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THE RESPONSE OF GUINEA PIGS TO INHALATION OF FORMALDEHYDE AND FORMIC ACID ALONE AND WITH A SODIUM CHLORIDE AEROSOL*

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Abstract—One hour exposures of guinea pigs to formaldehyde or formic acid produced an increase in resistance and a decrease in compliance which were statistically significant at concentrations as low as 0·3 p.p.m. A decrease in respiration rate and minute volume also occurred, but these factors did not change significantly until concentrations above 10 p.p.m. were used. An increase in respiratory work was observed, the greater proportion of the increase being in the resistive component. These changes became statistically significant at 1–3 p.p.m. Evaluated by these criteria, formic acid is a more potent respiratory irritant than formaldehyde. The resistance values returned to pre-exposure control values by the end of an hour after exposure except at the highest concentrations used (40–50 p.p.m.).

When the protective effect of the upper airway was by-passed with a tracheal cannula, the response to a given concentration of formaldehyde was increased. The resistance change was again reversible in an hour after the exposure ended.

The response to formaldehyde was potentiated by the presence of an aerosol of sub-micron particle size sodium chloride which was itself inert. The response to this combination was greater than the response to the gas alone breathed through a tracheal cannula. A tracheal cannula used with the gas-aerosol combination produced an increase exactly accounted for on the basis of the increased response to that concentration of gas alone. The sub-micron sized aerosol would not be removed by the upper respiratory tract, hence the potentiation due to the aerosol entity would be the same in normal and tracheotomized animals. The resistance remained elevated an hour after the exposure was terminated in animals exposed to the combination of formaldehyde and aerosol.

Formaldehyde was used with three different aerosol concentrations, 3, 10, and 30 mg/m³. The potentiation observed increased with increasing aerosol concentration, but attained a limiting value at each aerosol level once a critical gas concentration was reached.

The response to formic acid during exposure was not potentiated by the aerosol, but the resistance values remained elevated an hour after the exposure ended when the combination was used.

1. INTRODUCTION

MEASUREMENT of the mechanics of respiration in unanesthetized guinea pigs before, during and after inhalation of respiratory irritants has proved to be a toxicological tool of sufficient sensitivity to permit the study of air pollutants at concentrations of the order of magnitude of those occurring in highly polluted urban areas. Of the various changes observed, the increase in pulmonary flow-resistance is the most sensitive criterion of response and the one most closely related to concentration of irritant. By using a standard exposure time, dose-response curves relating increase in resistance to concentrations of irritant may be constructed. Thus various irritants may be compared and the effect of an inert aerosol on the response to various irritant gases over a wide range of concentrations may be studied.

The possibility of interaction of gaseous and particulate pollutants to produce a

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more toxic response is a matter of great concern to air pollution toxicology. Little experimental animal work has been done in this field and that has perforce employed the high concentrations necessitated by the classical toxicological methods. Once the guinea pig preparation had become a standardized research tool in this laboratory, its potentialities as a bioassay method for examining the toxicology of such mixtures became apparent. One advantage lay in the sensitivity, since a measurable response could be detected over a thousandfold or greater concentration range (i.e., from 0·1 to 100 p.p.m.) so that a given system could be tested over a wide range of gas-aerosol concentration ratios. This permits a comparison of the degree of response observed in the animals exposed to various combinations of gases and aerosols with the kinetic behavior of the gas-aerosol system in a manner that is not possible if toxicological data are not available for the low concentrations. A forthcoming paper (GOETZ) will be an example of such a treatment.

In the gas-aerosol work done so far by means of this bioassay method, the experimental design has been to study the effect of a single aerosol (NaCl) on several different irritant gases. The response during exposure to a given concentration of sulfur dioxide or formaldehyde was greater in the presence of the aerosol, whereas the response to acetic and formic acids remained unchanged. With all four irritants however, the response continued after the end of the exposure when the combination of gas and aerosol had been used. The results with sulfur dioxide have been reported in detail elsewhere and the over-all results with the other irritants have been summarized briefly (AMDUR 1957, 1959).

The present paper reports in detail the physiological response to formaldehyde and formic acid and the effect of the sodium chloride aerosol upon this response. While this work was in progress, the author had occasion to discuss the results with Dr. ALEXANDER GOETZ of the California Institute of Technology. As a result of these discussions it soon became apparent that sufficient parallelism existed between the toxicological approach and the application of physical theory to warrant a joint effort. Further experimental animal work was done with the formaldehyde-sodium chloride system to permit the examination of the postulated physical theory at three different aerosol concentrations. This paper and the subsequent one by GOETZ were planned essentially as a unit and should be so considered.

2. METHODS

The physiological technique developed for studying the mechanics of respiration in unanesthetized guinea pigs has been published in detail (AMDUR and MEAD, 1958). Three measurements are necessary, intrapleural pressure, tidal volume, and rate of flow of gas in and out of the respiratory system. Intrapleural pressure is measured by means of a fluid-filled catheter inserted into the chest cavity. Tidal volume is measured by recording the pressure changes produced in a body plethysmograph. The rate of flow is obtained by electrical differentiation of the volume signal with respect to time. Simultaneous tracings of these three measurements were made.

Intrapleural pressure has a resistive component which relates to resistance to flow of gas in the lungs and airways and an elastic component which relates to elastic recoil of the lungs. By relating the volume and flow rate changes to intrapleural pressure changes at specific points during the respiratory cycle, these two components can be evaluated separately. The pulmonary flow-resistance is expressed in cm H_2O ml/sec

and the compliance, which relates to the elastic recoil, is expressed in ml/cm H_2O. A decrease in compliance indicates that the lungs are behaving as a less elastic system.

In the experimental design used, respiratory measurements were made every five minutes during a control period of half an hour, then the substance under study was added to the airstream of the exposure chamber for one hour, during which period respiratory tracings were again made every five minutes. Each animal thus served as its own control. Post-exposure periods varied from one to three hours.

For establishing the concentrations of irritants a dynamic exposure chamber was used. The concentrations were determined by chemical analysis of an air sample collected during each exposure period.

The formaldehyde vapor was generated by passing air through 37% formaldehyde in a sintered glass bubbler. Formic acid vapor was generated by passing air over constant-boiling formic acid in a flask. Air samples for analysis were collected in demineralized water. The formaldehyde concentration was measured colorimetrically with Schiff's reagent as recommended by ELKINS (1950) for concentrations above 1 p.p.m. and by the chromotropic acid method of MACDONALD (1954) for lower concentrations. Formic acid concentrations were measured by the increase in conductivity of the resulting solution compared with a standard curve.

Sodium chloride aerosol was generated by aerosolizing a 1% sodium chloride solution in a Dautrebande generator (DELAUNOIS *et al.*, 1956). For determination of the concentration, the aerosol was collected on a millipore filter, the filter was soaked in demineralized water and the conductivity of the solution was measured. When the aerosol was used in combination with formaldehyde or formic acid, the filter preceded the midget impinger used for gas sampling.

Samples of the aerosol were collected with an electrostatic precipitator for particle sizing. Measurements made from electron micrographs indicated a geometric mean size of 0·04μ (σg 3·3). Particle size measurements were also made by Dr. Alexander Goetz at California Institute of Technology using the same aerosol generator and the same temperature and humidity conditions. These measurements, made with the aerosol spectrometer, would indicate more nearly the actual size of the airborne droplets than would the electron microscope.

3. RESULTS AND DISCUSSION

(*a*) *Formaldehyde and Formic Acid Alone*

The respiratory changes produced by a 1-hr exposure to formaldehyde are shown in Table 1. The lowest concentration used, 0·05 p.p.m., produced no detectable effect on the animals. At concentrations of 0·3 to 50 p.p.m. an increase in resistance accompanied by a slight decrease in compliance was observed.* Such changes suggest bronchial constriction as the physiological mechanism of response. As the concentration of formaldehyde was increased the change in resistance also increased.

This is further illustrated in Fig. 1, which shows the time course of the resistance change for 1 hr exposures to 0·58, 11, and 49 p.p.m. formaldehyde. At concentrations of 0·58 and 11 p.p.m., the resistance had returned essentially to control values an hour

* Since resistance change is the most useful criterion of response, in Table 1 and all subsequent tables the difference between control and exposure resistance and the standard error of this difference are given. For other factors the level of significant difference when present is merely indicated.

TABLE 1. RESPONSE OF GUINEA PIGS TO ONE-HOUR EXPOSURE TO FORMALDEHYDE

HCHO—P.P.M.		0·05 ± 0·02	0·31 ± 0·08	0·58 ± 0·08	1·22 ± 0·23	3·6 ± 0·3	11·0 ± 2·9	49 ± 9
Number of Animals		18	13	23	4	10	10	11
Resistance—cm H_2O/ml/sec	control	0·71	0·64	0·64	0·80	0·68	0·62	0·65
	exposure	0·76	0·73[d]	0·79[a]	1·03[b]	0·97[a]	1·03[b]	1·62[a]
$\bar{x}$-Exposure-Control		+0·05	+0·09	+0·15	+0·23	+0·29	+0·41	+0·97
$S\bar{x}$-Standard Error of diff.		0·030	0·039	0·024	0·028	0·044	0·053	0·135
Compliance—ml/cm H_2O	control	0·24	0·29	0·25	0·27	0·24	0·23	0·21
	exposure	0·21	0·26[d]	0·21[b]	0·22	0·21[d]	0·17[a]	0·13[a]
Tidal Volume—ml	control	1·24	1·74	1·69	1·74	2·06	1·83	1·46
	exposure	1·26	1·73	1·64	1·97	2·08	1·91	1·92[c]
Frequency—Breaths/min	control	113	104	111	102	102	99	105
	exposure	112	102	107	88	94	74[c]	72[a]
Minute Volume—ml/min	control	139	182	184	177	215	181	154
	exposure	138	175	175	172	192	136[d]	134[d]
Elastic Work—g-cm/min	control	359	590	653	605	915	739	589
	exposure	410	621	730	890[d]	961	765	1020[b]
Resistive Work g-cm/min	control	570	891	868	1020	876	880	658
	exposure	609	1148	995	1263[c]	1419[b]	1129	1297[b]
Total Work—g-cm/min	control	929	1481	1521	1625	1791	1619	1247
	exposure	1019	1769	1725	2153[c]	2380[c]	1894	2317[b]

Different from control at $P<$: $a = \cdot001$, $b = \cdot01$, $c = \cdot02$, $d = \cdot05$.

after the exposure ended. In the case of animals exposed to 49 p.p.m., the recovery was not complete in 1 hr, but the resistance had decreased markedly from the values obtained during exposure. The 1 hr post-exposure resistance values for all groups are given in Table 2. Only the 49 p.p.m. group differed statistically from the control resistance values 1 hr after exposure. This indicates that the changes occurred before the level of irreversible change was reached.

While the standard exposure time in these studies was 1 hr, three animals were exposed for 4 hrs to 50 p.p.m. The time course of the resistance is shown in Fig. 2. Quadrupling the exposure time produced no further response; in fact, the resistance during the fourth hour was somewhat lower than during the first hour. This is different from the situation seen with six animals exposed for 3 hrs to 24 p.p.m. sulfur dioxide, in which the resistance continued to increase during the exposure (AMDUR and MEAD, 1955). It is obvious that further work should be done with longer exposures to the various irritants.

As shown in Table 1, formaldehyde also produced a slight decrease in respiratory

rate and a decrease in minute ventilation. These changes, however, did not become statistically significant until a concentration of above 10 p.p.m. was used. At 49 p.p.m. there was a slight increase in tidal volume which was statistically significant. The changes in the mechanical behavior of the lungs on the other hand were detectable at 0·3 ppm.

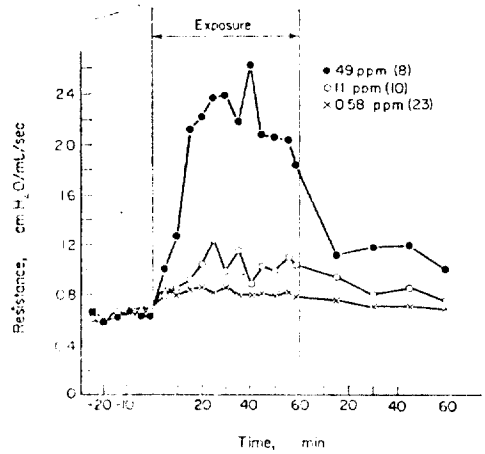

FIG. 1. Time course of the resistance change produced by formaldehyde. The numbers indicate the number of animals in each group.

The data available on resistance, compliance, tidal volume, and frequency permit the estimation of the elastic and resistive work done per minute in ventilating the lungs according to formulae developed by Otis *et al.* (1950). As shown in Table 1, in general, the exposure to formaldehyde produced an increase in respiratory work, the larger portion of which occurred in the resistive component. The increase was not graded with increasing concentration as was the case with the other factors considered. For example, the increase in work was greater at 1·22 and 3·6 p.p.m. than at 11 p.p.m. and the

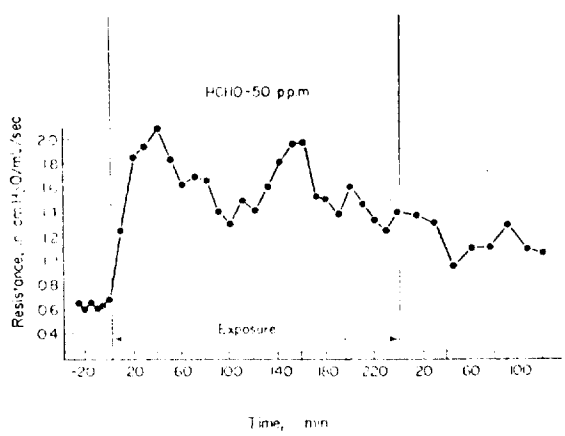

FIG. 2. Time course of resistance change produced in three animals by a four-hour exposure to 50 p.p.m. formaldehyde.

TABLE 2. RESISTANCE VALUES ONE HOUR AFTER END OF EXPOSURE

HCHO								
HCHO P.P.M.		0·05	0·31	0·58	1·22	3·5	11·0	49
Number of Animals		18	13	23	4	10	10	8
Resistance	control	0·71	0·64	0·64	0·80	0·68	0·62	0·65
cm H_2O/ml/sec	1 hr. post exposure	0·82	0·62	0·71	0·93	0·82	0·75	1·00
$\bar{x}$-Post Exposure	control	+0·11	−0·02	+0·07	+0·13	+0·14	+0·13	+0·37
$S\bar{x}$-Standard Error of Difference		0·05	0·056	0·038	0·125	0·080	0·071	0·06[illegible]
Level of Significance $P<$		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	0·01
HCHO—Tracheal Cannula								
HCHO P.P.M.		0·90	5·2	20	50		1·14	3·6
NaCl-mg/m³		—	—	—	—		10	10
Number of Animals		7	7	10	6		8	6
Resistance	control	0·40	0·31	0·44	·048		0·38	0·44
cm H_2O/ml/sec	1 hr. post exposure	0·46	0·41	0·87	1·04		0·98	1·24
$\bar{x}$-Post Exposure	control	+0·06	+0·10	+0·34	+0·56		+0·60	+0·80
$S\bar{x}$-Standard Error of Difference		0·036	0·049	0·213	0·198		0·207	0·196
Level of Significance $P<$		N.S.	N.S.	N.S.	0·05		0·05	0·01
HCOOH								
HCOOH P.P.M.		0·34	1·0	2·8	6·6	13·5	42·5	
Number of Animals		7	8	8	16	8	7	
Resistance	control	0·78	0·71	0·77	0·77	0·81	0·86	
cm H_2O/ml/sec	1 hr. post exposure	0·75	0·94	1·02	1·05	0·98	1·54	
$\bar{x}$-Post Exposure	control	−0·03	+0·23	−0·25	+0·38	+0·17	+0·68	
$S\bar{x}$-Standard Error of Difference		0·090	0·154	0·146	0·077	0·096	0·222	
Level of Significance $P<$		N.S.	N.S.	N.S.	0·01	N.S.	0·02	

changes at the two lower concentrations were statistically significant. At 49 p.p.m. the work had again increased and the changes were again statistically significant. This pattern is explicable when one considers the fact that a decrease in frequency of breathing, which first appears at 11 p.p.m., would tend to lower the minute-work despite the added increase in resistance and decrease in compliance. While the order of magnitude of the changes in respiratory work was not great, such changes have been a consistent finding for all the irritants studied (AMDUR 1958, 1959).

The amount of a given atmospheric concentration of irritant gas which actually reaches the lungs depends to a large extent on the solubility of the gas. In the case of a very soluble gas such as formaldehyde, a considerable amount is removed during passage through the upper respiratory tract. In their classification of irritant gases, HENDERSON and HAGGART (1943) consider the physiological action of formaldehyde to be limited entirely to the upper respiratory tract. The functional lung changes

TABLE 3. RESPONSE OF GUINEA PIGS WITH TRACHEAL CANNULA TO ONE-HOUR EXPOSURE TO FORMALDEHYDE

HCHO-P.P.M.		0·90 ± 0·08	5·2 ± 0·07	20 ± 3·8	50 ± 8	1·14 ± 0·08	3·6 ± 0·5
NaCl—mg/m³		—	—	—	—	10	10
Number of Animals		7	7	10	6	8	6
Resistance	Control	0·40	0·31	0·44	0·48	0·38	0·44
cm H_2O, ml/sec	Exposure	0·66[a]	0·78[b]	1·36[a]	1·75[b]	1·01[b]	1·34[b]
$\bar{x}$-Exposure-Control		+0·26	+0·47	+0·92	+1·27	+0·63	+0·90
$S\bar{x}$-Standard Error of Difference		0·034	0·089	0·120	0·247	0·121	0·184
Compliance	Control	0·31	0·25	0·29	0·28	0·28	0·31
ml/cm/H_2O	Exposure	0·21[b]	0·17[d]	0·17[a]	0·11[a]	0·20[c]	0·19[b]
Tidal Volume	Control	1·43	1·41	1·69	1·46	1·56	1·64
ml	Exposure	1·26	1·41	1·55	1·61	1·50	1·78
Frequency	Control	104	136	103	139	128	129
Breaths/min	Exposure	93	94[c]	75[c]	76[b]	123	69[b]
Minute Volume	Control	146	190	162	195	201	205
ml/min	Exposure	113	125[c]	108[b]	119[b]	176	129[b]
Elastic Work	Control	353	570	470	516	598	542
g-cm/min	Exposure	356	547	504	978[c]	742	715
Resistive Work	Control	387	514	483	771	680	801
g-cm/min	Exposure	449	600	607	1131[d]	1530[b]	904
Total Work	Control	740	1084	953	1286	1278	1344
g-cm/min	Exposure	805	1147	1101	2116[c]	2272[b]	1619

Different from control at P: a = ·001, b = 0·01, c = 0·02, d = 0·05

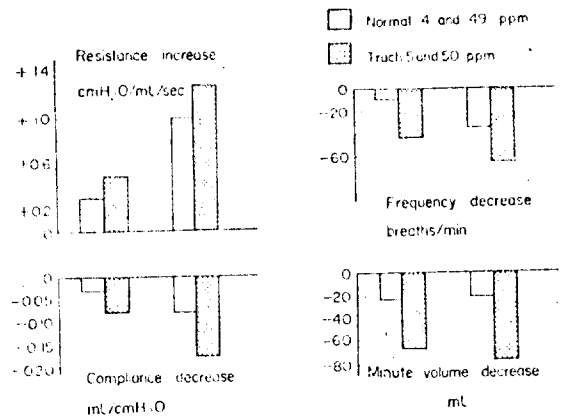

FIG. 3. Comparison of response of normal and tracheotomized animals to formaldehyde.

discussed above indicate, however, that formaldehyde cannot be dismissed as merely an upper respiratory irritant.

As was indicated elsewhere (AMDUR and MEAD 1958, 1955), it is possible to place a small polyethylene cannula in the trachea, thus by-passing the scrubbing effect of the upper airway. Table 3 presents the respiratory response to 1-hr exposures to formaldehyde in tracheotomized animals. The same pattern of changes was present, but the effect at a given concentration was greater when the protection of the upper airway was thus removed. This is illustrated in Fig. 3 in which the changes in resistance, compliance, frequency, and minute volume are compared at two different concentrations of formaldehyde.

The control resistance values for the animals breathing through a tracheal cannula averaged 0·3–0·4 cm H_2O/ml/sec whereas the animals breathing normally had a resistance of 0·6–0·8 cm H_2O/ml/sec. (This difference results from the low resistance of the cannula used compared to the resistance of the nasal passages of the guinea pig.) Thus if the resistance increases were compared in terms of per cent. change, even a slight change in the tracheotomized animals would appear to be greater than the

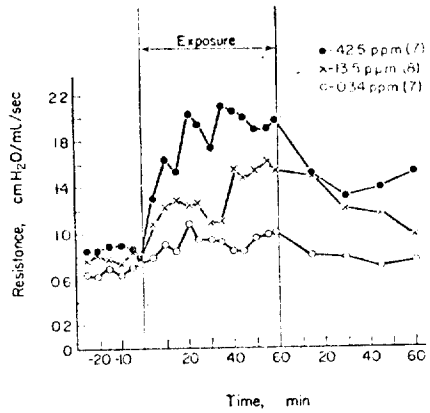

FIG. 4. Time course of the resistance change produced by formic acid.

change in the normal animals. The results shown in Table 3 and Fig. 3, however, indicate that the actual change in absolute terms was greater in the tracheotomized animals.

The 1-hr post-exposure resistance values for the tracheotomized animals are shown in Table 2. Only at the 50 p.p.m. concentration was there any significant difference between the control value and the value an hour after the exposure ended. Even though the changes were greater, they were reversible as were the changes resulting when the gas was breathed through the upper airway.

The respiratory changes produced by a 1-hr exposure to formic acid are shown in Table 4. The pattern of changes was the same as that seen with exposure to formaldehyde. A statistically significant increase in resistance and decrease in compliance were observed at concentrations as low as 0·3 p.p.m., whereas changes in rate and frequency did not appear until the higher concentrations were reached. Fig. 4 shows the time course of the resistance change for 1-hr exposures to 0·34, 13·5, and 42·5 p.p.m. formic acid. At 42·5 p.p.m. the recovery was not complete in 1 hr, but the resistance

TABLE 4. RESPONSE OF GUINEA PIGS TO ONE-HOUR EXPOSURE TO FORMIC ACID

HCOOH-P.P.M.		0·34±0·02	1·0±0·08	2·8±0·15	6·6±0·5	13·5±0·9	42·5±3·8
Number of Animals		7	8	8	16	8	7
Resistance	Control	0·78	0·71	0·77	0·77	0·81	0·86
cm H_2O/ml/sec	Exposure	1·01[b]	1·02[b]	1·28[b]	1·40[a]	1·56[a]	2·08[a]
$\bar{x}$-Exposure-Control		+0·23	+0·31	+0·51	+0·63	+0·75	+1·22
$S\bar{x}$-Standard Error of Difference		0·053	0·059	0·165	0·049	0·100	0·157
Compliance	Control	0·26	0·23	0·24	0·25	0·21	0·20
ml/cm H_2O	Exposure	0·21[d]	0·18[b]	0·19[c]	0·19[a]	0·14[b]	0·13[b]
Tidal Volume	Control	1·85	1·91	1·88	1·84	1·70	1·70
ml	Exposure	1·83	1·71	2·12	1·82	1·82	1·92
Frequency	Control	95	96	101	100	107	100
Breaths/min	Exposure	89	101	90	99	98	58[b]
Minute Volume	Control	176	183	183	184	183	170
ml/min	Exposure	165	172	175	179	173	110[b]
Elastic Work	Control	624	762	744	677	736	722
g-cm/min	Exposure	710	820	1065[c]	974[c]	1160[c]	822
Resistive Work	Control	990	980	1140	1070	1100	1020
g-cm/min	Exposure	1100	1250	1917[b]	1860	2040[b]	1060
Total Work	Control	1614	1742	1884	1747	1836	1742
g-cm/min	Exposure	1810	2070	2982[b]	2834[c]	3200[b]	1882

Different from control at $P<$: a = ·001, b = ·01, c = ·02, d = ·05

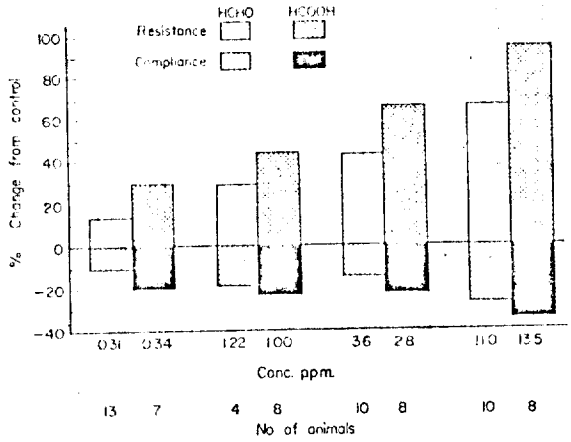

Fig. 5. Comparison of changes in resistance and compliance produced by equal concentrations of formaldehyde and formic acid.

had decreased from exposure values. At the two lower concentrations the resistance had essentially returned to control values. The 1-hr post-exposure resistance values are given in Table 2 for all concentrations. There was no significant difference from the control values except in the case of the 42·5 and 6·6 p.p.m. groups. The fact that the post-exposure resistance values tended to be higher than those for formaldehyde and the fact that the difference was significant at 6·6 p.p.m. (the largest group) suggest that possibly the response to formic acid was reversed somewhat more slowly than that to formaldehyde.

Formic acid is a more potent irritant than formaldehyde. In Fig. 5 the changes produced in resistance and compliance are compared for the groups exposed to essentially equal concentrations of the two irritants. At all concentrations the increase in resistance and decrease in compliance were greater with formic acid than with comparable exposures to formaldehyde. In Fig. 6 the increase in respiratory work is compared at two concentrations. Again the formic acid had produced a greater response than had the corresponding exposure to formaldehyde.

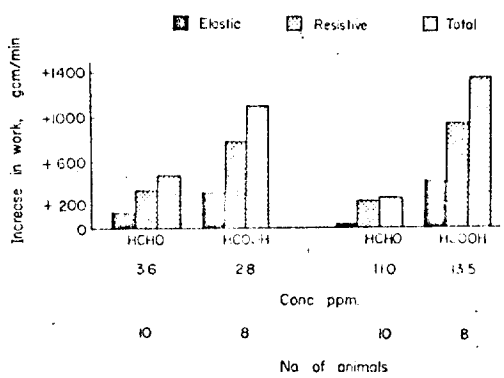

FIG. 6. Comparison of the changes in respiratory work produced by equal concentrations of formaldehyde and formic acid.

(b) Formaldehyde and Formic Acid Plus Aerosol

As was indicated in the introduction, the gas-aerosol studies have so far been limited to one aerosol. Sodium chloride was chosen because it was a known chemically pure compound, presumably devoid of any irritant action when inhaled. A concentration of the order of magnitude of 10 mg/m³ may be considered as the standard concentration since it has been used with all the irritant gases studied. It was possible to hold this concentration constant while varying the gas concentrations over the range desired, hence it was arbitrarily adopted. This concentration is considerably lower than the aerosol concentrations used by LA BELLE *et al.* (1955) which were of the order of magnitude of 2 g/m³ or the 50–100 mg/m³ of smoke used by PATTLE and BURGESS (1957). Formaldehyde was also studied at sodium chloride concentrations of 3 and 30 mg/m³.

In Table 5 are shown the results of 1-hr exposures to 12 and 42 mg/m³ of the sodium chloride aerosol alone. No statistically significant change was observed in resistance,

TABLE 5. DATA FROM ONE-HOUR EXPOSURE OF GUINEA PIGS TO SODIUM CHLORIDE AEROSOL

NaCl—mg/m³		12	42
Number of Animals		7	15
Resistance cm H_2O/ml/sec	Control	0·62	0·63
	Exposure	0·66	0·69
$\bar{x}$ Exposure-Control		+0·04	+0·06
$S\bar{x}$-Standard Error of Difference		0·028	0·039
Compliance ml/cm H_2O	Control	0·24	0·30
	Exposure	0·20	0·27
Tidal Volume ml	Control	2·20	1·99
	Exposure	2·14	1·80[d]
Frequency Breaths/min	Control	84	111
	Exposure	78	109
Minute Volume ml/min	Control	191	218
	Exposure	149	193[d]
Elastic Work g-cm/min	Control	970	749
	Exposure	942	679
Resistance Work g-cm/min	Control	872	1244
	Exposure	805	1189
Total Work g-cm/min	Control	1842	1993
	Exposure	1747	1868

d-Difference significant $P < 0·05$

compliance, frequency, or respiratory work at either concentration. The higher concentration produced a slight decrease in tidal volume and in minute volume which was significant at the 5 per cent level.

The respiratory changes produced by the combination of 10 mg/m³ sodium chloride and concentrations of formaldehyde from 0·07 to 47 p.p.m. are shown in Table 6. In the presence of the aerosol there was a statistically significant increase in resistance even at 0·07 p.p.m. The increase produced by 0·05 p.p.m. formaldehyde alone was 0·05 cm H_2O/ml/sec (47%) and this difference was not statistically significant. In the presence of the aerosol 0·07 p.p.m. formaldehyde produced an increase of 0·14 cm H_2O/ml/sec (+19%). Over the entire concentration range, the resistance change was greater in the presence of the aerosol, a point which will receive further discussion later.

The resistance increase was again accompanied by a decrease in compliance. As with formaldehyde alone, reduction in frequency and minute volume did not appear at

TABLE 6. RESPONSE OF GUINEA PIGS TO ONE-HOUR EXPOSURE TO FORMALDEHYDE +10 mg/m³ NaCl

HCHO-P.P.M.		0·07±0·01	0·32±0·02	0·76±0·27	2·6±0·3	10·8±0·5	27±6	47±13
NaCl—mg/m³		7·5±0·7	11·3±1·6	12·8±2·0	8·7±0·9	10·7±1·7	12·1±2·1	10·0±1·9
Number of Animals		4	8	8	8	8	12	8
Resistance	Control	0·72	0·78	0·85	0·75	0·80	0·81	0·77
cm H_2O/ml/sec	Exposure	0·86[c]	1·10[b]	1·40[a]	1·40[a]	1·70[a]	2·19[a]	2·32[a]
$\bar{x}$-Exposure-Control		+0·14	+0·32	+0·55	+0·65	+0·90	+1·38	+1·55
$S\bar{x}$-Standard Error of Difference		0·030	0·062	0·040	0·060	0·128	0·163	0·169
Compliance	Control	0·21	0·23	0·22	0·25	0·24	0·23	0·23
ml/cm H_2O	Exposure	0·18	0·16[a]	0·15[b]	0·19[b]	0·18[b]	0·19[b]	0·16[b]
Tidal Volume	Control	1·09	1·97	2·08	2·02	2·01	1·91	1·86
ml	Exposure	1·06	1·66	1·95	2·16	2·14	2·34[b]	2·62[b]
Frequency	Control	115	108	95	104	99	103	105
Breaths/min	Exposure	119	115	97	94	80[b]	76[b]	64[b]
Minute Volume	Control	129	211	196	211	195	196	196
ml	Exposure	128	192	181	199	164[a]	176[d]	163[b]
Elastic Work	Control	338	910	948	878	829	829	854
g-cm/min	Exposure	381	998	1252	1164[d]	966	1156	1280[c]
Resistive Work	Control	482	1266	1474	1427	1428	1384	1272
g-cm/min	Exposure	624	1746[d]	1971[d]	2202[d]	1905[c]	2679[b]	2334[b]
Total Work	Control	820	2176	2422	2305	2257	2213	2126
g-cm/min	Exposure	1005	2744[d]	3223[d]	3366[c]	2871[d]	3835[b]	3614[b]

Different from control at $P<$: $a=\cdot001$, $b=\cdot01$, $c=\cdot02$, $d=\cdot05$

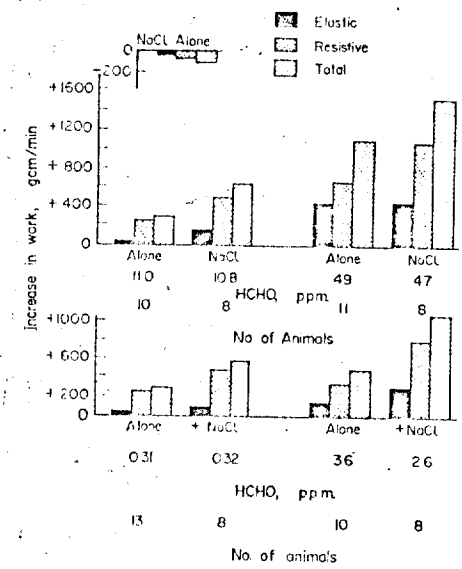

FIG. 7. Comparison of the changes in respiratory work produced by formaldehyde alone and plus 10 mg/m³ sodium chloride.

levels below 10 p.p.m. Changes in respiratory work, however, were statistically significant at concentrations of 0·3 p.p.m. and above in the presence of the aerosol. In Figure 7 the increases in work produced by four equivalent concentrations of formaldehyde alone and plus the aerosol are shown. The response to the combination was greater in every case than the response to the gas alone.

The time course of the resistance change for four of the groups of animals exposed to formaldehyde plus aerosol is shown in Fig. 8. A comparison of these curves with those for the gas alone (Fig. 1) indicates that the initial response is more rapid in the presence of the aerosol. When the exposure was to the gas alone, the resistance had

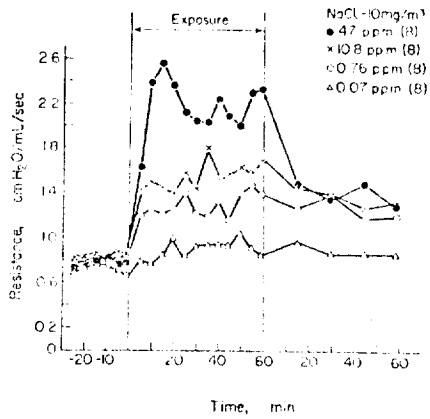

FIG. 8. Time course of the resistance change produced by formaldehyde plus 10 mg/m³ sodium chloride.

returned to control values an hour after the exposure ended with the exception of the group exposed to 49 p.p.m. Although the resistance had decreased from exposure levels, it had not returned to the control values when the aerosol was present. The post-exposure data for all groups are given in Table 7. From 0·32 to 47 p.p.m. these 1-hour post-exposure resistance values were all in the vicinity of 1·2–1·3 cm H_2O/ml/sec which represented an elevation of 40 to 60 per cent over the control values. A delayed recovery has been a consistent finding for all gas-aerosol combinations studied (AMDUR, 1959).

The accumulated evidence presented above for the greater response to formaldehyde in the presence of the aerosol is consistent with the finding of LA BELLE *et al.* (1955) that the toxicity of formaldehyde was enhanced by the presence of inert aerosols and extends it to much lower concentrations of both gas and aerosol. In considering the mechanism of this potentiation, one simple assumption would be that since formaldehyde is a soluble gas normally removed in large part by the moist surfaces of the upper respiratory tract, the sub-micron aerosol which is not thus removed produces its effect simply because more gas reaches the lungs.

TABLE 7. RESISTANCE VALUES ONE HOUR AFTER END OF EXPOSURE

HCHO + NaCl

HCHO-P.P.M.		0·07	0·32	0·76	2·6	10·8	27	47
NaCl—mg/m³		7·5	11·3	12·8	8·7	10·7	12·1	10·0
Number of Animals		4	8	8	8	8	12	8
Resistance	Control	0·72	0·78	0·85	0·75	0·80	0·81	0·77
cm H_2O/ml/sec	1 hr. post exposure	0·86	1·21	1·19	1·29	1·29	1·28	1·29
$\bar{x}$-Post Exposure-control		+0·14	+0·42	+0·34	+0·54	+0·44	+0·47	+0·51
$S\bar{x}$-Standard Error of Difference		0·065	0·088	0·110	0·120	0·112	0·122	0·120
Level of Significance $P<$		N.S.	·01	·02	·01	·01	·01	·01

HCOOH + NaCl

HCOOH-P.P.M.		0·23	1·8	6·4	28·2
NaCl—mg/m³		8·7	10·2	12·5	11·2
Number of Animals		6	7	10	11
Resistance	Control	0·71	0·64	0·63	0·72
cm H_2O/ml/sec	1 hr. post exposure	1·00	0·83	0·94	0·94
$\bar{x}$-Post Exposure-Control		0·29	0·19	0·31	0·23
$S\bar{x}$-Standard Error of Difference		0·091	0·045	0·089	0·074
Level of Significance $P<$		0·05	0·01	0·01	0·02

The degree of potentiation observed, however, cannot be accounted for on this basic alone since the actual amount of additional gas reaching the lungs carried on the aerosol would indeed be very slight. Another possibility is that gas reaching the lung surface carried on the aerosol, thus setting up high local concentrations of irritant, produces a far greater response than a similar increment in the amount of free gas molecules inhaled. The potentiation thus would not result from an increased amount of irritant *per se*, but from a different manner of administration of even a minute amount of irritant. Such a suggestion was made by VORWALD (1952) as purely a speculation. The data presented here support such an interpretation of the biological mechanism of the potentiation.

