not in accordance with the data from AUERBACH and MOSER (1953), who found the highest

mutation frequency in the first brood. This is perhaps due to a difference in sensitivity of our standard strain and to the fact that our larvae remained their whole larval lifetime on the treated medium (older larvae incorporate more of the substances than younger ones). All these explanations assume that the males mate only once with the M-5 females within one brood, as suggested by MANNING (1962).

The fact that AUERBACH (1949) did not succeed in mutation induction with formaldehyde vapour is possibly due to a higher concentration employed, which did not allow a longer time of treatment than 2 hrs.

During the larval feeding experiments, it was observed that addition of neutral formaldehyde to the nutrient medium caused a pH decrease, thus producing a mutagenic effect similar to that obtained with "a"-formaldehyde. We suggested earlier (STUMM-TEGETHOFF, 1964) that a part of the formaldehyde is oxidized to formic acid, which is also mutagenic.

In the larval feeding experiments, and in some of the vapour experiments, the most sensitive germ cell stages are the spermatocytes I. Probably, DNA replication in these cells is disturbed by the agents (NATTI and AUERBACH, 1964).

SOBELS (1954, 1958, 1963) suggested that the formation of peroxides was the cause of mutations, a result which is confirmed by other investigators for lower organisms (DICKEY, CLELAND and LOIZ, 1949; JENSEN, KIRK, KOLMARK and WESTERGAARD, 1951). Normally, *Drosophila* larvae contain catalase of high activity. Inhibition of catalase by acids or acidic formaldehyde solutions must lead to an accumulation of mutagenic peroxides. If acids and combinations of formaldehyde with these acids are used in vitro (STUMM-TEGETHOFF and VAN DER LOO, 1969), the resulting catalase inhibition shows a good correlation to the mean mutation frequency obtained in vivo. These findings suggest that, as a consequence of catalase inhibition, peroxides and free radicals are responsible for the mutation. In this regard, BUTLER and CONWAY (1953) proved that OH-radicals are able to break the connection between sugar and phosphate or sugar and base in DNA, and that they are able to oxidize the sugar or the base of nucleosides. In addition, FREESE (1961) found in T-phages that the formation of transversions or transitions by depurinating agents is dependent on a low pH, and can be imitated by a buffer of the same low pH. There may be some relationship between these reports and our results. The absence of a mutagenic effect in the experiments with a buffered medium of pH 7.5 can also be due to the formation of a glycine-formaldehyde complex.

With regard to the larval feeding experiments and the results obtained from the 3rd brood of the vapour experiments, formaldehyde and/or acids may inhibit catalase, as a consequence peroxides and free radicals accumulate; by this process DNA synthesis is disturbed, which leads to mutation. Mutations occurring in the 1st and 2nd broods of the vapour experiments may occur as a consequence of a disturbance of chromosome segregation during meiosis, thus producing repeats and deletions. Other theories of the mutagenic activity of formaldehyde are, however, also compatible with our findings (ALDERSON, 1960a, 1960b, 1961, 1964, 1967).

Acknowledgements

I am indebted to Miss L. VAN SCHELTINGA for her skilled assistance during the course of the experiments. Thanks are also due to Prof. Dr. S. J. GLERTS for critically reading the manuscript and to Prof. Dr. A. SCHWARTZ for checking the English text.

Zusammenfassung

Mit Hilfe des Muller-5-Tests wird bei *Drosophila melanogaster* die mutagene Aktivität von Formaldehyd, Ameisensäure, Essigsäure und Salzsäure allein und in verschiedenen Kombinationen von Formaldehyd mit den Säuren geprüft. Alle Kombinationen sind mutagen. Da keine Mutagenität mehr nachweisbar ist, wenn die Fliegen mit Formaldehyd mit einem pH $\geqq$ 7 behandelt werden, ist anzunehmen, daß die mutagene Wirkung von Formaldehyd von einem sauren Milieu abhängig ist. Die untersuchten Substanzen werden den Tieren entweder in der Gasphase oder durch Beimischung zum Nährboden verabreicht. Im ersten Fall werden alle Keimzellstadien beeinflußt, während die Applikation im Nährboden nur die Spermatocyten I angreift. Der Wirkungsmechanismus der untersuchten Stoffe wird als eine Katalase-Hemmung gedeutet, die zur Bildung von u. a. freien Radikalen und damit — über Störung der DNS-Synthese — zum Entstehen von Mutationen führt.

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Received August 1969

Communicated by H. F. LINSKENS

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Hydrogen Phosphide and Ethyl Formate: Fumigation of Insects Infesting Dates and Other Dried Fruits[1]

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ABSTRACT

The relative toxicity of hydrogen phosphide was determined for the life stages of *Carpophilus mutilatus* Erichson; the driedfruit beetle, *C. hemipterus* (L.); the pineapple beetle, *Urophorus humeralis* (F.); the yellowish nitidulid, *Haptoncus luteolus* (Erichson); the sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.); the Indian meal moth, *Plodia interpunctella* (Hübner); and the raisin moth, *Cadra figulilella* (Gregson). The relative toxicity of ethyl formate was determined for the life stages of *C. hemipterus*, *O. surinamensis*, *C. figulilella*, and *P. interpunctella*. In general, with hydrogen phosphide the egg stage of each species was the most difficult to kill, followed by the pupal stage, while the larval and adult stages were the most susceptible. The pupal stage was the most resistant to ethyl formate fumigation, whereas the adult stage was the most susceptible. With the exception of the driedfruit beetle, the egg stage was more susceptible than the larval stage to ethyl formate fumigation.

Dates may be infested with 4 species of nitidulid beetles, namely *Carpophilus mutilatus* Erichson; the driedfruit beetle, *C. hemipterus* (L.); the pineapple beetle, *Urophorus humeralis* (F.); and the yellowish nitidulid, *Haptoncus luteolus* (Erichson). Normally these nitidulid beetles do not continue to breed under packing-house conditions as they usually require soil in which to pupate. The sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.); the Indian meal moth, *Plodia interpunctella* (Hübner); and the raisin moth, *Cadra figulilella* (Gregson) also infest dates, but they differ from the nitidulid beetles in that they continue breeding in the packing house and in the packaged product. The nitidulid beetles are usually associated with dates of high moisture content and spoilage, whereas the other 3 insects can develop also in unspoiled fruit of low moisture content. Under conditions where harvesting is delayed until the entire bunch is removed, the dried dates are vulnerable to attack in the field by the 2 moth species and the sawtoothed grain beetle.

To prevent entry of these insects into the packing house, fumigation of all incoming fruit is essential. Methyl bromide is the fumigant currently used on processed dates. In seeking more effective fumigants laboratory experiments were conducted to determine the effectiveness of hydrogen phosphide and ethyl formate against this group of insects.

Hydrogen phosphide (HP) has been used as a fumigant in the United States for control of stored-grain insects since its introduction approximately 7 years ago (Lindgren et al. 1958, Lindgren and Vincent 1966, Vincent and Lindgren 1971). The commercial compound Phostoxin[2] is manufactured as a highly compressed tablet or pellet composed of aluminum phosphide and ammonium carbamate, which on exposure to moisture decomposes to hydrogen phosphide, aluminum hydroxide, ammonia, and carbon dioxide. The rate of decomposition is dependent on the relative humidity and temperature of the air. According to the manufacturer, tablets require approximately 48–72 hr and pellets 12–48 hr for complete decomposition. A tablet weighs 3 g and a pellet 0.6 g, of which approximately 32% by weight is evolved as hydrogen phosphide.

Ethyl formate ($HCO_2C_2H_5$) is a colorless liquid having a boiling point of 54.5°C, thus it is a fumigant that can be handled as a liquid at ordinary temperatures. Ethyl formate was tested by Neifert et al. (1925), Cotton and Roark (1929), Roark and Cotton (1929), Simmons et al. (1935), Shepard et al. (1937), and Simmons and Fisher (1945). Mayer and Nelson (1955) found that ethyl formate was not effective for fumigating beans and peas in cellophane bags under practical operating conditions.

Materials and Methods.—Stock cultures of the 4 species of nitidulid beetles were maintained in gallon jars on untreated dates at 26.7°C and 70% RH. Moist sand was placed in each jar to a depth of 5 cm to provide pupation sites. Dates were placed on the surface of the sand, and adults of the respective species were introduced.

Eggs of the nitidulid beetles were obtained by exposing to adults 2 pieces of plastic 2.5×7.6 cm with a piece of moist blotting paper in between. A crevice between the blotting paper and plastic was obtained by placing a small piece of filing card 0.6×2.5 cm between the blotting paper and plastic, the entire unit being held together with a rubber band. This crevice allowed the insect to insert its ovipositor between the blotting paper and plastic and deposit its eggs (Fig. 1). Nitidulid larvae, pupae, and adults were obtained by starting a culture from known-age eggs. These were reared on a paste consisting of ground dates and active yeast which was allowed to ferment for several days prior to use.

The sawtoothed grain beetle was reared on a medium consisting of rolled oats, raisins, and walnut meats. A procedure similar to that for the nitidulid beetles was used to obtain eggs. Larvae, pupae, and adults were obtained from known-age cultures.

The 2 moth species were reared on a medium consisting of bran, honey, glycerin, yeast, and wheat germ. Eggs were obtained by confining approximately 100 adults within a pint jar containing a small amount of food medium, with a 20-mesh screen cover, the whole being inverted over a petri dish. Any eggs laid by the moths dropped through the screen into the petri dish and were collected daily.