It thus becomes of interest to compare the response obtained in animals breathing the gas-aerosol combination with the response obtained when the scrubbing effect of the upper airway was by-passed with a tracheal cannula. The data for these two situations are presented in Tables 3 and 6. In Fig. 9 the dose-response curves are shown for formaldehyde alone, formaldehyde alone breathed through a tracheal cannula, and

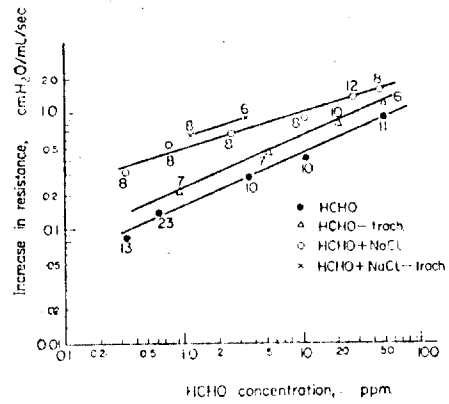

FIG. 9. Dose-response curves for formaldehyde alone and plus 10 mg/m³ sodium chloride in normal and tracheotomized animals. The increase in resistance at the end of 1-hour exposure is the criterion of response and the concentration is expressed as p.p.m.

formaldehyde plus 10 mg/m³ sodium chloride, plus the response of two groups of animals breathing the combination through a cannula. These curves were obtained by plotting the increase in resistance produced by a 1-hour exposure against the concentration of formaldehyde expressed in p.p.m. At all concentrations tested the response to the gas breathed through the tracheal cannula was greater than that to the gas breathed normally, as was indicated above. The response, however, was not as great as that produced by the gas-aerosol combination. Furthermore, the curves for the gas alone breathed normally and breathed through the cannula are parallel while the addition of aerosol has shifted the slope of the dose-response curve. Throughout the entire range a given concentration of formaldehyde breathed through a tracheal cannula produced the same response as 2·4 times that concentration breathed normally, i.e. 1 p.p.m. = 2·4 p.p.m. and 20 p.p.m. = 48 p.p.m. In the case of the gas-aerosol combination, this ratio increased as lower concentrations were approached, i.e. at

10 p.p.m. formaldehyde plus aerosol the response was equivalent to 50 p.p.m. formaldehyde alone, but at one p.p.m. formaldehyde plus aerosol the response was equivalent to 12 rather than to 5 p.p.m. formaldehyde alone. Such a shift in slope of the dose-response curve would be produced by a definite increment in response, related to the irritant carrying aerosol, and constant throughout the concentration range.

Further evidence that the response to the gas-aerosol combination represents the effect of an irritant aerosol superimposed upon the response to the gas was obtained from the animals exposed to the combination breathed through a tracheal cannula. A sub-micron sized aerosol such as the one used in these studies would not be removed in the upper respiratory tract, hence the response to the irritant-carrying aerosol should be the same in normal and tracheotomized animals. This being the case, the only effect of the cannula on the combination of gas and aerosol would be to increase the amount of the gas reaching the lungs. The increase in resistance produced by 3·5 p.p.m. formaldehyde plus aerosol breathed through the tracheal cannula was 0·90 cm H_2O/ml/sec and that to the same mixture breathed normally was 0·76 cm H_2O/ml/sec, increment of 0·14. The response to 3·5 p.p.m. of formaldehyde alone breathed through the cannula was +0·44 cm H_2O/ml/sec and the corresponding increase in the normal animals was +0·30 cm H_2O/ml/sec, again an increment of 0·14.

If, as evidenced above, the potentiation is related to the aerosol entity as such, then increasing or decreasing the aerosol concentration would increase or decrease the increment produced by the aerosol. In Fig. 10 are shown the dose-response curves for formaldehyde alone and in the presence of three levels of sodium chloride. (The

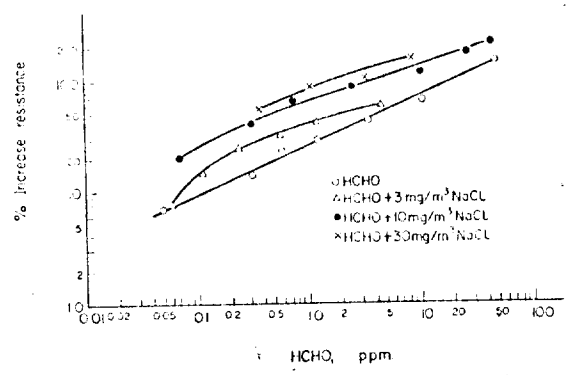

FIG. 10. Dose-response curves for formaldehyde alone and plus 3, 10, and 30 mg/m³ sodium chloride. The per cent increase in resistance at the end of the 1-hour exposure is the criterion of response and the concentration is expressed as p.p.m.

detailed data on resistance values for formaldehyde plus 3 and 30 mg/m³ sodium chloride are given in Table 8). These curves were obtained by plotting the per cent increase of the resistance over the control values against the concentration of formaldehyde. With the exception of the exposures to 0·05 p.p.m. alone and 0·06 p.p.m. plus 3 mg/m³ sodium chloride, all changes were statistically significant. As the concentration of aerosol was increased at a given formaldehyde concentration, the response also increased.

Further study of the curves indicates that for each level of aerosol concentration, the increment added by the aerosol attains an essentially constant value. This point is

illustrated in Fig. 11, which shows the *difference* in response between the gas plus aerosol and the gas alone for the three different aerosol levels. For example, a concentration of 0·07 p.p.m. formaldehyde plus 10 mg/m³ sodium chloride gave a change from control resistance of +20 per cent, the corresponding value for 0·07 p.p.m. formaldehyde alone read from the curve in Fig. 10 is +8 per cent; the difference 20 − 8 = 12 appears in Fig. 11. When the aerosol level was 3 mg/m³, the difference

TABLE 8. RESISTANCE INCREASE PRODUCED BY ONE-HOUR EXPOSURE

HCHO + 3 mg/m³ NaCl

HCHO-P.P.M.		0·06 ± 0·01	0·11 ± 0·02	0·24 ± 0·07	0·58 ± 0·09	1·2 ± 0·2	4·8 ± 0·6
NaCl – mg/m³		3·5 ± 0·2	3·9 ± 0·4	4·3 ± 1·3	3·0 ± 0·4	3·5 ± 0·2	4·1 ± 0·6
Number of Animals		13	13	10	7	6	5
Resistance	Control	0·64	0·62	0·67	0·76	0·69	0·69
cm H_2O/ml/sec	Exposure	0·69	0·72[b]	0·84[a]	1·02[b]	0·97[b]	1·09[b]
$\bar{x}$-Exposure-Control		+0·05	+0·10	+0·17	+0·26	+0·28	+0·40
$S\bar{x}$ Standard Error of Difference		0·028	0·025	0·029	0·049	0·046	0·075

HCHO + 30 mg/m³ NaCl

HCHO-P.P.M.		0·34 ± 0·08	1·01 ± 0·12	3·5 ± 0·5	9·6 ± 1·2
NaCl – mg/m³		35 ± 8	22 ± 1	26 ± 3	30 ± 2
Number of Animals		8	7	15	7
Resistance	Control	0·63	0·63	0·70	0·72
cm H_2O/ml/sec	Exposure	0·98[a]	1·17[b]	1·34[a]	1·70[a]
$\bar{x}$-Exposure-Control		+0·35	+0·54	+0·64	+0·98
$S\bar{x}$-Standard Error of Difference		0·048	0·108	0·084	0·128

HCOOH + 10 mg/m³ NaCl

HCOOH-P.P.M.		0·23 ± 0·02	1·8 ± 0·09	6·4 ± 0·4	28·2 ± 3·0
NaCl – mg/m³		8·7 ± 0·7	10·2 ± 0·9	12·5 ± 1·1	11·2 ± 0·8
Number of Animals		6	7	10	11
Resistance	Control	0·71	0·64	0·63	0·72
cm H_2O/ml/sec	Exposure	0·97[a]	1·11[a]	1·19[a]	1·58[a]
$\bar{x}$-Exposure-Control		+0·26	+0·47	+0·56	+0·86
$S\bar{x}$-Standard Error of Difference		0·075	0·038	0·066	0·113

Different from Control at $P<$: $a = 0·001$, $b = 0·01$, $c = 0·02$, $d = 0·05$

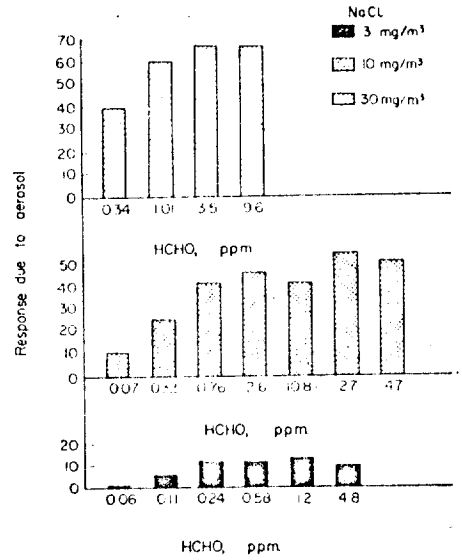

FIG. 11. Increment in response to formaldehyde produced by presence of 3, 10, and 30 mg/m³ sodium chloride.

reached a constant value of about 10. With 10 mg/m³ this value was 40–50 and with 30 mg/m³ it is 60–65. The difference had reached its plateau for 3 mg/m³ aerosol at a formaldehyde concentration of 0·24 p.p.m., but with 10 mg/m³ the difference had not become constant until 0·76 p.p.m. formaldehyde was used.

The response to formic acid in the presence of 10 mg/m³ sodium chloride was also studied. The dose response curves for formic acid alone and plus the aerosol are given in Fig. 12 and the detailed resistance data for the gas plus aerosol are given in Table 8. This aerosol concentration, which had a marked potentiating effect on formaldehyde, produced essentially no change in the degree of response to formic acid.

The recovery was, however, delayed in animals receiving the combination of formic

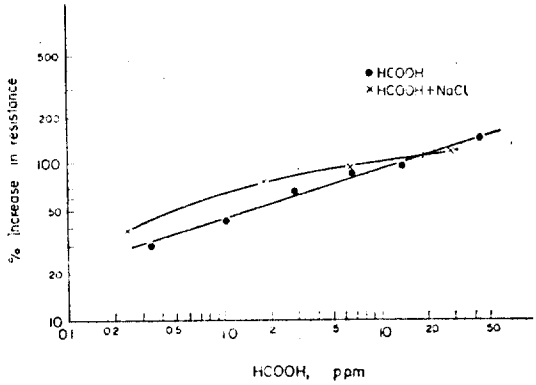

FIG. 12. Dose-response curves for formic acid alone and plus 10 mg/m³ sodium chloride. The per cent increase in resistance at the end of 1-hour exposure is the criterion of response and the concentration is expressed as p.p.m.

acid and aerosol as was shown in Fig. 13. At all four concentrations the 1-hour post-exposure resistance was higher than the control values and these changes were statistically significant as is shown in Table 7. When the vapor was used alone (Fig. 4 and Table 2) the resistance had returned to control values 1 hour after the exposure except

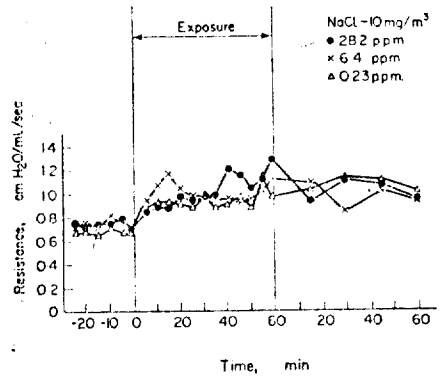

FIG. 13. Time course of resistance change produced by formic acid plus 10 mg/m³ sodium chloride.

in the case of the higher concentrations. The prolongation of the response has been observed with all four gas-aerosol combinations tested.

The bioassay technique thus has yielded the following information:

(1) The response to formaldehyde is potentiated by the presence of an aerosol of sodium chloride, which is itself inert.
(2) The potentiation appears to be mediated through the formation of an irritant aerosol entity.
(3) The effect increases with increasing aerosol concentration.
(4) For a given sodium chloride concentration, however, a maximum value is attained for the increment in response added by the aerosol.
(5) Similar results are not obtained when the aerosol is administered with formic acid.
(6) In the case of both formaldehyde and formic acid the presence of the aerosol prolongs the response.

These biological findings suggest that a portion of the formaldehyde becomes associated with the aerosol particles and that at a certain point for each aerosol concentration this association reaches a maximum beyond which increasing the formaldehyde concentration produces no further increase in the amount of irritant aerosol produced. The formic acid-aerosol system does not follow this pattern of behavior. If the observed biological response can be correlated with physical theories governing the behavior of such gas-aerosol systems, such a dual approach should aid in the over-all understanding of the phenomenon observed. The subsequent paper by GOETZ (1960) presents a physical theory which we believe to be adequate to relate the biological effects presented in this paper to the kinetics of the gas-aerosol system.

REFERENCES

AMDUR M. O. (1957) *Amer. Industr. Hyg. Assoc. Quart.* **18**, 149.
AMDUR M. O. (1959) *Int. J. Air Poll.* **1**, 170

AMDUR M. O. and J. MEAD (1955) *Proc. 3rd Nat. Air Pollution Symposium* p. 150 Pasadena, California.
AMDUR M. O. and J. MEAD (1958) *Amer. J. Physiol.* **192**, 364.
DELAUNOIS A. L., L. DAUTREBANDE and C. HEYMANS (1956) *Arch. Int. Pharmacodyn.* **108**, 238.
ELKINS H. B. (1950) *Chemistry of Industrial Toxicology* p. 316, John Wiley New York.
GOLTZ A. (1960) *Int. J. Air Poll.* (In press.)
HENDERSON Y. and HAGGARD H. W. (1943) *Noxious Gases* 2nd ed. p. 128, Reinhold, New York.
LABELLE C. W., LONG J. M. and CHRISTOFANO E. E. (1955) *Arch. Indust. Health* **11**, 297.
MACDONALD W. E. (1954) *Amer. Industr. Hyg. Assoc. Quart.* **19**, 196.
OTIS A. B., FENN W. O. and RAHN (1950) *J. Appl. Physiol.* **2**, 952.
PATTLE R. E. and BURGESS F. (1957) *J. Path and Bact.* **73**, 411.
VORWALD A. J. (1952) *Proc. U.S. Technical Conference on Air Pollution*, p. 485, McGraw Hill, New York.

THRESHOLD LIMIT VALUES FOR 1955

Adopted at the Seventeenth Annual Meeting of the American Conference of Governmental Industrial Hygienists, Buffalo, April 24-28, 1955

VALUES are given in the following tabulation for the maximum average atmospheric concentration of contaminants to which workers may be exposed for an eight-hour working day without injury to health.

These values are based on the best available information from industrial experience, from experimental studies, and, when possible, from a combination of the two. They are not fixed values but are reviewed annually by the Committee on Threshold Limits for changes, revisions, or additions as further information becomes available. Threshold limits should be used as guides in the control of health hazards and should not be regarded as fine lines between safe and dangerous concentrations. They represent conditions only within which it is felt that workers may be repeatedly exposed, day after day, without their health being adversely affected. It is felt, at the present time, that workers should not be exposed to a working environment containing any of these substances in excess of the value indicated.

These values are not intended for use, or for modification for use, in the evaluation or control of community air pollution or air pollution nuisances.

ESTABLISHED VALUES

GASES AND VAPORS

SUBSTANCE	PPM*	SUBSTANCE	PPM*
Acetaldehyde	200	Carbon dioxide	5,000
Acetic acid	10	Carbon disulfide	20
Acetic anhydride	5	Carbon monoxide	100
Acetone	1,000	Carbon tetrachloride	25
Acrolein	0.5	Cellosolve (2-ethoxyethanol)	200
Acrylonitrile	20	Cellosolve acetate (hydroxyethyl acetate)	100
Ammonia	100	Chlorine	1
Amyl acetate	200	Chlorobenzene (monochloro [illegible])	75
Amyl alcohol (isoamyl alcohol)	100	[illegible]	[illegible]
Aniline	5	Chloroform (trichloromethane)	[illegible]
Arsine	0.05	1-Chloro-1-nitropropane	20
Benzene (benzol)	35	Chloroprene (2-chlorobutadiene)	25
Bromine	1	[illegible]	5
Butadiene (1,3-butadiene)	1,000	Cyclohexane	400
Butanone (methyl ethyl ketone)	250	Cyclohexanol	100
Butyl acetate (n-butyl acetate)	200	Cyclohexanone	100
Butyl alcohol (n-butanol)	100	Cyclohexene	[illegible]
Butyl cellosolve (2-butoxyethanol)	200		

* Parts [illegible] per million parts of [illegible]

SUBSTANCE	PPM*
Cyclopropane	400
o-Dichlorobenzene	50
Dichlorodifluoromethane	1,000
1,1-Dichloroethane	100
1,2-Dichloroethylene	200
Dichloroethyl ether	15
Dichloromonofluoromethane	1,000
1,1-Dichloro-1-nitroethane	10
Dichlorotetrafluoroethane	1,000
Diethylamine	25
Dimethylaniline (N-dimethylaniline)	5
Dimethylsulfate	1
Dioxane (diethylene dioxide)	100
Ethyl acetate	400
Ethyl alcohol (ethanol)	1,000
Ethylamine	25
Ethyl benzene	200
Ethyl bromide	200
Ethyl chloride	1,000
Ethyl ether	400
Ethyl formate	100
Ethyl silicate	100
Ethylene chlorohydrin	5
Ethylene dibromide (1,2-dibromoethane)	25
Ethylene dichloride (1,2-dichloroethane)	200
Ethylene oxide	100
Fluorine	0.1
Fluorotrichloromethane	1,000
Formaldehyde	5
Gasoline	500
Heptane (n-heptane)	500
Hexane (n-hexane)	500
Hexanone (methyl butyl ketone)	100
[illegible] (methyl isobutyl ketone)	100
[illegible]	5
[illegible]	10
Hydrogen bromide	3
Hydrogen selenide	0.05
[illegible]	20
[illegible]	1
[illegible]	25
[illegible]	50
Methyl acetate	200
Methyl alcohol (methanol)	[illegible]
[illegible]	20
[illegible] solvent (carbon [illegible]	25
Methyl cellosolve acetate (ethylene glycol monomethyl ether acetate)	25
Methyl chloride	100
Methylal (dimethoxymethane)	1,000
Methyl chloroform (1,1,1-trichloroethane)	500
Methylcyclohexane	500
Methylcyclohexanol	100
Methylcyclohexanone	100
Methyl formate	100
Methylene chloride (dichloromethane)	500
Naphtha (coal tar)	200
Naphtha (petroleum)	500
Nickel carbonyl	0.001
Nitrobenzene	1
Nitroethane	100
Nitrogen dioxide	5
Nitroglycerin	0.5
Nitromethane	100
2-Nitropropane	50
Nitrotoluene	5
Octane	500
Ozone	0.1
Pentane	1,000
Pentanone (methyl propyl ketone)	200
Perchlorethylene (tetrachloroethylene)	200
Phenol	5
Phosgene (carbonyl chloride)	1
Phosphine	0.05
Phosphorus trichloride	0.5
Propyl acetate	200
Propyl alcohol (isopropyl alcohol)	400
Propyl ether (isopropyl ether)	500
Propylene dichloride (1,2-dichloropropane)	75
Sprays	0.1
Stoddard solvent	500
Styrene monomer (phenyl ethylene)	200
Sulfur monochloride	1
Sulfur dioxide	10
1,1,2,2-Tetrachloroethane	5
Toluene	200
o-Toluidine	5
Trichloroethylene	200
Turpentine	200
Vinyl chloride (chloroethene)	500
Xylene	200

THRESHOLD LIMIT VALUES

TOXIC [illegible]

SUBSTANCE	MG. PER CU. M.†
Antimony	0.5
Arsenic	0.5
Barium (soluble compounds)	0.5
Cadmium	0.1
Chlorodiphenyl	1
Chromic acid and chromates as CrO_3	0.1
Cyanide as CN	[illegible]
Dinitrotoluene	1.5
Dinitro-o-cresol	0.2
Fluoride	2.5
Iron oxide fume	[illegible]
Lead	0.15
Magnesium oxide fume	15
Manganese	6
Mercury	0.1
Parathion (O,O-Diethyl O-*p*-nitrophenyl thiophosphate)	0.1
Pentachloronaphthalene	0.5
Pentachlorophenol	0.5
Phosphorus (yellow)	0.1
Phosphorus pentachloride	1
Phosphorus pentasulfide	1

SUBSTANCE	MG. PER CU. M.†
[illegible]	0.1
Sulfur [illegible]	[illegible]
Tellurium	0.1
Tetryl (2,4,6-trinitrophenyl [illegible])	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
Trinitrotoluene	1.5
Uranium (soluble compounds)	0.05
Uranium [illegible]	0.25
Zinc oxide fumes	15

[illegible] "Maximum Permissible Concentration in Air and Water," Handbook 52, [illegible] Natural Bureau of Standards, March 1953, [illegible] Department of Commerce, [illegible] Bureau of Standards, Sept. 24, 1954.

† Milligrams of dust, fume, or mist [illegible]

MINERAL DUSTS

SUBSTANCE	MPPCF‡
Alundum (aluminum oxide)	50
Asbestos	5
Carborundum (silicon carbide)	50
Dust (nuisance, no free silica)	50
Mica (below 5% free silica)	20
Portland cement	50
Talc	20

SUBSTANCE	MPPCF‡
Silica	
High (above 50% free SiO_2)	5
medium (5 to 50% free SiO_2)	20
Low (below 5% free SiO_2)	50
Slate (below 5% free SiO_2)	50
Soapstone (below 5% free SiO_2)	20
Total dust (below 5% free SiO_2)	50

‡ Millions of particles per cubic foot of air.

TENTATIVE THRESHOLD LIMIT VALUES

The following values are suggested for further consideration before being presented for adoption [illegible] materials added this year and for which bibliographic [illegible] been prepared. The other materials appeared in [illegible] that the entire [illegible] will be presented for adoption at the meeting of the American [illegible] Governmental Industrial Hygienists in [illegible], if no reason to the contrary is forthcoming.

[illegible]

[illegible]	10	mg/M³
[illegible]	[illegible]	mg/M³
[illegible] 1,1,1-trichloroethane	20	ppm
[illegible]	0.1	ppm
§ [illegible]		
[illegible]	[illegible]	mg/M³
[illegible]	100	ppm
§ [illegible]	20	ppm
[illegible]	0	mg/M³
[illegible]	[illegible]	ppm
[illegible]	10	ppm
§ [illegible]	[illegible]	ppm
§ [illegible]	1	mg/M³
§ [illegible]	2	ppm
[illegible]	[illegible]	ppm
§ [illegible]	1	ppm
§ [illegible]	5	ppm
§ [illegible]	1	ppm
§ [illegible]	2	mg/M³
§ [illegible]	5	ppm
§ [illegible]	0.2	mg/M³
§ [illegible]	0.5	mg/M³
[illegible]	15	mg/M³
[illegible]	15	mg/M³
Methoxychlor [illegible] methoxyphenyl-1,1,1-trichloroethane	1,000	ppm
§ Methyl acetylene	25	ppm
Methyl bis(2-hydroxyethyl) carbinol (methyl vinyl alcohol)	50	ppm
Methyl [illegible]		
Molybdenum	5	mg/M³
§ [illegible]	15	mg/M³
§ [illegible]	1	ppm
[illegible]	0.01	mg/M³
Organic [illegible]	0.1	ppm
Pentachloro [illegible]	5	ppm
Phosphorus [illegible]	0.1	mg/M³
Propylene [illegible]	25	ppm
§ [illegible]	10	ppm
Ex [illegible]	0.1	ppm
§ [illegible]	2	mg/M³
[illegible]	1,000	ppm
[illegible]	0.025	ppm
§ [illegible]	0.2	mg/M³
[illegible] pyrophosphate	0.05	mg/M³
[illegible]	10	ppm
§ [illegible]	75	ppm
§ [illegible]	1	ppm
§ [illegible]	15	mg/M³
[illegible]	1,000	ppm
§ [illegible]		
[illegible]	0.5	mg/M³
[illegible]	0.1	mg/M³
[illegible]	5	mg/M³

§ [illegible] the literature which report a limit [illegible] Berylliosis, [illegible] Van Ordstrand, H. S.; [illegible] Sterner, J. H., and Eisenbud, M.: [illegible] **10**: [illegible] **1**:[illegible] (Aug.) 1951. Confirming [illegible] Threshold Limit [illegible] for the establishment of a definite value.

Reference material has been prepared on each of the above substances and, though in [illegible] substances rather meager, is available for distribution. The committee would welcome suggestions of substances to be added and also comments, additional references, or reports of experience with these materials.

William L. Bale	A. J. Vorwald
L. T. Fairhall	Louis F. Weller
Kingsley Kay	Allan L. Coleman, *Chairman*
H. F. Smyth	

Anon. 1963
Standard Methods of Chemical Analysis
D. Van Nostrand Co., Inc., Princeton, New Jersey
2: 588-590

21

Food and Drugs

PARTS 10 TO 129

Revised as of April 1, 1973

CONTAINING
A CODIFICATION OF DOCUMENTS
OF GENERAL APPLICABILITY
AND FUTURE EFFECT

AS OF APRIL 1, 1973

With Ancillaries

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

as a Special Edition of
the Federal Register

Food and Drugs

TYLOSIN IN ANIMAL FEED—Continued

Principal ingredient	Grams per ton	Combined with—	Grams per ton	Limitations	Indications for use
7. Tylosin	500-1,000			For broiler chickens; as tylosin phosphate; withdraw 5 days before slaughter; administer in feed to chickens 0 to 5 days of age, follow with second administration in feed for 24-48 hours at 3 to 5 weeks of age.	To aid in the control of chronic respiratory disease caused by *Mycoplasma gallisepticum*.
8. Tylosin	20-50			For laying chickens.	Improving feed efficiency.
9. Tylosin	1,000			For replacement chickens; as tylosin phosphate; withdraw 5 days before slaughter; administer in feed to chickens 0 to 5 days of age, follow with second administration in feed for 24 to 48 hours at 3 to 5 weeks of age.	To aid in the control of chronic respiratory disease caused by *Mycoplasma gallisepticum*.

(e) To assure safe use, the label and labeling of the additive, any combination of additives, and any feed additive supplement, feed additive concentrate, feed additive premix, or complete feed prepared therefrom shall bear, in addition to the other information required by the act, the following:

(1) The name of the additive or additives.

(2) A statement of the quantity or quantities of each contained therein.

(3) Adequate directions and warnings for use.

(f) Section 121.1049 establishes the limitations for residues of the additive in food for human consumption.

(Sec. 512(i), 82 Stat. 347; 21 U.S.C. 360b(i))

[30 F.R. 15845, Dec. 23, 1965, as amended at 31 F.R. 2834, Feb. 17, 1966; 31 F.R. 6274, Apr. 23, 1966; 34 F.R. 5929, Mar. 29, 1969; 35 F.R. 5220, Mar. 28, 1970; 35 F.R. 16630, Oct. 27, 1970; 36 F.R. 7647, 7650, Apr. 23, 1971; 36 F.R. 24214, Dec. 22, 1971; 37 F.R. 17709, Aug. 30, 1972]

§ 121.218 Diuron.

A tolerance of 4 parts per million is established for residues of diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) in dried citrus pulp for livestock feed when present therein as a result of the application of the pesticide in the production of citrus fruits.

§ 121.219 Formic acid.

Formic acid may be safely used as a preservative in hay crop silage in an amount not to exceed 2.25 percent of the silage on a dry weight basis or 0.45 percent when direct-cut. The top foot of silage stored should not contain formic acid and silage should not be fed to livestock within 4 weeks of treatment.

[37 F.R. 5816, Mar. 22, 1972]

§ 121.220 Nystatin.

The food additive nystatin may be safely used in animal feed in accordance with the following prescribed conditions:

(a) Nystatin is the antibiotic substance produced by growth of Streptomyces noursei (Fam. Actinomycetaceae) or the same antibiotic substance produced by any other means, and for the purpose of this section refers to nystatin or feed-grade nystatin.

(b) The antibiotic activities authorized are expressed in this section in terms of the weight of the appropriate antibiotic standard, except that 1 gram of nystatin is equivalent to 2,800,000 units of activity.

(c) Permitted uses of nystatin alone and with certain other additives in medicated feeds are described in tabular form in this section. These tables are to be read as follows:

(1) The numbered line items establish the required limitations and indications for use of the principal ingredient as the medicament alone, or with another ingredient added.

(2) The lettered line items establish the required limitations and indications for use of secondary ingredients that may be added to the principal ingredient. Where principal and secondary ingredients have been mixed, the applicable "limitations" and "indications for use" from both the numbered items and lettered items apply. If duplicated limitations occur, these may be appropriately combined.

(3) Permitted combinations of principal ingredient and secondary ingredients are individually listed. Unless specifically provided by the regulations, the principal ingredient may not be mixed with one or more secondary ingredients.

Association of Official Agricultural Chemists. 1960
Methods of Analysis of the AOAC, 9th edition
AOAC, Washington, D.C.
pp. 171-172, 236-238

Association of Official Agricultural Chemists. 1965
Methods of Analysis of the AOAC, 10th edition
AOAC, Washington, D.C.
pp. 205-206, 274-279

Acta pharmacol. 1947, 3, 312—322.

From the Department of Biochemistry, University of Aarhus, Denmark.
(Professor Fritz Schönheyder, M.D.).

On the Excretion of Formic Acid in Experimental Poisoning with Methyl Alcohol.

By

J. Th. Bastrup.

(Received June 12, 1947).

The object of the present paper has been to extend knowledge of the conversion of methyl alcohol to formic acid in rabbits and dogs. It is shown that in rabbits formic acid excretion is smaller in relation to the methyl alcohol administered than in dogs. As rabbits are able to burn considerable amounts of formic acid, the percentage excretion of formic acid is hardly an expression of the fraction of methyl alcohol converted to formic acid. A few experiments have confirmed ASSER's finding (1914) that ethyl alcohol exerts a very great inhibition on the excretion of formic acid in urine after administration of methyl alcohol.

Although the first experiments on the excretion of formic acid in animals poisoned with methyl alcohol were carried out as long ago as 1893 (POHL), only few authors have been engaged on this problem. POHL (1893), ASSER (1914) and BENEDICT and HARROP (1922) found a very marked rise in formic acid excretion in dogs after the administration of methyl alcohol. ROST and BRAUN (1925) carried out experiments on cats and found similar conditions. ASSER (1914) also investigated the excretion of formic acid in rabbits but found that the rise after administration of methyl alcohol to these animals was very capricious and was sometimes completely absent.

Technique.

In order to obtain the total amount of urine excreted, the rabbits were placed in metabolic cages with wire gauze bottom. Below this was a large funnel for collecting the urine. Dog's urine was collected by a permanent catheter, which was attached to a rubber-bladder. The bladder was kept in place under the abdomen of the dog by means of a leather bandage. The dogs soon got used to the bandage and apparently were not appreciably inconvenienced.

The methyl alcohol was administered by stomach tube. Determinations of methyl alcohol in urine were carried out in basic solution as described by WIDMARK (1932).

Formic acid was determined by the technique described by BASTRUP (1947). In some instances the formic acid was also determined by the MACFADYEN specific methods of DROLLER (1932) and MACFADYEN (1945).

Own Investigations.

The animals used in the experiments were mainly rabbits. In addition, three experiments on dogs were carried out.

A. Normal Formic Acid Excretion in Rabbits and Dogs.

Analyses were first carried out on the urine from normal rabbits on a diet consisting only of turnips, and on dogs on a diet consisting of black bread and butter. The results of those experiments are to be seen in Table 1.

It is seen that the excretion of formic acid in urine varies very much from one individual to the other. In the following experiments a correction for normal excretion of formic acid (i.e. no additional administration) is introduced, the individual value found for the animal immediately before the administration of methyl alcohol being used.

B. Formic Acid and Methyl Alcohol Excretion in Rabbits after Administration of Methyl Alcohol.

Eight experiments were carried out with rabbits receiving increasing amounts of methyl alcohol. The results are given in Table 2. In each animal the formic acid excretion was followed until normal values in the urine were observed. The total amounts excreted minus the normal average excretion of formic acid come

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Table 1.

The Normal Excretion of Formic Acid in the Urine of Rabbits and Dogs.

Animal		Period hours	Urine ml	HCOOH mg %	HCOOH mg	HCOOH mg per 24 hours
Rabbit	S	36	750	0.28	2.10	1.40
	-	24	500	0.30	1.50	1.50
	H	14	155	1.15	1.84	3.15
	-	17	260	1.50	3.90	5.50
	R_1	16½	250	0.70	1.75	2.54
	-	24	230	1.45	2.33	2.33
	R_2	16	250	0.90	2.25	3.37
	-	28	400	1.15	4.60	3.95
	R_3	16	230	2.60	5.97	8.95
	-	16	280	1.00	2.80	4.20
	R_4	17	270	3.70	10.0	14.1
Dog	D_1	11	100	22.4	22.4	49.0
	D_2	22	180	2.9	5.2	5.8
	D_3	13	160	2.0	3.2	6.0

from the methyl alcohol administered. One of the experiments will be described in detail.

Experiment 1. (R_2, weight 2560 g), 6 g methyl alcohol per kg body weight. On the first day the rabbit was lying on its side, but now and then stumbled about a little in the cage. It did not eat. In the course of the 2nd day it apparently became completely normal and ate well. The formic acid excretion in the urine is shown in Fig. 1.

C. Formic Acid Excretion in Rabbits after Administration of Methyl Alcohol + Ethyl Alcohol.

One experiment is recorded for the elucidation of the effect of ethyl alcohol upon the formic acid excretion in rabbits poisoned with methyl alcohol. It was necessary to give the ethyl alcohol in several doses because it is eliminated about 6 times as fast as methyl alcohol.

Experiment 9. (R_2, weight 2470 g), 6 g methyl alcohol + 2 g ethyl alcohol per kg body weight were given at the beginning of the experi-

Table 2.

Formic Acid and Methyl Alcohol in Urine after Administration Varying Amounts of Methyl Alcohol to Rabbits.

In column 4 is given the amounts of methyl alcohol administered in g per kg body weight; in column 5 the time of excretion in hours; in column 6 the total excretion of formic acid during the time of excretion in column 7; the normal excretion of formic acid in the same interval calculated from the normal value for the corresponding period; in column 8 is given the difference between total and normal excretion of formic acid; in column 9 the same value computed in mg per kg body weight; in column 10 is given the percentage of methyl alcohol excreted in the urine as formic acid; column 11 shows the total amount of methyl alcohol excreted in urine; column 12 gives the excretion of methyl alcohol as percentage of methyl alcohol given.

Exp. No.	Rabbit mark	Weight kg	CH_3OH g/kg	Time hours	Excretion of Formic Acid: total mg	normal mg	net mg	net mg/kg	% of HCOOH corr. to CH_3OH	Total Excret. of Methyl Alcohol: CH_3OH g	% of CH_3OH admin.
1	S	2.35	2	61½	19.6	3.7	15.9	7	0.24		
2	H	2.85	2	61½	16.9	10.5	6.4	3	0.11	0.82	14.0
3	R_1	2.63	4	60½	36.0	6.1	29.9	11	0.20	1.62	15.4
7	S	2.35	5	75	73.5	4.5	69.0	29	0.49	2.33	19.8
4	R_2	2.56	6	79½	232.7	12.1	220.6	86	1.00	3.21	26.9
8	H	2.75	7	142½	174.4	25.6	148.8	53	0.53	2.60	13.2
5	R_3	2.45	8	78	314.9	21.4	293.5	119	1.04	3.37	17.2
6	R_4	2.35	10	47	391.5	27.4	363.8	155	1.08	3.28	14.0*)

*) The rabbit died with methyl alcohol in the blood (about 5 p. p. m.) and would therefore have excreted a somewhat higher percentage in the urine, had it survived the experiment.

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ment. 4 g ethyl alcohol per kg body weight were given at 6 hours and 4 g ethyl alcohol per kg body weight at 30 hours after the beginning of the experiment.

After a few minutes the rabbit lost consciousness. The following day the condition was almost unchanged, but later in the day the animal recovered. After the last administration of ethyl alcohol the animal

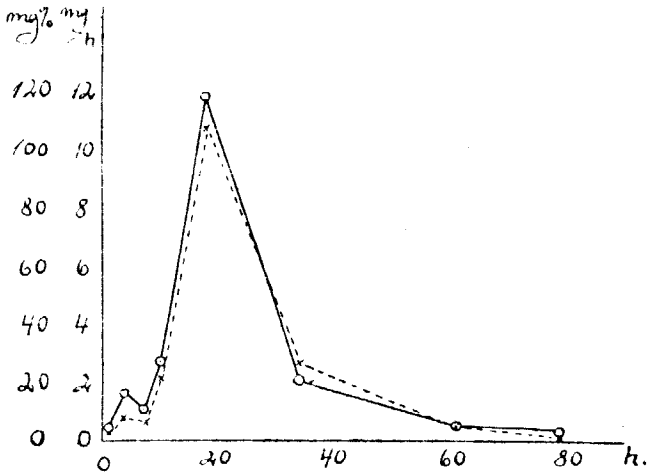

Fig. 1. Formic acid excretion in rabbit R_2 (Exp. 4)
Ordinate: x — — — x ; mg% formic acid in urine.
o - - - o; mg/h: amount of formic acid excreted per hour.
Abscissa: time in hours from the beginning of the experiment.

became moribund and died on the 3rd day (about 48 hours after the first administration).

In the course of the 48 hours the rabbit excreted a total of 2.9 mg formic acid. A fortnight previously the same animal after the same dose of methyl alcohol excreted 214 mg formic acid in about the same interval (44 hours).

D. Formic Acid Excretion in Rabbits after Administration of Formic Acid (per injectio).

One experiment is presented to illustrate the capability of the organism to burn formic acid. For this purpose several injections of an isotonic formate solution (0.7 % formic acid neutralized with NaOH) were given in the dorsal musculature of the rabbits and the formic acid concentration of the urine

was determined. There was no apparent toxic effect on the rabbit in these experiments.

Experiment 10. (R_3, weight 2700 g). At the time of the injection, at 24 and 50 hrs after the beginning of the experiments the rabbit received 0.55 g formic acid intramuscularly.

113 hours after the beginning of the experiment normal values of formic acid were found in the urine. Up to this time the animal excreted 220 mg formic acid. Without formic acid injection the animal is assumed to excrete 16 mg in the same period. Formic acid recovered: 204 mg, i.e. 12 % of acid injected.

E. Experiments on Dogs, after Administration of Methyl Alcohol.

These experiments were performed on two male dogs which were on a diet of black bread + butter + water ad lib.

Experiment 13. (D_1, weight 17 kg). 1 g methyl alcohol per kg body weight. On the first day the animal was a little tired and did not eat or drink. On the second day the animal was despondent and still did not eat or drink. On the 3rd day the animal began to drink and eat a little, but not until several days later did it show normal appetite. The experimental results are given in Table 3.

Experiment 14. (D_2, weight 6.8 kg). 2 g methyl alcohol per kg body weight. On the first day the dog was a little uncertain in its movements, but was otherwise well. Then rather suddenly it became weak and had diarrhoea. The dog did not eat or drink. On the 3rd day there was still diarrhoea and distinct weakness, the animal lying very quiet. At the end of the 3rd day the animal began to drink and then recovered slowly. The diarrhoea continued for another two days. Then the animal drank better, but ate very little. Not until about 1 month after the poisoning did its normal appetite return. The results are given in Table 3.

F. Experiment on Formic Acid Excretion in a Dog after Administration of Methyl + Ethyl Alcohol.

A single experiment is presented to supplement the rabbit experiments on this subject.

Experiment 15. (D_2, weight 6.3 kg). Methyl alcohol and ethyl alcohol were administered in the following way:

g methyl alcohol per kg body weight:	2	0	0	0	0	0
g ethyl alcohol per kg body weight:	2	0.63	1.26	1.5	0.63	1.26
Hours after beginning of the experiment:	0	22½	30	51	71½	78½

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Table 3.

Formic Acid Excretion in Dogs after Administration of Methyl Alcohol.

Exper. No.	Dog mark	Weight	CH_3OH g/kg	Time hours	Excretion of Formic Acid: total mg	normal mg	net mg	net mg/kg	% of HCOOH corresp. to CH_3OH	Excretion of Methyl Alcohol: Total CH_3OH g	% of CH_3OH admin.
13	D_1	17.0	1	95	1505	194	1311	77	5.4	0.87	5.1
14	D_2	6.8	2	95½	2890	23	2867	421	14.7	1.17	8.5

10 to 20 min. after the administration the dog was very weak. There was a violent flow of saliva, but no vomiting. On the following day the animal was also 4 months, drank well and ate a little; it was at first somewhat affected by the methyl alcohol. There was no diarrhoea throughout the period of poisoning. In 101½ hours 1834 mg formic acid were excreted. In a previous experiment (Exp. 14) the same animal was given the same dose of methyl alcohol without ethyl alcohol. It then excreted 2867 mg formic acid in 95½ hours, cf. Table 3.

Discussion.

It appears from Table 2 that formic acid excretion, in mg per kg body weight, increases with the amount of methyl alcohol administered and apparently relatively more formic acid is excreted at the larger methyl alcohol doses than at the smaller ones. However, formic acid excretion in all experiments with rabbits seems only slight in comparison with the amount of methyl alcohol given. Only 1 %, or less of the amount of formic acid corresponding theoretically to the methyl alcohol is excreted.

It must be pointed out that the relatively high excretion of formic acid at the larger methyl alcohol doses does not necessarily mean that an increasing percentage of the methyl alcohol is converted to formic acid. When there is a higher formic acid concentration in the blood, there is a possibility that a greater percentage of the acid will be excreted in the urine.

On the basis of the experiments performed, it is not justifiable, however, to draw the conclusion that only about 1 % of the methyl alcohol administered to rabbits is converted to formic acid. From the experiments mentioned in Section D it appears that only part of the formic acid administered was recovered in the urine (11—12 %). The rest is probably oxidized to CO_2 and water. Even though the experiments in Section D are few, they indicate that in the rabbit probably far more than 1 % of the methyl alcohol is converted to formic acid.

A certain comparison between experiments in Section B and D may be made. Experiment 4 and Experiment 10 show some points of resemblance, the maximum formic acid concentration in urine (107 mg % and 91 mg % respectively) having practically

much as one month after the poisoning and the dog did not increase in weight until after this period. A similar effect, but not nearly as pronounced, was observed in Dog D_2.

Possibly the strong effect of methyl alcohol on dogs may be due to a greater conversion of methyl alcohol to formic acid or a lowered capability to burn this substance. Future studies on this problem will have to be carried out.

Finally some remarks should be made about the experiments in sections C and F, which show that in rabbits ethyl alcohol exercises a marked inhibition on formic acid excretion after administration of methyl alcohol. In dogs only one experiment with ethyl alcohol was carried out. Here, too, an inhibition was observed, but not as pronounced as in rabbits. Two previous authors (ASSER 1914; ROE 1943) each put forward a theory about this inhibition. The former claims that he has observed an increasing oxidation of formic acid in the presence of ethyl alcohol; the latter maintains that the formation of formic acid from methyl alcohol is inhibited.

Summary.

1. A short survey is given of the work of previous authors who have studied formic acid excretion after administration of methyl alcohol.

2. Some experiments are presented on normal formic acid excretion in rabbits and dogs, and it is shown that the urine of these animals contains very varying and small amounts of formic acid under normal conditions.

3. In rabbits and dogs there is a considerable increase in formic acid excretion after administration of methyl alcohol. In rabbits the amount of formic acid excreted is always small, not exceeding about 1% of the amount of methyl alcohol administered. In experiments on dogs it is found that a considerably larger fraction of methyl alcohol is excreted as formic acid (up to 14—15%) in the urine.

4. By administration of formic acid to rabbits it is shown to be probable that rabbits are able to burn large amounts of

the same value in the two experiments. In Experiment 4 there was found an excretion of formic acid equivalent to 1% of the methyl alcohol administered; in Experiment 10 the value was 12% (1/8) of the formic acid injected. One may therefore expect that the excreted formic acid constitutes about 1/8 of the methyl alcohol converted to formic acid, i.e. about 8% of the methyl alcohol given should be converted to formic acid.

It appears from Table 2 that in rabbits about 16% of the methyl alcohol given is excreted unchanged in the urine. If about 8% is converted to formic acid, then about 76% of the methyl alcohol remains and may be excreted through the respiratory air. In this connection it is of interest to mention that HAGGARD and GREENBERG (1939) have found that in rats 76% of the methyl alcohol administered is eliminated in the respiratory tract.

The most striking difference between the rabbit and the dog is (cf. Tabl. 2 and 3) that dogs excrete a much larger percentage of methyl alcohol as formic acid. On the other hand the excretion of unchanged methyl alcohol seems to be larger in rabbits.

On the assumption that equal fractions of methyl alcohol are eliminated through the respiratory tract in the two species of animal, the differences mentioned above may be explained in the following way. Either the dog forms formic acid more easily than the rabbit or burns it with more difficulty; possibly a combination of both factors occurs.

Formic acid excretion in the animals investigated proceeds in the same way, with maximum values on the 2nd and 3rd day somewhat after administration of methyl alcohol, varying somewhat with the size of the dose administered.

It seems from the course of the rabbit experiments that when the effect of the methyl alcohol no longer asserts itself the rabbit is apparently quite normal. This does not, of course, exclude the possibility that the rabbits used underwent pathological and anatomical changes, for instance in the retina. Compared with the rabbit experiments it was striking how differently Dog D_2 reacted to methyl alcohol. Lack of appetite lasted for as

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formic acid. From this fact it is concluded that in rabbits a larger amount of methyl alcohol is formed than is indicated by the excretion of formic acid in urine.

5. In both rabbits and dogs there is a pronounced inhibitory effect of ethyl alcohol on formic acid excretion after administration of methyl alcohol.

6. The above mentioned results are discussed

REFERENCES

Asser, E.: *Ztschr. exp. Path. u. Therapie*, 1914, **15**, 322.

Bastrup, J. Th.: *Acta Pharmacol.* 1947, **3**, 303.

Benedict, E. M. and G. A. Harrop: *J. Biol. Chem.* 1922, **54**, 443.

Deoller, H.: *Ztschr. f. physiol. Chem.* 1932, **211**, 57.

Haggard, H. W. and L. A. Greenberg: *J. Pharmacol.* 1939, **66**, 479.

MacFadyen, D. A.: *J. Biol. Chem.* 1945, **158**, 107.

Neymark, M.: *Skand. Arch. f. Physiol.* 1936, **73**, 227.

Pohl, J.: *Arch. exp. Path. u. Pharmakol.* 1893, **31**, 281.

Rost, E. and A. Braun: *Arbeit. a. d. Reichsgesundheitsamt.* 1926, **57**, 580

Roe, O.: *Acta med. Scand.* 1943, **113**, 558

Widmark, E. M. P.: *Fortschr. d. Naturwiss. Forsch., Neue Folge*, Heft 11, 1932.

COTTON MADE RESISTANT TO MICROBIOLOGICAL DETERIORATION USING FORMIC ACID COLLOID OF METHYLOLMELAMINE

W. Norbert Berard, Ethel K. Leonard, and Wilson A. Reeves

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It is well known that cotton will deteriorate rapidly when exposed to microbiological and photochemical degradation, and considerable research has been devoted to solve these problems. A number of additive type fungicides, which have been developed in recent years, have extended the useful life of cotton products in many end uses. However, these chemicals have drawbacks such as lack of durability and unacceptable coloration of the fabric.

Another approach to this problem has been through chemical modification of the cellulose by reaction with various organic chemicals. The material retains completely the appearance of cotton but acquires new properties which have been of interest to the textile industry. Investigations by Goldthwait *et al.* (1946) and Cooper *et al.* (1952) have shown that cotton chemically modified by partial acetylation is resistant to rotting and mildewing and withstands soil-burial tests for much longer periods than does cotton treated with additive type fungicides. Partial acetylation is the process of attaching acetyl radicals onto cellulose molecules. Figure 1 is a schematic representation of a substituted cellobiose unit which illustrates a degree of substitution of unity (D.S. of 1.0).

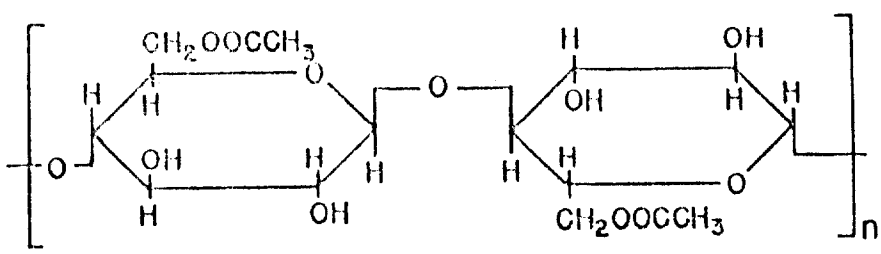

Fig. 1. Partially acetylated cellulose.

Another chemical modification that has been investigated is cyanoethylated cotton, a product of the reaction of acrylonitrile with cotton to yield a cyanoethylcellulose ether. The studies of Compton *et al.* (1953) have shown that cyanoethylated cotton can withstand soil burial exposure for extended periods without significant loss in strength. The schematic representation for cyanoethylated

cotton with D.S. of 0.5 is shown in Fig. 2. Acrylonitrile polymerized in cotton fabrics is also an effective means of protecting cotton from microorganism attack (Gottlieb and Jeffries, 1957; Haydel *et al.*, 1957).

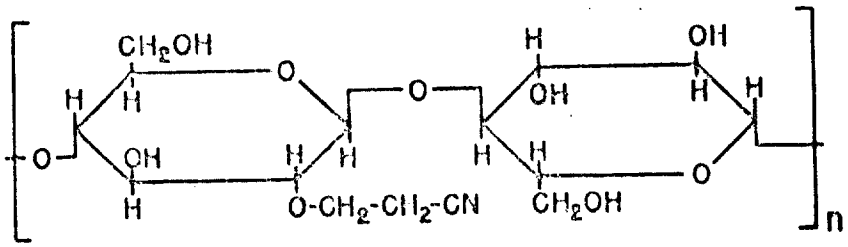

Fig. 2. Partially cyanoethylated cellulose.

Other chemical modifications of cellulose have been prepared which show some resistance to microbiological attack. Studies by Guthrie (1947) demonstrated that 2-aminoethylsulfuric acid reacts with cellulose under alkaline conditions. Upon subsequent treatment with a mercury salt, the aminized fabric showed rot resistance in a soil-burial test. The amino groups serve as effective means for attachment of various metals.

Partial carboxymethylation of cellulose reported by Reid and Daul (1947) does not protect it from microbiological attack. However, addition of various metals to form insoluble salts of carboxymethylcellulose imparts considerable resistance to such attack.

Investigations by Frick *et al.* (1957) have shown that cellulose can be reacted with acrylamide to give modified cotton having good fabric properties. Carbamoylethylated cotton as prepared showed a slight degree of rot resistance, but this could probably be increased with a higher degree of substitution.

Recent studies on the reaction of chloromethylphosphonic acid with cellulose, described by Hobart *et al.* (1959) showed that phosphonomethylated cotton in the copper salt form was superior in rot resistance to the sodium and ammonium salt forms and to untreated cotton. However, the degree of rot resistance is not comparable to that imparted by commercial rot-resistant treatments.

In all of the above work on chemically modified cottons, partially acetylated and cyanoethylated cottons had excellent resistance to rot. Due to the cost of these products they have not been utilized commercially as rot resistant cotton fabrics. The other chemically modified cottons depend upon a subsequent reaction with metal salts to produce rot resistance, equivalent to that obtained with additive type fungicides now available commercially.

In considering all of the above factors it was evident that a durable, colorless, and inexpensive rot-resistant treatment that would be easy to apply by textile finishing companies, was needed for cotton. The use of aminoplast resins seemed to offer good possibilities, as previous investigations (Nute, 1945; White and Siu, 1947) with melamine formaldehyde have demonstrated that resin-impregnated cotton fabrics were resistant to microbiological deterioration when subjected to soil-burial tests. However, these results were not as good as those obtained by partial acetylation or cyanoethylation. In all of the above work, the melamine

formaldehyde and other aminoplast resins were applied by the "conventional process." "Conventional process" means the application of an aqueous system of essentially a monomeric-resin former and a latent acid catalyst to a textile followed by a dry and a cure at elevated temperatures. Recent studies at this laboratory have shown that an acid colloid of trimethylolmelamine can make cotton quite resistant to degradation in the soil bed (Berard *et al.*, 1959).

The purpose of the present paper is to report additional studies on application techniques of methylolmelamine resins to cotton, and to reveal a practical method for producing cotton fabric with excellent rot and good weather resistance. The structural formula for trimethylolmelamine is shown in Fig. 3. This method

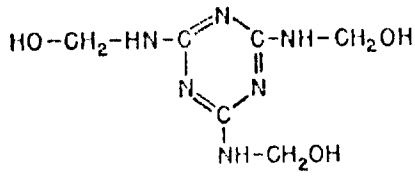

Fig. 3. Trimethylolmelamine.

is based on the use of a formic acid colloid of methylolmelamine (Berard *et al.*, 1959). The method of application of the colloid is essentially the same as the conventional method for resin finishing; that is, pad, dry, and cure. The main difference is the use of formic acid in the treating solution to serve as the catalyst and most important to produce a colloid with the trimethylolmelamine. The term "acid colloid" as used in this paper refers to methylolmelamine molecules and polymers of methylolmelamine which are associated and stabilized by polar acid groups. All of these colloids possess light-scattering properties.

Acid colloids of aminoplast resin formers have been used before, but according to the way they were produced and applied to textiles, they did not impart much rot resistance. Nute (1949) has shown that colloids of methylolmelamine are unique textile finishing materials—they produce excellent shrinkage control and durable stiffness, with no loss in tensile strength. In the earlier studies the colloids were permitted to age, often as much as 24 hr, thus allowing rather large polymers or colloidal particles to form and deposit on the surface of the cotton fiber. Cooke (1959) observed that a cotton fabric impregnated with an aged acid colloid of methylolmelamine completely deteriorated when exposed to a 2-week soil-burial test. This was a much shorter deterioration time than that observed when the resin was applied by the conventional method.

MATERIALS AND METHODS

Fabric Description

Most of the experimental work was done with a desized, scoured and bleached 80 × 80 cotton print cloth weighing approximately 3.12 oz/yd^2. Other fabrics used included a desized and scoured 48 × 48 cotton sheeting, and a scoured 8-oz cotton army duck.

Chemicals Used

The N-methylol compounds used were commercial products sold under various trade names. The compounds were unmethylated methylolmelamine (Resloom HP*, Aerotex Resin UM Special*). Formic acid (90% purity) and lactic acid (85% purity) were commercially available products.

Processing Procedure

In each case the resin-forming monomer, and crosslinking agent, were first dissolved in water at room temperature, and then the acid catalyst was added. In most cases the solutions were used within 10 min; in a few instances, where indicated, the solution containing formic acid was allowed to stand one or more hours after the acid was added before it was used. Samples of cotton fabric were impregnated on a laboratory padder, dried 4 min at 80 C, and cured for the same length of time in a forced draft oven, at the temperatures indicated below. Following the curing step, samples were washed at 100 F with Tide* as the detergent in a typical agitator-type home automatic machine on the modern-fabric cycle. The cloth samples were then tumbled dry in a gas-heated home dryer.

Test Methods

The following properties were determined by the usual ASTM procedures: breaking strength, strip method (1a) (except the strips of print cloth were raveled to 80 threads, the sheeting to 48 threads, and the army duck to 55 threads), and soil burial (1b). Samples were leached with water according to Fed. Spec. CCC-T-191b, Method 5830.

Weather exposure tests were conducted in New Orleans, Louisiana, in an unshaded location, with the samples exposed on open-backed wooden rack facing south at an angle of 45° from the vertical and at a minimum distance of 30 in. above the ground.

RESULTS

Effect of Aging of Colloids on Rot Resistance

Previous work at this laboratory (Berard *et al.*, 1959) on application techniques of methylolmelamine resins to cotton revealed that cotton fabric with outstanding rot resistance and good weather resistance could be produced. Print cloth samples were treated with solutions containing 17% methylolmelamine and 20% formic acid (a molar ratio of about 1:5), which gave 12% resin add-ons, to explore the value of formic acid colloids and the effects of aging colloid solutions. The fabric was impregnated immediately (within 10 min) after preparing the colloidal solution, and then at 2-, 4-, 5-, 6-, and 7-hour intervals. Table 1 shows breaking strengths in pounds of the different samples as treated and at the indicated periods of soil burial. The sample treated with freshly prepared colloid solution retained all of its original strength after 21 weeks of soil burial, and the fabric impregnated with the solution aged for 2 hr had a breaking strength of 26.0 lb, compared to its original strength of 33.3 lb, for the same period of exposure. The rest of the fabrics showed a decreasing resistance as the colloid solution

*Use of a company and/or product named by the Department does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

was allowed to age. These results demonstrate that freshly prepared acid colloid resin solutions (and those aged less than 2 hr) impart to cotton fabrics excellent resistance to microbiological deterioration. Fabrics treated with the aged colloids offered much less resistance to microorganisms. In previous studies (Berard *et al.*, 1959), fabric treated with trimethylolmelamine by the conventional process to contain 12% resin add-on, had completely deteriorated after 6-weeks soil burial.

Table 1. Effect of Aging of Colloids on Rot Resistance

Age of colloid used to produce 12% resin in finished fabric	Breaking strength in pounds, before and after soil burial						
	Orig.	2 Wks.	4 Wks.	6 Wks.	12 Wks.	18 Wks.	21 Wks.
Freshly prepared	32.3	36.1	39.4	34.6	40.9	40.9	32.6
2 Hours	33.3	38.0	39.0	36.5	38.1	30.6	26.0
4 Hours	36.9	36.5	40.5	36.7	22.5	7.4	-
5 Hours	36.4	39.9	41.1	28.9	14.4	9.3	-
6 Hours	37.7	39.2	38.4	28.8	7.8	2.7	-
7 Hours	39.0	37.8	34.0	24.4	6.0	-	-

Influence of Colloid Add-on upon Rot Resistance of Treated Fabric

As the above work had been confined to using 12% add-ons of the trimethylolmelamine resin, it seemed desirable to determine if lower concentrations of resin would afford similar resistance to microbial attack. Therefore, print cloth was treated to contain from 3% to 12% resin add-on. For the 12% resin add-on, the fabric was treated with a solution containing 17% trimethylolmelamine and 20% formic acid. To obtain the lower resin add-ons, more dilute solutions of trimethylolmelamine were used, but the concentration of formic acid was held constant. For example, 3% resin add-on was obtained by using a solution containing 3.4% trimethylolmelamine and 20% formic acid.

Table 2 gives the breaking strengths in pounds of the samples with the various resin add-ons as treated and after the indicated periods of soil burial. The sample with 12% resin retained all of its breaking strength after 18-weeks soil burial, and the sample with 10% resin add-on had retained approximately 2/3 of its strength for the same period of exposure. The samples containing still lower resin add-ons retained even less strength which was directly related to resin add-on. The sample with 3% resin was completely deteriorated in 2 weeks of soil burial. The results show that excellent resistance to microorganisms was obtained when the cloth was treated with freshly prepared acid colloid solutions so as to impart 10–12% resin add-on.

It should be noted in the above two tables that there is an increase in breaking strengths of the treated fabrics after various periods of soil burial. This phenomenon cannot be attributed to fabric shrinkage and thereby breaking of more warp yarns, as all samples were stripped to a specific thread count before the soil-burial tests. Buras and coworkers (1954) report that a small gain in strength

Table 2. Influence of Add-on of Formic Acid Colloid Upon Degree of Rot Resistance

Resin Add-on in %	Breaking strength in pounds before and after soil burial							
	Orig.	2 Wks.	4 Wks.	6 Wks.	9 Wks.	12 Wks.	15 Wks.	18 Wks.
12	26.6	37.5	36.0	38.3	38.3	40.6	38.5	39.4
10	31.2	31.8	35.3	33.8	36.3	22.4	21.4	21.6
8	33.4	35.4	10.9	8.8	5.8	1.3	1.1	-
5	34.3	11.8	2.1	-	-	-	-	-
3	35.1	-	-	-	-	-	-	-

is often observed in acetylated cotton during the early stages of soil-burial tests; this effect is paralleled in heat tests as well. It is evident that additional studies are needed before a satisfactory explanation can be given of these unusual increases in breaking strengths.

Effect of Curing Temperatures and Water Leaching on Rot Resistance of Acid Colloid — Finished Print Cloth

In the above work it was found that the freshly prepared formic acid colloid could be cured in print cloth adequately at 140 C to provide a high degree of re-

Table 3. Effect of Curing Temperatures and Water Leaching on Rot Resistance of Acid Colloid — Finished Print Cloth

12% resin in finished fabric*	Cure Temp. (°C)	Breaking strength in pounds, before and after soil burial					
		Orig.	2 Wks.	4 Wks.	6 Wks.	12 Wks.	15 Wks.
Unleached	-	42.9	45.5	48.5	35.2	2.3	-
Leached	-	35.3	44.8	42.9	27.0	2.1	-
Unleached	80	40.4	44.1	46.6	49.1	25.8	11.2
Leached	80	36.7	42.3	48.0	51.3	18.7	9.8
Unleached	100	31.9	39.7	43.0	43.8	26.7	26.4
Leached	100	31.9	38.9	42.0	44.0	29.2	22.6
Unleached	120	32.3	40.5	41.3	44.6	28.0	22.9
Leached	120	34.0	39.8	41.2	42.8	42.0	31.2
Unleached	140	31.5	36.2	40.8	38.4	39.4	35.7
Leached	140	33.9	32.4	41.8	42.2	33.6	40.4
Untreated, control	-	51.4	-	-	-	-	-

*All samples dried 4 min at 80 C.

sistance to microbiological attack. Therefore a study was made to establish the minimum curing temperature needed for optimum rot resistance. Print cloth samples were treated with an acid colloid solution containing 17% trimethylol-

melamine and 20% formic acid to give 12% resin add-ons and dried 4 min at 80 C. One sample was not cured while other samples were cured at 80, 100, 120, and 140 C for 4 min.

To determine the durability of this finish, part of each of the above samples was leached in water at a rate of about 5 changes/hr for a period of 24 hr.

Table 3 shows the breaking strengths in pounds of the different samples as treated, and after leaching, and at the indicated periods of soil burial. After 15-weeks soil burial, the fabric cured at 140 C, unleached or leached, had retained all of its strength. The fabrics cured at the lower temperatures retained less breaking strength. Samples cured at the lowest temperatures retained the least strength. The sample not cured completely deteriorated in the 15-weeks soil burial. The leaching had practically no effect upon the samples as most breaking strengths were about the same as the unleached samples. These results demonstrate that a cure temperature of at least 140 C is necessary to produce excellent rot-resistant cotton, and also that the finish is durable and not affected by a severe water-leaching test.

Preparation of Stable Acid Colloids of Trimethylolmelamine by Use of Other Acids

Since stable acid colloids of trimethylolmelamine had been obtained with formic acid, investigations were conducted to determine if glycolic and lactic acids would also produce stable colloids with trimethylolmelamine, and if such

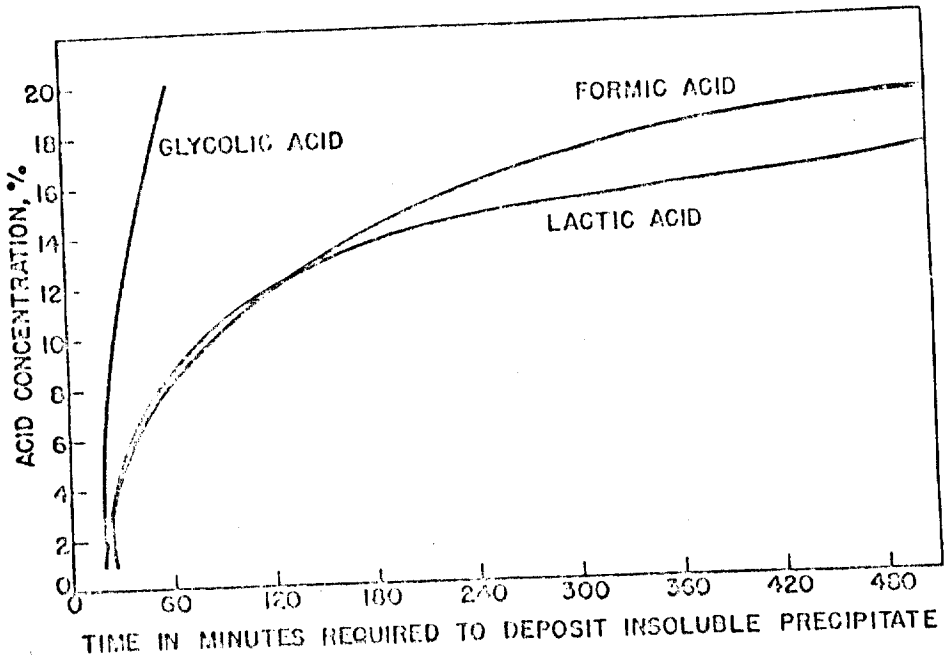

Fig. 4. Time of stability of the colloidal solutions with varying amounts of formic, lactic, and glycolic acids.

colloids could be used to impart the degree of rot resistance obtained with formic acid. A series of solutions were made in which the methylolmelamine concentration was held constant at 17% while the amount of formic, lactic, or glycolic acids was varied up to 20% of the weight of the total solution. The time required for a precipitate or gel to occur was noted.

Figure 4 gives the time of stability of the colloidal solutions of the three acids, as judged by the appearance of a visible precipitate. The solutions with 4% or less acid began to deposit a precipitate within 25 min. At these low concentrations, the three acids act primarily as polymerization catalysts. Higher concentrations of formic and lactic acids have a stabilizing effect and also act as polymerization catalysts. The colloid solutions containing 20% formic acid and 18% lactic acid were stable for at least 8 hr, but did form clear gels sometime before 24 hr. The glycolic acid was not effective in producing a stable colloid even at 20% concentration, as an insoluble precipitate formed in just over one hour. Obviously, the larger amounts of formic and lactic acids have a marked stabilizing effect on the colloid.

Rot Resistance of Print Cloth Finished With Formic and Lactic Acid Colloids of Trimethylolmelamine

As the stability curves for the formic and lactic acids were very similar, it seemed desirable to compare the microbiological resistance of fabric finished with these two colloidal solutions. Print cloth samples were treated with the formic acid and lactic acid colloids of trimethylolmelamine to give approximately 12% resin add-ons. The samples were dried 4 min at 80 C and cured for the same length of time at 140 C.

Table 4 gives the breaking strengths in pounds of the samples as treated and after the indicated periods of soil burial. The sample finished with the formic

Table 4. Rot Resistance of Print Cloth Finished With Formic and Lactic Acid Colloids of Trimethylolmelamine

Acid colloid used, 12% resin in finished fabric	Breaking strength in pounds, before and after soil burial					
	Orig.	2 Wks.	4 Wks.	6 Wks.	12 Wks.	15 Wks.
Formic Acid	31.5	36.2	40.8	38.4	39.4	35.7
Lactic Acid	39.8	44.2	31.1	51.0	28.3	15.6

acid colloid had retained all of its original breaking strength after 15-weeks soil burial, while the sample finished with the lactic acid colloid had retained a little less than half of its original breaking strength. This would indicate that the formic acid colloid imparts higher resistance to microbial attack than the lactic acid colloid.

Viscosity measurements were made, using a capillary tube, on the freshly prepared formic acid and lactic acid colloids, and after aging for various periods of time. The viscosity of the lactic acid colloid increased at a more rapid rate than the formic acid colloid. The higher viscosity of the lactic acid colloid at the time of application probably caused more resin deposition on the fiber surface,

similar to the deposition of resin previously reported for aged colloids, and afforded less resistance to microbial attack.

Weather Resistance of Cotton Fabric Treated with Formic Acid Colloid of Trimethylolmelamine

As previous investigations on the aged acid colloid of methylolmelamine had not included weather exposure evaluations, it seemed desirable to evaluate fabric finished with the freshly prepared formic acid colloid. Samples of 8-oz duck and 48×48 sheeting were treated with acid colloid solutions to give approximately 12% and 6% resin add-ons for each fabric.

Figure 5 shows the breaking strengths obtained on the treated 8-oz duck samples and untreated control after weathering for periods up to 12 months. The two treated fabrics possessed very similar strength retentions and had retained all of their original breaking strengths after 12-months exposure. The curves indicate that the sample containing 12% resin add-on had a slight advantage over the

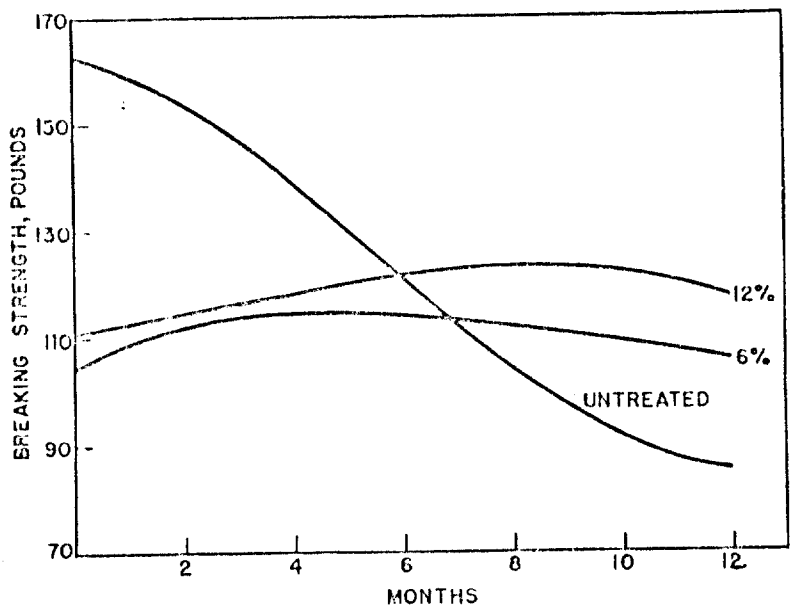

Fig. 5. Weather resistance of 8-oz duck treated with formic acid colloid of methylolmelamine at 12% and 6% resin add-ons, and untreated control.

fabric containing 6% resin add-on. This is probably due to the fact that duck fabrics retain moisture for a longer period of time than lightweight cotton fabrics, and the higher resin add-on afforded more resistance to microbiological attack. Although the untreated control fabric had a higher initial breaking strength, after 6–7 months of exposure the breaking strength was about equal to that of the treated fabrics. After 12-months exposure the strength of the control fabric was much lower than that of the acid colloid – finished samples.

Figure 6 shows the breaking strengths of the acid colloid – treated 48×48 sheeting, and untreated control, after weather exposure for periods up to 12 months. The breaking strengths for the 12% and 6% resin add-on samples are essentially the same for the various exposure periods, with practically no loss of strength after 12-months weathering. For lighter weight fabrics which dry

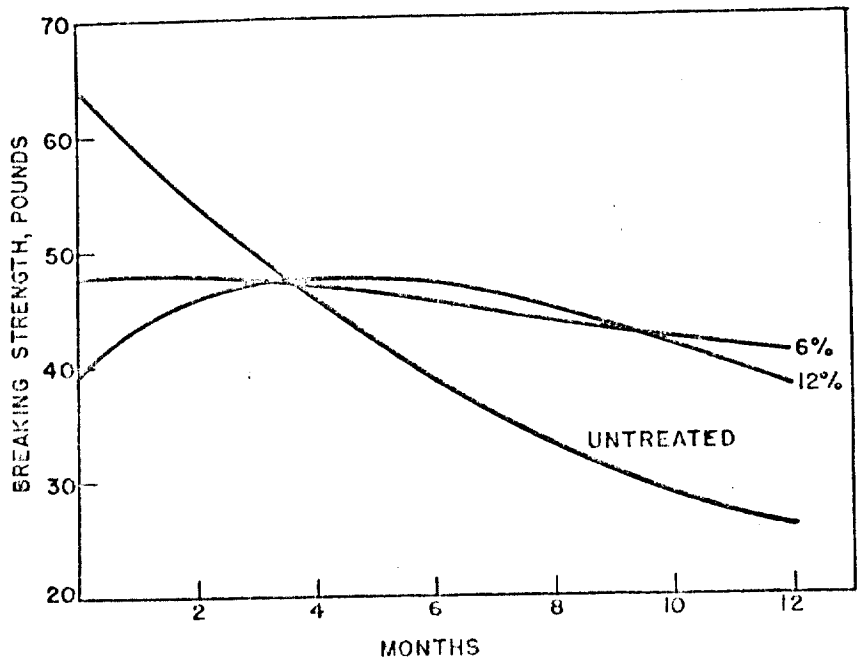

Fig. 6. Weather resistance of 48×48 sheeting treated with formic acid colloid of methylolmelamine at 12% and 6% resin add-on, and untreated control.

out relatively fast after a heavy dew or rain, these results would indicate that the 6% resin add-on would be satisfactory as a weather-resistant finish. The untreated sheeting had a higher initial breaking strength, but was about equal in strength to the acid colloid – finished fabrics at the 4-month period. After 12 months the untreated fabric had a much lower breaking strength than the treated fabrics.