All fumigations were conducted in 1-liter glass flasks at 26.7°C. The dosage of hydrogen phosphide applied to these flasks was measured as a gas with a glass syringe from a supply obtained by the decom-

[1] Received for publication Feb. 28, 1972.

[2] Phostoxin is manufactured by Degesch-Deutsche Gesellschaft für Ungeziefervertilgung, Germany. American supplier: Hollywood Chemic Control Co.

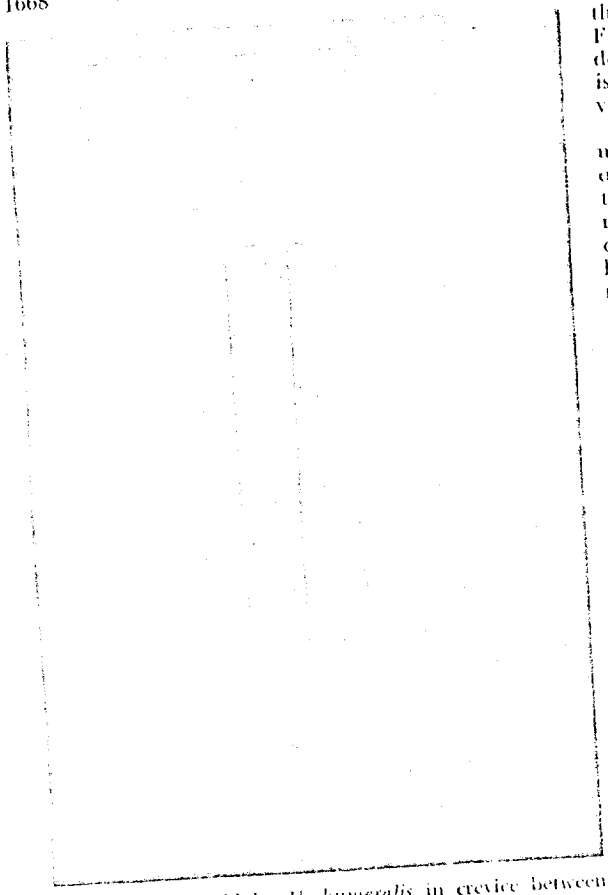

FIG. 1.—Eggs laid by *U. humeralis* in crevice between plastic and blotting paper.

position of a Phostoxin tablet in a 20-liter glass bottle. The concentration of hydrogen phosphide in the 20-liter supply bottle was determined by gas chromatography, and the dosages desired for the 1-liter flasks were calculated from this concentration. By this method the test insects were exposed to a constant concentration of hydrogen phosphide for the duration of the 24-hr exposure period. There was very little or no sorption of hydrogen phosphide by the glass fumigation chamber.

Ethyl formate was measured as a liquid, using a microsyringe and ejected onto a small piece of filter paper within the fumigation flask to facilitate evaporation. There was very little or no sorption by the glass fumigation chamber during the 6-hr exposure period as determined by gas chromatography.

To prevent desiccation of the test insects during the exposure period a small piece of moist blotting paper was placed in each fumigation chamber prior to introduction of the fumigant. Following fumigation the test insects were held at 26.7°C and 70% RH. Mortality counts of adults and larvae were made 7 days following fumigation; eggs were observed until no further hatching occurred, and the pupal counts were based on the number of adults emerging.

Fifty or more eggs of each species were used for each test. Twenty-five or more larvae, pupae, or adults were used in each test with the exception of the 2 moth species in which 10 adults were u[illegible] Five or more points were obtained to establi[illegible] dosage-mortality curve, and each point on the c[illegible] is the average of at least 5 replications. The [illegible] values were estimated from eye fitted lines.

RESULTS.—*Hydrogen Phosphide.*—Table 1 giv[illegible] ng per liter of hydrogen phosphide require[illegible] obtain 95% mortality of the life stages of the i[illegible] tested. All stages of the Indian meal moth [illegible] more difficult to kill than comparable stages o[illegible] other species tested. In rating the species as t[illegible] highest concentration required to kill 95% o[illegible] most tolerant stage the order is: *P. interpunc[illegible]* *C. mutilatus*>*C. pusillella*>*H. luteolus*>*O. s[illegible]* *mensis*>*C. hemipterus*=*U. humeralis*. The egg [illegible] with the exception of *O. surinamensis* and *C. h[illegible]* *terus* (egg=pupa), was the most tolerant to [illegible] gen phosphide. The adults, in general were th[illegible] susceptible stage to hydrogen phosphide tanki[illegible] with the exception of *O. surinamensis* and *C.* [illegible] *ella*. In most cases the larvae were more su[illegible] to hydrogen phosphide than the pupae.

Ethyl Formate.—In ranking susceptibility o[illegible] test insects to ethyl formate fumigation (Tab[illegible] *C. hemipterus* was the most resistant followed [illegible] sawtoothed grain beetle, while the Indian meal [illegible] and the raisin moth were the most susceptible. [illegible] adults, in general, were the most susceptible sta[illegible] ethyl formate fumigation, and the pupal stag[illegible] the most tolerant. There was very little dif[illegible] in susceptibility of the egg and adult stages o[illegible] sawtoothed grain beetle. With the exception of [illegible] dried fruit beetle, the egg stage was more susc[illegible] than the larval stage to ethyl formate fumigati[illegible]

In making comparisons between the 2 fumig[illegible] will be noted that while *C. hemipterus* is the [illegible] tolerant to ethyl formate it is one of the mo[illegible] ceptible species to hydrogen phosphide, whi[illegible] *interpunctella* is the most tolerant species to hy[illegible] gen phosphide, but it is one of the most susceptib[illegible] to ethyl formate.

Where comparisons can be made, the pupae of *surinamensis* were the most resistant stage to [illegible] fumigants, while with the other species, the egg s[illegible] with the exception of *C. hemipterus* (egg=p[illegible] was the most resistant stage to hydrogen phosp[illegible]

Table 1.—Results of fumigation tests at 26.7°C [illegible] hydrogen phosphide and ethyl formate on various s[illegible] of several stored product insects.

Insect	LC_{95} (mg/liter)			
	Egg	Larva	Pupa	Ad[illegible]
Hydrogen phosphide, 24-hr exposure				
C. hemipterus	0.031	0.008	0.032	0.0[illegible]
C. mutilatus	.199	.020	.029	[illegible]
U. humeralis	.031	.014	.018	[illegible]
H. luteolus	.125	.007	.006	[illegible]
O. surinamensis	.038	.017	.049	[illegible]
C. pusillella	.110	.018	.087	[illegible]
P. interpunctella	1.960	.060	.320	[illegible]
Ethyl formate, 6-hr exposure				
C. hemipterus	48.0	34.0	72.0	[illegible]
O. surinamensis	22.3	33.0	48.5	[illegible]
C. pusillella	9.0	23.2	29.0	[illegible]
P. interpunctella	9.0	26.5	36.0	[illegible]

and pupae were the most resistant to ethyl formate. These results indicate that the tolerance of the stages varies with the fumigant being tested.

Although the LC_{50} for ethyl formate was obtained with a 6-hr exposure and that for hydrogen phosphide with a 24-hr exposure, a comparison of the toxicity between the 2 fumigants can be made by using the concentration × time product ($ct = K$). The product K, obtained from results in Table 1, indicates that hydrogen phosphide is more toxic than ethyl formate to the stages of the insects tested.

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The Aliphatic Acids and Their Esters—Toxicity and Potential Dangers

The Saturated Monobasic Aliphatic Acids and Their Esters

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Formic Acid and Esters: Formic Acid

Chemistry.—Formic acid, methanoic acid, HCOOH, is a colorless liquid of pungent odor, of the molecular weight 46.03 and of the specific gravity 1.220 at 20/4 C. It solidifies at 0 C, melts at 8.40 C, and boils at 100.8 C. It is miscible with water, alcohol, ether and glycerol.* The salts of formic acid are generally well soluble in water, except lead formate which is soluble with difficulty, and calcium formate which is soluble to the extent of 14.66 gm. in 100 gm. of water at 40 C (Seidell, 1919).

With an excess of lead acetate, formic acid forms a double salt of lead acetate and lead formate which is easily soluble in water but insoluble in alcohol. Zinc formate is also insoluble in alcohol (Rosenthaler, 1923).

Formic acid is easily oxidized to carbon dioxide and water, and when heated with sulfuric acid it is decomposed with the formation of carbon monoxide.

It reduces silver nitrate to silver, mercuric chloride to mercurous chloride, and gold chloride and platinum chloride to gold and platinum. Nascent hydrogen reduces fromic acid to formaldehyde.

As the aldehyde of carbonic acid, formic acid yields colored compounds with various phenols in sulfuric acid solution. Thus phenol gives a yellow-pink color, resorcinol a golden-yellow, o-cresol a pinkish-yellow, and pyrogallol a brownish-yellow color (Fenton and Barr, 1917; Krauss and Tampke, 1921; Ekkert, 1933).

Formic acid gives with ferric chloride a blue-red color, and, when heated, a precipitate of basic iron salt (Smith, 1921).

Received for publication June 15, 1959.

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*Unless otherwise stated data on the physical-chemical properties of the various chemicals are quoted from Lange's *Handbook of Chemistry*, Ed. 9, 1956, or *The Merck Index*, 1952.