Microscopic Examination of Acid Colloid – Finished Cotton

Microscopic studies were made on fibers treated with the freshly prepared formic acid colloid of methylolmelamine, with aged acid colloid, and by the conventional technique, by treating ultrathin sections of these fibers with cupriethylenediamine hydroxide. Figure 7 shows a schematic drawing of cross sections of these fibers with the location of resin deposition. The cotton fiber treated with the fresh acid colloid had the resin deposited primarily in the outer portion of the cell wall. The innermost section of the fiber responded to cupriethylenediamine hydroxide by inhomogeneous solution in a manner similar to that observed

for cotton having small amounts of bound formaldehyde. Examination of the aged acid colloid – treated fibers showed the resin was deposited only on the surface of the fiber. The conventionally processed fibers were found to be uniformly penetrated by the resin.

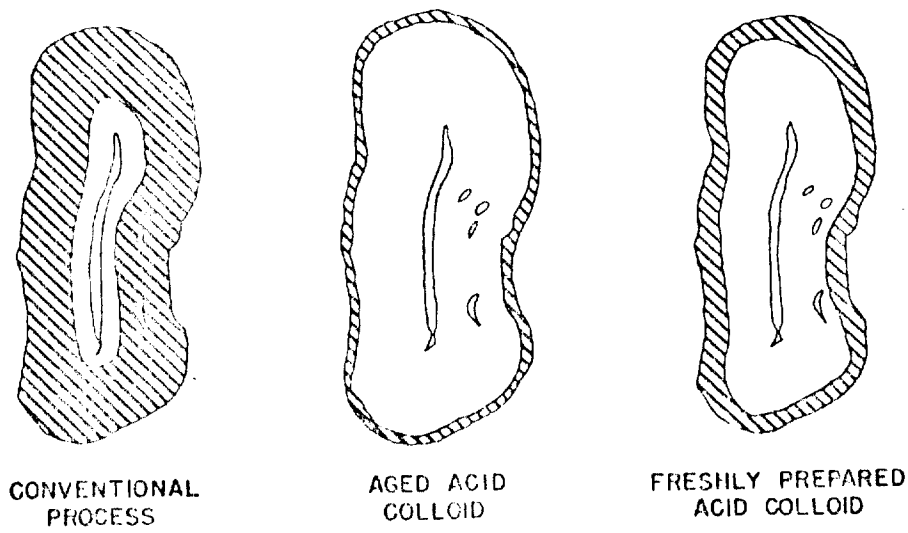

Fig. 7. Schematic drawing of cross sections of cotton fibers showing location of resin deposition.

DISCUSSION

Cotton fabric was made rot- and weather-resistant by treating with a freshly prepared formic acid colloid of methylolmelamine. The method of finishing consisted of the usual pad, dry, and cure procedure. Best rot resistance was obtained by using acid colloids within 2 hr after preparation. Cotton print cloth finished with 12% resin imparted by use of a fresh acid colloid retained 100% strength after 21 weeks of soil burial. These results were substantially better than those reported for the same trimethylolmelamine applied by the conventional technique, and is much better than is known for additive-type fungicides. Previous work with similar acid colloids, aged at least 12 hr, and as long as 24 hr, did not impart rot resistance to cotton fabric.

Different curing temperatures had a marked effect on the rot resistance of the fabric. The results showed that a cure temperature of at least 140 C is required to produce excellent rot resistance. A severe water leaching test had practically no effect on the resistance to microorganisms in soil-burial tests, indicating excellent durability of the acid colloid finish.

Stable acid colloids were obtained when the colloidal solutions contained about 1 mole of trimethylolmelamine to 5 moles of formic acid. Colloidal solutions prepared with lactic and glycolic acids were not as effective for this purpose as formic acid. Viscosity measurements of a formic acid colloid and a lactic acid

colloid, as measured through a capillary tube, showed that the lactic acid colloid had a higher viscosity than the formic acid colloid. This probably caused more of the resin to be deposited on the fiber surface, thereby imparting less resistance to microbial attack.

The outdoor weathering resistance of fabric was improved by the acid colloid finish. The results show that two different weights of cotton fabric, each at two levels of resin add-ons, retained all of their breaking strengths after 12-months weather exposure, while the untreated samples lost 50% or more of their breaking strengths for the same period of exposure.

Microscopic observations on fibers treated with the formic acid colloid of methylolmelamine showed that the resin was deposited primarily in the outer portion of the cell wall of the cotton fiber—which portion did not respond to swelling in cupriethylenediamine hydroxide; the innermost section of the fiber showing inhomogeneous solution in this reagent in a manner similar to that observed for cotton having small amounts of bound formaldehyde. Fibers treated with an aged acid colloid had a coating of resin on the surface of the fiber, and fibers treated by the conventional technique were quite uniformly penetrated by the resin.

It has been suggested that the microbiological degradation of cotton can be prevented by an inert physical barrier which interrupts the contact between the organisms and the cellulosic substrate. According to this mechanism, any cracks in the barrier or resin film should permit the fungi to penetrate to the cellulose, and cause deterioration of the cotton. To test this mechanism, print cloth containing 12% acid colloid resin was stretched through a number of cycles to 50% of the fabric breaking load to produce ruptures in the "resin film," and then subjected to soil-burial tests. The fabric still maintained excellent rot-resistant properties, indicating that the acid colloid finish does not act as an inert physical barrier but rather is a chemical barrier to protect the cotton from microbiological attack.

SUMMARY

Cotton fabric has been made rot- and weather-resistant by treating with a freshly prepared formic acid colloid of methylolmelamine. Previous investigations have shown that acid colloids allowed to age at least 12 hr, and as long as 24 hr, do not impart much resistance to microbiological deterioration. The method of application of the freshly prepared acid colloid is essentially the same as conventional procedures used for resin finishing, that is, pad, dry, and cure. The main difference is the use of formic acid in the treating solution to produce a colloid.

Cotton fabric finished with the acid colloid of methylolmelamine at about 12% resin add-on had excellent resistance to microorganisms compared to cloth finished with methylolmelamine in the conventional manner at the same level of resin add-on. A curing temperature of at least 140 C is required to obtain excellent resistance to microbiological attack.

In microscopic studies of fibers treated with the formic acid colloid of methylolmelamine made by treating ultrathin sections with cupriethylenediamine hydroxide, the resin was found deposited primarily in the outer portion of the cell wall of the cotton fiber, which portion did not swell in the reagent; the innermost

section of the fiber responded to cuprietheylenediamine hydroxide by inhomogeneous solution in a manner similar to that observed for cotton having small amounts of bound formaldehyde.

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REFERENCES

A.S.T.M. (a) D39-49; (b) D884-54; American Society for Testing Materials, Philadelphia.

Berard, W.N., Gautreaux, G.A., and Reeves, W.A. 1959 Formic acid colloid of methylolmelamine as a weather and rot resistant finish for cotton. Textile Research J. 29, 126.

Buras, E.M., Jr., Cooper, A.S., Keating, E.J., and Goldthwait, C.F. 1954 Practical partial acetylation of cotton. Am. Dyestuff Reptr. 43, 203.

Compton, J., Martin, W.H., Word, B.H., Jr., and Thompson, D.D. 1953 New textile fibers created by reaction of cotton with acrylonitrile. Textile Inds. 117, No. 10, 138-A.

Cooke, T.F. 1954 Resistance to microbiological deterioration of resin-treated cellulosic fabrics. Textile Research J. 24, 137.

Cooper, A.S., Voorhies, S.T., Jr., Buras, E.M., Jr., and Goldthwait, C.F. 1952 Partial acetylation of cotton. Textile Inds. 116, No. 1 97.

Frick, J.W., Reeves, W.A., and Guthrie, J.D. 1957 Carbamoylethylation of cotton with acrylamide. Textile Research J. 27, 294.

Goldthwait, C.F., McLaren, J., and Voorhies, S.T., Jr. 1946 Acetylated cotton highly resistant to rotting. Textile World 96, No. 2, 117.

Gottlieb, I. and Jeffries, F. 1957 Acrylonitrile and iron make a stronger cotton. Chem. Week 80, No. 11, 112.

Guthrie, J.D. 1947 Introduction of amino groups into cotton fabric by use of 2-aminoethylsulfuric acid. Textile Research J. 17, 625.

Haydel, C.H., Jansen, H.J., Neal, J.E., Vix, H.L.E., and Gastrock, E.A. 1957 Vapor phase method for preparation of polyacrylonitrile coated cotton yarn and physical properties of the product. Textile Research J. 27, 97.

Hobart, S.R., Drake, G.L., Jr., and Guthrie, J.D. 1959 The preparation and properties of partially phosphonomethylated cotton. Textile Research J. 29, 844.

Nute, A.D. 194[illegible] Some recent aspects in the finishing of textile fabrics with urea and melamine resins. Am. Dyestuff Reptr. 34, 2[illegible].

Nute, A.D. *et al.* 1949 Acid colloids of resins. Am. Dyestuff Reptr. 38, 842.

Reid, J.D. and Paul, G.C. 194[illegible] The partial carboxymethylation of cotton to obtain swellable fibers I. Textile Research J. 17, 564.

White, W.L. and Siu, R.G.H. 1947 Resistance of resin-impregnated cotton fabrics to microorganisms. Ind. Eng. Chem. 39, 1628.

German Translation

Formic Acid Therapy

H. Biehl

Source: Quart. J. Crude Drug Res. 8(1): 1170-72 1968

Ants have been used in therapeutics already since antiquity. Early Indian surgeons already made use of the large Bengal ants for closing the edges of wounds during operations. The ants were placed tightly side by side around the wound until their tongs had bitten like clamps into the edges of the wound. The bodies of the ants were then separated from their heads and only the tongs remained. This curious method not only offered the advantage of an excellent "suture material", but formic acid also acted at the same time as an antiseptic against infections.

Another method of using ants was described 1000 years ago by the holy Hildegard of Bingen in her "Physika". She recommended their use in depressions, melancholia and nervous weakness, shortly in conditions known today under the collective term "vegetative dystonia". In those days, however, pure formic acid was not used, but rather alcoholic extracts and boiling mixtures obtained from whole ants. These tinctures were primarily used as rubbing ointments and in baths, but were also given internally.

Apart from the fact that the collection and processing of ants is subject today to legal rules, formic acid can be obtained with much less effort and in a more simple way by chemical means. There seems to be no significant difference in the therapeutic effect of synthetic formic acid and natural formic acid obtained from secretions of Formica rufa.

Pure formic acid is an oxidation product of methyl alcohol with the

formula HCOOH; it is a colorless product with a stinging and caustic odor. In nature, formic acid is found, apart from ants, also in various caterpillar species, in bees, stinging nettles, honey and in small amounts in many mineral waters. It is also present in sweat and urine. To many of our patients formic acid should less likely be known as a therapeutic agent, but rather as a frequently used preservative.

In medicine formic acid has the disadvantage, however, of being effective only under certain conditions when given per os, and relatively large amounts must be administered over a long time period to achieve approximately the same effect as when given by injection.

The technique of formic acid injection is quite simple, since it is mostly given subcutaneously. When using one of the available commercial preparations, such as Formisoton, Acirufan or Formidium, which consist of isotonic solutions, the injection is absolutely painless. In children, the injection site can previously be anesthetised with chlorethyl spray or rubbed with an anesthetic ointment. The injection site recommended for subcutaneous administration is the skin above the shoulder blades or the lateral surface of the thigh and (or) lower or upper part of the arm. In general, the location of the body at which the injection is made is of no importance in regard to the effect of formic acid, except when practicing definite neural and segment therapy. Intravenous injection exerts a somewhat more rapid effect in acute cases, but cannot always be carried out. It should never be used with children, who are exposed to unnecessary fright from the syringe, needle and the practitioner.

The problem of dosage is much more important. Dosage depends in the first place upon whether the sickness considered is an acute or chronic

case. Basically, the rules of homoeopathy are applicable here, namely that diseases with an acute course require lower dilutions at frequent intervals, while in a chronic case high and even the highest dilutions must be administered at long intervals. Formic acid displays its optimum therapeutic effect approximately from the D_5 dosage up. We may have any opinion whatsoever about the high potencies of homoeopathy, the fact is that formic acid is still effective at a D_{200} dosage, sometimes acting even more rapidly and longer than at low and medium dosages.

Dr. A. Reuter in his book "Formic acid as therapeutic agent" sets the following general rules for formic acid treatment:

1. Healthy tissue requires the highest dosages in order that a reaction may be detected.

2. Acute inflammatory tissue reacts towards medium and small dosages.

3. Chronically diseased tissue reacts towards small and very small dosages.

The injection intervals thus depend primarily on the reaction situation of the patient (or on the way the patient is able to react). In acute cases, injections can be given twice a day, while in chronic cases injections should be given once a month or once every 3 months.

The preferred areas in which formic acid is indicated as a therapeutic agent include primarily allergic diseases, particularly asthma (as long as it does not have a cardiac cause), eczemas, psoriasis, urticaria, hay fever, further all varieties of exudative diathesis, rheumatic diseases, many types of migraine, pregnancy vomiting, mucitis of the gastrointestinal tract and chronic kidney inflammations.

In all these cases, it is not sufficient, however, to carry out the treatment only for a short period of time; actual recovery is achieved mostly only when formic acid is used over a period of many months and even years. Certainly, this is a long time, and both patient and therapeutist must have a lot of patience; yet, formic acid therapy affords the chance of true recovery. Whoever wants to go further can combine (bind) formic acid with autogenous blood, whereby intramuscular injection is preferable in this case.

There are no definite counterindications in the use of formic acid, only in cases of hypertonia, coronary sclerosis and angina pectoris, high dosages should be avoided. Many patients exhibit a very sensitive reaction to formic acid; however, only seldom does this extend beyond the desired measure of initial aggravation (change for the worst). In such cases, the dosage selected was mostly too high, and an attempt should be made, after symptoms have decreased, to use again a lower dose or a higher homeopathic potency. When one of the commercial formic acid preparations mentioned above are used, no or very slight side-effects are noted if the attached dosage directions are observed.

In conclusion, I would like to make one further therapeutic proposal which provides an opportunity for every therapeutist to become convinced about the good results achieved by formic acid therapy. How often do we see small children with milk crust during our office hours, and precisely here does our treatment often become a painful ordeal, not only for our small patients which are exposed to all the bitter drops and foul-smelling ointments, but also for us, when we finally do not know what to do any further. Here, we should immediately give a formic acid injection at a

D_{12}, or better still, at a D_{30} dosage. In most cases, a strong initial aggravation is observed after several days, which can last for some time, and then a striking improvement is noted. A further injection is given, and then, after another slight aggravation, recovery sets in. In many cases, 2 or 3 injections are enough to free our small and pitiful patient forever from his suffering.

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("Naturheilpraxis, July 1967)

AMEISENSÄURE IN DER THERAPIE

Von H. Biehl

Schon seit ältesten Zeiten werden die Ameisen in der Heilkunde angewandt. Bereits die frühen indischen Chirurgen bedienten sich der großen bengalischen Ameisen, um bei Operationen Wundränder zu schließen. Die Ameisen wurden dabei dicht nebeneinander gesetzt, bis sich ihre Zangen wie Klammern um die Wundränder verbissen hatten. Anschließend trennte man die Ameisenkörper von ihren Köpfen und nur die Zangen blieben zurück. Diese einfallsreiche Methode hatte nicht nur den Vorteil eines ausgezeichneten "Nahtmaterials", sondern die Ameisensäure wirkte gleichzeitig als Antiseptikum gegen Infektionen.

Eine andere Anwendungsart der Ameisen beschrieb vor rund 1000 Jahren die heilige Hildegard von Bingen in ihrer "Physika". Sie empfahl sie bei Depressionen, Melancholie und Nervenschwäche, kurz bei dem Zustand, den wir heute mit dem Sammelbegriff "Vegetative Dystonie" umschreiben. Zu jener Zeit bediente man sich allerdings noch nicht der reinen Ameisensäure, sondern alkoholischer Auszüge und Abkochungen ganzer Ameisen. Man benutzte diese Tinkturen dann vorwiegend zu Einreibungen und Bädern, jedoch wurden sie auch innerlich verabreicht.

Abgesehen davon, daß das Sammeln und Verarbeiten von Ameisen heute gesetzlichen Bestimmungen unterliegt, läßt sich die Ameisensäure viel müheloser und einfacher auf chemischem Wege herstellen. Ein wesentlicher Unterschied in der Heilwirkung zwischen synthetisch hergestellter Ameisensäure und der natürlichen Ameisensäure aus dem Sekret der Formica rufa scheint nicht zu bestehen.

Die reine Ameisensäure ist ein Oxydationsprodukt des Methylalkohols mit der Formel HCOOH. Sie ist farblos und verbreitet einen stechenden, ätzenden Geruch. In der Natur kommt Ameisensäure außer in Ameisen noch in verschiedenen Raupenarten vor, ferner in Bienen, Brennnesseln, im Honig und in geringen Mengen in manchen Mineralwässern. Ebenso findet man sie im Schweiß und im Harn. Vielen unseren Patienten dürfte die Ameisensäure bekannt sein, wenn auch weniger als Heilmittel, so doch als häufig verwendeter Konservierungsstoff.

In der Heilkunde hat Ameisensäure allerdings den Nachteil, bei peroraler Verabreichung nur bedingt wirksam zu sein, und man muß schon relativ große Mengen über einen längeren Zeitraum hinweg verabreichen, um die annähernd gleiche Wirkung von Injektionen zu erzielen.

Die Injektionstechnik ist denkbar einfach, zumal die Einspritzung überwiegend subkutan erfolgt. Nimmt man dazu eines der im Handel befindlichen Fertig-Präparate, wie etwa Formisoton, Acirufan oder Formidium, welche aus isotonischen Lösungen bestehen, so ist die Injektion absolut schmerzlos. Bei Kindern kann man die Einstichstelle vorher mit etwas Chloräthylspray anästhesieren oder mit einer Anästhesinsalbe einreiben. Als Injektionsstellen bei subkutaner Verabreichung wählt man am besten die Haut oberhalb der Schulterblätter oder die laterale Fläche des Oberschenkels bzw. des Unter- oder Oberarmes. Im übrigen dürfte es hinsichtlich der Wirkung bedeutungslos sein, an welcher Stelle des Körpers man die subkutane Injektion vornimmt, es sei denn, man betreibt ausgesprochene Neural- und Segment-Therapie. Die intravenöse Injektion wirkt bei akuten Fällen etwas rascher, läst sich jedoch nicht immer durchführen. Bei Kindern sollte man sie gar nicht anwenden, sie bekommen nur unnötige Angst vor Spritze und Behandler.

Von weitaus größerer Bedeutung jedoch ist die Frage der Dosierung. Diese hängt in erster Linie davon ab, ob wir es bei der vorliegenden Erkrankung mit einem akuten oder aber chronischen Fall zu tun haben. Grundsätzlich gelten auch hierbei die Regeln der Homöopathie, daß nämlich akut verlaufende Krankheiten niedrigere Verdünnungen in häufigeren Abständen erfordern, bei chronischem Verlauf jedoch hohe und höchste Verdünnungen in langen Zeitabständen verabreicht werden. Ihre besten Heilwirkungen entfaltet Ameisensäure etwa ab der D_5. Man mag zu den Hochpotenzen der Homöopathie stehen wie man will, Tatsache ist, daß Ameisensäure auch noch in der D_{200} wirkt, ja oftmals schneller und nachhaltiger als Tief- und Mittelpotenzen.

Dr. A. Reuter stellt in seinem Buch "Ameisensäure als Heilmittel" folgende allgemeine Regeln für die Ameisensäure-Behandlung auf:

1. Gesundes Gewebe bedarf höchster Dosen, um eine Reaktion erkennen zu lassen.
2. Akut entzündliches Gewebe reagiert bereits auf mittlere und kleine Dosen.
3. Chronisch erkranktes Gewebe reagiert schon auf kleine und kleinste Dosen.

Die Injektionsabstände richten sich also in erster Linie nach der Reaktionslage des Patienten. In akuten Fällen kann man ohne weiteres 2mal täglich injizieren, in chronischen Fällen 1mal monatlich bis 1mal vierteljährlich.

Zu den bevorzugten Indikationsgebieten gehören vor allem die aller-

gischen Erkrankungen, insbesondere Asthma (sofern es nicht kardial bedingt ist), Ekzeme, Psoriasis, Urticaria, Heuschnupfen, ferner der gesamte Formenkreis der exsudativen Diathese, rheumatische Erkrankungen, manche Migränearten, Schwangerschaftserbrechen, Schleimhautentzündungen des Magen-Darm-Traktes und chronische Nierenentzündungen.

In all diesen Fällen genügt es jedoch nicht, die Behandlung nur über einen kurzen Zeitraum hinweg durchzuführen, meist gelingt eine wirkliche Heilung nur bei einer Anwendung über Monate und sogar Jahre hinaus. Gewiß, eine lange Zeit, und Patient und Behandler müssen viel Geduld aufbringen, doch bietet die Ameisensäure-Therapie dafür die Chance einer echten Heilung. Wer ein weiteres tun will, kann die Ameisensäure auch mit Eigenblut verbinden, wobei hier allerdings der intra-muskulären Einspritzung der Vorzug zu geben ist.

Ausgesprochene Kontraindikationen bei der Anwendung der Ameisensäure bestehen nicht, lediglich bei Hypertonie, Koronarsklerose und Angina pectoris sollte man keine zu hohen Dosen verabreichen. Manche Patienten allerdings reagieren sehr empfindlich auf Ameisensäure, jedoch nur selten über das gewünschte Maß einer Erst-Verschlimmerung hinaus. Meist war in diesem Fall die Dosis zu hoch gewählt, und man versuche es nach Abklingen der Erscheinungen nochmals mit einer niedrigeren Dosis, bzw. höheren homöopathischen Potenz. Nimmt man zur Injektion eines der eingangs erwähnten Fertig-Präparate, so treten bei Einhalten der beigefügten Dosierungsanweisungen gar keine oder nur sehr geringe Nebenerscheinungen auf.

Zum Abschluß bringe ich noch einen Therapievorschlag, der jedem Behandler Gelegenheit gibt, sich von den guten Heilerfolgen der Ameisensäure-Therapie zu überzeugen. Wie häufig bringt man uns Kleinkinder mit Milchschorf in die Sprechstunde, und gerade hier wird unsere Behandlung oftmals zu einer Quälerei, nicht nur für die kleinen Patienten, die all die bitteren Tropfen und schlecht riechenden Salben über sich ergehen lassen müssen, sondern auch für uns, wenn wir am Ende nicht mehr weiter wissen. Hier injiziere man gleich Ameisensäure in der D_{12}, besser noch D_{30}. In den meisten Fällen wird nach einigen Tagen eine starke Erst-Verschlimmerung eintreten, welche einige Zeit anhalten kann, um dann anschließend in eine auffallende Besserung überzugehen. Man injiziere dann nochmals und kann erleben, daß nach einer erneuten leichten Verschlimmerung die Heilung eintritt. In vielen Fällen genügen 2 oder 3 Injektionen, um den kleinen bedauernswerten Patienten für immer von seinem Leiden zu befreien.

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('Naturheilpraxis', Juli 1967)

Chemical Rubber Company. 1967
CRC Handbook, 3rd Edition
Chemical Rubber Company, Cleveland, Ohio
p. 190

Committee on Specifications. 1972
Food Chemicals Codex
Committee of Food Protection, National Academy of Sciences,
National Research Council, Eashington, D.C.

FORMIC ACID

C.A., 56, 15904e, (1962)

191

Formic acid content of roasted coffee, chicory, soluble coffee extracts, and coffee substitutes. J. Deshusses. *Mitt. Gebiete Lebensm. Hyg.* 52, 425-30 (1961).—In the course of roasting coffee and coffee products HCOOH is formed. In roasted coffee HCOOH ranges from 135 to 220, in chicory 500, in coffee extn. with and without caffeine 200-770, in exts. from coffee substitutes 350-900 mg. %. Eugene E. Gansel

Detection

German Translation

Formic Acid Treatment of Female Genital Inflammatory Diseases

Dr. D. Eisenstadter, Chief Gynecologist.

(From the Zagreb General Public Foundation Hospital,

Director: Dr. I. Majnaric)

Source: Zentralbl. f. Gynak. 56:161-62, Jan. 16, 1932.

On the basis of the concept of Arndt-Schulz's law, the concept of oligodynamic action and that of irritation (or stimulation) delay according to Bier (see note*), and supported by the experience gained by the older Krull and in recent years by Reutter, I have started to use microdosages of formic acid in the treatment of inflammatory diseases.

Since I have been using this method of treatment for just about over one year and since my tests on the effect of formic acid on experimental inflammations have not yet been concluded, the following case description should be considered as a preliminary communication. Nevertheless, my experience has progressed so far that I can recommend with a clear conscience the further checking of this method.

At first, formic acid was used in a case, the success of which prompted me to conduct further tests. A female patient, who had suffered almost 7 years from painful, fist-size adnexa (uterine appendage) tumors, which could not be relieved by any common and known treatment methods, was unable to perform even the lightest house work due to frequent

* Translator's note: This statement is ambiguous. It could also mean: "stimulation delay after beer", since the German word for "beer" is "Bier", which can also be a proper name.

aggravations. Although this patient was finally ready to undergo an operation as the only possible recovery method, I still decided first to perform a test with formic acid injections. The success achieved was surprising: pain disappeared after 2 weeks, and after a further 6 weeks only prune-size, indolent resistances could be palpated. Menorrhagia, which in the last pre-treatment time often lasted up to 14 days, had stopped. Since that time, menstruation lasts 5 days and is moderately strong; no aggravation was noted during the following menstruation periods. Lumbar pains, weakness, psychic depression, pale coloring of the face have vanished. The patient was also able to endure bodily efforts, such as dancing and riding in a horse cart along a rough village street, without showing the slightest deterioration.

In a second case, where the patient was bed-ridden for almost 5 months in the hospital and was released with a tumor the size of a man's fist, I was also able to achieve the same success, so that this patient took a car trip of several days, against my advice, without suffering the slightest injury.

On December 27, 1930, a patient was admitted with a tumor the size of a man's head, following a curettage performed 4 weeks earlier apparently as a result of bleeding. The uterus was imbedded in the tumor, so that it was impossible to determine either the position of the uterus or on which side the tumor was located. The parametriums were free. Hardly one week after the formic acid injection, the tumor, which extended under the navel up to 3 cross fingers, could no longer be palpated from outside. After the second injection (after 12 days), the findings were as follows: retroverted, small uterus, with a palpable tumor the size of a hen's

egg on the right side and a tumor the size of a goose egg on the left side. On 28 January 1931, only a soft, completely insensitive tumor, almost the size of a hen's egg, was palpable on the left side. Further observation was unfortunately impossible since the patient was released upon her own request on February 2.

Parametric exudates and infiltrates exhibited the same behavior. Thus, an exudate of a size larger than a double fist had completely vanished in 5 weeks, and not even the slightest resistance was palpable in this case.

I have used formic acid in the case of perimetritis adhesions and fixed retroflexion, and shall also report on my findings in the near future.

Further tests will show to what extent and whether gonorrhoea can be helped (abetted) by this method, since progress is also in progress in this direction.

Results of the treatment have recently been compared with the sedimentation rate of red blood corpuscles. According to the experience gained so far, which however do not permit arriving at a final judgement, the sedimentation would approach normal values according to recovery. This fact could possibly also be an indicator of how long and whether the injections should be continued.

In none of the at least 50 cases treated with formic acid did an aggravation take place during menstruation, and also in no case did a failure occur. At this point, however, I must cite 2 cases which apparently did not respond to formic acid. In one case, I was able to detect, during the operation, a strongly adherent pyosalpinx (pus tube)

with extremely thick walls and larger than a double fist. It was removed, and the patient was released, completely cured, after 15 days. The second case, a puerperium patient admitted to the clinic of Prof. Durst, had an immobile hard tumor extending up to the navel, which did not respond to treatment after 3 weeks, so that injections were stopped.

We have practically never performed local treatment, although diathermy was used in some cases, because the patients were not satisfied that they were getting enough treatment, and, on the other hand, also to find out whether this would speed up recovery. No difference could be found.

Formic acid treatment is extremely simple. Intravenous injections, 1 cc of a 1:1,000,000 solution (equal to D6), are given into the arm vein, one injection every 10-14 days. It is advisable to use a glass syringe and a platinum or Contracid canula. The injection site should be disinfected with dilute alcohol. Seldom, and only in acute and subacute cases, did I inject 0.5-1 cc of a 1:100,000 (equal to D5) solution. In acute cases, injections can be given at shorter intervals, even at 5-day intervals, but in that case a D6 dilution should be used, i.e. a 1:1,000,000 solution. In rare cases was a moderate increase in size of the tumor and a slight temperature rise noted, up to a maximum of 38.5°C for 1-2 days. This must be considered either as a reaction or, although it might seem a paradox, as an overdosage. In each patient, however, a definite decrease of pains and general improvement were often observed already after the first injection. The duration of the treatment was never more than 10 weeks. In some cases, after recovery, I gave the patient a few prophylactic injections, namely a subcutaneous injection,

every 4-5 weeks, of 0.5-1 cc of a D_{12} solution. Subcutaneous injections of formic acid are painful. Ampuls of the acid ready for use were obtained from the firm of Dr. Wilmer Schwabe, Leipzig 029.

Aus dem Allgemeinen öffentlichen Fondspitale in Zagreb
Direktor: Dr. I. Majnarić

Ameisensäurebehandlung entzündlicher Erkrankungen des weiblichen Genitales

Von Dr. D. Eisenstädter, Primarius für Gynäkologie

Ausgehend von der Lehre des Arndt-Schulz'schen Gesetzes, der Lehre der oligodynamischen Wirkungen und jener des Reizverzuges nach Bier, gestützt durch die Erfahrungen des älteren Krull und in den letzten Jahren von Reutter, begann ich in der Behandlung der entzündlichen Erkrankungen Mikrodosen von Ameisensäure zu verwenden.

Da ich erst etwas über 1 Jahr diese Behandlungsmethode durchführe und meine Versuche über die Wirkung der Ameisensäure auf experimentell erzeugte Entzündung noch nicht abgeschlossen sind, sollen folgende Ausführungen als vorläufige Mitteilung betrachtet werden. Immerhin aber bin ich mit meinen Erfahrungen so weit gekommen, daß ich mit ruhigem Gewissen diese Methode zur Nachprüfung empfehlen darf.

Zuerst wurde das Mittel bei einem Fall verwendet, dessen Erfolg mich zu weiteren Versuchen ermutigt hatte. Eine Kranke, die fast 7 Jahre an schmerzhaften, faustgroßen Adnextumoren litt, welche durch keine der üblichen und bekannten Behandlungsmethoden zu beeinflussen waren, war durch öftere Exazerbationen selbst an der leichtesten Hausarbeit gehindert. Obwohl die Pat. zur Operation endlich als der einzigen Heilungsmöglichkeit entschlossen war, machte ich doch vorher den Versuch mit Ameisensäureinjektionen. Der Erfolg war überraschend. Nach 2 Wochen schwanden die Schmerzen, und nach weiteren 6 Wochen waren nur mehr pflaumengroße, indolente Resistenzen zu tasten. Menorrhagien, die in der letzten Zeit oft bis zu 14 Tagen dauerten, haben aufgehört. Die Menstruation ist seither 5tägig, mäßig stark. Keine Exazerbation während der folgenden Menstruationen. Kreuzschmerzen, Mattigkeit, psychische Depressionen, blasse Gesichtsfarbe sind verschwunden. Die Pat. konnte auch körperliche Anstrengungen, wie Tanzen und Wagenfahrt auf einer Dorfstraße, vertragen, ohne daß die geringste Verschlimmerung eingetreten wäre.

In einem zweiten Falle, der fast 5 Monate auf einer Abteilung lag und mit einem mannsfaustgroßen Tumor entlassen wurde, konnte ich denselben Erfolg verzeichnen, so daß die Pat. nach 8 Wochen gegen meinen Rat eine Autofahrt von mehreren Tagen machte, ohne den geringsten Schaden zu erleiden.

Am 27. XII. v. J. wurde eine Pat. mit kopfgroßem Tumor, nach einer angeblich wegen Blutungen vor 4 Wochen erfolgten Curettage aufgenommen. Der Uterus war in den Tumor eingemauert, so daß man weder die Lage des Uterus noch die Seitenzugehörigkeit des Tumors bestimmen konnte. Parametrien waren frei. Kaum 1 Woche nach der Injektion war der Tumor, welcher bis 3 Querfinger unter den Nabel reichte, von außen nicht mehr zu tasten. Bei der zweiten Injektion — nach 12 Tagen — konnte man folgenden Befund erheben: Uterus klein, retrovertiert, rechts ein hühnerei- und links ein gänseeigroßer Tumor zu tasten. Am 28. I. 1931 nur links ein fast hühnereigroßer, weicher Tumor tastbar, vollkommen unempfindlich. Leider war die weitere Beobachtung nicht möglich, da die Pat. am 2. II. auf eigenes Verlangen entlassen wurde.

Parametrane Exsudate und Infiltrate zeigten dasselbe Verhalten. So war ein über zweifaustgroßes Exsudat in 5 Wochen gänzlich verschwunden, ja es war sogar in diesem Falle nicht die geringste Resistenz mehr zu tasten.

Bei perimetritischen Adhäsionen und fixierter Retroflexio machte ich von Ameisensäure Gebrauch und werde demnächst auch darüber berichten können.

Inwiefern und ob die Heilung der Gonorrhöe durch dieses Verfahren unterstützt wird, werden die weiteren Versuche zeigen, da bereits auch in dieser Richtung gearbeitet wird.

Die Ergebnisse der Behandlung werden seit kurzem mit der Senkungsgeschwindigkeit der roten Blutkörperchen verglichen. Nach den bisherigen Erfahrungen, die aber noch kein endgültiges Urteil erlauben, soll sich entsprechend der Heilung die Sedimentation der Norm nähern. Dies könnte auch möglicherweise der Indikator werden, wie lange und ob mit den Injektionen prophylaktisch fortgesetzt werden soll.

In keinem von den mindestens 50 mit Ameisensäure behandelten Fällen kam es zu Verschlimmerung während der Menstruation, ebenso kann in keinem Falle von einem Mißerfolg gesprochen werden. Hier muß ich jedoch zwei Fälle erwähnen, die scheinbar auf Ameisensäure nicht ansprachen. Bei einem konnte ich bei der Operation eine über zweifaustgroße, besonders dickwandige, stark verwachsene Pyosalpinx feststellen. Exstirpation, nach 15 Tagen geheilt entlassen. Bei der zweiten, an der Klinik des Herrn Prof. Durst liegenden Puerpera, handelte es sich um einen bis zum Nabel reichenden, unbeweglichen, harten Tumor, der nach 3 Wochen nicht reagierte, so daß die Injektionen ausgesetzt wurden.

Lokalbehandlung übten wir so gut wie gar nicht. In einigen Fällen Diathermie, weil die Pat. unzufrieden waren, daß man sie zu wenig behandelte. Andererseits auch deshalb, um zu sehen, ob damit die Heilung beschleunigt wird. Es konnte kein Unterschied festgestellt werden.

Die Ameisensäurebehandlung ist ungemein einfach. Intravenöse Injektionen in die Armvene, 1 ccm der Lösung 1 : 1000000 (= D6), alle 10—14 Tage eine Injektion. Es ist ratsam, Glasspritze und Platin- bzw. Contracidkanüle zu verwenden. Die Injektionsstelle ist mit Alkoh. dilutus zu desinfizieren. Selten, und nur in akuten und subakuten Fällen, gab ich ½—1 ccm der Lösung 1 : 100000 (= D5). Bei akuten Fällen können die Injektionen auch in kürzeren Abständen verabreicht werden, sogar bis zu 5tägigen Intervallen, dann aber nur die Verdünnung D_6, i. e. 1 : 1000000. In seltenen Fällen sah man nach der ersten Injektion mäßige Vergrößerung des Tumors und leichte Temperatursteigerung, höchstens bis 38,5 für 1—2 Tage. Das ist entweder als Reaktion oder, wenn es auch paradox scheinen mag, als Überdosierung aufzufassen. Bei jeder Kranken aber war schon oft nach der ersten Injektion deutliches Nachlassen der Schmerzen zu beobachten, wie auch Besserung des Allgemeinbefindens. Die Dauer der Behandlung war nie länger als 10 Wochen. In einzelnen Fällen gab ich nach erfolgter Heilung noch einige prophylaktische Injektionen, und zwar ½—1 ccm der Lösung D_{12}, alle 4 bis 5 Wochen eine subkutane Injektion. Die subkutanen Injektionen sind schmerzhaft.

Gebrauchsfertige Ampullen bezog ich von der Firma Dr. Willmar Schwabe, Leipzig O 29.