Occurrence and Uses.—Formic acid is used as decalcifier, reducing agent in dying wool-fast colors, in dehairing and plumping of hides, in tanning, for sizes, in electroplating, for coagulation of rubber latex, and as aid in regeneration of old rubber. It occurs in many plant and animal poisons and is partly responsible for their irritant effect. Small quantities of formic acid are normally excreted with the human urine, as demonstrated by Gréhant and Quinquaud (1887), Dakin and Wakeman (1911), Dakin, Janney, and Wakeman (1913), Strisower (1913), and Kapeller-Adler and Lauda (1933).

Determination in Air.—Flury and Zernik (1931) suggested that formic acid be determined by passing a measured volume of the contaminated air through an absorber containing 0.1 N sodium hydroxide solution and back-titrating the alkali. According to Snell and Snell (1937) the formate content of such solutions can be determined colorimetrically with fuchsin reagent after reduction of formic acid to formaldehyde by means of mercuric chloride, which is reduced to mercurous chloride (calomel) by formic acid.

Determination of Formic Acid in Biological Material.—Gréhant and Quinquaud (1887) determined formic acid in urine by distilling the acidulated urine in vacuo and neutralizing the distillate. After evaporation of the latter to a small volume, the formate is decomposed with sulfuric acid with the formation of carbon monoxide, the amount of which is then determined. Pirie (1946) steam-distilled the acidulated urine into a solution of sodium hydroxide, oxidized the

formate to carbon dioxide, and determined the latter manometrically in a van Slyke apparatus. Kapeller-Adler and Lauda (1933) separated formic acid by vacuum distillation, oxidized the formate with potassium permanganate, destroyed the excess of the latter with a measured amount of oxalic acid, and back-titrated the excess of the latter with N/10 potassium permanganate solution. Dakin, Janney and Wakeman (1913) extracted the acidulated urine with ether, neutralized the extracted acid with alkali, and, after acidification of the latter with phosphoric acid, separated the formic acid by steam-distillation. They determined the formic acid by oxidation with mercuric chloride, and measured the amount of calomel formed gravimetrically. Modifications of this procedure were used by Riesser (1896), Autenrieth (1919), Benedict and Harrop (1922), Utkin-Ljubozoff (1923), and Bastrup (1947, *a*). The two latter determined the amount of mercurous chloride formed iodometrically.

For the determination of formic acid in blood, De Eds (1924) hemolyzed the blood sample with distilled water, precipitated the proteins with trinitrophenol, and determined the formic acid in the filtrate by means of mercuric chloride, determining the amount of mercurous chloride formed iodometrically. Droller (1932) reduced the protein- and sugar-free blood filtrate with metallic magnesium, and determined the amount of formaldehyde formed colorimetrically with acid fuchsin. A similar procedure was advocated by Ballotta (1936), who identified the formaldehyde by the violet color formed upon addition of a solution of morphine in concentrated sulfuric acid.

Antiseptic Properties.—As shown by Schulz (1885), formic acid has antiseptic properties superior to other fatty acids. According to Lebbin (1906) concentrations of 0.10% to 0.15% are sufficient for the preservation of food stuffs. Fleig (1907) stated that concentrations of 60 mg. per liter prevent the growth of Bacillus pyocyaneous and Bacillus anthracis and that the organism of chicken cholera is very sensitive to a trace. As shown by Croner and Seligmann (1907), concentrations of 0.1% do not interfere with the fermentation of yeast, but concentrations of 0.2% prevent both growth and fermentation. In the opinion of Bach (1932) formic acid is a potent antiseptic having sterilizing properties even in concentrations of 1:50,000 to 1:100,000, and in some instances even in concentrations of 1:1,000,000, the antiseptic action being due to the un-ionized molecule. Lebbin (1906) and Weingarten (1932) saw no objections to the use of formates for the preservation of foods, but they are not used for this purpose.

Irritant Action.—Formic acid causes definite irritation. Its application to the shaved skin of rabbits has resulted in the destruction of the cutis and in corrosion (Schulz, 1883). According to Oettel (1936) its contact with the human skin causes moderate pungent pain, some erythema, and hyperemia; later the skin assumes a cooked appearance, and after 24 hours parchment-like necroses with a slow tendency to heal. As indicated by the experiments of Mayer (1886), Fleig (1907), and others with various experimental animals, the oral administration of formic acid, as well as of formate, causes irritation of the gastrointestinal tract resulting in vomiting and diarrhea. Croner and Seligmann (1907) found that in rabbits oral doses of 2 cc. of 5.7% and 10% solutions of formic acid cause irritation and corrosion of the gastric walls.

Absorption, Fate and Excretion.—The absorption of formic acid may take place from the gastrointestinal tract, through the lungs, and as shown by Schulz (1883), through the intact skin, and also from the urinary bladder (Lund, 1948, *b*).

As to the fate of formic acid in the organism, it appears that it is partly oxidized in the organism and partly excreted unchanged with the urine. The amounts isolated from dogs' urine after oral doses of 20, 5, and 1 gm., respectively, have been given by Schotten (1883) as 26%, by Gréhant and Quinquaud (1887) as 65%

and by Eppinger (1913) as 8% to 9%. The latter stated that in rabbits the urinary excretion was 14.2% to 19.3% after doses of 1 gm. per kilogram. Similarly Bastrup (1947, *b*) and Lund (1948, *a*) found that rabbits may oxidize large amounts of formic acid. With intravenous administration of 4 gm. of sodium formate in 20 cc. of water to dogs, Gréhant and Quinquaud (1887) found that 55% of the sodium formate is excreted unchanged within the first 48 hours. Pohl (1893) found that in dogs small doses (0.143 gm. per kilogram) are completely oxidized, whereas larger doses are partly excreted unchanged. In man Autenrieth (1919) found that, of 20 gm. of sodium formate taken over a period of two days, 18% was excreted undecomposed, and, according to Weingarten (1932), 25% to 50% was excreted unchanged after intravenous injection of 4 gm. of sodium formate in 20% solution. It appears, therefore, obvious that the amount of unchanged formate excreted with the urine will be influenced by other factors than the dose, such as the amount of food and fecal material in the intestinal tract at the time of the oral administration, the concentration in which the formate is given, and, in the case of intravenous administration, with the rate of the injection, as pointed out by Fleig (1907). In addition, it appears that some formic acid excreted with the urine may escape the determination because of reabsorption from the urinary bladder, as demonstrated by Lund (1948, *b*).

As to the site of oxidation of formic acid, Battelli (1904) showed that liver extract oxidizes formate to carbon dioxide and water in the presence of hydrogen peroxide, and Fleig (1907) demonstrated that extracts of intestinal mucosa, spleen, kidney, lungs, and blood oxidize a certain amount of formate under similar conditions. The same author points out that Popoff (1875) and Hoppe-Seyler (1876) had shown that certain bacteria may destroy formic acid with the formation of carbon dioxide and water. It appears, therefore, that with oral administration part of the formic acid administered may be destroyed in the gastrointestinal tract.

The Effect on the Nervous System.—Mayer (1886) reported that in rabbits the intravenous injections of 0.46, 0.61, 0.82, and 1.25 gm. per kilogram of formic acid, given as 8% and 10% neutralized solution, caused distinct depression of the central nervous system, and that larger doses caused convulsions, some animals being more resistant than others. In dogs the subcutaneous injection of 0.8 gm. per kilogram of sodium formate caused staggering gait and weakness lasting 24 hours. The subcutaneous injection of 0.4 gm. per kilogram caused in cats somnolence, and larger doses staggering, and finally death in about three hours. Similar results in rabbits were reported by Croner and Seligmann (1907). Leo (1925) noted in mice paralyses, slowed respiration, dyspnea, and death after rectal administration of 1 cc. of a 2.5% solution of formic acid, corresponding doses of acetic acid having no effect. He found that the administration of formates had qualitatively the same effect, the toxicological picture being, however, more protracted and lasting several days. These animals showed a slow dyspneic respiration, disappearance of the auditory reflexes without involvement of the spinal reflexes, and coma. Following intravenous fatal doses, Fleig (1907) observed in dogs and rabbits clonic and less often tonic convulsions, progressive dyspnea, and paralysis of the medulla. The stimulation of the respiration after intravenous administration of formic acid is of acidotic nature.

Effect on the Circulation.—With regard to the effect of formic acid on the heart, Livierato (1900) found that the administration of formic acid causes injury of the myocardium, the intensity of which increases with the dose. This is characterized by a moderate primary increase and subsequent progressive decrease of the systole, slowing of the heart beat, irregularities of the contraction, arrhythmias, and diastolic

arrest. He found that with adequate doses formates produce the same effect as formic acid. Sammartino (1933, *a*) studied the effect on the isolated frog heart, and found that concentrations of 1:1,000,000 cause a slight increase of the amplitude and the heart beat. With concentrations of 1:500,000 this stimulant effect is less marked, and with 1:100,000 there is some slowing and some decrease in amplitude. With ten times this concentration these effects become more marked; the arrest occurs promptly, and is irreversible. He considered formic acid seven times more toxic than formaldehyde, and 187 times more toxic than methanol in this respect.

With regard to the effect of formic acid on the blood vessels, Fleig (1907) found that the intravenous injection of formic acid causes immediate vasoconstriction, whereas sodium formate causes vasodilatation in liver, brain, and kidneys, paralleled by a vasoconstriction in the limbs. Sammartino (1933, *b*) studied the vascular effects of formic acid in the hind leg of the frog using the Läwen-Trendlenburg technique and found that concentrations of 1:1,000,000 caused slight vasodilatation, 1:500,000 vasoconstriction and 1:1,000 complete blocking of the circulation.

As pointed out by Fleig (1907), the intravenous injection of formic acid is followed by a rise of the blood pressure during the period of vascular constriction, but larger doses have a hypotensive effect.

As to the effect of formic acid on the blood, Fleig (1907) showed that in sufficient concentrations both the free acid and its salts delay the clotting of blood, presumably because of formation of calcium formate; and that formates cause formation of methemoglobin. This was also noted by Croner and Seligmann (1907) in rabbits after the repeated injection of 0.5 cc. of a 2% solution of formic acid.

Effect on the Digestive and Secretory Organs.—According to Fleig (1907), formic acid stimulates the gastric secretion and the peristaltic activity of the intestine, and favors the absorption of peptone from the gut. As shown by Schulz (1883) and Lebbin (1906), the administration of single large doses, or the repeated administration of small doses of formates, will cause injury of the kidneys. According to Eppinger (1913), rabbits appear to be more sensitive than other animals, and in this species the diet influences considerably the toxic action in that animals fed exclusively oats are more susceptible than those fed green food. The question whether the diuretic action is due to vasodilatation as assumed by Fleig (1907), or due to osmotic changes, appears to be open to discussion.

Toxicity for Laboratory Animals.—Fleig (1907) showed that oral doses of 4 gm. per kilogram of formic acid are fatal to dogs and slightly larger doses fatal to rabbits; the symptoms being repeated vomiting in dogs, and in dogs and rabbits clonic convulsions and progressive dyspnea, death being due to respiratory paralysis. The same author found that with slow intravenous injection 3 gm. per kilogram are fatal to dogs, whereas Sammartino (1933) gave the fatal dose as 0.239 gm. per kilogram for rabbits. The difference between these findings is presumably due to differences in the rate of injection and the concentration used, and, to a lesser degree, to differences in susceptibility between the two species. The chronic toxicity of formic acid was studied by Sollmann (1921), who found that concentrations of 0.01% to 0.25% in drinking water, corresponding to 0.2 cc. per kilogram of free acid, had no toxic effect on growing rats over a period of two to four months, whereas with 0.5% solutions, corresponding to 0.36 cc. per kilogram of free acid daily, appetite and growth were materially, immediately, and progressively diminished.

According to Harnack (1912), the toxicity of formic acid is due to its peculiar position among the aliphatic acids, in that it may act by its carboxylic group * or its

* OH
|
H—C=O

aldehydic group,† its marked reducing action being due to its aldehydic character. In equimolecular doses it is more toxic than its higher homologues, acetic and propionic acid.

Toxicity for Man.—According to Lebbin (1906), daily ingestion of 0.5 gm. of formic acid in the form of lemonade over a period of four weeks caused no toxic effects, and Weingarten (1932) stated that human subjects can tolerate intravenous injections of 20 cc. of a 20% solution of sodium formate (equal to 4 gm.) without untoward subjective and objective effects. On the other hand, Stern (1906) stated that larger doses of formate (2 to 3 gm.) several times daily may cause vertigo, nausea, vomiting, albuminuria, hematuria, tenesmus, dyspnea, and lowering of the body temperature. Similarly, Eppinger (1913) reported that ingestion of 3 to 4 gm. of sodium formate caused in one out of three human subjects albuminuria, and, 24 hours later, hematuria which cleared up after five days.

Severe and fatal poisoning from the ingestion of formic acid are not exceptional, and such cases were reported by Lutz (1913), Schneider (1928, *a* & *b*), Karunakaran and Pillai (1944), and Holzer (1952-54). The clinical picture is characterized by salivation, vomiting, burning sensation in mouth and pharynx, bloody vomiting, diarrhea, and agonizing pain. In severe poisoning the patient suffers from shock, his pulse becomes rapid and soft, the skin cold and clammy, and the blood pressure drops; later he may develop respiratory embarrassment and cyanosis, and die within 48 hours. He may develop albuminuria with casts, hematuria, and subsequently anuria, and may die from uremia, circulatory failure, or pneumonia.

Industrial poisoning from formic acid was reported by Lutz (1913), and Koisorov and Petrov (1934). The former reported on a worker who had suffered splashes of hot formic acid in his face, which reddened immediately; he developed marked dyspnea, difficulties in swallowing, and inability to speak, and died six hours later. According to Koisorov and Petrov (1934) exposure to the vapors of formic acid or the dust of formates may cause pallor, hyperemia of conjunctiva and sclera, and gastrointestinal disturbances simulating in two instances gastric ulcers.

As mentioned by Prosser White (1934), contact of the skin with formic acid will cause burns with blisters, and, in many instances, keloid formation at the site of the burn. Maschka (1881) observed black-brown acid burns around the face and mouth of an infant which presumably were produced postmortally by bites of ants and due to formic acid.

Prophylactic Measures.—Because of its corrosive action, contact of the skin with formic acid should be avoided. Contamination of the air with vapors of formic acid or dust of formates should be controlled by proper ventilation.

Treatment of Formic Acid Injuries.—Formic acid burns should be treated by rinsing with ample amounts of water, the application of lime water, and covering with a sterile dressing. Poisoning from the ingestion of formic acid should be treated by neutralizing the gastric contents by the administration of limewater, gastric lavage, and the administration of demulcents. Special attention should be paid to the kidney function, and adequate diuresis should be maintained by administration of fluids. The irritation of the respiratory tract following inhalation of formic acid vapors may be alleviated by inhalation of a mist prepared from a 5% solution of sodium bicarbonate.

Pathological Changes in Formic Acid Poisoning.—As reported by Lutz (1913) and Schneider (1928, *a* & *b*), the pathological changes after ingestion of formic acid are similar to those produced by other corrosive acids. The tongue, palate, pharynx, and esophagus are hyperemic, mark-

† $H-C(=O)-OH$

edly swollen, and covered with necrotic spots. The stomach may contain bloody material, its walls may be edematous with numerous necrotic areas, or it may be dry and brittle. Similar but less severe changes may be seen in the jejunum. The liver may contain hemosiderin deposits and the kidneys are hyperemic; they may contain hemorrhages and present the picture of nephritis. The larynx and trachea may be edematous and inflamed with hemorrhages, and there may exist bronchitis or pneumonia.

Methyl Formate

Chemistry.—Methyl formate, $HCOOCH_3$, has the molecular weight 60.05; it is a colorless flammable liquid of ethereal odor which boils at 31.8 C and is solid below −99.0 C. Its specific gravity is 0.974 at 20.4 C, and it is soluble in water to the extent of 30 parts per 100 at 20 C, miscible with ethanol, and soluble in methanol. Its flash point is −2 F (−18.89 C) (closed cup), and its lower explosive limit is 5% and the upper 22.7%. The autoignition temperature is 840 F (448.89 C), and its vapor density is 2.07 (air=1).

Uses.—Methyl formate is used to some extent as larvicide and fumigant; it is a good solvent for fats, oils, fatty acids, acetyl cellulose, collodion, and celluloid (Gnamm, 1943).

Insecticidal Action.—According to Jones (1938) methyl formate is less toxic for the red flour beetle (Tribolium castaneum) than methyl bromide, but the toxicity may be considerably increased by the addition of carbon dioxide. According to David (1944) it may have some value as a fumigant for the destruction of body lice in garments.

Determination in Air.—Schrenk and associates (1936) determined the concentration of methyl formate in air by passing a measured volume of the contaminated air through air-equilibrated activated charcoal, and determining the gain in weight of the latter. In this case it is, however, necessary to modify the customary absorption train by removal of the soda lime, since the latter will cause hydrolysis of the methyl ester. Methyl formate can also be determined by adsorption on silica gel, subsequent hydrolysis with alkali, and determining either the formic acid or the methanol formed in this way.

Determination in Biological Materials.—Gettler (1940) determined methyl formate in various organs by distilling the ground tissue, hydrolyzing the distillate, and determining the amount of methanol formed.

Absorption, Fate, Distribution, and Excretion.—Methyl formate is absorbed through the lungs, from the gastrointestinal tract, and, evidently, also through the skin (Gettler, 1940). As shown by this author, considerable amounts may be stored in the brain and smaller quantities in the liver. According to Ciaranfi (1941), the oxygen consumption of slices of liver, kidney, striated muscle, spleen, cortex of the brain, Rous sarcoma, chicken lympho-sarcoma, and Ehrlich's adenosarcoma is considerably increased by methyl formate but not by sodium formate. He showed that only the formic acid fraction of methyl formate is oxidized by liver and striated muscle but not by kidney slices, whereas the methanol fraction remains intact. The relation between the amount of carbon dioxide formed by oxidation of methyl formate, and the amount of oxygen used simultaneously, was 2:1, indicating that the formic acid of the ester molecule is completely oxidized. According to Duquenois and Revel (1935), methyl formate may be present in small quantities in the blood of animals killed by inhalation of its vapors.