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FORMIC ACID

C.A., 62, 11075c (1965)

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Honey. Jean Estienne. *France Parfums* 7(41), 383-90 (1964)(Fr). The chem. compn. of honey: H_2O 20.64, glucose 35.40, fructose 34.90, sucrose 6.39, dextrins 0.80, albuminoids 0.50, minerals 0.20, invertase 0.25, HCOOH 0.20%, and vitamins trace. Characteristics of the components are discussed, including properties and physiol. effects. Examples of the therapeutic value of honey are presented. M. Kenigsberg

DETECTION

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INACTIVATING DNA ALTERATIONS INDUCED BY PEROXIDES AND PEROXIDE-PRODUCING AGENTS

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SUMMARY

Agents containing a free-NOH group such as hydroxylamine, *N*-methylhydroxylamine, hydroxyurea, hydroxyurethan, and hydrazines produce H_2O_2 on exposure to oxygen. All these agents, H_2O_2 itself, and disuccinyl peroxide, predominantly induce inactivating DNA alterations, whereas their mutagenic effect (per lethal hit) on transforming DNA is small. Chemicals containing NH_2 in place of NOH, such as urea and urethan (ethylcarbamate) do not inactivate DNA.

Formaldehyde and its oxidation products, formic acid, performic acid and dihydroxymethyl peroxide do not inactivate or mutate transforming DNA at a significant rate.

INTRODUCTION

Chemical alterations of DNA have been operationally divided into mutagenic and inactivating alterations[19]. Both alterations can give rise eventually to cellular mutations but on the whole, inactivating alterations are more frequently lethal for the cell than mutagenic ones. Furthermore, the mechanism of mutation induction is quite different for the two alterations. Whereas mutagenic DNA alterations do not prevent DNA replication across the altered site and give rise to point mutations, inactivating alterations do block DNA replication and produce mostly chromosomal breaks or large chromosomal aberrations, except when they are repaired or when replication occasionally proceeds across the alteration giving rise to a point mutation.

This paper shows by means of transforming DNA that agents which produce H_2O_2, H_2O_2 itself, and certain organic peroxides mainly inflict inactivating DNA alterations. This effect apparently involves the intermediate formation of radicals because it is inhibited by chelating agents or radical scavengers.

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MATERIALS AND METHODS

Transformation. DNA donor strain: 60009 (= S B 19 Romig) = prototroph. Recipient strain: 60087 = tryptophan⁻. Transformation to tryptophan independence and production of fluorescent mutants were assayed as described previously[14]. DNA was isolated as described by FREESE AND FREESE[15].

Treatment of DNA. DNA (100 μg/ml) in 2 *M* NaCl was diluted 10-fold in the ice cold reaction mixture, mixed well, and a control sample diluted 50-fold into ice cold stopping mixture (0.05 *M* Tris + 5 μg/ml catalase). All reaction mixtures were buffered by 0.02 *M* sodium phosphate, pH 7.5, except when other pH's are stated. The reaction tube was placed in a water bath at the desired temperature and after different times, aliquots were diluted 50-fold into the ice cold stopping mixture. For transformation the DNA was diluted another 10-fold. All points of a given curve were determined using the same batch of both DNA and transformable bacteria.

Production of hydrogen peroxide was measured by the titanium sulfate method (for detail see FREESE AND FREESE[15]).

Chemicals. Most of the chemicals were prepared by the Aldrich Chemical Company, Milwaukee, Wisconsin: *N*-methylhydroxylamine, *O*-methylhydroxylamine = *O*-methoxylamine, carboxymethoxyamine, *N*-hydroxyurethan = ethyl *N*-hydroxycarbonate, hydroxyurea and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine and nitrosopiperidine.

Urethan = ethylurethan = ethyl-carbamate, hydrazine, hydrogen peroxide and formic acid were bought from Fisher Scientific Co. Formaldehyde was also purchased from this Company as a 30% solution in 15% methanol; it was obtained in pure form by fractional distillation at 45°, with a −70° trap after the condensing column, and collected in deionized and distilled water. Hydroxylamine HCl was bought from the Fluka Chemical Co., Buchs, Switzerland, urea from J. T. Baker, Chem. Co., peroxidase from Sigma Chem. Co., and catalase from Worthington Biochem. Corp. Disuccinylperoxide was prepared by the method of CLOVER AND HOUGHTON[9] and di-hydroxymethyl peroxide by the method of WIELAND AND WINGLER[20]; both compounds were 4 times recrystallized and freeze dried to remove all traces of H_2O_2. Performic acid was prepared by the method of HIRS[22].

RESULTS

Reactions of transforming DNA with H_2O_2

(1) Inactivation. The inactivation of the tryptophan marker of a given preparation of transforming DNA by different H_2O_2 concentrations (pH 7.5, 45°) is shown in Fig. 1. The inactivation rates increased with the H_2O_2 concentration.

DNA prepared by different methods showed greatly different inactivation rates in H_2O_2. Fig. 2 shows, for example, the inactivation by H_2O_2 of 2 differently prepared DNA samples, of which one (prep. I) was barely inactivated by 10^{-2} *M* H_2O_2 alone but responded strongly to the addition of $FeSO_4$ (10^{-5} *M*). (This preparation was not used for other experiments in this paper, because even with 10^{-2} the rate of inactivation was much smaller than that observed for our ordinary DNA preparations (prep. II).) DNA prepared by our usual method did not exhibit an increased inactiva-

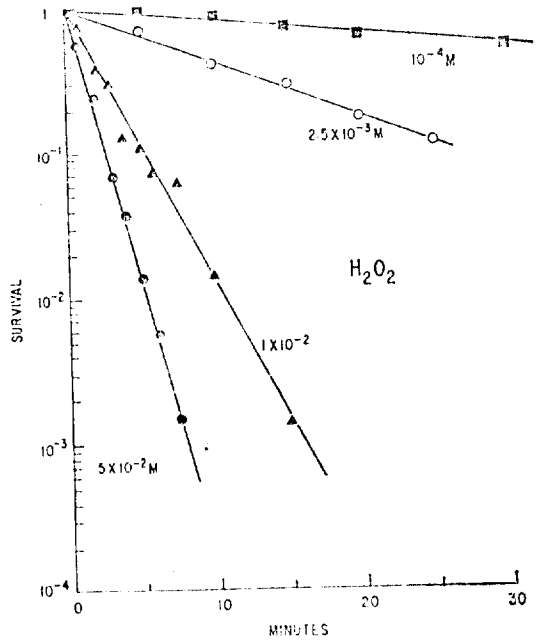

Fig. 1. Inactivation of the tryptophan marker of *B. subtilis* transforming DNA by different H_2O_2 concentrations.

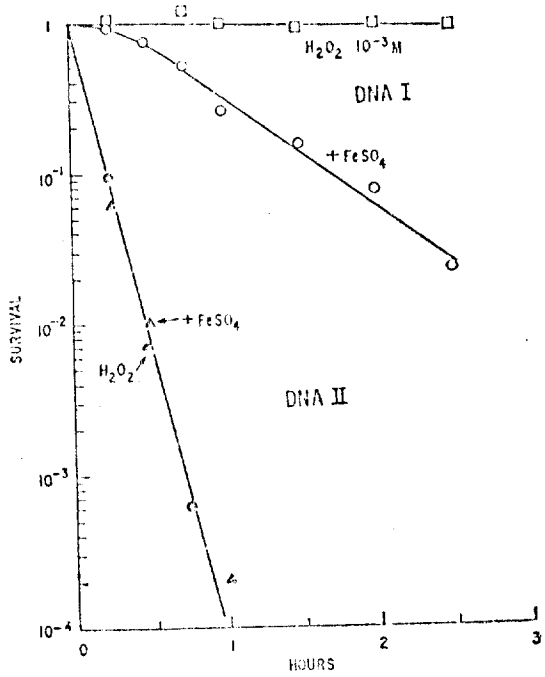

Fig. 2. Inactivation of H_2O_2 of 2 differently prepared DNA samples.

tion rate when $FeSO_4$ was added (Fig. 2), presumably because it contained enough trace metals.

Sodium pyrophosphate (0.05 *M*), KCN (10^{-2} *M*), $FeCl_3$ ($2 \cdot 10^{-3}$ *M*) and EDTA (10^{-2} *M*) reduced the inactivation rate (see Fig. 3), whereas catalase (10 mμ/ml) and peroxidase (10 mμ/ml) completely abolished the inactivation (Fig. 4).

(2) Mutation. The induction of mutations by H_2O_2 was measured by the frequency of fluorescent colonies among the tryptophan independent transformants. The

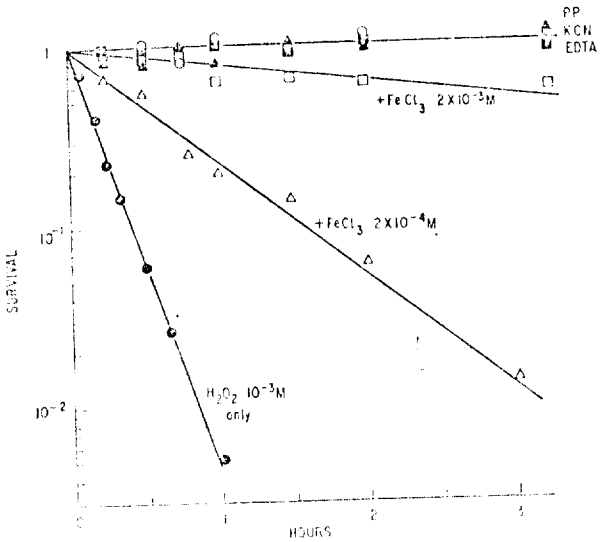

Fig. 3. DNA inactivation by H_2O_2: inhibition by KCN = ○; EDTA = □; pyrophosphate (PP) = △; $FeCl_3$ ($2 \cdot 10^{-3}$ *M*) = □; $FeCl_3$ ($2 \cdot 10^{-4}$ *M*) = △.

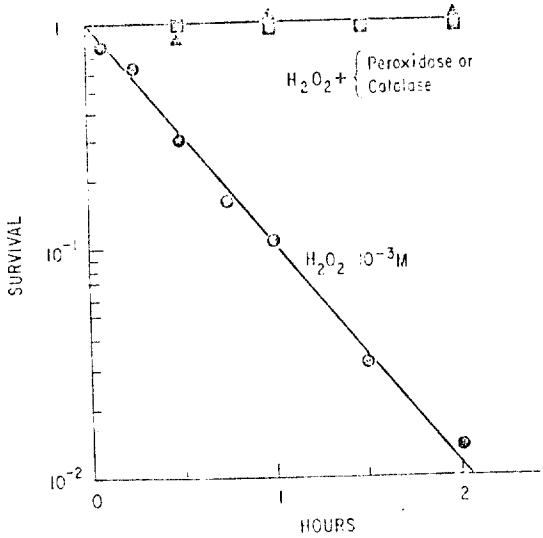

Fig. 4. DNA inactivation by H_2O_2: inhibition by catalase (10 μg/ml) and peroxidase (10 μg/ml).

frequency of these linked forward mutations increased barely measurably (about 0.04% mutations per lethal hit, see Table I).

TABLE I

RATE OF MUTATION INDUCTION PER LETHAL HIT FOR DIFFERENT AGENTS

Agent	*Molar concentration*	*pH*	*Temp. in °C*	*Mutation % per lethal hit*
Hydroxylamine = HA	1	7.5	70	6
	10^{-2}	7.5	70	~0.02
N-Me-HA	1	7.5	70	6
	10^{-2}	7.5	70	~0.02
O-Me-HA	1	7.5	70	3
Carboxymethoxyamine	1	7.5	70	3
Hydroxyurethan	1	6.2	75	0.15
	10^{-2}	6.2	75	~0.03
Hydroxyurea	10^{-2}	7.5	45	≲0.01
Hydrazine	1	5–7.5	45	~0.02
	10^{-2}	7.5	45	≲0.01
Hydrogen peroxide	10^{-2}	7.5	45	~0.04
Disuccinyl peroxide	10^{-3}	7.5	45	~0.1
N-Methyl-*N*'-nitro-	0.05	5	60	0.3
N-nitrosoguanidine	0.05	7	60	0.11

H_2O_2 production by different agents

Many agents react with oxygen, producing radicals, organic peroxides, and hydrogen peroxide. The hydrogen peroxide production of several agents, which

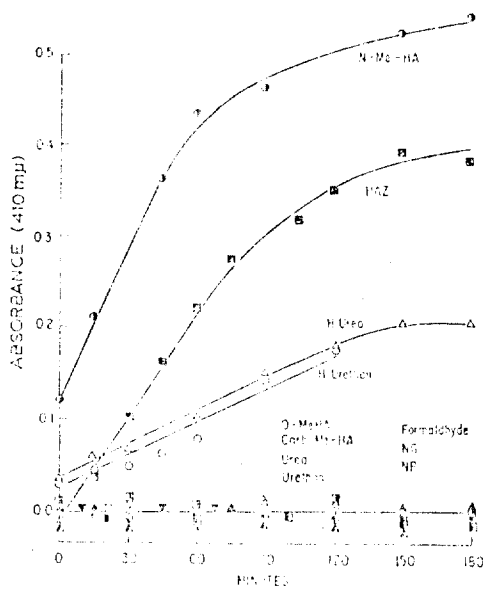

Fig. 5. Production of H_2O_2 by different chemicals at 10^{-2} *M* concentration: ◑, *N*-methylhydroxylamine; ⊡, hydrazine; △, hydroxyurea; ○, hydroxyurethan; ◐, *O*-methylhydroxylamine; ⊿, carboxymethoxyamine; ▽ urea; ●, urethan; ▲, formaldehyde; ◧, nitrosoguanidine; ▮, nitrosopiperidine.

inactivate DNA, has been measured previously, as shall be mentioned in the discussion. For other agents hydrogen peroxide production was measured by the titanium sulfate method.

Fig. 5 shows that peroxide production was observed for 10^{-2} *M* concentrations of hydrazine, hydroxyurea, *N*-hydroxyurethan, and *N*-methylhydroxylamine, but not for *O*-methyl-hydroxylamine, carboxymethoxyamine, urea, urethan, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, and nitrosopiperidine.

Reactions of transforming DNA with hydrogen peroxide producing agents

In order to see whether there was a correlation between the production of H_2O_2 (*i.e.* radicals) and the reaction of transforming DNA, different agents were tested for their inactivating and mutagenic effect.

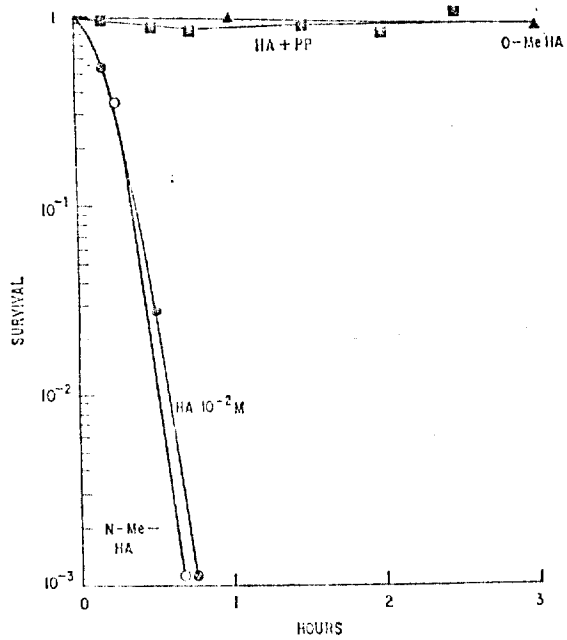

Fig. 6. Inactivation by 10^{-2} *M* hydroxylamine (HA), *N*-methylhydroxylamine (*N*-Me-HA), *O*-methylhydroxylamine (*O*-Me-HA), and HA + PP (sodium pyrophosphate 0.05 *M*).

(1) Inactivation. (a) 10^{-2} *M* hydroxylamine or 10^{-2} *M* *N*-methyl-HA rapidly inactivated DNA whereas 10^{-2} *M* *O*-methyl-HA did not. The inactivation was abolished when 0.5 *M* pyrophosphate was present.

(b) Hydroxyurea (10^{-2} and 10^{-1} *M*) inactivated DNA rapidly, while urea did not. In the presence of 0.5 *M* pyrophosphate hydroxyurea also did not inactivate DNA (Fig. 7).

(c) 10^{-2} *M* *N*-hydroxyurethan inactivated DNA whereas 10^{-2} *M* urethan or hydroxyurethan together with pyrophosphate showed no inactivation (Fig. 8).

(d) 10^{-2} *M* hydrazine inactivated DNA; this effect was inhibited by 10 m*µ*/ml

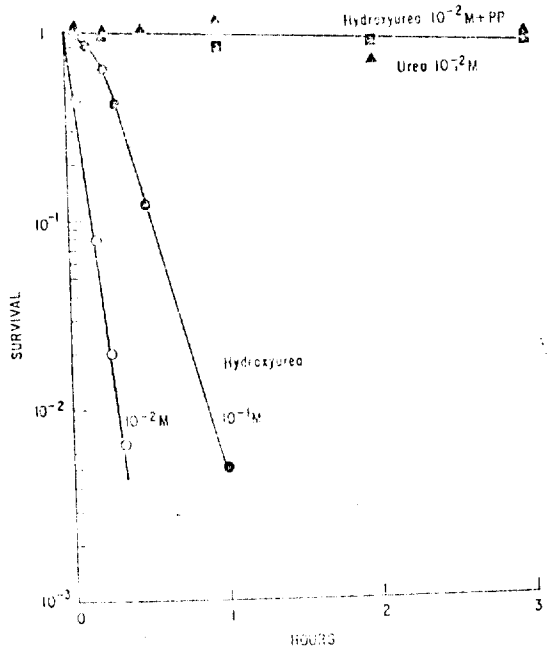

Fig. 7. Inactivation by hydroxyurea and urea.

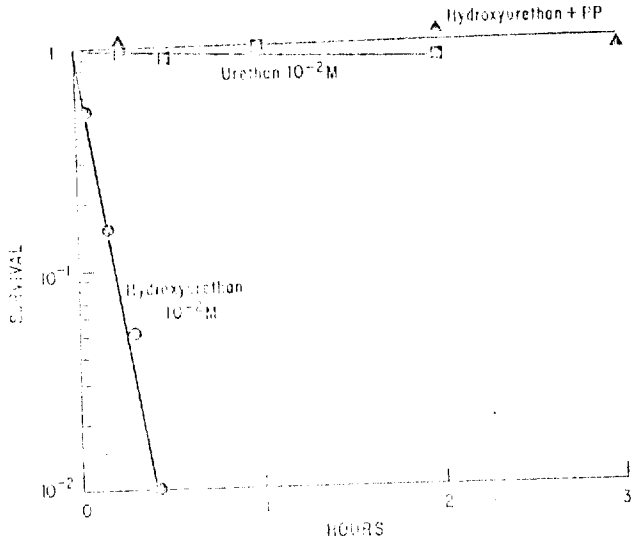

Fig. 8. Inactivation by hydroxyurethan and urethan.

catalase or 10^{-4} M EDTA, but not by 10 mμ/ml peroxidase or 0.5 M pyrophosphate. In the presence of nitrogen some inactivation still took place but the inactivation rate later decreased (Fig. 9).

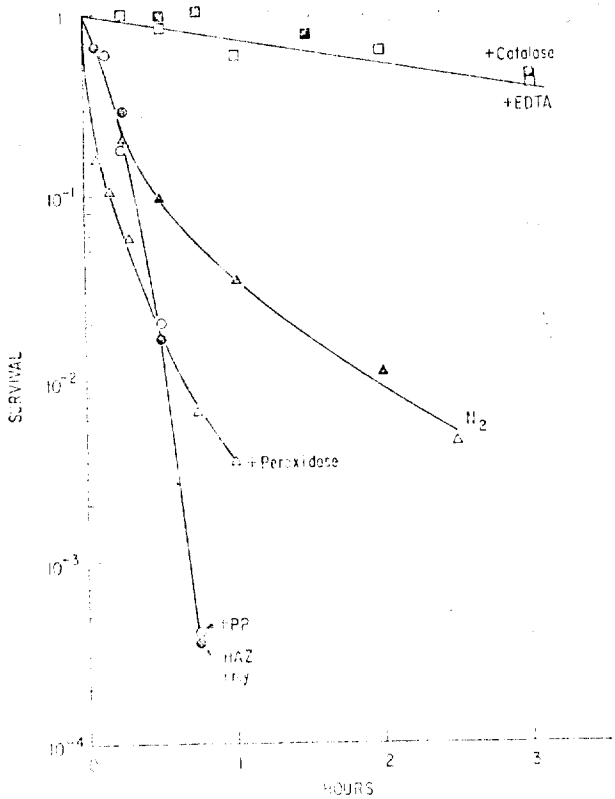

Fig. 9. Inactivation by hydrazine and its inhibition by ▪ catalase; ○ EDTA; ▲ N_2 atmosphere; and △ peroxidase.

(c) 10^{-3} *M* *N*-methyl-*N*-nitro-*N*-nitrosoguanidine showed almost no inactivation of DNA and did not influence the inactivation by H_2O_2.

(2) Mutations. The induction of fluorescent mutants has been measured for all agents for which inactivation was analyzed. A high rate of mutation induction was observed with 1 *M* concentrations of HA, *N*-methyl-HA, *O*-methyl-HA and carboxymethoxyamine. At concentrations of 10^{-2} *M*, however, at which many agents both produced peroxide and inactivated DNA at a high rate, the mutation induction per lethal hit was very low (see Table I).

Reactions of transforming DNA with formaldehyde and organic peroxides

(a) Formaldehyde at concentrations of 10^{-1} and 10^{-2} *M* did not inactivate DNA. A mixture of 10^{-2} *M* formaldehyde and 10^{-1} *M* H_2O_2 incubated at 45° for 1 h, then catalase added for ½ h to destroy the unreacted H_2O_2, also did not inactivate DNA. Finally, 10^{-2} *M* H_2O_2 inactivated DNA at the same rate whether 10^{-2} *M* formaldehyde had been added or not (see Fig. 10).

(b) Formic acid and performic acid (10^{-1} or 10^{-2} *M*) did not inactivate DNA.

(c) Di-hydroxymethyl peroxide (10^{-1} or 1 M) did not inactivate DNA (Fig. 10), if it had been sufficiently purified (4 × recrystallization + freeze drying). (After 2 × recrystallization some residual inactivation, which soon leveled off, had still been ob-

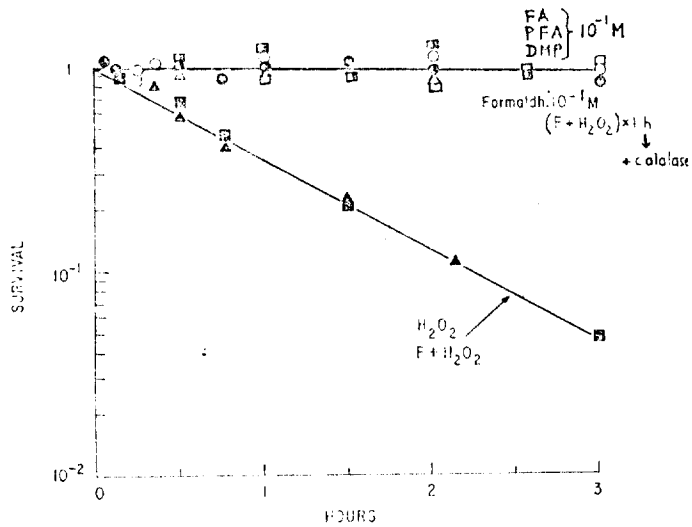

Fig. 10. Absence of DNA inactivation by formaldehyde ● and its oxidation products: formic acid (FA) ■; performic acid (PFA) ▲; and di-hydroxymethyl peroxide (DMP) □.

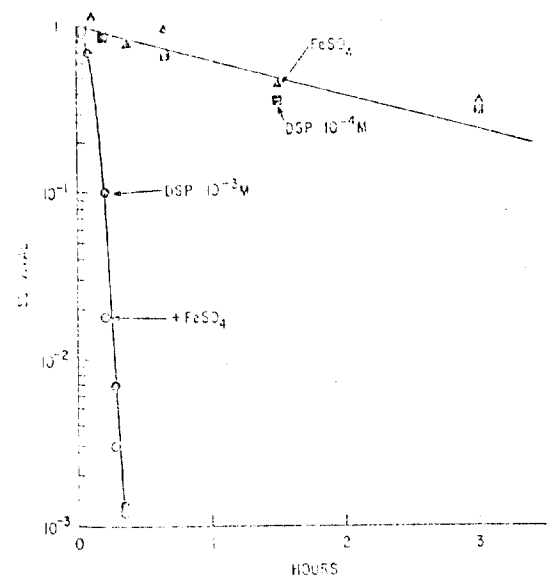

Fig. 11. Inactivation of DNA by disuccinyl peroxide (DSP): ●, DSP 10^{-3} M; ○, DSP 10^{-3} M + $FeSO_4$ 10^{-5} M; ■, DSP 10^{-4} M; ▲, DSP 10^{-4} M + $FeSO_4$ 10^{-5} M.

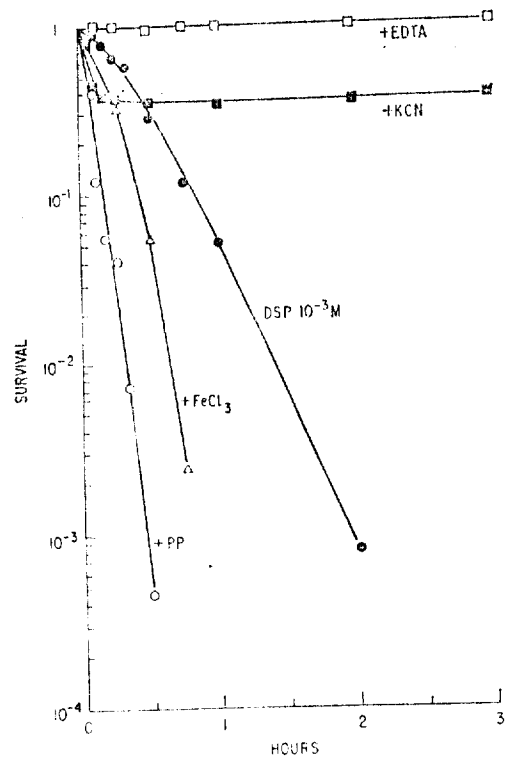

Fig. 12. Inhibition of DNA inactivation by disuccinyl peroxide (DSP): inhibition by KCN and EDTA.

served, apparently caused by H_2O_2 which had been left over from the reaction of formaldehyde with H_2O_2.)

(d) Disuccinyl peroxide (10^{-3} *M* or 10^{-4} *M*) inactivated DNA at a rate which was not influenced by the addition of $FeSO_4$ (Fig. 11). The inactivation could not be inhibited by pyrophosphate, $FeCl_3$, peroxidase, or catalase (catalase and albumin had the same small effect). However, EDTA and KCN prevented the reaction (Figs. 12 and 13). Disuccinyl peroxide at 10^{-3} *M* did not induce forward mutations at a significant rate (see Table I).

DISCUSSION

Reactions with transforming DNA

We have shown that several agents containing free NOH groups and hydrazine produce, in the presence of oxygen, hydrogen peroxide. Earlier papers have analyzed the production of hydrogen peroxide by isoniazid (4-NC_5H_4·CO·NH·NH_2, a hydrazine derivative)[50], methylhydrazine compounds[49], and by hydroxylamines[18]. An oxygen effect has been reported for the chemical reactions of hydroxylamine[21] and hydrazine[6] with DNA or its components.

All peroxide producing agents that were examined, and H_2O_2 itself, showed a

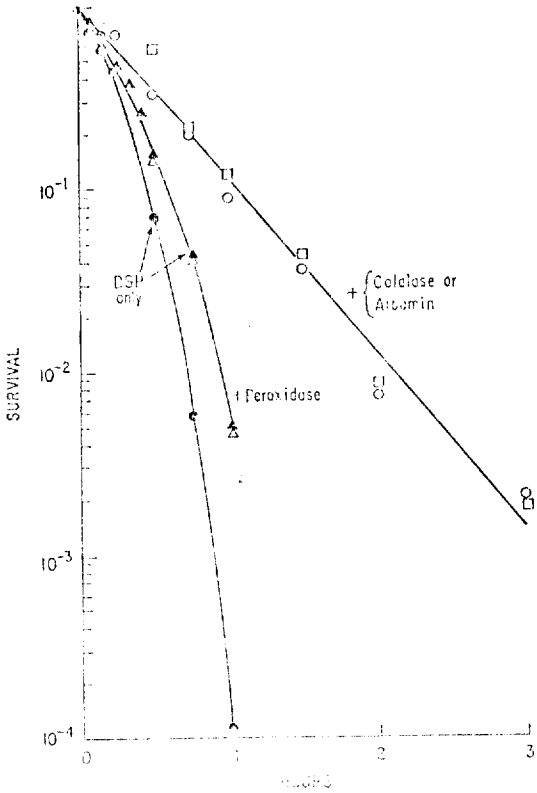

Fig. 13. Inactivation of DNA by disuccinyl peroxide (DSP) in the presence of peroxidase (10 μg/ml) (△); catalase (10 μg/ml) (▲); or albumin (10 μg/ml) (□).

trong inactivating effect on transforming DNA which was accompanied by the production of only very few mutations per lethal hit (see Table I). The inactivation of transforming DNA has been earlier reported for hydrogen peroxide[32,28,32] for hydroxylamine and *N*-methylhydroxylamine[16,15]; hydrazine[19]. Mutations were induced at a high rate only by high concentrations (1 *M*) of hydroxylamines by a reaction which has previously been shown to be oxygen independent[16]. Hydrazine has been observed to inactivate phages more rapidly at low (10^{-1} *M*) than at high (1 *M*) concentrations[13], presumably because hydrazine both produces and destroys radicals and H_2O_2, similar to hydroxylamine[12].

Both the peroxide production and the DNA inactivation by hydroxylamines were inhibited by cyanide, excess of oxidized Fe^{3+}, EDTA, pyrophosphate, or catalase. It is therefore apparent that they required the formation of radicals. Most DNA preparations could react with H_2O_2 without the addition of trace metals. Some DNA preparations, however, reacted only when (reduced) Fe^{2+} had been added. As a polyanion, DNA tends to be covered with metals which can be removed only by exchange with other cations. Some trace metals seem to be required therefore as catalysts for the radical reactions.

TABLE II

CORRELATION BETWEEN THE PRODUCTION OF H_2O_2 AND INACTIVATION OF DNA BY DIFFERENT AGENTS

Molar concentration	*Agent*	*H_2O_2 production pH 9, 70°*	*Inactivation pH 7.5, 45*	*Mutation pH, temp. see Table I*
10^{-2}	Hydroxylamine = HA	+	+	—
10^{-2}	*N*-Me-HA	+	+	—
10^{-2}	*O*-Me-HA	—	—	—
10^{-2}	Carboxymethoxyamine	—	—	—
10^{-2}	Hydroxyurea	+	+	—
10^{-2}	Urea	—	—	—
10^{-2}	Hydroxyurethan	—	+	—
10^{-2}	Urethane	—	—	—
10^{-2}	Hydrazine		+	—
10^{-3}	Hydrogen peroxide	+	+	—
10^{-3}	Disuccinylperoxide	—	+	(+)
10^{-2}	Formaldehyde	—	—	—
$5 \cdot 10^{-2}$	Nitrosoguanidine	—	—	(—)

The inactivation of DNA by hydrazine was also inhibited by catalase and EDTA but not by pyrophosphate. The inhibition of the earlier-mentioned reactions by pyrophosphate is not clear: Pyrophosphate is known to chelate certain metals, but its effect might also be due to the rupture of pyrophosphate bonds by the radicals, thus acting as a radical scavenger by decreasing the available free energy of the resulting radicals. The autoxidation of hydrazine may not be inhibited by hydrazine either because it requires trace metals that cannot be chelated by pyrophosphate or because some of the produced radicals may have too low energy to split the pyrophosphate bond but high enough energy to attack DNA. *O*-methylhydroxylamine, carboxymethoxyamine, urea and urethan neither produced H_2O_2 nor did they inactivate transforming DNA.

Formaldehyde autoxidizes slowly[18]; the concentration of radicals present at any time apparently is very low because the rate of DNA inactivation is negligibly slow. This ineffectiveness of low concentrations ($< 10^{-1}$ *M*) of formaldehyde on DNA has already been reported by ZAMENHOF *et al.*[52]. At higher concentrations (0.5 *M*) formaldehyde reacts with RNA (ref. 11) or denatured DNA (ref. 43), forming *N*-hydroxymethyl groups ($-NH-CH_2OH$) with free amino groups. Native DNA shows a very small reaction which becomes significant only at high formaldehyde concentrations (4%) and causes the inactivation of transforming DNA (ref. 52). At such high concentrations (12%) formaldehyde slowly (24 h) causes crosslinking of DNA (ref. 21). These reactions do not seem to be responsible for the *in vivo* effect of formaldehyde in *Drosophila* or *Neurospora*, because the effective formaldehyde concentration near the chromosome should be much too small. In fact, the *in vivo* mutagenic effect of formaldehyde is greatly enhanced by the addition of H_2O_2 to formaldehyde, indicating that some oxidation product of formaldehyde may be responsible (see SOBELS[11]). We have therefore examined the inactivating and mutagenic effect of oxidation products of formaldehyde: formic acid, performic acid, and dihydroxymethyl-peroxide, finding all 3 compounds ineffective. Presumably, one of these organic peroxides, which are not attacked by catalase (that renders H_2O_2 ineffective), causes the *in vivo* reaction by enzymic conversion into an active radical. It remains open whether this radical induces mutations by direct alteration of DNA or indirectly.

A similar enzymic activation seems to be responsible for the effect of other organic peroxides which have not shown any effect on transforming DNA but do induce mutations or inactivate cells *in vivo*: di-*t*-butyl peroxide or cumene peroxide (refs. 28, 24).

Disuccinyl peroxide, however, seems to have a sufficiently low activation energy for decomposition that it is effective *in vitro*. The inactivation of transforming DNA by this agent has been reported already by LATARJET *et al.*[28] and LUZZATI *et al.*[32]. The radical involvement is indicated by the inhibition of the inactivation by EDTA and KCN. Being an organic peroxide, catalase cannot inhibit its effect. Pyrophosphate also does not inhibit the reaction. The reason for this ineffectiveness may be the same as that for hydrazine mentioned above.

Effects in cellular organisms

Many of the radical producing agents have been shown to be lethal to microorganisms: e.g. hydrogen peroxide inactivates *Staphylococcus*[31] and *Escherichia coli*, the latter more rapidly in a strain which lacks catalase[1]. Disuccinyl peroxide inactivates *E. coli*[7]. *Mycobacterium tuberculosum* is inactivated by isoniazid[30] and *E. coli* by hydrazine[29] and hydroxyurea[38]. It has not been established whether death was caused by the alteration of DNA or some other cell components. But at least in the case of hydroxyurea a drastic alteration of bacterial DNA has been observed after prolonged exposure of the cells[38].

The induction of chromosomal breaks has been shown for some of the agents: hydroxylamine and many of its derivatives having free NOH groups induce chromosomal breaks in mammalian cells[36,37,17]. Urethan induces chromosomal alterations in *Oenothera*, *Vicia faba*[39] and in *Drosophila*[16], presumably by enzymic conversion to hydroxyurethan[40] and consequent production of radicals. Maleic hydrazide induces chromosomal breaks in *Vicia faba*[25]. *Tert.*-butylhydroperoxide induces chromosomal breakage and rearrangements in *Vicia faba*[41].

Most hydroxylamines having free NOH groups or hydrazines (see TARNOWSKI *et al.*[11]) and many peroxides (see KOTIN AND FALK[26]) are carcinogenic if they carry an organic residue, which presumably is needed to permit passage of cellular (and nuclear) membranes.

Several of the radical-producing chemicals examined in this paper have been reported to be mutagenic in different organisms (in bacteria: WYSS *et al.*[51], CHEVALIER AND LUZZATI[7], LINGENS[29,30]; *Neurospora*: DICKEY *et al.*[10], WAGNER *et al.*[17], JENSON *et al.*[31]; *Aspergillus*: VAN ARGEL[15]; higher plants: OEHLKERS[33,34]; *Drosophila*: RAPOPORT[35], VOGT[16], AUERBACH[3], ALTENBURG[2], SOBELS[10,11]; phages: FREESE *et al.* refs. 12, 13). In *Drosophila* recessive lethals were measured; these could have been caused by chromosomal breaks or point mutations, whereas the induction of reverse mutations presumably was caused by point mutations in *Neurospora*, bacteria, and phages. In most cases the mutagenic efficiency, *i.e.* mutations per lethal hit has not been measured, but where it has been determined, in bacteria or phages, it was low. The treatment of *Neurospora conidia*[31] showed a seemingly high mutagenic efficiency, but the lethal effect may have been masked because the *conidia* were multinucleate: the different nuclei can cancel a lethal effect by crossfeeding. It is therefore not clear at this time whether most of the induced mutations were caused by occasional mutagenic DNA alterations or rather by the predominant effect of inactivating DNA alterations.