Toxicity for Laboratory Animals.—Weber (1902) found that in rabbits methyl formate has irritant and narcotic properties causing in sufficient concentrations deep narcosis, marked dyspnea, convulsions with opisthotonus and trismus, and finally coma and death. Flury and Neumann (1927) made similar studies with cats, and Nuckolls (1933) and Schrenk, Yant, Chorynak, and Patty (1936) with guinea pigs, which are summarized in Table 1. Duquenois and Revel (1935) made similar observations, and reported that frogs exposed to air saturated with methyl formate vapors developed nystagmus, miosis, irritation of the eyes, forced respiration, incoordination, progressive depression, and death, the heart stopped in diastole and death was followed by rapid onset of rigor. It appears, as stated by Schrenk and associates (1936), that concentrations of 50,000 p.p.m. are fatal within 20 to 35 minutes, that 15,000

TABLE 1.—*Effect of Inhalation of Methyl Formate Vapors on Laboratory Animals*

Concentration Mg/L.	Concentration P.p.m.	Duration, Min.	Symptoms	Author
			A. Cats	
25	10,200	20	Marked irritation of eyes and nose; no signs of paralysis.	Flury and Neuman (1927)
25	10,200	120	After 90 min. ataxia, after 105 min. beginning narcosis; after 120-180 min. pulmon. edema and death.	
14.5	5,916	60	After 50 min. irritation of eyes and nose, staggering, after 60 min. fatal pneumonia in some animals.	
			B. Guinea Pigs	
20-25	9,000-10,000	1	Intense irritation of eyes and nose.	Nuckolls (1933)
		30	Beginning narcosis, severe lacrimation, no fatalities during or after exposure.	
		60-120	All animals became stuporous, intense irritation of skin and mucosae, recovery within 10 min. No death.	
49-61.4	20,000-25,000	5	Intense irritation of eyes and nose, dyspnea, cough, beginning narcosis, recovery after 10 min., No death.	
		30	The same symptoms more marked; recovery after 10 min.; no death.	
		60	⅗ of animals died during exposure, ⅕ recovered very slowly.	
		120	All animals in stupor, tremors, some died within 18 hours after exposure, 2 recovered very slowly.	
122.7	50,000	25-35	Fatal to all animals in 20-30 min.	Schrenk et al. (1936)
61.4	25,000	50-72	Fatal to all animals, dangerous in 30-60 min.	
25	10,000	2-3	Nasal irritation, lacrimation.	
		6-15	The same + retching.	
		120-135	Incoordination.	
		120-150	Narcosis, death in 150-175 min.	
8.6	3,500	480	Irritation of eyes & nose, retching.	
3.68	1,500	480	Moderate nasal irritation.	

to 25,000 p.p.m. are dangerous to life in 30 to 60 minutes, that 5,000 p.p.m. is the maximum amount which can be tolerated for 60 minutes without serious disturbances, and that the maximum concentration which can be tolerated for several hours without serious disturbances is 1,500 to 2,000 p.p.m. From experiments reported by Gross (1938) it appears that methyl formate is a cumulative poison.

Pathological Changes Observed in Laboratory Animals.—The most outstanding pathological findings in animals exposed to methyl formate refer to the lungs. As reported by Schrenk and associates (1936) these may show with high concentrations (50,000 and 25,000 p.p.m.) intense congestion, emphysema, and edema; and there may be hemorrhages, as reported by Nuckolls (1933). Liver, kidney, and adrenal cortex, as well as the meningeal vessels, are hyperemic, and, according to Duquenois and Revel (1935), there is a rapid onset of rigor mortis. According to Gross (1938), the subcutaneous injection of 0.5 cc. per kilogram causes tissue necrosis. Fühner and Neubauer (1907) determined the minimal hemolytic concen-

tration for bovine erythrocytes as 8.80 gm. % (1.47 mol/l.).

Toxicity for Man.—According to Schrenk and associates (1936), inhalation of a concentration of 1,500 p.p.m. (3.68 mg/l.) of methyl formate for one minute causes no signs of irritation, but as pointed out by Flury and Wirth (1936) higher concentrations are very irritant, and, if repeated, may lead to convulsions and death. According to Duquenois and Revel (1935) workers exposed to the vapors of a solvent containing 30% of methyl formate, in addition to ethyl formate and methyl and ethyl acetate, complained about irritation of the mucous membranes, oppression in the chest, and dyspnea of varying intensity. Some felt exhilarated, others depressed, and some suffered from temporary loss of memory. Several complained about visual disturbances which disappeared after discontinuation of the exposure. One patient, however, developed optic injury and became completely blind, but recovered after about two weeks following venesection, blood transfusions and spinal puncture.

Gettler (1940) reported a fatal case of methyl formate poisoning in a nineteen-month-old child whose scalp had been treated with a parasiticide, evidently containing methyl formate, and subsequently covered for 20 minutes with a rubber cap. When the latter was removed the child collapsed, became cyanotic, and breathed slowly. Upon administration of stimulants there was a temporary improvement, but later the child became more and more depressed, the reflexes became negative, and it died from cardiac arrest. Brain, lungs, liver, kidneys, and spleen were found highly congested.

Precautionary Measures.—From the foregoing it is apparent that methyl formate is a highly toxic material which should be handled with adequate precaution. Contact of the skin should be avoided, and the concentration of its vapors in air should be controlled by proper ventilation. According to the Conference of Governmental Industrial Hygienists (1955), the maximum acceptable concentration of methyl formate in air is 100 p.p.m. Premises contaminated with methyl formate should only be entered with masks which allow inhalation of uncontaminated air, and with goggles to protect the eyes from irritant vapors.

Treatment of Poisoning.—In the treatment of methyl formate poisoning prompt discontinuation of exposure is of paramount importance. The contaminated skin should be washed with soap and water. In case the ester is inhaled, the patient should be removed to fresh air, and after heavy exposure kept at rest. The irritation of the respiratory tract may be reduced by inhalation of a mist from a 5% solution of sodium bicarbonate, and the patient should be watched for the development of pulmonary edema. In case this should become manifest, oxygen should be administered against a pressure of 3 to 4 cm. of water, and in severe cases venesection may become necessary. In case the central nervous system is involved, spinal puncture may alleviate the symptoms. Otherwise the treatment has to be symptomatic and supportive.

Ethyl Formate

Chemistry.—Ethyl formate, $HCOOC_2H_5$, formic acid ethyl ester, has the molecular weight 74.08, and is a colorless liquid of aromatic odor which has the specific gravity 0.923 at 20/4 C. It is solid below −79.4 C and boils at 54.2 C. It is soluble in water to the extent of 11 parts per 100 parts at 18 C, and miscible with alcohol and ether. Its flash point is −4 F (−20 C) (closed cup method) and 10 F (−12.22 C) (open cup method). The lower explosive limit is 3.5% and the upper 16.5%.

Uses.—Ethyl formate is used as a flavoring agent for lemonades and essences, and for the manufacture of artificial rum and arrack. It is a good solvent for oils and fats, nitrocellulose, and cellulose acetate, and, to a lesser degree, for waxes. It is used as a fungicide and larvicide for tobacco, cereals, and dry fruits, and it is also used in organic synthesis. (*Merck Index*, 1952; Durrans, 1944; Gnamm, 1943; Simmons and Fischer, 1945).

Determination in Air and Biological Material.—Ethyl formate may be determined in air and biological specimens by the same procedures as mentioned under methyl formate.

Absorption, Fate and Excretion.—Ethyl formate is absorbed through the lungs and

from the gastrointestinal tract, but it is questionable whether and to what extent it is absorbed through the skin. Its fate in the organism and its excretion have evidently not been studied.

Toxicity for Laboratory Animals.—According to Smyth, Carpenter, Weil, and Pozzani (1954) ethyl formate causes no irritation of the rabbit skin. As shown by these authors and by Gross (1938) it causes, however, irritation of the eyes and of the mucous membranes. The former found its oral toxicity quite low, the LD_{50} for rats being 4.290 grams per kilogram (3.070-5.980) counting fatalities occurring within 14 days. In contrast to methyl formate, the subcutaneous injection of 1.0 cc. per kilogram is well tolerated, causing neither local nor systemic reactions (Gross, 1938). Similarly, Wachtel (1920) stated that the intravenous injection of 430 to 500 mg. given as 5% solution caused no visible toxic effects in rabbits, but, as shown by Vogel (1897), the intrajugular injection of 28 mg. per kilogram of the undiluted ester causes an increase of the respiratory volume without depressing the central nervous system.

According to Duquenois and Revel (1935), inhalation by guinea pigs of air saturated with vapors of ethyl formate results within a few minutes in tremors, progressive depression of the central nervous system, and death from circulatory and respiratory failure. In contrast to methyl formate, ethyl formate does not produce nystagmus or rigor in frogs. Smyth and associates (1954) stated that air saturated with ethyl formate vapors can be inhaled for five minutes by rats without fatalities, whereas the inhalation of a concentration of 8,000 p.p.m. (24 mg/l.) killed five of six animals within four hours. Flury and Neumann (1927) reported that exposure of cats to concentrations of 32 mg. per liter (10,560 p.p.m.) causes after 20 minutes moderate irritation of the mucous membranes of the eye and respiratory tract, after 80 minutes progressive narcosis, and after 90 minutes death with pulmonary edema. Concentrations of 44 mg. per liter (14,520 p.p.m.) caused after 17 minutes marked irritation of the mucous membranes, severe dyspnea, and staggering. When the exposure was discontinued after 20 minutes one animal recovered, but the other died from pulmonary edema. Similarly, with concentrations of 40 to 130 mg. per liter, Gross (1938) noted in rabbits and guinea pigs marked depression of the central nervous system, and frequently the development of pneumonia. It appears, therefore, that ethyl formate is rather toxic with inhalation, especially with regard to the depression of the central nervous system. Fühner and Neubauer (1907) determined the minimal hemolytic concentration of ethyl formate for bovine erythrocytes as 5.25 gm. per liter (0.71 mol per liter).

Toxicity for Man.—There are no reports on accidental or occupational poisoning from ethyl formate in man. According to Flury and Neumann (1927) concentrations of 32 mg. per liter (10,560 p.p.m.) cause progressive irritation of the eyes and mucous membranes which may last four hours after discontinuation of the exposure.

Prophylactic Measures.—According to the American Conference of Governmental Industrial Hygienists (1955) the maximum acceptable concentration of ethyl formate in air is 100 p.p.m. for eight hours' exposure daily. In view of the rather high toxicity of this material, it should be handled with adequate precautions. Rooms contaminated with ethyl formate should only be entered with proper gas masks, and the eyes should be protected by goggles.

Treatment of Poisoning.—This should follow the same principles as discussed with methyl formate.

Propyl Formate

Chemistry.—Propyl formate, formic acid propyl ester, $HCOOC_3H_7$, has the molecular weight 88.10. It is a colorless liquid of aromatic odor, has the specific gravity 0.901 at 20/4 C, solidifies below −92.9 C, boils at 80.9 C, is soluble to the extent of 2.2 parts in 100 parts of water at 22 C,

and miscible with alcohol and ether. Its flash point is 27 F (−3 C) by the closed cup method.

Isopropyl formate, formic acid isopropyl ester, $HCOOCH(CH_3)_2$, has the molecular weight 88.10. It is a colorless liquid of the specific gravity 0.873 at 20/4 C which boils at 68-71 C at 751 mm. Hg. It is soluble in water to the extent of 2.1 parts in 100 at 22 C. Its flash point is 22 F (−5.56 C) by the closed cup method.

Neither of these compounds appears to have extensive application and no studies have been reported regarding their toxicity and potential dangers. According to Simmons and Fischer (1945) propyl formate is used as a fumigant for dried fruit, killing insects and their eggs.

n-Butyl Formate

Chemistry.—n-Butyl formate, $HCOO(CH_2)_3CH_3$, has the molecular weight 102.13; it is a liquid which boils at 106.9 C and which has the specific gravity 0.911 at 0 C. It is very slightly soluble in water but miscible with alcohol and ether. Its flash point by the closed cup method is 64 F (17.78 C).

Uses.—n-Butyl formate is used occasionally as solvent for cellulose nitrate, cellulose acetate, ester gum, copal ester, coumarone, and mastic; it partly dissolves shellac (Durrans, 1944).

Absorption, Fate and Excretion.—n-Butyl formate is absorbed through the lungs and from the gastrointestinal tract, but it has not been established to what extent it is absorbed through the skin. Its metabolic fate and its excretion have evidently not been studied.

Toxicity for Animals.—According to Flury and Neumann (1927), cats exposed to static concentrations of 43.5 mg. per liter of air (10,418 p.p.m.) show signs of increasing irritation of the eyes, salivation, and, after 20 minutes, somnolence: after 45 minutes they tolerate side position, after 60 minutes they are deeply narcotized, and die after 70 minutes' exposure with pulmonary edema. A dog exposed to this concentration developed irritation of the eyes, salivation, and, after exposure for 30 minutes, vomiting and staggering; but it recovered after discontinuation of the exposure. Cats exposed to a flowing vapor-air mixture of 17 mg. per liter (3,941 p.p.m.) showed salivation, lasting irritation of the eyes, and, after one hour, staggering.

Toxic Effects in Man.—As shown by Flury and Neumann (1927) concentrations of 43 mg. per liter (10,418 p.p.m.) cause in man immediately severe irritation of the eyes and blepharospasm, and become intolerable after a few inhalations. There are no reports on industrial or accidental poisoning with this compound.

Prophylactic and Therapeutic Measures.—These should be the same as discussed with methyl formate.

Amyl Formate

Chemistry.—n-Amyl formate, $HCOO(CH_2)_4CH_3$, has the molecular weight 116.16. It is a colorless liquid of the specific gravity 0.902 at 0 C which is solid below −73.5 C, boils at 132 C, and is very slightly soluble in water but miscible with alcohol and ether.

i-Amyl formate, $HCOO(CH_2)_2CH(CH_3)_2$, has the molecular weight 116.16, and is a colorless liquid of the specific gravity 0.882 at 20/4 C, which is solid below −93.5 C, boils at 123.5 C, is soluble in water to the extent of 0.3 parts per 100 at 22 C, and is miscible with alcohol and ether.

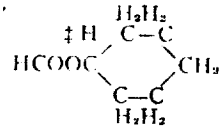
Uses.—According to Durrans (1944) amyl formate is a good solvent for cellulose nitrate, colophony, ester gum, copal ester, benzyl abietate, cumarone, and mastic; it is miscible with linseed oil, caster oil, and hydrocarbons.

Absorption, Fate, and Excretion.—No information is available regarding its fate in the organism or its excretion.

Toxicity for Animals and Man.—No information is available regarding its toxicity for animals or man, except a statement by Lehmann (1913) to the effect that, according to preliminary experiments, its narcotic action is three times as great as that of amyl acetate. There are no reports of accidental or industrial poisoning from this material.

Cyclohexyl Formate

Chemistry.—Cyclohexyl formate ‡ has the molecular weight 128.10. According to Lehmann and Flury (1938/1943), it is a liquid of the specific gravity 1.010 at 20/4 C which boils at 162.5 C and has a flash point of 123.8 F (51 C).



Uses.—As stated by Gnamm (1943) it is a high boiling solvent for nitrocellulose, but it is of moderate industrial importance.

Toxicity for Animals.—According to Lehmann (1913) it is twice as volatile as cyclohexyl acetate and is about as toxic as the latter, and according to Gross (1938/1943) the toxicity is of the same type and intensity as that of amyl acetate.

Toxicity for Man.—There are no reports on accidental or industrial poisoning from this material.

Methylcyclohexyl Formate

Chemistry.—Methylcyclohexyl formate § has the molecular weight 142.12. It is, according to Lehmann and Flury (1938/1943), a liquid of the specific gravity 0.957 at 20/4 C, which boils at 176-180 C and has a flash point of 147.2 F (64 C).

Uses.—According to Gnamm (1943) it is a high boiling solvent for nitrocellulose and of moderate industrial importance.

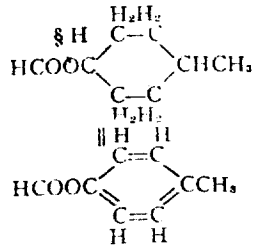
Toxicity for Animals.—As stated by Lehmann (1913) it is about one and one-half times as volatile as cyclohexyl acetate, and according to Gross (1938/1943) its toxicity equals about that of amyl acetate.

Toxicity for Man.—There are no reports of industrial or accidental poisoning from this material.

Benzyl Formate

Chemistry.—Benzyl formate ‖ has the molecular weight 136.14, and is a colorless liquid of the specific gravity 1.081 at 23 C, which solidifies below 3.6 C and boils at 202-3 C at 747 mm. Hg. It is insoluble in water, soluble in alcohol, and miscible with ether.

Uses.—According to Durrans (1944) it is an excellent solvent for cellulose nitrate and acetate, ester gum, copal ester, benzyl abietate, cumarone, and glycerylphthalate resin. It is miscible with castor oil, linseed oil, and aromatic and petroleum hydrocarbons.



Toxicity for Animals and Man.—No studies on its toxicity for animals have been reported, nor industrial or accidental poisoning in man.

Allyl Formate

Chemistry.—Allyl formate, $HCOOCH_2CH{=}CH_2$, has the molecular weight 89.09. It is a liquid of strong mustard-like odor which has the specific gravity 0.948 at 18 C, boils at 83.6 C at 768 mm. Hg, and is insoluble in water but soluble in alcohol.

Uses.—It is used in organic chemistry.

Toxicity for Animals.—Haas (1941) showed that allyl formate is a protoplasmic poison, in that concentrations of 1:500 cause injury of the eggs of the pond mussel, Anodonta, and concentrations as low as 1:100,000 cause injury of their nuclei.

According to Casals and Olitsky (1946) the intraperitoneal injection of 15 mg. per kilogram of allyl formate in saline is fatal to mice, 4 out of 21 animals dying within 24 hours, 10 in 24 hours, and some within 24 and 30 hours. It is therefore apparent that allyl formate is a highly toxic material.

Gloggengiesser (1944) showed that in rabbits, dogs, and guinea pigs the administration of allyl formate produces three types of reactions in the liver, namely (1) a serous inflammation which passes into complete necrosis, (2) degeneration of the parenchyma, and (3) unspecific changes of the vascular apparatus characterized by dilatation and hyperemia of the capillaries and basophilia of the liver cells. According to Casals and Olitsky (1946) congestion of the sinoids is visible in rats after one to two hours, and the congestion may be associated with hemorrhages. After six to eight hours, periportal necroses may become visible, which increase in severity during the first 24 hours. As shown by Rosin and Doljanski (1946), the latter is preceded by hydropic degeneration of increasing intensity. According to Kaunitz and Selzer (1937) the liver of guinea pigs poisoned with allyl formate showed, after a temporary increase, a much lower oxygen consumption than that of untreated animals, but the activity of neither succinic acid nor citric acid dehydrogenase was seriously

affected. Spiess-Bertschinger (1944) showed that in thyroidectomized animals feeding of thyroid increases the serous inflammation of the liver, which increasingly becomes more severe and irreversible, progressing to severe necrosis. Benda, Locker and Rissel (1951) confirmed the findings of Kaunitz and Selzer as to the effect of small doses of allyl formate on the oxygen metabolism of the liver, and showed that there is in the liver tissue a distinct aerobic glycolysis and that addition of glucose to the substrate reduces the intensity of the hepatotoxic action. These authors assumed that the inhibition of the oxygen metabolism is due to structural changes in the liver tissue and to inhibition of certain ferments, whereas the primary stimulation is due to liberation of ferments because of loosening the parenchymal structure. As shown by Krüskemper and Hartmann (1955) rats poisoned by repeated injections of 50 mg. per kilogram of allyl formate suffered a loss of serum albumin, with the onset of the serous inflammation of the liver which parallels the increased oxygen consumption, triacetin cleavage, and phosphatase activity of this organ; whereas the cholinesterase activity is reduced. However, with increasing injury the first three functions are also reduced. It is assumed that the different behavior of these ferments with regard to time and intensity is affiliated with their relation to different cells.