Since most of the above agents give rise to OH radicals, the chemical reactions of H_2O_2 with DNA and its components have also been examined and will be discussed in different papers[36,37]. It has been found that pyrimidine rings are broken frequently, all four bases are liberated less frequently, and the DNA backbone is broken as a consequence of these reactions. All these effects constitute inactivating DNA alterations. In addition, adenine (A) is occasionally altered to a base analog A_1. The possibility that this effect constitutes a mutagenic DNA alteration is under investigation.

REFERENCES

1 ADLER, H. J., Catalase, hydrogen peroxide and ionizing radiation, *Radiation Res.*, Suppl. 3 (1963) 110–129.

2 ALTENBURG, E. S., The production of mutations in *Drosophila* by tertiary-butyl hydroperoxide, *Proc. Natl. Acad. Sci. (U.S.)*, 40 (1954) 1037–1040.

3 AUERBACH, C., Mutation tests on *Drosophila melanogaster* with aqueous solutions of formaldehyde, *Am. Naturalist*, 86 (1952) 330–332.

4 BERNTSEN, K., M. KOELLER, W. BOLLAG, P. ZELLER, A. KAISER AND A. LANGEMANN, Der prooxidative Effekt tumorhemmender Methylhydrazin-Verbindungen, *Helv. Chim. Acta*, 46 (1963) 2157–2167.

5 BERNTSEN, K., M. KOELLER AND W. BOLLAG, The influence of chelating agents on the prooxidative effect of a hydrogen peroxide producing methylhydrazine compound, *Experientia*, 20 (1964) 73–74.

6 BROWN, D. M., A. D. MCNAUGHT AND P. SCHELL, The chemical basis of hydrazine mutagenesis, *Biochem. Biophys. Res. Commun.*, 24 (1966) 997–974.

7 BORESTEIN, D., M. KEIN AND A. BENTSCH, Chromosomal alterations induced by hyponitrite and hydroxylamine derivatives, *J. Natl. Cancer Inst.*, 32 (1964) 607–676.

8 CHEVALLIER, M. R., AND D. LOZZATI, Action mutagène spécifique de trois peroxydes organiques sur les mutations de deux loci de *E. coli* 15 T⁻ 4–13, *Compt. Rend.*, 258 (1964) 1572–1574.

9 CLOVER, A. M., AND A. C. HOUGHTON, The action of hydrogen peroxide upon anhydrides and the formation of organic acid, peroxides and peracids, *Am. Chem. J.*, 32 (1904) 43–68.

10 DEMERY, F. H., G. P. CITTERIO AND C. LOZZI, The role of organic peroxides in the induction of mutations, *Proc. Natl. Acad. Sci. (U.S.)*, 45 (1959) 581–586.

11 FRANCOIS CONRAT, H., Reaction of nucleic acid with formaldehyde, *Biochim. Biophys. Acta*, 15 (1954) 377–379.

12 FREESE, E., E. B. FREESE AND E. BAUTZ, Hydroxylamine as a mutagenic and inactivating agent, *J. Mol. Biol.*, 3 (1961) 133–143.

13 FREESE, E., E. BAUTZ AND E. B. FREESE, The chemical and mutagenic specificity of hydroxylamine, *Proc. Natl. Acad. Sci. (U.S.)*, 47 (1961) 345–355.

14 FREESE, E., AND H. B. SLACK, Induction of mutations in transforming DNA by hydroxylamine, *Proc. Natl. Acad. Sci. (U.S.)*, 48 (1962) 1796–1804.

15 FREESE, E. B., AND E. FREESE, The rate of DNA strand separation, *Biochemistry*, 2 (1963) 707–715.

16 FREESE, E. B., AND E. FREESE, Two separable effects of hydroxylamine on transforming DNA, *Proc. Natl. Acad. Sci. (U.S.)*, 52 (1964) 1289–1297.

17 FREESE, E. B., The effects of urethan and hydroxyurethan on transforming DNA, *Genetics*, 51 (1965) 953–960.

18 FREESE, E., AND E. B. FREESE, The oxygen effect on deoxyribonucleic acid inactivation by hydroxylamines, *Biochemistry*, 4 (1965) 2439–2443.

19 FREESE, E., AND E. B. FREESE, Mutagenic and inactivating DNA alterations, *Radiation Res.*, Suppl. 6 (1966) 97–40.

20 FREESE, E., E. B. FREESE AND S. GRAHAM, The oxygen-dependent reaction of hydroxylamine with nucleotides and DNA, *Biochim. Biophys. Acta*, 123 (1966) 17–25.

21 FRIEDLANDER, D., AND P. F. DAVISON, Physicochemical studies on the reaction between formaldehyde and DNA, *Biochem. J.*, 3 (1963) 49–53.

22 HEIS, C. H. W., The oxidation of ribonuclease with performic acid, *J. Biol. Chem.*, 210 (1950) 611–621.

23 HSU, F. C., AND C. E. SOMERS, Effect of 5-bromodeoxyuridine on mammalian chromosomes, *Proc. Natl. Acad. Sci. (U.S.)*, 47 (1961) 399–403.

24 JOHNSON, K. A., J. KIRK, G. KOMMERK AND M. WUTTI RUBARD, Chemically induced mutations in *Neurospora*, *Cold Spring Harbor Symp. Quant. Biol.*, 19 (1951) 245–251.

25 KHELIAN, B. A., Factors affecting the production of chromosome alterations by chemicals, *J. Biophys. Biochem. Cytol.*, 2 (1956) 545–555.

26 KOTIN, P., AND H. L. FALK, Organic peroxides, hydrogen peroxide, epoxides, and neoplasia, *Radiation Res. Suppl.* 3 (1963) 193–211.
27 LAFARJET, R., Effects of radiation and peroxides on viral and bacterial functions linked to DNA specificity, In G. E. W. WOLSTENHOLME AND C. M. O'CONNOR (Eds.), *Ciba Foundation Symposium on Ionizing Radiations and Cell Metabolism*, Little, Brown, Boston, 1959, pp. 275–299.
28 LAFARJET, R., N. REBENROTTE AND P. DEMEREMAN, Action de peroxydes organiques sur un facteur transformant du pneumocoque, in M. HAISSINSKY, (Ed.), *Organic Peroxides in Radiobiology*, Pergamon, Oxford, 1958, pp. 61–72.
29 LINGENS, F., Mutagene Wirkung von Hydrazin auf *E. coli* Zellen, *Naturwissenschaften*, 48 (1961) 480.
30 LINGENS, F., Erzeugung biochemischer Mangelmutanten von *E. coli* mit Hilfe von Hydrazin und Hydrazinderivaten, *Z. Naturforsch.*, 19b (1964) 151–156.
31 LOVELESS, A., Quantitative aspects of the chemistry and biology of radiomimetic (mutagenic) substances, *Nature*, 167 (1951) 338–342.
32 LUZZATI, D., H. SCHWEITZ, M. L. BACHARD AND M. R. CHEVALLIER, Action du peroxyde succinique sur l'ADN, *J. Chim. Phys.*, 58 (1961) 1021–1027.
33 OEHLKERS, F., Die Auslösung von Chromosomenmutationen in der Meiosis durch Einwirkung von Chemikalien, *Z. Vererbungslehre*, 81 (1943) 315–341.
34 OEHLKERS, F., Die Auslösung von Mutationen durch Chemikalien bei *Antirrhinum majus*, *Z. Vererbungslehre*, 87 (1956) 584–589.
35 RAPOPORT, I. A., Carbonyl compounds and the chemical mechanism of mutations (in Russian), *Compt. Rend. Acad. Sci. U.R.S.S.*, 54 (1949) 65–67.
36 RHAESE, H., E. FRIESE AND E. FRIESE, Chemical analysis of DNA alterations, I. Base liberation and backbone breakage of DNA and oligodeoxyadenylic acid induced by hydroxylamine, in preparation.
37 RHAESE, H., E. FRIESE AND M. MELZER, Chemical analysis of DNA alterations, II. Alteration and liberation of bases of deoxynucleotides and deoxynucleosides, in preparation.
38 ROSENKRANZ, H. S., AND H. S. CARR, Studies with hydroxyurea, *J. Bacteriol.*, 92 (1966) 178–185.
39 ROSENKRANZ, H. S., An anomalous DNA component in hydroxyurea-treated *E. coli*, *Biochim. Biophys. Acta*, 129 (1966) 618–621.
40 SOBELS, F. H., Organic peroxides and mutagenic effects in *Drosophila*, *Nature*, 177 (1956) 1517–1518.
41 SOBELS, F. H., Peroxides and the induction of mutations by X-rays, ultraviolet, and formaldehyde, *Radiation Res. Suppl.* 3, (1963) 171–184.
42 SOBELS, F. H., AND T. C. HSU, Chromosome damage induced by hydroxylamine in mammalian cells, *Proc. Natl. Acad. Sci. (U.S.)*, 48 (1962) 937–943.
43 STAEHELIN, M., Reaction of tobacco mosaic virus nucleic acid with formaldehyde, *Biochim. Biophys. Acta*, 20 (1956) 449–450.
44 TARNOWSKI, G. S., W. KREIS, F. A. SCHMID, J. S. CAPPUCCINO AND J. H. BURCHENAL, Effect of hydroxylamine and related compounds on growth of transplanted animal tumors, *Cancer Res.*, 20 (1960) 1279–1[illegible].
45 VAN ARKEL, G. A., *Mutagenesis of Ultraviolet and Formaldehyde Mutagenesis in Aspergillus nidulans*, Thesis, Utrecht, 1958.
46 VOGT, M., Mutationsauslösung bei *Drosophila* durch Äthylurethan, *Experientia*, 4 (1948) 68–69.
47 WAGNER, R. P., C. H. HANDOS, R. FUERST AND W. R. STONE, The effect of irradiated medium, cyanide and peroxide on the mutation rate in *Neurospora*, *Genetics*, 35 (1950) 237–248.
48 WALLING, C., *Free Radicals in Solution*, Wiley, New York, 1957.
49 WIELAND, H., AND A. WINGLER, Über den Mechanismus der Oxydationsvorgänge V., *Ann. Chem.*, 431 (1923) 301–322.
50 WINDER, G., AND J. M. DENNEBY, Metal catalyzed auto-oxidation of isoniazid, *Biochem. J.*, 73 (1959) 505–507.
51 WYSS, O., J. B. CLARK, E. HAAS AND W. S. STONE, The role of peroxide in the biological effects of irradiated broth, *J. Bacteriol.*, 56 (1948) 51–57.
52 ZAMENHOF, A., H. E. ALEXANDER AND G. LEIDY, Studies on the chemistry of the transforming activity, *J. Exptl. Med.*, 98 (1953) 373–395.

Gleason, Marion N., Robert E. Gosselin,
Harold C. Hoage and Roger P. Smith. 1969
Clinical toxicology of commercial products, 3rd edition
Williams and Wilkins Co., Baltimore

Arch. oral Biol. Vol. 1. 397–421, 1966. Pergamon Press Ltd. Printed in Gt. Britain.

KINETICS OF ENAMEL DISSOLUTION DURING FORMATION OF INCIPIENT CARIES-LIKE LESIONS

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SUMMARY—The kinetics of enamel dissolution and mechanism of lesion formation were studied using a simple *in vitro* chemical method for producing lesions in human dental enamel comparable to natural incipient carious lesions, known as white spots. Enamel was exposed to a decalcification medium consisting of an aqueous solution of an acidic buffer plus a soluble organic polymer, hydroxyethyl cellulose, which protects the surface from acid attack during subsurface dissolution. The extent of the lesion was [illegible] by measuring the amount of calcium and phosphate dissolved from enamel and by microscopic examination of sections from the enamel. Enamel dissolution was investigated by a differential method to give the initial rate and by an integral method to give the approach to equilibrium. Five buffer acids (chloroacetic, dichloroacetic, lactic, formic, and acetic) were examined at pH's of 3–6 and buffer concentrations of 0·0–0·5 M. The initial rate increased with decreasing pH, decreasing acid dissociation constant, and nearly linearly with increasing buffer concentration. The initial rate of enamel dissolution during white spot formation was expressed quantitatively by a single equation for all these acids, in terms of undissociated acid concentration, hydrogen ion concentration, acid dissociation constant, and the dissociation constant of the acid-calcium complex of calcium ion. This equation is similar to that for surface dissolution demonstrating the interrelation of the two processes. The rate of enamel dissolution is controlled principally by the rate of diffusion of undissociated acid into enamel and to a lesser extent by pH and acid strength. Free calcium ion inhibits dissolution and can affect even the initial dissolution rate. Some acids complex calcium, although weakly, enough to eliminate this inhibition and are somewhat more effective in white spot formation.

INTRODUCTION

CARIOUS lesions of enamel are generally believed to be the end result of acid attack, although many other factors also enter into the total description of the process. The present work is concerned with the kinetics of the acid dissolution of enamel during the early stages of the decay process, aptly called "incipient carious lesions" and commonly known as "white spots".

The effect of acid in dissolving enamel was well appreciated by early investigators (MAGITOT, 1878; MILLER, 1889) and the determination of the various factors influencing the reaction has continued to the present. The reaction has been shown to behave like most dissolution reactions in having a typical rate curve which approaches an apparent equilibrium value (VOLKER, 1940; SUESS and FOEDICK, 1951). The dissolution rate of enamel in acidic buffers has also been shown to increase with decreasing pH and increasing buffer capacity (GRAY, 1962). Calcium and phosphate ions present in the acid solution retard or prevent dissolution (GRAY, 1962). STRÅLFORS (1958) first posed

that the reaction is diffusion controlled and was partially successful in deriving a mathematical expression for the rate of dissolution. GRAY (1962) was able to correlate all these factors in a single study and express the initial dissolution rate as a function of pH, buffer concentration, and buffer dissociation constant in a semi-empirical equation. Using only theoretical considerations, HIGUCHI *et al.* (1965) derived an equation for the initial dissolution rate that fits the data of GRAY (1962) very well.

The enamel dissolution results described so far were concerned only with the surface attack of enamel which bears little resemblance to the development of a carious lesion where the enamel is dissolved from the interior and this decalcified region is overlain by a relatively sound outer layer. During this period, however, investigations were also directed toward attempts to produce caries-like lesions by the use of acid. MAGITOT (1878) described experiments which resulted in caries-like lesions and, from what is known today, these were good facsimiles. HALS, MØRCH and SAND (1955) made an extensive study of lesions in enamel produced by exposure to lactic acid, defined the surface attack "outer spot" and subsurface attack "inner spot", showed a correlation in decalcification with diffusion rate of acid, and attributed the sound surface layer to hypermineralization of the original surface layer. COOLIDGE, BASIC and JACOBS (1955) produced incipient caries-like lesions using acidic solutions of calcium and phosphate to simulate the chemical environment found under natural conditions. They were able to define some of the concentration requirements for producing the lesions and showed that calcium and phosphate aid in preserving the sound surface layer over a white spot. DARLING (1956) produced lesions with solutions of lactic acid alone and found them to be comparable to natural lesions. In describing the nature and growth of incipient carious lesions, he suggested that preservation of the enamel surface over a white spot might be the result of protection from acid attack by the enamel cuticle. BRUDEVOLD (1962) later attributed the preservation of the surface layer to differences in chemical composition from the deeper layer of enamel caused by uptake of trace materials like fluoride during exposure to the oral environment. WEISSEL and BROWN (1963) as well as MANLY, EMERSON and KAPUR (1961) have produced lesions in tooth sections cemented between glass slides. However, surface damage to the enamel was present in nearly all of these experiments, thus differing from the smooth surface white spots found naturally. Based on the enamel dissolution studies, GRAY, FRANCIS and GRIEBSTEIN (1962) described a mechanism for white spot formation founded on an organic coating to preserve the surface, diffusion control, and dependence on total acid strength similar to that found for dissolution of the enamel surface in acid. KAPUR, FISCHER and MANLY (1962), employing tooth sections as mentioned above, measured the rate of growth of such lesions and found that the rate is proportional to the total acid strength.

SPERBER and BUONOCORE (1963a) found that white spots can be produced by aqueous solutions of acidic buffers alone by maintaining the pH in the range of 4·5–5·5 and using no agitation. Surface attack can be reduced further and production of white spots improved by adding certain acid anions to the acidic buffers. It was proposed that these ions are attracted to the surface forming a protective cloud or coating. SPERBER and BUONOCORE (1963b) also reported that the presence of the natural surface of

enamel, after only a pumicing, does prolong the period before the surface was attacked, but that equally good white spots can be made with enamel samples with the natural surface completely ground off.

GRAY and FRANCIS (1963) reported an *in vitro* system for making lesions that uses subsurface enamel and still produces a true white spot without incurring surface damage. The system is simple, consisting of an organic, nonionic polymer (hydroxyethyl cellulose) and an acidic buffer used over a wide range of pH values and buffer concentrations. The rate of enamel dissolution during formation of the lesions was measured as a function of pH, buffer concentration, calcium concentration, and temperature. The rate is, as a good first approximation, directly proportional to the total acid concentration (dissociated hydrogen ion plus undissociated acid), and is little affected by temperature. Calcium ion (as $CaCl_2$) added to the decalcification system reduces the rate but increases the extent of the relatively sound outer layer. Several other cations also reduce the rate. Light and electron micrographs and microradiographs showed that the lesions produced *in vitro* are similar to natural specimens and that the attack proceeds into the enamel between the rods and then laterally into the rods much as has been described for natural lesions and lesions produced by other *in vitro* methods (DARLING, 1956; SPERBER and BUONOCORE, 1963 a and b). The organic content was also found to be greater in the lesion than in normal enamel, again as described for natural lesions (STACK, 1954).

EXPERIMENTAL METHOD

The present work is an extension of the study by GRAY and FRANCIS (1963) mentioned above. Samples of enamel were exposed to mixtures of hydroxyethyl cellulose (HEC), water, and acid with varying pH, acid type, and acidic buffer concentration. The solutions were analysed for calcium and phosphate to determine the amount of enamel dissolved. The enamel was first examined visually for surface damage and white spot formation. Sections of the enamel samples were then examined by various microscopic techniques.

The enamel samples were prepared by cutting circular or square blocks from human teeth under water using a rotating diamond instrument (Horico Diamond, Hopf, Ringleb & Co., Berlin-Lichterfelde Gardeschützenweg 82, Germany) or a jewelers' circular saw blade. The teeth, extracted by local dentists, were selected at random (usually maxillary canines and incisors) and until used were stored in 3% formaldehyde solution adjusted to pH 7·5. The enamel samples were cemented to the ends of ½-in. dia. plastic rods with a dental plastic (DuraBase, Reliance Dental Manufacturing Company, Chicago, Illinois), covering all sides except the surface and were stored in a desiccator over 5% formaldehyde solution.

The enamel surface was then ground away in a mechanized device using light pressure and a 600 grit silicon carbide-water slurry against a plastic plate in order to remove the organic cuticle coating and the original enamel surface. A final high mirror finish was imparted by polishing against wet billiard felt successively first with 600 grit silicon carbide and then with gamma alumina followed by a wipe-off with paper towels or a cloth soaked in water to remove any adhering abrasive. The mirror finish permitted

detection of any surface dissolution or attack when examined visually or with a metallurgical microscope using top illumination.

The original hydroxyethyl cellulose (Cellosize WP-300, Union Carbide Chemicals Company, New York, N.Y.) as obtained from the manufacturer contained small amounts of sodium acetate and ethylene glycol. These materials were removed by six washes with isopropyl alcohol containing 17·5% water and one wash with ethyl ether or acetone; the product was dried under a heat lamp. The purified hydroxyethyl cellulose (HEC) was used in an amount of 6 g per 100 ml of aqueous acid solution, referred to hereafter as 6% HEC. The pH was adjusted with HCl or NaOH as required. The aqueous acidic solutions were prepared by weight from reagent grade compounds, and the solutions were checked by titration with NaOH. After mixing the HEC with the acid solutions, air bubbles were removed by centrifugation before the mixtures were placed in small vials (Fig. 1). Different size vials were used, depending on the amount of enamel that would dissolve, with the vial size increasing as the dissolution rate increased. When a constant amount of the initial dissolution rate was desired, the volume of the decalcification medium had to be kept sufficiently large so that accumulation of reaction products did not become great enough to affect the rate of enamel dissolution. On the other hand, the amount of medium used had to be kept to a minimum for maintaining precision of the chemical analyses for dissolved calcium and phosphate. The proper amount of decalcification medium was determined by experimentation and was checked by calculation of amounts of products formed. The amount of decalcification medium ranged from 2 to 100 g and was used in vials of 14–56 mm dia. The enamel sample was placed in the HEC–acid medium, the sample rod being supported by a rubber stopper and care being taken that no bubbles were trapped against the enamel surface. The system was kept at 37°C in an air thermostat for the desired time interval. No stirring was permitted because of the increase in surface dissolution caused by agitation (GRAY, 1962; SPERBER and BUONOCORE, 1963 a and b).

At the end of the decalcification period, the enamel sample was removed and the medium was diluted to a known volume with water for analysis of phosphate (LUCENA-CONDE and PRAT, 1957) and calcium (WHARTON and CHAPMAN, 1964). The enamel surface was examined visually and under a metallurgical microscope for white spot formation and surface damage. Sections 30–100 μ thick were then cut (GRAY and OPDYKE, 1962) from the enamel samples using no embedding or demineralization. Microradiographs were taken of the sections using a tungsten target at 26·5 kV, and the sections were mounted (Permount, Fischer Scientific Company, Pittsburgh, Pennsylvania) and examined by light microscopy using ordinary transmitted light and polarized light with a first order red filter. In selected cases, sections cut with a diamond knife were examined with the electron microscope using techniques reported previously (GRAY and FRANCIS, 1963).

The analyses for calcium and phosphate were converted to weight of enamel using the composition given by ORBAN (1957), which was checked further by analyses of known weights of enamel. The rate was expressed as mg of enamel dissolved per cm² of enamel surface exposed in order to provide a common basis for comparison among the different samples and experiments. The surface area of the enamel samples used in

the experiments was about 13 mm². The precise area was determined by photographing the enamel samples at 6·3 × on a dimensionally stable Mylar film and measuring the enamel surface area on the photographic film with an areagraph (No. 4850 Areagraph Chart, Charles Bruning Co., Inc., 1800 W. Central Road, Mount Prospect, Illinois).

The coefficient of variation for the rate measurement across all variables was about ±7%.

RESULTS

Appearance of lesions

The general characteristics of the lesion produced by this *in vitro* method and the similarity to natural incipient carious lesions has been covered rather thoroughly in a previous publication (GRAY and FRANCIS, 1963). The beginning of the lesion, either natural or artificial, is recognized by the appearance of a milky white spot on the normally translucent enamel (Fig. 1). It is characterized by the loss of mass from within the bulk of the enamel with little or no loss from the enamel surface. The growth of the lesion follows the macro and micro structures of enamel, such as the inter-rod region, the striae of Retzius, and the enamel cross striations.

In a light micrograph of a section through a lesion produced by the present *in vitro* method (Fig. 2a), the area of decalcification was distinguished by the disruption of the optical continuity of the enamel structure and the enhancement of the enamel cross striations and the striae of Retzius. The use of microradiography (Fig. 2b) proved that a considerable amount of enamel material had been lost. Furthermore, a layer of more or less sound enamel existed between the main region of damage and the surface, and this layer is hereafter referred to as the relatively sound outer layer. Obviously, the acid had to pass through this outer layer and the preservation of this layer of enamel is a point of major importance in the process.

The lesion shown in Figs. 2a and b was of a particular type with a narrow relatively sound outer layer and an intense region of subsurface decalcification. In natural lesions, the subsurface decalcification is frequently less concentrated with a broad relatively sound outer layer. Similar lesions, but somewhat neater in appearance, could be produced by the present *in vitro* method (Fig. 3). The controlling factors of this aspect of the lesion form appeared to be length of exposure and the morphology of the enamel sample (described by DARLING, 1956), such as the alignment of enamel rods and striae of Retzius and the original location of the lesion on the tooth. Nearly all results in this presentation were obtained from samples such as used in Fig. 2.

It should also be emphasized that these incipient caries-like lesions were produced with subsurface enamel. The original enamel surface, with all the trace elements and organic material acquired naturally during exposure to the oral environment that might contribute to protection against acid attack, had been entirely removed before exposure to the decalcification medium.

Rate of incipient caries-like lesion formation

Effect of time, integral method. Most of the rate measurements were done by a differential method, but an integral method was also used to support and extend the information obtained by the differential method. As opposed to the differential method,

which is used primarily to measure the initial rate, the integral method is used to give the whole solubility curve as a function of time showing the approach to equilibrium. Experimentally, the sample size of decalcification medium was kept small to allow the reaction products to accumulate and concentrate while at the same time reactant was being depleted by the reaction. The integral method was also used in another case where the sample size of the decalcification medium was purposely increased at the longer exposure times to prevent this approach to equilibrium.

In Fig. 4, the amount of dissolved enamel is plotted versus length of exposure time using a constant amount of decalcification medium consisting of 2 g (lower curve). Dashed lines are used whenever the curve was drawn by eye to fit the data points.

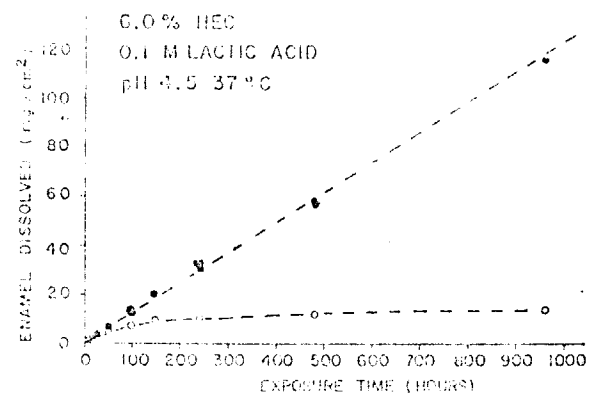

FIG. 4. Enamel dissolution is shown as a function of time during incipient caries-like lesion formation (i.e., integral method). The lower curve shows the typical rate curve approaching equilibrium. The upper curve shows the linear behaviour obtained when the volume of reactant (i.e., decalcification medium) is increased to prevent inhibition of dissolution due to loss of reactant and accumulation of reaction products such as occurred in the lower curve.

Solid lines are used for curves calculated from equations fitted to the data. The medium consisted of 0·1 M lactic acid at pH 4·5 plus 6% hydroxyethyl cellulose. Each point is a separate experiment with a fresh enamel sample. These results gave a typical rate curve which gradually approached an equilibrium. However, if the sample size of the medium was increased to reduce accumulation of products and depletion of acid, the rate of dissolution remained almost linear (Fig. 4, upper curve). This linear relation was also a tangent to the lower equilibrium curve at or near zero time. The fact that a linear relationship was obtained demonstrates the validity of the later practice of using a 96-hr exposure with an adequate amount of decalcification medium as representative of the initial dissolution rate. Some appreciation for the reproducibility of the measurements can be gained from the several replicate experiments which were done over a period of 1 year (Fig. 4 or Table 1).

Effect of pH and buffer concentration, differential method. The differential method was used to determine as nearly as possible the initial rate of enamel dissolution before

TABLE 1. REPRODUCIBILITY OF ENAMEL DISSOLUTION FOR DIFFERENT EXPOSURE TIMES IN 6% HEC-0·1 M LACTIC SOLN AT pH 4·5

Exposure time (hr)	Enamel dissolved (mg/cm²)
4	0·8, 0·7, 0·7
16	2·3, 2·1
24	3·0, 3·2, 3·4
96	12·2, 10·9, 12·1, 13·4, 12·5
240	30·3, 29·2, 28·6
480	57·5, 54·0

the reaction products could accumulate in sufficient concentration to retard the reaction. Such a method involves measuring the instantaneous dissolution rate at a single time, preferably at zero time or as close to zero time as possible. This was accomplished in the present study by using the 0–96-hr time interval during which, as just described, the rate remained constant and was, for all practical purposes, the same as the initial rate. Also, the amounts of decalcification medium used were of sufficient size to keep the reaction products at concentrations too low to affect the dissolution rate. The minimum amount was determined experimentally by measuring the rate of enamel dissolution using different quantities of decalcification medium until the rate became constant. The time interval of 96 hr was selected for reasons of adequate amount of subsurface dissolution, sufficient amounts of dissolved enamel for analyses, and experimental convenience.

The initial rate of enamel dissolution during the formation of these *in vitro* caries-like lesions is presented in Figs. 5–7 for lactic, acetic, formic, chloroacetic, and dichloroacetic acids as a function of total buffer concentration at several different pH values. Lactic acid was selected because it has been the acid implicated most often in dental caries. The other acids were chosen to provide a range of acid dissociation strengths of 10^{-2}–10^{-5} (Table 2) but with minimum possibility for side reactions.

Lactic acid, which was studied most extensively, is quite representative for showing the general effects of acidic buffers, and results are presented for a concentration range of 0–0·5 M and a pH range of 3–6 in Fig. 5 and up to 3·0 M at pH 6·0 in Fig. 6. Three

TABLE 2. DISSOCIATION CONSTANTS FOR THE ACID AND CALCIUM COMPLEX FORMS

Acid	Acid dissociation* constant K_A	Calcium complex dissociation† constant K_c
Dichloroacetate	$5{\cdot}0 \times 10^{-2}$	—
Chloroacetate	$1{\cdot}25 \times 10^{-3}$	—
Lactate	$1{\cdot}345 \times 10^{-4}$	0·038
Formate	$1{\cdot}75 \times 10^{-4}$	0·158
Acetate	$1{\cdot}72 \times 10^{-5}$	0·170

* Extrapolated values for 37°C from Kortüm, Vogel and Andrussow (1961).
† Values at 37°C from Bonnar, Dunsmore and Smith (1957).

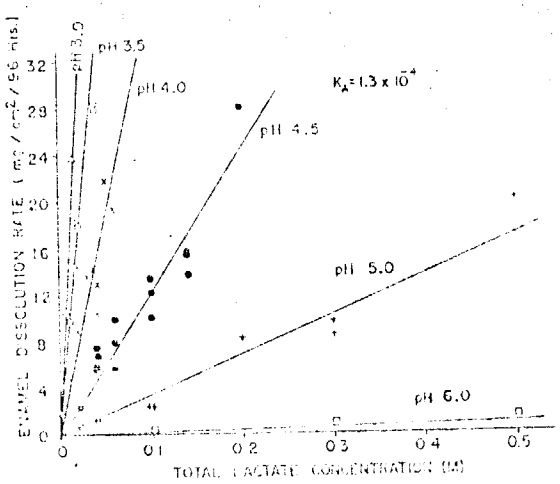

FIG. 5. The enamel dissolution rate during incipient caries-like lesion formation with 6% hydroxyethyl cellulose + lactic acid solutions is shown as a function of pH and lactate concentration at 37°C (differential method). The solid curves were calculated from a rate equation developed to fit the experimental results. The rate increases with decreasing pH and increasing buffer concentration. The symbols used for pH curves throughout this paper are pH 6·0 □—□, pH 5·0 + — +, pH 4·5 ● — ●, pH 4·0 × — ×, pH 3·5 ○ — ○, pH 3·0 △ — △.

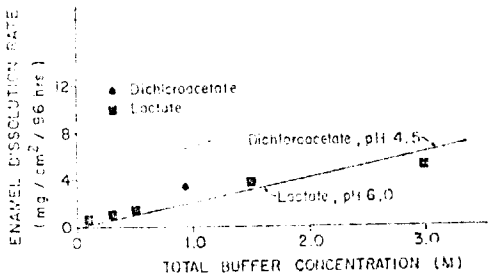

FIG. 6. The enamel dissolution rate is shown for high concentrations of lactic and dichloroacetic acids beyond the scale of Figs. 5 and 7. The solid curve shows the calculated values.

general relations are apparent. The dissolution rate increased linearly with increasing lactate concentration, especially at lower pH values. The rate also increased with decreasing pH. Finally, the dissolution rate when there was no buffer capacity, as in the case of an acid like hydrochloric, was almost zero at pH values above 3·0. These same relationships can be seen in Fig. 7, which shows a comparison of the results of the other four acids with decreasing acid dissociation constant, beginning with dichloroacetic at about 10^{-2}, chloroacetic at 10^{-3}, formic at 10^{-4}, and acetic at 10^{-5}. The formic acid constant is very close to that of lactic acid. The data for dichloroacetate at pH 4·5 is shown in Fig. 6. There was quite definitely a progressive shift in the slope of the rate

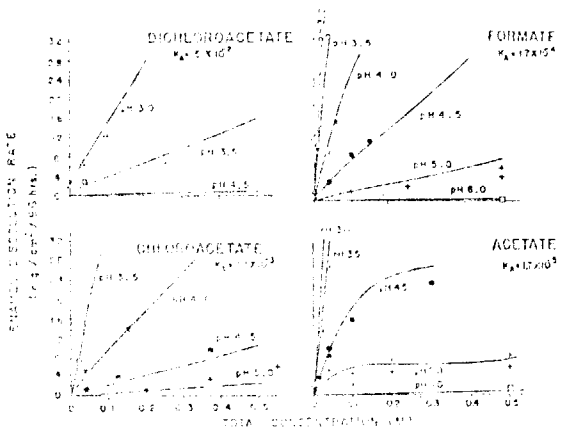

FIG. 7. The enamel dissolution rate during caries-like lesion formation in 6% hydroxyethyl cellulose + acid solutions is shown for four acids, with decreasing dissociation constants. The solid lines are calculated values from a single equation fitted to the results.

curves for each pH value as the acid dissociation constant was decreased. Generally, at constant pH and acid buffer concentration, the rate increased with decreasing acid dissociation constant. There were some deviations from this general relationship, but, as will be discussed, this resulted from secondary reactions.

It should also be mentioned that true white spots fulfilling the characteristics of natural lesions have been made at all pH values between 3 and 6, although exposure time becomes long at the higher pH. There were some slight differences among the acids in regard to the preservation of the enamel surface. Using acetic acid, the enamel surface was perfectly preserved over almost all the conditions examined. In some cases where the rate of dissolution was very high, use of lactic acid resulted in a very slight attack of the surface, while with formic acid there was obvious dissolution of the surface, but still of a low magnitude. Usually, surface damage could be expected when the rate of enamel dissolution exceeded 20 mg/cm²/96 hr.

Effect of calcium and other cations. Adding the reaction product, calcium, and, indeed, many cations to the decalcification medium retarded the dissolution rate quite markedly. The results of using a medium with lactic acid containing 0·05 M $CaCl_2$ is shown in Fig. 8. Comparison with Fig. 5 shows that the presence of calcium chloride decreased the rate, changing the slope of the curve for each pH value and introducing a lactate concentration range where the rate was zero or nearly so. The magnitude of the inhibition by calcium, both on a percentage and an absolute basis, appeared to be dependent on pH, being larger at higher or, perhaps, intermediate pH values. Whitening of lesion can be completely arrested by the presence of sufficient concentration of calcium ion. The effect is not peculiar to calcium as zinc, indium, tin(II),

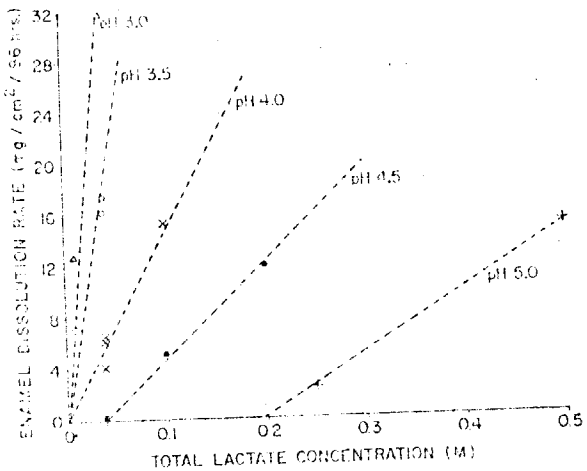

FIG. 8. Adding calcium ions as $CaCl_2$ to the decalcification medium (6% hydroxyethyl cellulose + lactic acid solutions) decreased the enamel dissolution rate during formation of the lesions, particularly at the high pH levels (cf. Fig. 5).

and indium ions also inhibited the rate of white spot formation. The order of decreasing effectiveness was In > Sn(II) > Ca > Zn > Li.

Composition of white spot enamel. Calcium analyses gave results for enamel dissolution which were parallel to those based on phosphate analyses, but in order to show further that the calcium was not removed preferentially over phosphate, the white spot enamel remaining was analysed. The white spot enamel was scraped off the sound enamel, dissolved in hydrochloric acid, and analysed for calcium and phosphate. The calcium to phosphate ratio was identical with normal enamel (Table 3), proving that no preferential dissolution of calcium or phosphate had occurred. Several different samples decalcified with different acids under a variety of conditions were examined.