Zawadski (1938) noted that allyl formate reduces the energy of the excised frog muscle, presumably because of an increased permeability of the muscular interfaces.

Toxicity of Allyl Formate for Man.—There are no reports on accidental or industrial poisoning with allyl formate, but from the above it is evident that it is a very toxic material which should be handled with greatest precaution. The treatment of such poisoning has to be symptomatic and supportive, and special attention should be paid to the function of the liver.

Ethyleneglycol Diformate

Chemistry.—Ethyleneglycol diformate, ethylene formate, $HCOOCH_2CH_2OOCH$, molecular weight 118.09, is a liquid which boils at 174 C, and is soluble in alcohol and ether.

Toxicity for Animals.—Smyth and associates (1941) determined its oral LD_{50} for rats and guinea pigs as 1.51 and 0.39 gm. per kilogram, respectively. It is therefore considerably more toxic than the ethyl ester, the only formate for which comparable data are available.

Toxicity for Man.—There are no reports on industrial or accidental poisoning from this material, but it should be handled with the same precautions as advocated for methyl formate.

TABLE 2.—*Physical-Chemical Properties of Alkyl Formates*

Ester	[2]Mol. Wt.	[1]Sp. Grav.	[1]Boil. Pt., C	Vapor Press.,[3] Mm. at 30 C	[1]Flash Pt., F.	[2]Solubil. Water, p/100	Acid Hydrol. Ks 25 C	[3]Surf. Tension at 20 Against Air or Vapor, Dynes/Cm.
Methyl	60.05	0.974 20/4 C	31.8	707.9	−2	30 20 C	0.145	25.08
Ethyl	74.08	0.923 20/4 C	54.2	297.5	−4	11 18 C	0.187	23.6
n-Propyl	88.10	0.901 20/4 C	80.9	101.1	27	2.2 22 C	0.195	24.5
i-Propyl	88.10	0.873 20/4 C	68-71	—	22	2.1 22 C	0.168	—
n-Butyl	102.13	0.911 0 C	106.9	—	64	v.sl.	0.187	—
i-Butyl	102.13	0.885 20/4 C	98.2	62 (35)	—	1.1 22 C	0.190	—
n-Amyl	116.16	0.902 0 C	132	—	—	v.sl.	—	—
i-Amyl	116.16	0.882 20/4 C	123.5	49 (50)	—	0.3 22 C	—	—
n-Hexyl	130.18	0.886 20/4 C	155.5	—	—	—	—	—
n-Heptyl	144.21	0.883 15/4 C	178.1	—	—	Insol.	—	—

1. Lange's *Handbook of Chemistry*, 1956.
2. Internat. Critical Table VII.
3. Hodgman, *Handbook of Chemistry and Physics*, 32d. Ed.

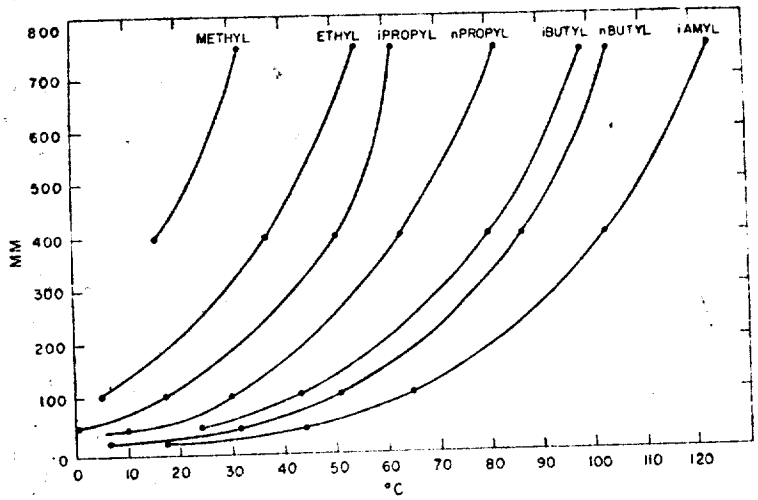


Fig. 1.—Vapor pressure of alkyl formates at various temperatures according to Hodgman (1950).

Comparison of Physical-Chemical and Toxicological Properties of Formic Acid Esters

Comparison of the physical-chemical properties of alkyl formates with their toxic properties is rendered difficult because of lack of comparable quantitative toxicological data, but the information available allows an approximate evaluation of their potential dangers.

As illustrated in Table 2, the specific gravity of these esters decreases with increasing molecular weight, that of the isocompounds being lower than that of the normal homologues. The boiling point increases and the vapor pressure decreases (Fig. 1) with increasing molecular weight, so that the inhalation hazard decreases with the higher homologues. Similarly, the flash point increases with the molecular weight, so that the higher homologues offer a smaller fire hazard. The isocompounds have a lower boiling point, lower flash point, and a greater vapor tension than the normal compounds. The solubility in water decreases, and the stability towards acid hydrolysis increases with the molecular weight. It is therefore apparent that the lower homologues, especially methyl formate, should have a greater irritant action than the higher homologues; which seems to be supported by the observations of Schrenk and associates (1936), and Flury and Neumann

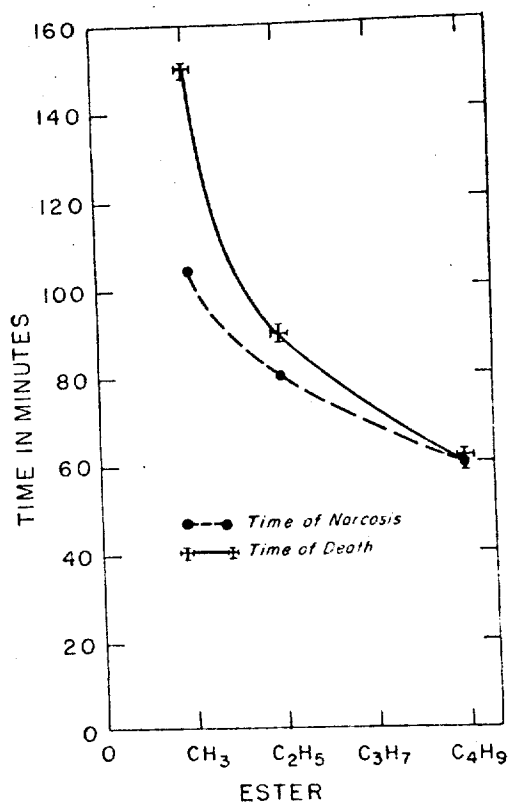


Fig. 2.—Narcotic and toxic action of alkyl formates as measured by onset of narcosis and death after exposure of cats to approximately equimolecular concentrations (approx. 0.00042 mol/L.) (Flury & Neumann, 1927).

(1927). The opposite holds true for the hemolytic action of the alkyl formates. As shown by Fühner and Neubauer (1907), the minimum effective hemolytic concentration of methyl formate is 1.47 mol per liter, that of ethyl formate 0.71, and that of the propyl ester 0.23 mol per liter. On the other hand, as illustrated in Figure 2, the data of the latter investigators show that the toxicity by inhalation of approximately equimolecular concentrations increases both as to narcotic and lethal effects with the molecular weight, and that, at the same time, the margin of safety between both functions becomes smaller. There is, however, one important difference between methyl formate and the higher homologues, in that with the former the animals die with convulsions in coma, i.e., with a picture similar to that produced by formic acid, and with the latter death occurs from central depression and pulmonary edema. It appears, therefore, that the higher homologues are fatal because of their narcotic action, and that in the case of methyl formate some other factor, namely the more easy hydrolysis of the molecule, may play a role, as indicated in the eighth column of Table 2. This secondary action is also indicated by the greater margin of safety between narcotic and fatal concentrations of equimolecular amounts of methyl formate as compared with that of the higher homologues. Because of this mechanism, and of the greater volatility of methyl formate as illustrated in Figure 1, this compound is definitely the most hazardous material in this group.

Department of Health, Education, and Welfare, Public Health Service.

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WORLD HEALTH ORGANIZATION

TECHNICAL REPORT SERIES

No. 309

SPECIFICATIONS FOR THE IDENTITY AND PURITY OF FOOD ADDITIVES AND THEIR TOXICOLOGICAL EVALUATION: FOOD COLOURS AND SOME ANTIMICROBIALS AND ANTIOXIDANTS

Eighth Report of the Joint FAO/WHO Expert Committee on Food Additives

Geneva, 8-17 December 1964

WORLD HEALTH ORGANIZATION

GENEVA

1965

2.2.2 *Conclusion*

The Committee wishes to reiterate here the point made in the fifth report (pp. 19-20) that evaluation of acceptability is the function of toxicologists, oncologists and food scientists. The application of their findings is the proper function of the authorities responsible for food legislation and public health. It is the duty of these authorities to ensure that the product used conforms to the appropriate specification and that analytical methods are available for effective control.