TABLE 3. CALCIUM AND PHOSPHATE CONTENT (DRY WEIGHT) OF INCIPIENT CARIES-LIKE LESIONS

Decalcification method	Rate of enamel dissolved (mg/cm²)	Ca (%)	PO_4 (%)	Ca/PO_4 (wt. ratio)
96 hr, 6% HEC 0·02 M Lactic acid, pH 3·5	14·5	35·4	50·5	0·70
96 hr, 6% HEC 0·04 M Dichloroacetic acid, pH 3·5	15·1	35·0	52·9	0·66
144 hr, 6% HEC 0·1 M Lactic acid, pH 4·5	19·7	39·1	51·7	0·76
200 hr, 6% HEC 0·1 M Lactic acid, pH 4·5	28·7	35·2	51·1	0·69
200 hr, 6% HEC 0·1 M Lactic acid, pH 4·5	30·3	34·2	51·0	0·67
Normal enamel	—	36·9	54·3	0·68

The percentage content of calcium plus phosphate in white spot enamel, however, was higher than for normal enamel, indicating that additional material had been lost or dissolved during lesion formation. This would most probably be carbonate. No analyses for carbonate were made, but such determinations might be interesting in light of reports (Cronquist and Lycons, 1957) that carbonate is lost preferentially during formation of incipient carious lesions.

Initial dissolution rate vs. pH or hydrogen ion concentration. The data for the lactic acid system are shown plotted against pH and hydrogen ion concentration, (H^+), in Fig. 9. This plot is included to show exactly how the enamel dissolution rate changes

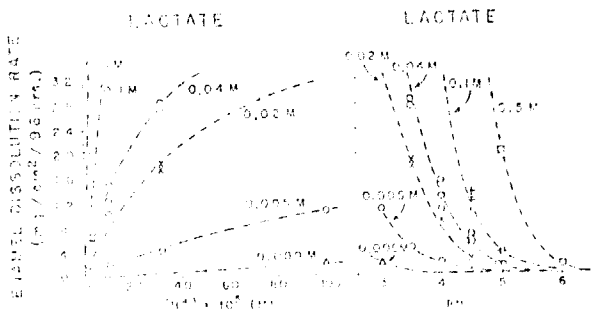

FIG. 9. The enamel dissolution rate (from Fig. 5) during lesion formation in HEC – lactic acid solutions are shown as a function of hydrogen ion concentration and its logarithmic function, pH, at different lactate concentrations. The plots were similar for the other acids of Fig. 7 and appear quite different from the plots vs. buffer concentrations of Figs. 5–7.

with these important variables, which is not readily discernible in the previous graphs. The rate increased in a nonlinear fashion with increasing hydrogen ion concentration. At lower buffer concentrations, the change in rate began to level off with increasing hydrogen ion concentration corresponding to the per cent of buffer in the undissociated acid form. It is also apparent that at very low buffer concentrations, increasing hydrogen ion concentration had a small effect while at high buffer concentrations, increasing the hydrogen ion concentration caused a tremendous increase in rate. With regard to the graph of rate versus pH, which is simply an inverse logarithm of the hydrogen ion axis of the preceding graph, the rate increased nonlinearly with decreasing pH. The rate change was especially marked at high buffer concentrations used at intermediate acidic pH values. This behaviour resulted in large changes in rate for small pH variations at high buffer concentrations and very small rate changes for pH variations at low buffer concentrations. The lactic acid system is fairly representative of the other acids in this study as to trends, although some shifts and deviations did occur.

Initial dissolution rate vs. total acid. It was concluded in previous studies (GRAY and FRANCIS, 1963) that the dissolution rate was determined primarily by the total available acid concentration, hydrogen ion plus undissociated acid. The initial dissolution rate

for the lactic acid system as a function of total acid is shown in Fig. 10 for all pH values and buffer concentrations taken from Fig. 5. The result is a fairly good linear relationship. As a good first approximation, the rate can be expressed as a function of the total acid concentration, which is, for all practical purposes, equal to the undissociated acid concentration. The free hydrogen ion concentration is nearly always much below a thousandth molar in any of these systems.

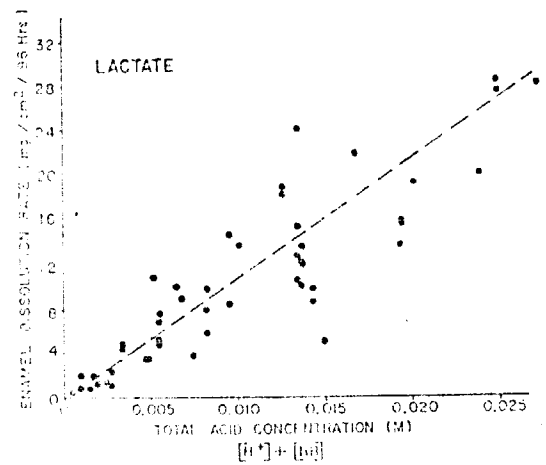

FIG. 10. The enamel dissolution rate (from Fig. 5) during lesion formation with the lactate decalcification medium is shown as a function of the total acid concentration, the major controlling factor. As a good first approximation, the rate is directly proportional to total acid concentration.

Additional study showed, however, that there was a regular progression within these data due to a pH effect, with the rate increasing with decreasing pH, as could be seen when the same data points were identified with pH levels (Fig. 11). The rate was still linear with respect to the total acid or undissociated acid concentration. Furthermore, an additional progression with respect to acid dissociation constant was also present when the different acids were compared (Fig. 12), the rate increasing with increasing dissociation constant. This plot versus total acid presents a quite different appearance for the comparison among acids from the previous plot versus total buffer concentration (Fig. 7). For instance, the acid with the largest dissociation constant, dichloroacetic acid, in the present graph (Fig. 12) gave the highest dissolution rate in terms of undissociated acid concentration, and the effect of pH was barely detectable. As the acid being used became weaker (i.e. dissociation constant decreased), the dissolution rates for equivalent total acid concentration decreased and there was an obvious dependency on pH. The initial dissolution rates for acetic acid were so low that the scale had to be reduced. This plot for acetic acid is important because it shows quite definitely a nonlinear relationship between the rate and total acid concentration, particularly at pH 4·5 and 5·0, and probably at pH 3·5. In fact, the results for formic acid also were not

all quite linear, based on subsequent developments, but the precision of the formic acid data alone did not justify such a conclusion. This deviation from linearity became very important in the development of an expression for the rate of subsurface dissolution.

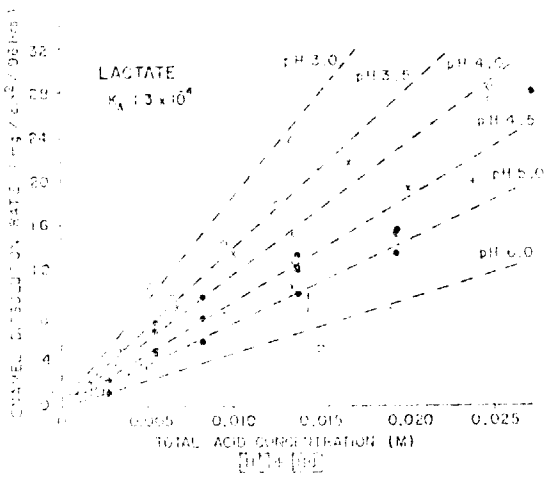

FIG. 11. The effect of pH on enamel dissolution rate plotted as a function of total acid can be seen when the data of Fig. 10 are identified with the pH of the decalcification medium. The rate is still a linear function of total acid concentration for any given pH.

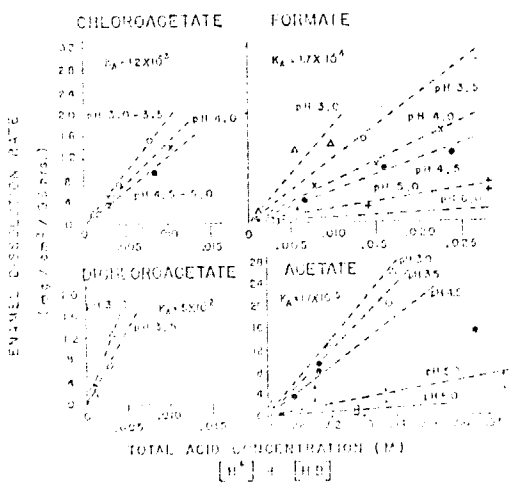

FIG. 12. The enamel dissolution rate as a function of pH and total available acid is shown for the 4 different acids of Fig. 7. The rate is pH dependent and linear with respect to total acid concentration except for acetate at high pH levels.

DISCUSSION

The reaction in the acid dissolution process of enamel, whether it be surface or subsurface, is between hydrogen ion and the inorganic portion of dental enamel, principally with hydroxylapatite. It was assumed for the remaining discussion that the carbonate was removed in the same proportions as the calcium and phosphate, and the results do not suggest that any large errors arose from this assumption. It has also been shown in previous studies (STRÅLFORS, 1956; GRAY, 1962; KAPER *et al.*, 1962; GRAY and FRANCIS, 1963) that the reaction is diffusion controlled, and the reaction, therefore, depends on how fast the hydrogen ions reach the apatite crystal surfaces. In the case of acidic buffers, the principal source of hydrogen ions is the undissociated acid buffer. Then, the rate of enamel dissolution also becomes dependent on the diffusion of undissociated acid buffer to the enamel crystal surfaces.

This study was initiated with the anticipation that a general physical-chemical relationship could be determined for the rate of subsurface dissolution of enamel during the formation of incipient caries-like lesions. This approach was encouraged by the fact that such a treatment had been possible for the surface dissolution of enamel (GRAY, 1962). The development of a logical mathematical expression for calculating the results is a real test of any concept, and there is the secondary utility of being able to interpolate and extrapolate results by the more accessible route of calculation.

Throughout this communication, various arguments have been presented to show that the controlling variables for reaction between enamel and acid are undissociated acid concentration, hydrogen ion concentration, and the acid dissociation constant. The fundamental reactant is hydrogen ion and all these variables mentioned can operate by affecting the hydrogen ion concentration at the reaction sites. Using these fundamental variables, an expression has been derived that expresses the rate at which dissolved enamel appears in solution depending on the acid composition of that solution. From this expression, it is possible to deduce or otherwise infer certain aspects of the mechanism that must have occurred between the initial exposure to the acid solution and the final appearance of the reaction products. Ultimately, a development from a mathematical model, as has been done for surface dissolution by HIGUCHI *et al.* (1965), must be used to provide an accurate description of the mechanism, but the foundation for the model will come from the understanding gained in studies such as the one described here.

The method used for development of the mathematical expression of the enamel dissolution rate is similar to that used successfully in the surface dissolution studies.

$$R = \frac{dEn}{dt} = f([H^+],[HB],K_A). \quad (1)$$

$$= f([H^+]) + f([H^+],[HB],K_A). \quad (2)$$

where:

$\frac{dEn}{dt} = R$ = rate of enamel dissolution (mg/cm²/96 hr).

$[H^+]$ = hydrogen ion concentration (moles/l).

$[HB]$ = undissociated acidic buffer concentration (moles/l).

K_A = dissociation constant of the acidic buffer, HB

$[B^-]$ = dissociated acidic buffer concentration (moles/l).

This expression states that the rate of enamel dissolution, R, is a function of hydrogen ion concentration, undissociated acid concentration, and the dissociation constant. The rate is a derivative with respect to time and could for more rigorous representation be expressed as the derivative dEn/dt where En is the amount of enamel dissolved and t is time. Two terms are required as in equation (2) in order that the whole expression does not go to zero when the buffer concentration becomes zero, as for example, in the case of hydrochloric acid.

It should be pointed out that in the previous development (GRAY, 1962), the form $f([HB],[B^-],K)$ was employed for the second term. However, in such a treatment there are four variables $[H^+]$, $[HB]$, $[B^-]$, and K_A which are interrelated through the acid dissociation expression:

$$K_A = \frac{[H^+][B^-]}{[HB]} \tag{3}$$

Only three variables of the four can be determinant because of the interdependence expressed by equation (3), and the determination of the controlling variables which are significant physically has to be accomplished by deduction or induction. Previously, $[B^-]$ was selected because it was thought at the time that the only pH effect resided in the first term and that the dissociated buffer anion was influencing the rate by controlling (i.e., inhibiting) the extent of dissociation. The present data (Figs. 11 and 12) show quite clearly that there is a pH effect with regard to the second term that is contrary to the expected inhibition by $[B^-]$. Therefore, from all points of reasoning, it appears that the dissolution rate for surface or subsurface enamel is determined by the variables used in the present paper. This discussion assumes, for the moment, that there is no complexing of calcium by the buffer anion as this effect can and should be incorporated separately by an additional term.

Equation (2) was given a functional form for evaluation by substituting for each variable the usual form of a rate expression of a constant (k) times the concentration, c, to the nth power (LAIDLER, 1950):

$$\frac{dc}{dt} = kc^n, \tag{4}$$

which gives:

$$R = k_0[H^+]^{n_0} + k_1 K_A^{n_1}[H^+]^{n_2}[HB]^{n_3} \tag{5}$$

The first term is the contribution due to hydrogen ion alone. The second term is the contribution due to undissociated acid which is affected by the pH level and the dissociation constant of the acid. For any single acid, $K_A^{n_1}$ could be absorbed into the constant k_1, but the inclusion of $K_A^{n_1}$ in the equation makes the equation universal for all acidic buffers.

To numerically evaluate the equation, the exponents or orders, n_0 through n_3, were determined first. Then, the rate constants k_0 and k_1 were determined by calculation from each experimental point and averaging the results. The exponents were determined

by taking the logarithm of both sides of equation (5) and making suitable logarithmic plots of the proper variables.

$$\log(R - k_0[H^+]^{n_0}) = \log k_1 + n_1 \log K_A + n_2 \log [H^+] + n_3 \log [HB] \tag{6}$$

This mathematical arrangement produces a form of the algebraic equation for a straight line, and the plots should, therefore, be straight lines with the slopes being equal to the exponent. The first term was quite small and could usually be ignored, such as at high pH values. At low pH values, the first term was transferred to the other side of the equation as shown in equation (6). Then a logarithmic plot of $(R - k_0[H^+]^{n_0})$ vs. $[HB]$ at constant K_A and $[H^+]$ gave n_3 as the slope. A similar plot vs. $[H^+]$ but at constant K_A (i.e., for each acid) and constant $[HB]$ gave n_2.

The rate was linear with respect to the undissociated acid concentration for most of the acids as shown in Figs. 11 and 12, which would make n_3 equal to one. However, the results for acetic acid did not follow this relation and, indeed, gave a value of $n_3 = 3/4$. It would have been possible to force a value of $n_3 = 1$ through the data and permit some deviation in the points at the outer concentration limits studied. However, the situation became untenable because the values of n_2 for both formic and acetic acids were different from the other three acids. In order to unify the results for all acids in a single equation, the values of n_2 and n_3 have to be the same for each acid. If the values of n_2 and n_3 vary with acid type, as they do, it means that the present equation is inadequate and the proposed mechanism has to be either extended or completely changed in order to maintain a single unifying expression. Otherwise, separate expressions would have to be used for the different acids.

One factor neglected in this expression was complexing of the calcium reaction product by the buffer anion, and adding a term for such an effect did unify the results of all the acids into a single rate equation. However, the term ultimately utilized was not an expression for any over-all increase in dissolution potential due to complexing, but rather an expression for prevention of inhibition of the dissolution rate by the calcium reaction product. Uncomplexed calcium reaction product retards dissolution by accumulating in the vicinity of the reaction sites. Calcium complexing when present prevents this accumulation and the concomitant inhibition of rate rather than increasing the absolute dissolution rate. SPERBER and BUONOCORE (1963a) have proposed a similar explanation for the difference in demineralization rate between acetic and lactic acids as well as the increased rate with complexing agents like ethylenediaminetetraacetate.

The support for the preceding concept begins with consideration of the results with formic and lactic acid. Formic acid and lactic acid have very similar acid dissociation constants (KORTÜM, VOGEL and ANDRUSSOW, 1961) (1·7 and $1{\cdot}3 \times 10^{-4}$, respectively) and therefore would be expected to produce about the same dissolution rate. At low pH values, they did give about the same dissolution rate, but at high pH values, formic acid gave lower dissolution rates. However, formic acid was similar to acetic acid with respect to this behaviour as shown by evaluation of the exponents n_2 and n_3 of equation (5). The one difference between lactic acid and formic acid and the one similarity between formic acid and acetic acid was the strength of their respective calcium com-

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ebert (Bjerrum, Schwarzenbach and Sillén, 1957). The dissociation constant for calcium lactate complex was about 0·04 while for calcium acetate and calcium formate complexes the dissociation constant was about 0·17. Such values show that all the calcium complexes of the acids used in this study are very weak, but in solutions with high acid anion to calcium ratios, these anions can have a significant and measurable complexing effect (Lassers, 1965).

An attempt was made to add a term to the equation that would express the increase in rate due to complexing of calcium ion, essentially an additive effect to that of the fundamental constant hydrogen ion. Mathematically, the equation would not fit the data. Theoretically, it should not because two fundamentally different steps cannot be rate controlling at the same time. If the term had been additive, then acetate and formate, because of little or no complexing effect for calcium, should have given the lowest rate for subsurface demineralization or enamel dissolution. Then, when the complexing effect became negligible, as for example at low pH, the rates for all acids should have paralleled that of acetate and formate. The reverse occurred; the acetate and formate acids showed the greatest rate and the chloroacetates. This effect could only be explained by an inhibition in rate for some acids at high pH values that disappeared at low pH values.

These conditions are satisfied by a term expressing the inhibition of dissolution rate by calcium ion (Fig. 6) and the prevention of such inhibition by complexing of the calcium product by the acid buffer. At low pH values, the inhibiting effect of calcium is much less than at higher pH levels (again as shown in Fig. 6), and complexing would not be necessary. This would result in the slower dissolution rates found for lactic acid and formic acid at low pH values. At high pH values, lactate complexes calcium ion enough to prevent inhibition of dissolution rate but the formate complex is too weak and the rate would be inhibited. This would cause formate to be different from the lactate system at high pH values.

Experimentally, the enamel dissolution rates were determined using sufficiently large volumes of medium to prevent accumulation of enough reaction products in the bulk medium to cause inhibition of the dissolution rate. However, in this static system where much of the reaction is occurring beneath the enamel surface, temporary local accumulation of reaction products around the reaction site inside the enamel is very probable. This would account for the effect of the calcium reaction product found in the present systems.

A term was added to express the inhibition due to the calcium reaction product and the moderation of this inhibition by complexing with acid buffer anion. The equation becomes:

$$R = k_a[H^+]^{m_a} + k_1K_A^{m_1}[H^+]^{m_2}[HB]^{m_3} + k_2[H^+]^{m_b}[Ca^{2+}]^{n_c} \tag{7}$$

where: $[Ca^{2+}]$ = free calcium ion concentration (moles/l).

The calcium concentration is for that in the neighbourhood of the reaction site, and the hydrogen ion term was included because the calcium ion effect was found experimentally to be pH dependent. The first two terms of the equation can be evaluated if it is assumed that the last term is zero for the lactate and chloroacetate systems. Then, the deviation

for the experimental values found for formic and acetic acid from this equation can be calculated and used to evaluate the last term.

The calcium inhibition term was evaluated by expressing the concentration of calcium ion as a function of the first part of equation (7), that is, the total calcium ion concentration was equal to the rate of enamel dissolution because calcium ion is one of the reaction products:

$$[Ca^{2+}]_{total} = k_1K_A^{m_1}[H^+]^{m_2}[HB]^{m_3} = R_1 \tag{8}$$

Since the rate will be decreased by the inhibition of calcium ion, the exponents may change, thus the use of the symbol "m" instead of "n". The first term $k[H^+]^m$ is small and negligible and was ignored in this part of the treatment. To find the free or dissociated calcium ion concentration, the expression for the dissociation constant was used.

$$K_c = \frac{[Ca^{2+}][B^-]}{[CaB^+]} \tag{9}$$

where: K_c = dissociation constant for the calcium complex of buffer acid, HB.

$[CaB^+]$ = concentration of the calcium complex of the buffer acid, HB.

Then by mass balance:

$$[CaB^+] = [Ca^{2+}]_{total} - [Ca^{2+}] = R_1 - [Ca^{2+}] \tag{10}$$

Combining 9 and 10 gave:

$$K_c = \frac{[Ca^{2+}][B^-]}{R - [Ca^{2+}]} \tag{11}$$

Solving for $[Ca^{2+}]$, the free calcium concentration near the reaction site in the presence of complexing by the acid buffer anion, gave:

$$[Ca^{2+}] = \frac{K_c R_1}{[B^-] + K_c} \tag{12}$$

The original form of R_1 from equation (8) was substituted.

$$[Ca^{2+}] = \frac{K_c R_1}{[B^-] + K_c} = \frac{K_c k_1 K_A^{m_1}[H^+]^{m_2}[HB]^{m_3}}{[B^-] + K_c} \tag{13}$$

The third term of equation (7) became:

$$k_a[H^+]^{m_b}[Ca^{2+}]^{n_c} = k_a[H^+]^{m_b}\left(\frac{K_c k_1 K_A^{m_1}[H^+]^{m_2}[HB]^{m_3}}{[B^-] + K_c}\right)^{n_c} \tag{14}$$

$$= k_a k_1 K_A^{m_1 n_c}[H^+]^{m_b + m_2 n_c}[HB]^{m_3 n_c}\left(\frac{K_c}{[B^-] + K_c}\right)^{n_c} \tag{15}$$

Not all the individual exponents can or need to be evaluated so that these can be represented as follows:

$$= k_a K_A^{m_4}[H^+]^{m_5}[HB]^{m_6}\left(\frac{K_c}{[B^-] + K_c}\right)^{n_7} \tag{16}$$

Finally:

$$R = k_a[H^+]^{m_a} + k_1K_A^{m_1}[H^+]^{m_2}[HB]^{m_3} + k_2K_A^{m_4}[H^+]^{m_5}[HB]^{m_6}\left(\frac{K_c}{[B^-] + K_c}\right)^{n_7} \tag{17}$$

[illegible] slope for the [illegible] exponents can not be determined from the experimental data.

This [illegible] for the first term. This represents the dissolution rate in pure acids where the [illegible] term would be zero. The dissolution rate for apatite, as for many solids dissolving in a liquid, is first order (CHEPELEVETSKII, 1957) so that n_0 was taken equal to 1. The value of k_0 was calculated from the data at pH 3·0 to be $2{\cdot}0 \times 10^{7}$ with the units of [mg cm^{-2} moles^{-1} l (96 hr)$^{-1}$]. The units are a little unusual because of the choice of using practical units for expressing the rate of enamel dissolution.

The values of the constants for the second term were determined from the data for lactic, chloroacetic, and dichloroacetic acid. It will be recalled from the previous discussion that there appeared to be no inhibiting effect by the calcium ion product for these acids and, therefore, the third term can be neglected. This reduces the equation to the previous form [equation (6)] which can be readily evaluated. Then [as described following equation (6)], from Figs. 5 and 7, $n_3 = 1$; from appropriate logarithmic plots, $n_1 = 1/4$ and $n_5 = 1/4$; and by calculation, $k_1 = 8{\cdot}14 \times 10^{7}$ with the units of [mg cm^{-2} moles$^{-3/2}$]$^{1/2}$ (96 hr)$^{-1}$].

The third term was evaluated from the acetate and formate data. First, values of R were calculated for acetic and formic acid from the first two terms of equation (17), which were now known from the preceding two paragraphs. The difference in this calculated value, R_c, from the experimental value, R, is equal to the third term:

$$R_c - R = k_7 K_A^{n_7} [H^+]^{n_1} [HB]^{n_5} \left(\frac{K_c}{[B^-] + K_c} \right)^{n_6} \tag{18}$$

Once again, logarithms were taken of both sides of the equation and suitable logarithmic plots were made to give straight lines whose slopes were equal to the exponents. Little data are available for making a logarithmic plot of $(R_c - R)$ vs. $(K_c/[B^-] + K_c)$ at constant K_A, $[H^+]$, and $[HB]$ for determination of n_7 as the slope. An approximation can be obtained by plotting data at constant $[H^+]$ and $[HB]$ for formic acid and lactic acid as these acids have similar values for K_A. It is necessary to assume some very low value of $R_c - R$ for lactic acid because the actual value of zero cannot be plotted logarithmically. A final value of $n_7 = 3/2$ was obtained by trial and error. All other exponents were determined from logarithmic plots giving $n_1 = 1$, $n_5 = 3/4$, and $n_6 = 2$. Then, k_7 was calculated to be $-7{\cdot}0 \times 10^{7}$ with the units of [mg cm^{-2} moles$^{-9/4}$ l$^{9/4}$ (96 hr)$^{-1}$].

$$R = 2{\cdot}0 \times 10^{7}[H^+] + 8{\cdot}14 \times 10^{7} K_A^{1/2} [H^+]^{1/2} [HB] - 7{\cdot}0 \times 10^{7} \frac{K_A [HB]^{3/4}}{[H^+]^{3/4}} \left(\frac{K_c}{[B^-] + K_c} \right)^{3/2} \tag{19}$$

or in terms of total buffer, it becomes:

$$R = 2{\cdot}0 \times 10^{7}[H^+] + 8{\cdot}14 \times 10^{7} K_A^{1/2} [H^+]^{1/2} [TB] \left(\frac{K_A^{1/4}}{K_A + [H^+]} \right) + 7{\cdot}0 \times 10^{7} [H^+]^{1/2} [TB] \cdot$$

$$\frac{K_A}{[H^+]^{3/4}} \left(\frac{K_c}{K_A [TB] + K_A [H^+] + K_A^2 K_c} \right)^{3/2} \tag{20}$$

[illegible] Note that the equation [illegible] pH term are properly self-limiting.

Note, too, that although the equation appears complicated as regards the number of terms, it is, in principle, relatively simple. The equation shows that the enamel dissolution rate is determined by the hydrogen ion concentration or pH, the undissociated acid concentration, and the acid strength or dissociation constant. Furthermore, the product of enamel dissolution, calcium ion, inhibits the reaction and the inhibition can be prevented by the complexing by the acid anion of complexing strength K_c.

The fit of the equation without the third term for calcium inhibition is shown in Fig. 13 for formic and acetic acids. It will be recalled that solid lines are used for curves

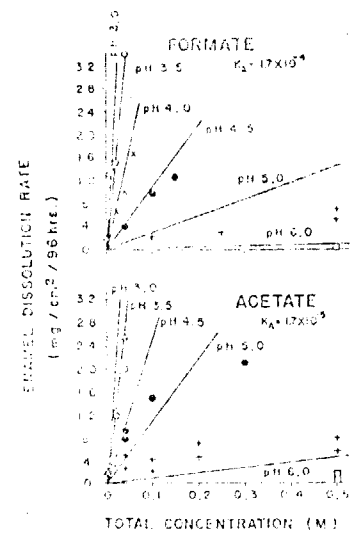

FIG. 13. The calculated enamel dissolution rate using no correction for calcium inhibition [equation (6)] shown by the solid lines fits the data for acetic and formic acids at low pH but not at high pH (e.g. pH 6·0 □ — □, pH 5·0 + — +, pH 4·5 ○ — ○). The better fit obtained by including the calcium inhibition correction [equation (22)] can be seen by comparison with Fig. 7.

calculated from equations fitted to the data, and dashed lines are used for curves drawn by eye through the points. First, values were calculated using only the first two terms (i.e., with no calcium correction) and the fit between calculated values and experimental data was good for lactic, chloroacetic, and dichloroacetic acids over all pH values and for formic acid at low pH values. At high pH values, there was a very considerable disagreement between the calculated and experimental values for formic and acetic acids (as can be seen in Fig. 13) when no correction for calcium complexing was used.

The calculated results using the whole equation with all three terms is shown in Figs. 6 and 7. The fit is now quite good for acetic acid which had the poorest agreement when only the first two terms were used. It was found that [illegible] the final form of the [illegible] equation was [illegible]

dichloroacetic acids. This required that a series of successive approximations be made using these new calculated values to recalculate more precise values for the constants k_3 and K_4. These are the values shown in equations (19) and (20).

Although the stability dissociation constants for the calcium complexes of chloroacetic and dichloroacetic acids are not available. The value for acetic was used for these acids because the [illegible] on chemical grounds to be weak and because the dissociation constants for other metal cations in common among the three ligands were nearly equivalent. The fit of the data is excellent for lactic acid and is quite good for the formic, chloroacetic, and dichloroacetic acids. The largest deviation between calculated and experimental values was obtained at low pH for acetic acid, but the deviation is [illegible].

It is interesting to note that the point at pH 4.5, 0.39 M acetate was run after the equation was developed in order to give a severe test of the equation, and the result fell close to the curve. It is also interesting that the equation shows a complexing [illegible] inhibition, even at low pH, for the chloroacetates and lactate, improving the agreement between calculated and experimental results even though the assumption of the existence of no such effect had to be used to develop the equation. The apparent contradiction of the form of the equation and the use of successive approximations overcame this assumption. This very agreement and reasonableness of the [illegible] obtained by the equation also provides additional justification for the many assumptions needed to derive the equation.

It should be emphasized that this is not necessarily the best fit that could be made [illegible]. In the [illegible] fitting operation, mathematical equations which [illegible] the observed curves are used, and the constants are adjusted until a good fit is made. By using enough terms and with the aid of a computer, these data could be made to correlate perfectly with some equation which would then have little or no physical meaning. In the semi-empirical approach used here, the variables observed experimentally are related in an expression using physical-chemical expressions and concepts with mathematical equations to explain on a logical basis the experimentally observed results. Some deviations can be expected if not all effects have been properly taken into account. For example, the bulk solution value of $[B^-]$ was used in the third term of equation (19) when a better value (as defined in the development of the third term) would probably be the $[B^-]$ concentration in the vicinity of the reaction site but which is not measurable at present. The conclusion, in this instance, would be that the major effects have been determined and adequately explain the rate of the subsurface dissolution process.

The final equation demonstrates quite clearly that the rate of enamel dissolution is dependent on all three factors, pH or hydrogen ion concentration, acidic buffer concentration, and acidic buffer strength (i.e. dissociation constant). Furthermore, the [illegible] interrelated, which has led to considerable confusion in the past when the effect of only one factor was studied. It is also interesting to compare the [illegible] of surface and bulk solution. The equation for surface [illegible] when from a previously [illegible] and rearranged gave:

$$R_{s} = [illegible]$$

Any calcium reaction product effect was eliminated completely in the surface dissolution studies so that for comparison in the absence of any calcium effect, the equation for subsurface dissolution was reduced to the first two terms.

$$R_{s\,(\mathrm{no\ Ca})} = k_2[H^+] + k_3 K_4^{1/2}[H^+]^{1/2}[HB]$$

It is obvious that the two results are quite similar, the only difference being in the exponent of the undissociated acidic buffer term. In retrospect, the similarity could be expected because the surface dissolution process must be the same as that in subsurface dissolution at the enamel crystal surfaces or reaction site. The difference lies in the extra diffusion step involved for reactant to diffuse through enamel to the reaction site and for reaction products to diffuse out of enamel. It would appear that this difference has been absorbed in the undissociated acid term.

From a practical application viewpoint, it is apparent that for caries activity, an acidic pH is necessary but hardly sufficient. Increasing buffer capacity and buffer strength would usually increase caries activity, therefore making the type of acid anion produced by bacteria an important consideration. This importance is heightened by the fact that acid anions which complex calcium, even only weakly, maintain a higher pH than acids with less calcium complexing strength. However, the mechanism is quite different from that proposed by proponents of chelation in that there is no primary attack of enamel structure by the calcium complexing anion. Instead, the complexing of calcium by the acidic buffer anion serves to remove the reaction product, calcium ion, from the vicinity of the reaction site where calcium ion can inhibit the progress of the reaction. The inhibition effect by calcium ion can under some conditions lead to a common ion effect, but under the present acidic conditions, it definitely is not a common ion effect at equilibrium and probably not during the approach to equilibrium. It has been shown (GRAY and FRANCIS, 1963) by solubility product measurements that the solution composition is in equilibrium with dicalcium phosphate rather than hydroxylapatite when enamel, dentin, bone, or synthetic apatite are equilibrated in acidic solution. This result can only mean that the enamel crystal surfaces have been converted to dicalcium phosphate. Furthermore, the rate of subsurface dissolution was also inhibited by the presence of other cations such as zinc, tin(II), lithium, and indium, and the rate of surface dissolution (GRAY, 1962) was inhibited by almost any cation. This, obviously, is not a common ion effect with hydroxylapatite. It has been proposed that surface deposits are formed from precipitation of these cations with the reaction products of dental enamel.

Many of the concepts are well illustrated by the very simplified diagram of Fig. 14 showing subsurface dissolution or white spot formation in progress. The HEC adsorbs on the surface (in the case of plaque, this could be bacterial products and salivary components) forming a protective coating against acid dissolution but otherwise is assumed to be inert. The buffer acid ions and molecules diffuse through the spaces between the rods, which have been enlarged by previous acid attack. These acidic buffer reactants migrate to reaction sites in the enamel and there react to form the ions shown. The products then must diffuse out and out into the bulk solution, that is, to a region of lower concentration. It is at this point that sequestering of calcium ion by

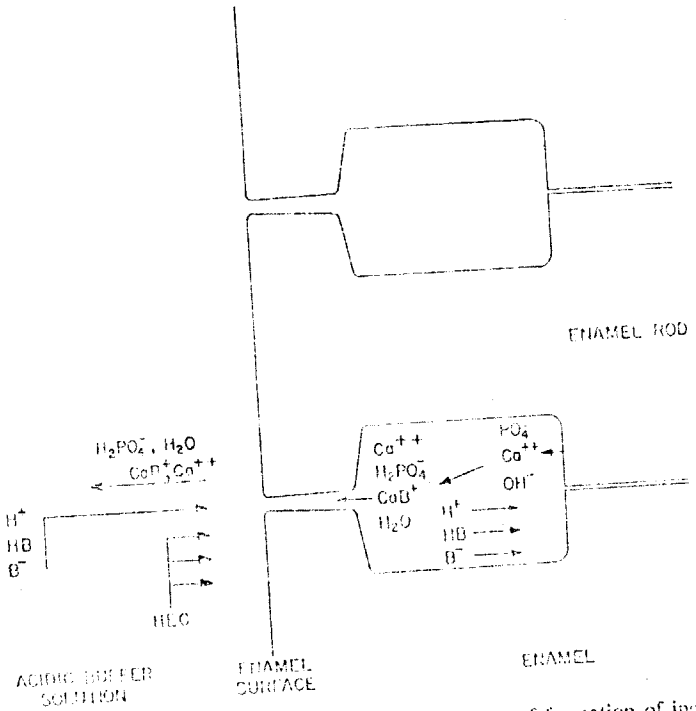

FIG. 14. A flow-sheet diagram to illustrate the process of formation of incipient caries-like lesions. The hydroxyethyl cellulose adsorbs on the surface while acidic buffer components diffuse into enamel for reaction with subsurface apatite crystals, and reaction products diffuse out into the bulk solution. In general, about 50% of the enamel is dissolved from the region of subsurface decalcification and the process then advances into the enamel.

the buffer anion becomes significant in tying up the calcium until it can diffuse out into the bulk solution.

The reasons for lack of further dissolution in the region partially destroyed are not completely clear. In general, about 50% of the enamel (calculated from amount of enamel dissolved and depth of lesion) is dissolved and then the process advances further into the enamel. Accumulation of the reaction products which can reprecipitate to form other less soluble compounds or reverse the reaction is certain to have an effect. It is also known that organic matter, insoluble in acid, accumulates in this region and may form an acid insoluble film over the remaining hydroxylapatite surfaces. The organic material can originate from external sources such as HEC and plaque and diffuse into enamel through the spaces enlarged by the dissolution. However, another source also may be present in the natural organic moiety of enamel. This finely [illegible] present in the enamel, during the formation of the lesion, could concentrate [illegible] such as seen by [illegible] and thus [illegible] the organic residues such as seen by

electron microscopy. It is undoubtedly a combination of these factors which serves to preserve the relatively sound outer layer and limit internal dissolution, just as it was found to be a combination of factors which controlled the rate of dissolution of enamel.

Acknowledgement—This work was presented in part before the 43rd meeting of the International Association for Dental Research, Toronto, Canada, July (1965).

Résumé—La cinétique de la dissolution de l'émail et le mécanisme de formation de la lésion sont étudiés en utilisant une méthode cinétique simple in vitro, permettant de produire des lésions de l'émail dentaire humain, comparable à des lésions carieuses naturelles débutantes, appelées taches blanches. L'émail est soumis à un milieu de décalcification, constitué par une solution aqueuse de tampon acide et d'un polymère organique soluble (cellulose d'hydroxyéthyle), qui protège la surface de l'attaque acide, pendant la dissolution de la sub-surface. L'étendue de la lésion est déterminée par l'analyse du calcium et du phosphate de l'émail dissout dans le milieu de décalcification et par examen microscopique des coupes de l'émail. La dissolution de l'émail est étudiée par une méthode différentielle donnant la vitesse initiale et par une méthode intégrale permettant l'étude du stade d'équilibre. Cinq acides tamponnés (Chloroacétique, dichloroacétique, lactique, formique et acétique) ont été testés à pH 3–6 et à une concentration de tampon de 0–0,5 M. La vitesse initiale augmente avec la diminution du pH, avec des constantes de dissociation acide décroissantes; elle augmente presque [illegible] avec la concentration [illegible] du tampon. La vitesse initiale de dissolution de l'émail, pendant la formation de la tache blanche, est exprimée quantitativement par une équation simple pour toutes les acides, sous la forme de concentration en acide non dissocié, concentration en ion hydrogène, constante de dissociation acide, et constante de dissociation du complexe anionique acide de l'ion calcium. Cette équation est similaire à celle de la dissolution de surface [illegible] les rapports des 2 processus. La vitesse de dissolution de l'émail est principalement contrôlée par la vitesse de diffusion des acides non dissociés dans l'émail et à un degré moindre par le pH et la force de l'acide. Des ions calcium libres inhibent la dissolution et peuvent même influencer la vitesse de dissolution initiale. Certains acides forment des complexes solubles avec le calcium, suffisant pour supprimer cette inhibition, et sont quelque peu plus actifs dans la formation des taches blanches.