The first report of the Joint FAO/WHO Expert Committee on Food Additives (p. 14) states that "the amount of an authorized additive used in food should be the minimum necessary to produce the desired effect." The present Committee emphasized the need to implement this limitation on levels of use, in accordance with good manufacturing practice. On page 15 of the same report attention is drawn to the need "to avoid a gradual and unrecognized increase in the amounts present in the total diet to a point at which an adequate margin of safety is no longer ensured."

The Committee therefore points out that for future toxicological work in this field it is essential to have available an adequate specification for each substance. In order to obtain a satisfactory specification it may be necessary to define the starting material from a botanical standpoint, as well as to describe the colouring matter in terms of chemical composition. This will probably involve the extraction and purification of the active principle or principles. A general toxicological evaluation can be based only on a knowledge of the biological properties of the chemically defined compound or compounds. The Committee recommends that, when the chemical data become available, a group of experts, which should include a pharmacognosist as well as chemists, should reconsider the possibility of drawing up specifications for food colours obtained from natural sources.

3. GENERAL CONSIDERATIONS ON SOME ANTIMICROBIALS AND ANTIOXIDANTS

3.1 General principles governing the establishment of specifications for identity and purity

The principles governing the establishment of specifications for food additives have been extensively dealt with in previous reports of the Joint FAO/WHO Expert Committee on Food Additives. It may, however, be useful to restate here the objectives:

(*a*) to identify the substance that has been subjected to biological and toxicological testing;

(*b*) to ensure that the substance is of the quality required for safe use in food;

(*c*) to reflect and encourage good manufacturing practice.

3.1.1 *Substances studied*

The Committee had before it a list of antimicrobials and antioxidants that had been considered by the Joint FAO/WHO Expert Committee on Food Additives that met in June 1961 but for which no specifications for identity and purity were available at that time. It was agreed that it would be advisable to delete from this list any substance that was not known to be in use as a food additive, or on which the information available to the Committee was insufficient for compilation of specifications or for toxicological evaluation. On this basis the following three substances were deleted from the list:

(*a*) Distearyl thiodipropionate. According to information available to the Committee, this antioxidant is not on any of the permitted lists.

(*b*) Gum guaiac. This additive being a natural substance does not lend itself to precise definition.

(*c*) Isopropyl citrate mixture. This additive is produced only by one company, which has a use patent, and the specifications have not been made available to the Committee.

The substances for which the Committee has prepared specifications are:

Diphenyl
✓Formic acid
Potassium nitrate
Sodium nitrate
Potassium nitrite
Sodium nitrite
Ortho-phenylphenol
Sodium-ortho-phenylphenol
Thiodipropionic acid
Dilauryl thiodipropionate.

3.1.2 *Impurities*

The nature of the impurities that might be present in a food additive has already been discussed. In the case of arsenic and lead, limits of 3 and 10 mg/kg, respectively, may be considered acceptable unless otherwise stated in the specifications. It is stressed that these limits are established to ensure good manufacturing practice and not, in most cases, because of any great toxicological concern, since food additives are commonly used in relatively

Annex 2

ACCEPTABLE DAILY INTAKE ZONES FOR MAN OF SOME ANTIMICROBIALS AND ANTIOXIDANTS

Compounds considered	*Unconditional* (mg/kg body weight)	*Conditional*
Butylated hydroxytoluene	—	0-0.5
Formic acid [a]	—	0-5
Gum guaiac	See section 3.1.1, page 16	
Hexamethylenetetramine [b]	—	—
Isopropyl citrate mixture	See section 3.1.1, page 16	
Nitrates of sodium and potassium [a]	0-5	5-10
Nitrites of sodium and potassium [a]	0-0.4	0.4-0.8
Nordihydroguiaretic acid [c]	—	—
Phosphoric acid [a]	0-5	5-15
Ortho-phenylphenol	0-0.2	0.2-1
Sodium ortho-phenylphenol	0-0.2	0.2-1
Sorbic acid [a]	0-12.5	12.5-25
Sulfur dioxide	0-0.35	0.35-1.5
Sodium sulfites (calculated as SO_2)	0-0.35	0.35-1.5
Sodium metabisulfite (calculated as SO_2)	0-0.35	0.35-1.5
Sodium hydrogen sulfite (calculated as SO_2)	0-0.35	0.35-1.5
Tartaric acid [a]	0-6	6-20
Thiodipropionic acid	0-3	3-15
Dilauryl thiopropionate	0-3	3-15
Diphenyl	0-0.05	0.05-0.25
Distearyl thiodipropionate	See section 3.1.1, page 16	

[a] Naturally occurring substances; the estimated acceptable intakes listed above do not include the amounts occurring naturally.

[b] Not considered suitable for use in food pending the result of work now in progress.

[c] Available scientific evidence inadequate for evaluation.

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WORLD HEALTH ORGANIZATION
TECHNICAL REPORT SERIES
No. 383

FAO NUTRITION MEETINGS REPORT SERIES
No. 44

SPECIFICATIONS FOR THE IDENTITY AND PURITY OF FOOD ADDITIVES AND THEIR TOXICOLOGICAL EVALUATION: SOME FLAVOURING SUBSTANCES AND NON-NUTRITIVE SWEETENING AGENTS

Eleventh Report of the Joint FAO/WHO Expert Committee on Food Additives

Geneva, 21-28 August 1967

Published by
FAO and WHO

WORLD HEALTH ORGANIZATION
GENEVA
1968

4.1 Flavouring substances

In its evaluations of flavouring substances, the Committee took into consideration not only the toxicological and biological data but also such aspects as levels of use and occurrence in natural foodstuffs.

The Committee was able to allocate unconditional acceptances on the basis of adequate toxicological information to the following compounds:

benzaldehyde	benzyl acetate
ethyl acetate	ethyl butyrate
ethyl vanillin	methyl salicylate
(−)- and (±)-menthol	γ-nonalactone
piperonal	vanillin
γ-undecalactone	

For benzaldehyde and benzyl acetate, the allocated ADI is given in terms of benzoic acid, representing total benzoate from all food additive sources.

Where a flavouring substance is also a therapeutic agent, as for menthol and for methyl salicylate, the Committee followed the usual practice of applying a safety factor to the therapeutic dose in order to calculate the ADI for the use of the substance as a food additive.

For the undermentioned substances, conditional acceptances were allocated:

trans-anethole	isoamyl butyrate
(+)- and (−)-carvone	cinnamaldehyde
citral	citronellol
ethyl heptanoate	✓ethyl formate
ethyl laurate	ethyl lactate
eugenol	ethyl nonanoate
α-ionone	geranyl acetate
linalol	β-ionone
methyl anthranilate	linalyl acetate
octanal	nonanal

For all these substances the further work required was specified. For eugenol, although a conditional ADI was allocated, the Committee was of the opinion that the additional work required should be made available within a reasonable period.

The conditional ADIs for ethyl formate and ethyl lactate have been established at the same levels as those of formic acid and lactic acid respectively.

Annex 2

ACCEPTABLE DAILY INTAKES FOR MAN OF SOME FLAVOURING SUBSTANCES AND NON-NUTRITIVE SWEETENING AGENTS *

Specifications have been prepared for all the substances designated in the following table (see page 4).

Substance	Unconditional ADI (mg/kg body-weight)	Conditional ADI (mg/kg body-weight)	Temporary ADI (mg/kg body-weight)
Isoamyl Butyrate		0–5	
trans-Anethole		0–1.25	
Benzaldehyde	0–5 *a*		
Benzyl Acetate	0–5 *a*		
(+)-Carvone and (–)-Carvone		0–1.25	
Cinnamaldehyde		0–1.25	
Citral		0–1	
Citronellol, 90% and 98% total alcohols		0–0.25	
Ethyl Acetate	0–25		
Ethyl Butyrate	0–15		
Ethyl Formate		0–5	
Ethyl Heptanoate		0–1	
Ethyl Lactate		0–100 *b*	
Ethyl Laurate		0–1	
Ethyl Methylphenylglycidate			0–0.6
Ethyl Nonanoate		0–1	
Ethyl Vanillin	0–10		
Eugenol		0–5	
Geranyl Acetate		0–5	
α-Ionone		0–0.1	
β-Ionone		0–0.1	
Linalol, 90% and 95%		0–0.25	
Linalyl Acetate, 90% and 96%		0–0.25	
Maltol			0–1
(+)-Menthol and (–)-Menthol	0–0.2	0.2–2	
Methyl Anthranilate		0–1.5	
Methyl Salicylate	0–0.5		
γ-Nonalactone	0–1.25		
Nonanal		0–0.1	
Octanal		0–0.1	
Piperonal	0–2.5		
γ-Undecalactone	0–1.25		
Vanillin	0–10		
Calcium Cyclamate			0–50
Sodium Cyclamate			
p-Phenetylcarbamide (Dulcin) *c*			
Saccharin			
Calcium Saccharin			
Sodium Saccharin	0–5	5–15 *d*	

a As total benzoic acid from all food additive sources.
b As total D(–) lactic acid from all food additive sources.
c The Committee recommended that this substance should not be used as a food additive.
d For dietetic foods only.

* Monographs containing biological data on, and toxicological evaluation of, these substances will be issued by FAO and WHO in a separate document entitled: *Toxicological evaluation of some flavouring substances and non-nutritive sweetening agents*. FAO Nutrition Meetings Report Series, 1968, No. 44A; WHO/Food Add./68, 33.