Zusammenfassung—Mit Hilfe einer einfachen chemischen Methode *in vitro* zur Entwicklung von Läsionen im menschlichen Zahnschmelz, die natürlichen, als weisse Flecke bekannten, beginnenden kariösen Läsionen vergleichbar sind, wurde die Kinetik der Schmelzauflösung und der Mechanismus der Defektbildung untersucht. Der Schmelz wurde einem demineralisierenden Medium ausgesetzt, das aus einer wässrigen Lösung eines sauren Puffers und einem löslichen organischen Polymer (Hydroxyäthyl-Cellulose) besteht, welches die Oberfläche vor der Säureeinwirkung während der Auflösung unter der Oberfläche schützt. Die Ausdehnung des Defektes wurde durch Analyse des Dekalzifikationsmediums auf vom Schmelz gelösten Kalzium und Phosphor und durch mikroskopische Untersuchung von Schmelzschnitten bestimmt. Die Schmelzauflösung wurde mit Hilfe einer Differentialmethode untersucht, welche die Anfangsrate angibt. Eine Integralmethode ergab die Annäherung an das Gleichgewicht. 5 Puffersäuren (Chloressigsäure, Dichloressigsäure, Milchsäure, Ameisensäure und Essigsäure) wurden bei pH's zwischen 3 und 6 und bei Pufferkonzentrationen von 0 bis 0,5 M untersucht. Die Anfangsrate wuchs mit abfallendem pH, abfallender Säuredissoziationskonstante und nahezu linear mit anwachsender Pufferkonzentration an. Die Anfangsrate der Schmelzauflösung während der Entwicklung des weissen Fleckes wurde quantitativ in einer einzigen Gleichung für alle diese Säuren als undissoziierte Säurekonzentration, Wasserstoffionen Konzentration, Säuredissoziationskonstante und Dissoziationskonstante des Säureanionenkomplexes von Kalziumionen ausgedrückt. Diese Gleichung ist derjenigen für die Oberflächenauflösung ähnlich, was die Beziehung zwischen den beiden Prozessen demonstriert. Die Schmelzauflösungsrate wird prinzipiell durch die Diffusionsrate der undissoziierten Säure in den Schmelz und in geringem Grade durch das pH und die Säurestärke bestimmt. Einige Säuren bilden lösliche Komplexe, die [illegible] diese Hemmung zu [illegible], und die [illegible] des weissen Fleckes etwas wirksamer sind.

REFERENCES

PERRIN, J., SCHWARZENBACH, G. and SILLÉN, L. G. 1957. *Stability Constants*, Part I: Organic Ligands. pp. 1–12. Soc. Chem. Ind., pp. 6. The Chemical Society, London.

BRUDEVOLD, F. 1962. Chemical composition of the teeth in relation to caries. In: *Chemistry and Prevention of Dental Caries* (Ed. by SOGNNAES, R. F.). Chap. 2, pp. 32–88. Charles C. Thomas, Springfield, Ill.

CHERNEVITCH, M. I. 1957. The theory of acid decomposition of minerals: Apatite. *Trans. Sci. Res. Institute Fertilizers Insecticides* (U.S.S.R.) 137, 10–33.

COOLIDGE, T. B., [illegible] and JACOBS, M. H. 1955. A microscope comparison of clinically and artificially produced changes in enamel. *Oral Surg.* 8, 1204–1210.

COOLIDGE, T. B. and JACOBS, M. H. 1957. Enamel carbonate in caries. *J. dent. Res.* 36, 765–768.

DARLING, A. I. 1956. Studies of the early lesion of enamel caries with transmitted light, polarised light and radiography. *Proc. roy. soc.* 101, 329–341.

FRANCIS, M. D. 1965. Solubility behaviour of dental enamel and other calcium phosphates. *Ann. N.Y. Acad. Sci.* 120, [illegible]–713.

GRAY, J. A. 1962. Kinetics of the dissolution of human dental enamel in acid. *J. dent. Res.* 41, 633–645.

GRAY, J. A., FRANCIS, M. D. and GREBETHUN, W. J. 1962. Chemistry of enamel dissolution. In: *Chemistry and Prevention of Dental Caries* (Ed. by SOGNNAES, R. F.). Chap. 5, pp. 164–179. Charles C. Thomas, Springfield, Ill.

GRAY, J. A. and CHOW, L. L. 1962. A device for thin sectioning of hard tissues. *J. dent. Res.* 41, 172–181.

GRAY, J. A. and FRANCIS, M. D. 1965. Physical chemistry of enamel dissolution. In: *Mechanisms of Hard Tissue Destruction* (Ed. by SOGNNAES, R. F.) Chap. 8, pp. 213–260, publication no. 75. American Association for the Advancement of Science, Washington, D.C.

HALS, E., MIKAEL, H. and [illegible] H. E. 1[illegible]. Effect of fluoride solutions on dental enamel *in vitro* as observed in polarising microscope. *Acta odontol. Scand.* 23, 55–[illegible].

HIGGINS, W. L., GRAY, J. A., WHITEHEAD, J. A. and FRANCIS, M. D. 1965. Mechanisms of enamel dissolution in acid buffers. *J. dent. Res.* 44, 339–341.

KAUFF, R. K., FEDERER, [illegible] and FRANKS, [illegible] S. 1962. Influence of type of acid, pH, molarity, and saturation on [illegible] penetration. *J. dent. Res.* 41, 760–770.

KOLTHOFF, G., V[illegible] 1[illegible]. Dissociation constants of organic acids in aqueous solutions. *Ind. Eng. Chem.* 1, [illegible]. Interscience, New York.

LOHMANN, K. J. 1[illegible]. *Chemical Kinetics*, pp. 1[illegible]. Prentice-Hall, New York.

LUCENA-[illegible], L. and [illegible], L. 1[illegible]. A new reagent for the colorimetric and spectrophotometric determination of [illegible]. *Anal. Chem.* 36, [illegible].

MOORE, L. [illegible]. *Proceedings of the Fluoride Investigations Circus*. Edited by [illegible]. Pp. [illegible]. Harvard Dental School, Boston, Mass.

MOORE, R. S., [illegible], R. R. 1[illegible]. A method for study of penetration of enamel by acid buffers. *J. dent. Res.* 45, [illegible].

MILLER, W. D. 1905. A study of certain questions relating to the pathology of the teeth. *Dent. Cosmos* 47, 19–[illegible].

ORBAN, B. J. 1957. *Oral Histology and Embryology* (4th Ed.). pp. 55–55. Mosby, St. Louis.

SELLER, G. H. and [illegible], M. G. [illegible]. Effect of different acids on character of demineralization of enamel surfaces. *J. dent. Res.* 45, 795–7[illegible].

SILVERSTONE, L. M. and [illegible], M. C. 1[illegible]. Dental surface in "white-spot" formation. *J. dent. Res.* 45, 72[illegible]–73[illegible].

SLACK, H. V. 1[illegible]. The organic content of relatively enamel". *Brit. dent. J.* 96, 73–75.

SIRAKOV, [illegible]. Rate of dissolution of dental enamel. *Trans. R. Sch. Dent., Stockh. & Umeå* [illegible], 52–[illegible].

SUISS, [illegible] L. S. 1[illegible]. The histology of normal and fluoridized enamel. *J. dent. Res.* 28, [illegible]–[illegible].

VOLKER, J. F. [illegible] Study of fluorosed enamel and dentine. *Proc. Soc. exp. Biol., N.Y.* 43, 643–645.

WOODWARD, L. W. and [illegible], L. R. [illegible]. Factors influencing the shape of the developing lesion. *[illegible] Res.* [illegible]–197.

WHITEHEAD, E. W. and CHRISTIAN, L. B. 1964. Application of spectro-photometric titrations to micrometer solutions of calcium, magnesium, oxalate, or sulfate. *Analyt. Chem.* 36, 1679–1681.

PLATES 1–3 OVERLEAF

Effects of Folic Acid Antagonist Therapy on Urinary Excretion of Formic Acid by Humans.* (23968)

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Although formic acid has been reported to occur in urine of normal human subjects on a mixed diet, the methods used for its determination have involved either extraction of urine with ether and subsequent distillation of volatile acids from acid solution(1,2), or direct distillation of the urine(3-5). Significance of values obtained using these methods is somewhat doubtful since many naturally-occurring substances are known to yield formic acid upon treatment with acid at elevated

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TABLE I. Formic Acid Excretion in Subjects without Leukemia.

Subject	Sex	Age	Clinical data	Urinary formic acid, μmoles/hr
Adults				
60	♂	32	Normal control	16.8, 13.8
61	♂	35	*Idem*	23.4, 13.8
62	♂	29	"	16.3, 19.8
82	♂	65	Arteriosclerotic	32.0
83	♂	65	Metastatic carcinoma	10.4
84	♂	60	? Neurologic disease	20.5
85	♂	59	Angina pectoris	12.3
86	♂	67	Emphysema	14.7
87	♂	19	Idiopathic thrombocytopenia	21.5
88	♂	68	Pneumonia	47.3
91	♀	72	Aortic stenosis, angina pectoris	8.5
92	♀	68	Acute myocardial infarct	15.0
93	♀	64	*Idem*	13.2
94	♀	64	Congestive heart failure	9.9
95	♀	70	Angiomyolipo-hepatoma	9.0
96	♀	60	Acute myocardial infarct	3.6
Children				
2	♂	12	Ulcerative colitis	1.9
5	♂	10	Streptococcal pharyngitis	8.5
8	♀	10	Duplication of ureteropelvic junction	3.8
45	♂	11	Rhabdomyosarcoma	5.0
46	♂	2	Nephroblastoma	4.5
90	♀	10	Fracture of fibula	15.7

temperatures. In animal studies with C^{14}-labeled substrates, sources of urinary formate have been shown to include arginine (7), α-carbon of glycine, glycolate, and glyoxylate, and β-carbon of serine, acetone, and methionine(8). A major fraction of formic acid presented to the mammal is further metabolized (9-11), but profound impairment in capacity to utilize formate has been demonstrated in folic acid-deficient rats(11,12). This undoubtedly accounts for the marked increase in urinary excretion of formate by rats on folic acid-deficient diet(13).

The work to be reported indicates that formic acid is present in detectable quantity in urine of all human subjects studied. The levels in adult urine are lower than previously reported values(1,5). In children with acute leukemia undergoing folic acid antagonist therapy, excretion of formic acid is markedly increased. This investigation has been made possible by recent development of a sensitive and specific enzymatic method for determination of formic acid(14).

Methods. Timed urine samples were obtained from 3 healthy adults and from 19 patients on medical and pediatric services of Beth Israel Hospital. In addition, specimens were obtained from 22 children undergoing therapy for acute leukemia at Children's Cancer Research Fn. Sixteen of the latter group were receiving the antifolic acid drug, amethopterin (Methotrexate®; 4-amino-4-deoxy-N^{10}-methylpteroyl glutamic acid) in amounts listed in Table III. Pertinent clinical data are summarized in Tables I-III. Urine samples were refrigerated immediately, and either analyzed for formic acid within 18 hours, or frozen within that time and kept at −10° until

TABLE II. Formic Acid Excretion in Children with Acute Leukemia not Receiving Amethopterin.

Subject	Sex	Age	Clinical data	Previous amethopterin and interval since last given	Urinary formic acid, μmoles/hr
21	♂	13	In remission on 50 mg of 6-mercaptopurine daily	Yes; 5 mo	7.8
23	♂	4	In remission; receiving adrenal steroids, chloramphenicol, and novobiocin	" 6 mo	16.8
24	♂	6	In relapse; receiving intrac. ACTH, chloramphenicol, and novobiocin	" 5 mo	22.1
26	♂	3	No previous therapy, receiving adrenal steroids	No	6.1
40	♀	7	Amethopterin omitted 24 days previously because of severe toxicity (stomatitis); partial remission	Yes; 14 days	11.2
50	♂	1	No previous therapy	No	3.6
53	♀	2	*Idem*	"	2.8

TABLE III. Formic Acid Excretion in Children with Acute Leukemia Receiving Amethopterin.

Patient	Sex	Age	Remission	Daily dosage of amethopterin, mg	Duration of treatment	Urinary formic acid, μmoles/hr
26	♂	3	Yes	1.25	4 wk	10.4
					5 "	8.3
					12 "	119
27	♀	5	"	1.25	3 mo	45.8
28	♂	4	"	2.5	6 wk	26.8
					18 "	85.8
29	♀	4	"	1.25	11 mo	49.5
30	♂	4	No	1.25 (& prednisone)	1 yr	32.0
33	♂	8	Yes	2.5 "	4 wk	8.4
					10 "	6.7
					12 "	6.7
					15 "	36.4
					18 "	66.1
34	♂	3	Partial	1.25	3 mo	123
					3½ "	165
			Amethopterin stopped 3 wk previously			9.7
			" " 4 " "			9.6
35	♂	3	Yes	2.5	6 mo	64
					8 "	117
					9 "	117
37	♀	7	No	2.5 (& prednisone)	2 "	14.0
41	♀	8	Yes	2.5 "	3 "	122
43	♂	9	"	2.5 "	2 wk	12.8
					12 "	72.1
					16 "	125
44	♀	5	"	1.25	6 "	10.5
					15 "	36.6
					22 "	265
47	♂	3	Early relapse	2.5	5 mo	60
					5½ "	204
			Amethopterin stopped 2 wk previously			72
			" " 3 " "			23.8
55	♂	3	In relapse	2.5	8 mo	301
			Amethopterin stopped 3 wk previously			10.5
56	♂	6	Incomplete (improving)	2.5	14 days	34.8
57	♂	10	Yes	2.5 & 5.0 on alternate days	1 mo	193

analyses were performed. Urines were brought to pH 7.0 with 5.0 *N* KOH, and assayed for formic acid according to the method of Rabinowitz and Pricer(14).

Results. Urinary excretion of formic acid by adults ranged from 3.6 to 47.3 μmoles/hour (Table I). Nonleukemic children and leukemic patients not receiving amethopterin excreted 1.5-22.1 μmoles/hour (Tables I and II). The 3 children with acute leukemia who had never received the folic acid antagonist excreted 2.5-6.1 μmoles/hour. Fifteen of 16 children receiving amethopterin excreted more than 32 μmoles/hour, and 9 patients excreted over 117 μmoles/hour (Table III). An increase in formic acid excretion was usually not apparent until after several weeks of amethopterin therapy, and often not until long after remission of the leukemic process. Thus in subject #33 formic acid excretion was only 6.7 μmoles/hour 12 weeks following start of treatment, but had increased by 10-fold after 18 weeks. A similar phenomenon was observed in patients 26, 43, and 44. This latent period may explain the low excretion of formic acid in patient 37, who had received amethopterin for 2 months. The level of urinary formic acid in treated children bore no apparent

relationship to clinical status of leukemia. Cessation of amethopterin administration to patients 34, 47, and 55 was followed by a sharp fall in formic acid excretion.

No evidence was found in urine of amethopterin-treated patients of substances that interfered with the formic acid assay. This was demonstrated by recovery experiments in which urine samples were reassayed after addition of known increments of formic acid; 100% recovery was obtained in all samples so tested.

Discussion. Our data demonstrate suitability of the enzymatic procedure of Rabinowitz and Pricer(14) for determination of urinary formic acid. Our studies indicate that in normal human subjects and in patients with a variety of disease states, formate may be detected in the urine, although in smaller quantity than previously reported(1,5). The variation in formic acid excretion from one patient to another, as well as in the same patient may be attributable to variations in dietary intake of formate precursors.

Participation of formic acid in metabolic processes is known to involve a folic acid derivative(15-18). Formation of this derivative has been shown to be inhibited in animals receiving folic acid antagonists(19). Hence, formic acid would be expected to accumulate in folic acid deficiency and in subjects receiving folic acid antagonist therapy. This explanation undoubtedly accounts for the formaturia reported in folic acid-deficient rats(13) and for that observed in our patients treated with folic acid antagonist, amethopterin. Increased excretion of formic acid was often not seen until after several weeks or months of therapy.

It is of considerable interest that in several patients a complete remission had taken place well in advance of an increase in formic acid excretion, or in formiminoglutamic acid excretion(20). Since animal studies indicate that increase in urinary formic and formiminoglutamic acids is a reflection of folic acid deficiency in the whole organism(13), our observations provide *in vivo* evidence for the thesis that leukemic cells are abnormally sensitive to folic acid deprivation(21).

Summary. An enzymatic procedure has been employed to demonstrate the presence of formic acid in small quantity in urine of normal human subjects and of patients with a variety of disease states. The excretion is markedly elevated in patients with acute leukemia receiving the folic acid antagonist, amethopterin.

1. Dakin, H. D., Janney, N. W., Wakeman, A. J., *J. Biol. Chem.*, 1913, v14, 341.

2. Riesser, O., *Z. Physiol. Chem.*, 1916, v96, 355.

3. Autenrieth, W., *München med. Wchnschr.*, 1919, v66, 862.

4. McNeal, M., Eldridge, C. J., *Am. J. Dis. Child.*, 1922, v23, 419.

5. Benedict, E. M., Harrop, G. A., *J. Biol. Chem.*, 1922, v54, 443.

6. Voit, K., Friedrich, H., *Klin. Wchnschr.*, 1935, v14, 1792.

7. Mackenzie, C. G., *J. Biol. Chem.*, 1950, v186, 351.

8. Weinhouse, S., Friedmann, B., *ibid.*, 1952, v197, 733.

9. Sonne, J. C., Buchanan, J. M., Delluva, A. M., *ibid.*, 1948, v173, 69.

10. Sakami, W., *ibid.*, 1948, v176, 695.

11. Plout, C. W. E., Bethell, J. J., Lardy, H. A., *ibid.*, 1950, v184, 795.

12. Friedman, B., Nakada, H. I., Weinhouse, S., *ibid.*, 1954, v210, 413.

13. Rabinowitz, J. C., Tabor, H., *ibid.*, in press.

14. Rabinowitz, J. C., Pricer, W. E., Jr., *ibid.*, 1957, v229, 321.

15. Gordon, M., Ravel, J. M., Eakin, R. E., Shive, W., *J. Am. Chem. Soc.*, 1948, v70, 878.

16. Greenberg, G. R., *Fed. Proc.*, 1951, v10, 192.

17. Buchanan, J. M., Schulman, M. P., *J. Biol. Chem.*, 1953, v202, 211.

18. Rabinowitz, J. C., Pricer, W. E., Jr., *J. Am. Chem. Soc.*, 1956, v78, 4176.

19. Nichol, C. A., Welch, A. D., Proc. Soc. Exp. Biol. and Med., 1950, v74, 403.

20. Hirt, H. H., Goldstein, M., Tabor, H., *J. Clin. Invest.*, in press.

21. Fischer, G. A., Welch, A. D., *Science*, 1957, v126, 1048.

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FORMIC ACID

CA 67, 10474e (1967)

7

10474e Major free fatty acids in Gouda cheese. Meena Iyer, T. Richardson, C. H. Amundson, and R. C. Tripp (Univ. of Wisconsin, Madison). *J. Dairy Sci.* 50(3), 385(1967)(Eng). Four samples of Gouda cheese were examd. for free fatty acid content by the methods of Wiseman and Irvin (*C.A* 51: 17019b) (formic and acetic acid analyses) and Iyer, *et al.* (*C.A* 66: 84846d) (butyric through linolenic acids). Total free fatty acids ranged from 1949 to 3454 mg. acid/kg. cheese. Ranges, in mg. acid/kg. cheese, for individual fatty acids were as follows: 1:0, 22–68; 2:0, 186–1073; 4:0, 57–101; 6:0, 22–62; 8:0, 15–36; 10:0, 38–57; 12:0, 84–109; 14:0, 154–252; 16:0, 432–591; 18:0, 193–277; 18:1, 370–751; 18:2, 64–141; and 18:3, 34–69. Substantial similarity was noted between Gouda and Cheddar cheeses, with respect to free fatty acid compn. E. H. Marth

DETECTION

Indian Medical Gazette. Dec. 1944

FORMIC AND ACETIC ACIDS AS POISONS*

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Historical

THE appearance of the volatile fatty acids, formic and acetic, in toxicology is very patchy. According to Autenrieth (1928) formic acid has 'hardly ever come up as poison'. Five cases have however been reported from the Federated Malay States (*Analyst*, 1940, p. 459), 4 from the Straits Settlements (*Analyst*, 1937, p. 742; 1939, p. 744), and 2 from Ceylon (*Analyst*, 1932, p. 575) during the period of 1931-42.

Although acetic acid has been used for suicide, and accidental poisonings have also been fairly common in certain countries of Europe, notably Russia and Germany from the beginning of the present century, Autenrieth (1928) and Owens (1935) have not mentioned any instance of poisoning with this acid. This might be because, as Hunt and Gettler say, 'in the United States and England, poisoning by acetic acid seems to be rare'. There were according to these authors 4 fatal cases in England and Wales in 1912, while in Germany there were 256 cases of acetic acid poisoning in 1909 and in St. Petersburgh in 1908 it accounted for 34 per cent of all suicides and 72 per cent of all cases of poisoning. Blyth and Blyth (1920) refer to 12 cases as having occurred in England and Wales during the five years ending in 1916. Two instances of cases of acetic acid poisoning have been mentioned in the Annual Report of the Medical Officer of the Ministry of Health, 1937. Fifteen cases have been reported from Ceylon during 1931–42 (*Analyst*, 1932, p. 575; 1937, p. 469; 1938, p. 501; 1939, p. 512 and 1940, p. 652).

Owens (1935), while stating that 'a few fatal cases of poisoning by this have been reported as having occurred in Europe', does not mention a single case anywhere in India. It has been referred to as poison only once during the last decade by the Chemical Examiner to the Government of Madras (Annual Report of the Chemical Examiner's Department, 1940, p. 15).

Cases in Travancore

The number of cases from which we have been able to isolate formic and acetic acids during the last seven years is significant and interesting. Although toxicological analysis was introduced in Travancore 51 years ago, these acids had not figured in our toxicological history prior to 1936. Since then we have had 71 cases out of a total of 428 cases of poisoning distributed as follows :—

	1935–36	1936–37	1937–38	1938–39	1939–40	1940–41	1941–42	1942–43	Total
Formic acid ..	..	6	8	13	5	2	..	..	34
Acetic acid ..	7	1	3	1	5	11	6	3	37
TOTAL ..	60	54	47	55	53	64	51	44	428

The regional distribution of these cases is significant. Administratively the State is divided into three districts, and in one respect the districts are strikingly different from each other.

Travancore is the biggest rubber producing area in India. Of the 136,605,671 acres under rubber in India, 10,446,517 acres are in Travancore. But the distribution of rubber among the districts is unequal. Trivandrum division has little rubber. Although both Quilon and

* A paper presented before the Indian Science Congress, 1944.

Kottayam have extensive acreage under rubber, most of the small holdings are in the Kottayam district. Of the 71 cases under review, Kottayam claimed 62, Quilon 9 and Trivandrum none.

The close association of rubber with poisoning by the two acids is remarkable. Indeed, it is the development of this industry that has brought these acids into common use in this country and led to their deliberate or accidental use as poisons. The high incidence of such cases in Ceylon and Malaya, two of the leading rubber producing countries of the world, corroborates this view.

During the last two years, there has been no case of poisoning by formic acid because the outbreak of war has made it unavailable. Acetic acid has also become increasingly costly and more difficult to obtain, and it is to a large extent replaced by a sulphuric acid preparation called 'Sulpho'ex'. Because these acids have become more precious, the owners are naturally taking greater care of their stocks than they did before.

The 71 cases come under three heads : (*a*) suicidal, (*b*) accidental, (*c*) overdose—when deliberately taken as a medicine.

	Suicidal	Accidental	Over-dose	Total
Formic acid ..	27	6	1	34
Acetic acid ..	28	7	2	37

No case of homicidal poisoning has been reported, obviously because they do not satisfy the primary properties which criminals desire in their poisons—*i.e.* smallness of dosage and absence of unpleasant smell and taste. The choice of a poison for suicide is governed by its easy availability as well as by the level of intelligence of the victim. An intelligent person's suicide is seldom a momentary impulse. It is generally the result of long thought and brooding, and a quick-acting poison not likely to cause much agony, such as the cyanides, opium or arsenic will be preferred. In the case of an illiterate labourer, the thought of suicide is often a sudden impulse, following, say, a domestic quarrel, discharge from employment, etc. He has neither the knowledge of the more powerful poisons nor the cunning or means to procure them. He uses what he can easily get and is not deterred by fear of suffering. This explains the large use of these acids for suicidal purposes in this country. Their extensive use in the rubber industry made them available to anyone, and the victims invariably belonged to the lower strata of society.

A parallel to this can be found in the use of acetic acid for suicide in Russia and Germany during the early part of this century, referred to above. Essence of vinegar containing 80 per cent of acetic acid had just come into use as a food preservative, and was freely available to all, and the poor people, probably not more advanced in intelligence than the labourers engaged in the rubber industry, used it for suicide.

Accidental consumption of any poison is due to its domestic storage along with receptacles containing medicine or alcoholic drinks. Accidental poisoning by carbolic acid and other disinfectants has often occurred in Great Britain in this manner, and by acetic acid in Germany and Russia. In Travancore, while no domestic storage of these acids is done in the large rubber estates, the small holders keep small quantities at home in bottles similar in size and appearance to those used for keeping various indigenous medicines or alcoholic drinks. The smell of some of these indigenous preparations is not very different from that of these acids, and this leads to accidental poisoning.

Although the conditions under which acetic and formic acids have been used for suicide or consumed accidentally are more or less similar everywhere, there is another group of cases which, although small, is of a unique type. In these cases the victims were reported to have been using small doses of acetic acid for the relief of their stomach pain. It may be noted here that the incidence of gastro-duodenal disease in Travancore is estimated to be 3 to 4 times that of other parts of India (Orr, 1936) and occasionally larger doses of acids than usual have been consumed either accidentally or deliberately with the hope of getting the relief which smaller doses had failed to give. In a fair proportion of suicides also, the desire to escape for ever from their incurable gastric pain appears to have been the motive for the crime.

Signs and symptoms

These are in general similar to those caused by mineral acids. The post-mortem appearances are also of the same nature. Vomiting, salivation and a burning sensation of mouth are present invariably. The vomit contains bloody fluid. The victim usually rolls about in agonizing pain. The pulse becomes rapid first and then slows down. Respiration also follows the same course of rise and fall. The skin is often cold and the face cyanosed, and there is rattling of the throat and irritation of the respiratory passages, although in the majority of cases the larynx and trachea were found normal. If concentrated acid is taken, the whole of the mouth and tongue may be corroded, the skin over the chin, front of the neck and on the upper part of the chest showing brownish desquamation if the acid has flowed down the mouth. Sometimes dark-stained streaks run down from the angles of the mouth and the middle of lower lip just under the chin, through the trickling down of the acid.

Fatal period

Death in acetic acid poisoning is stated to occur in 48 hours by Reid Hunt and Gettler.

We have reliable information about this point in 14 cases of formic acid and 21 of acetic acid poisoning, and this is given below :—

	Deaths in less than 2 hours	2–4 hours	4–6 hours	6–12 hours	12–24 hours	24–48 hours	More than 48 hours
Formic acid ..	2	2	1	3	4	2	..
Acetic acid ..	1	2	4	4	6	1	3

Of the three victims of acetic acid who survived for more than 48 hours, one survived for 3 days, another for 7 days and the last for 14 days. The second was a young adult male (24) who had swallowed the acid with his stomach loaded with toddy.

Post-mortem appearances

They included the presence of blood-stained froth in the mouth and nostrils. The pupils were usually dilated. The right side of the heart was full with blood, and the left empty. The lungs were often congested and crepitant; in some cases they were normal. The liver, kidney and spleen were generally congested. If the concentrated acid was swallowed on an empty stomach, the latter was often distended and dark in appearance and contained dark thick fluid matter. With large doses the coats of the stomach had a dark and burnt appearance. This dark appearance was not noticed when the acid was taken on a loaded stomach. The coats might be normal, although a congested appearance was sometimes seen in such cases. If the dose was heavy enough, the intestines might also be dark in colour and distended in appearance. The bladder in formic acid poisoning cases was empty. This being so, examination of the urine which would have thrown light on the rate of absorption of the acid into the system and excretion could not be carried out. The membranes of the brain, the brain substance and ventricles were often congested, the latter containing sometimes cerebro-spinal fluid. Death appeared to result from asphyxia.

Quantities detected

In formic acid poisoning the quantities detected have ranged from 0.04, 0.17, and 0.55 to 31.8 grammes of anhydrous acid, and in acetic acid cases from a trace to 2.20 and 2.40 grammes of anhydrous acid.

Toxicity and fatal dose

The two acids, being corrosive liquids, destroy the epithelial tissues, formic acid being more irritant than acetic acid. It may be noted here that in the use of the two acids for the coagulation of rubber, 1 ounce of formic acid is enough for 18 lb. of latex whereas 1 ounce of acetic acid coagulates only 11 lb. of latex. In none of the cases of poisoning by formic acid did the victim survive; even 4 ounces of a 50 per cent diluted acid proved fatal. In two of the cases of acetic acid poisoning in which 2 ounces of undiluted acid were swallowed, recovery was seen. Death was however reported within 9 hours in a case in which the victim, a man of 35, took 2 ounces of the concentrated acid.

Separation, detection and estimation

For quantitative estimation of acetic acid, a known weight of the well-comminuted viscus (100 grammes) is taken in a litre flask with a long neck. 30 c.cm. of 25 per cent phosphoric acid is added, together with 200 c.cm. of water to make the volume up to 300 c.cm. A few glass beads are introduced into the flask. A still head is used and the distillation is conducted with vigorous boiling. The volume of the liquid is kept constant by adding distilled water drop by drop from a separating funnel, distillation being carried on until about 1,500 c.cm. of the distillate have passed over, when usually the point of neutrality to litmus is reached. Stir an excess of calcium carbonate with water and add to the distillate. Bring at once to the boil, and then evaporate in a flat dish on the water bath to dryness. Dry the whole residue for an hour at 100°C. Add 50 c.cm. of hot water, keep for a few minutes and filter, washing the dish several times with warm water and passing the liquid each time through the filter. Cool. Make up to 500 c.cm. A portion of this solution is evaporated to dryness on the water bath, dried at 100°C. and the residue is heated in a hard glass test tube with arsenious acid. The presence of acetic acid is inferred from the smell of cacodyl produced. The ethyl acetate test can be applied to a portion of the residue; but often the presence of organic matter prevents the smell being noted. In such cases, the cacodyl test answers the purpose very well.

Take 25 c.cm. of the above solution in a long-necked flask of 500 c.cm. capacity and proceed as follows: Add 30 c.cm. of water free from CO_2 and 10 c.cm. of phosphoric acid (sp. gr. 1.7). The volume of the liquid in the flask is kept constant by adding CO_2 free distilled water. The distillation is carried on for 1½ to 1¾ hours, the distillate being collected in a flask containing excess of a known volume of standard NaOH, to which access of CO_2 is prevented by providing a bent tube containing soda lime. Ninety-five per cent of the acid is evolved during the first hour. The excess of NaOH is titrated with standard H_2SO_4, using phenolphthalein as indicator. Each c.cm. of N/10 NaOH represents 0.006 grammes acetic acid.

For the quantitative estimation of formic acid, the method suggested by Autenrieth (1928) may be followed. The presence of organic matter interferes with the usual reduction test (with silver nitrate). This can be avoided if the residue is treated with magnesium and dilute sulphuric acid in a distilling flask, the contents distilled, and the distillate tested for formaldehyde by the usual reagents namely phloroglucinol and resorcinol (Henry Leffmann in Allen's *Commercial Organic Analysis*, Vol. I, 1924).

Summary

Formic and acetic acids have figured in the toxicology of Travancore in a remarkable manner during the last 8 years. Prior to 1936 there were no recorded cases of poisoning due to these acids, but since then 71 cases have occurred out of a total of 428 cases in which poison was detected. Formic acid does not appear to have found a place in toxicology elsewhere in India, while acetic acid has been mentioned only once in the last decade by the Chemical Examiner to the Government of Madras.

The regional incidence of these cases shows their close association with the rubber industry, in particular with the distribution of the small rubber holders whose store room is a part of the house they live in. The scarcity of the acids resulting from the outbreak of the war has considerably reduced the incidence of poisoning by these acids.

In 77 per cent of cases the acids were swallowed for suicide; in 18 per cent it was accidental. There were no instances of their use for homicide, but in 3 cases death followed deliberate use for relief of stomach pain.

Death may occur within a few hours or up to 14 days after swallowing. While 2 ounces of the concentrated acid have been found generally fatal, in a few cases of acetic acid poisoning, that dose did not cause death. All cases of formic acid poisoning were fatal.

The signs and symptoms are those of irritant poisoning, and post-mortem appearances are suggestive of death from asphyxia.

The technique adopted by the authors for the qualitative and quantitative estimation of the acids is described.

REFERENCES

AUTENRIETH, W. (1928). *Detection of Poisons.* J. and A. Churchill, London.

BLYTH, A. W., and BLYTH, M. W. (1920). *Poisons: Their Effects and Detection.* Charles Griffin and Co., Ltd., London.

MINISTRY OF HEALTH (1937). *Ann. Rep. Chief Med. Officer*, 1936. His Majesty's Stationery Office, London.

ORR, I. M. (1936) .. *Travancore Med. J.*, **1**, 387.

OWENS, T. F. (1935) .. *Lyon's Medical Jurisprudence for India.* Thacker, Spink and Co. (1933), Ltd., Calcutta.

FORMIC ACID

C.A., 64, 20523c, (1966)

235

Quantitative analysis of the major free fatty acids in Swiss cheese. J. E. Langler and E. A. Day (Oregon State Univ., Corvallis). *J. Dairy Sci.* 49(1), 91–3(1966)(Eng). Examn. of 5 different Swiss-type cheeses (pH 5.4 to 5.8) indicated the presence of the following free fatty acids (in mg. acid/kg. cheese): formic 0 to 314; acetic 1835 to 3724; propionic 2181 to 5919; butyric 86 to 337; 2-methylbutyric 12 to 100; 3-methylbutyric 0 to 47; caproic 37 to 225; caprylic 38 to 216; capric 40 to 190; lauric 74 to 310; myristic 258 to 966; palmitic 587 to 3091; stearic 97 to 650; oleic 363 to 2026; linoleic 47 to 272; and linolenic 62 to 231. 2-Methylbutyric acid is believed to be an essential component of the flavor complex, since there was a direct relation between its concn. and flavor intensity of the cheese. When the proportions of the free fatty acids of Swiss cheese are compared to those of the esterified fatty acids of milk fat, the percentages of butyric through linolenic are quite comparable, suggesting that these acids arise from a nonspecific hydrolysis of milk fat. E. H. Marth

Detection
Metabolism
CH

Acta pharmacol. 1948, 4, 205—212.

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Excretion of Methanol and Formic Acid in Man after Methanol Consumption.

By

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(Received March 24, 1948).

Methanol poisoning occurs with some frequency and is generally due to confusion of methanol with ethanol, two closely related alcohols. Nevertheless the literature available on formic acid excretion after methanol poisoning is exceedingly sparse; furthermore, it is often not open to a critical estimate, because precise statements are lacking about the amount of methanol consumed and the time of the consumption.

Previous Investigations.

Some figures for formic acid excretion in 2 cases of methanol poisoning have been set out in Table 1. Pohl's figures indicate the *additional* excretion of formic acid, the normal formic acid concentration in the urine having been deducted. The figures in column B, on the other hand, indicate the entire concentration of formic acid in the urine. Apparently the excretion of formic acid runs the same protracted course in man as in dogs (Lund, 1948 b), whereas the total output seems to be far smaller in man. Administration of 20 g methanol resulted in an additional excretion of 0.575 g formic acid, representing only about 1.6% of the total alcohol.

Table 1.

Formic acid excretion in the urine of human subjects poisoned with methanol.
A: Man, aged 31, weight 62 kg, consumed 25 ml (20 g) methanol (POHL 1893).
B: Woman, aged 25, consumed about 80 g methanol (HARROP and BENEDICT 1920).

Days after intake	excreted formic acid in urine: mg in 100 ml		mg total	
	A	B	A	B
1	2.3	—	37.4	—
2	32.5	126	420	—
3	6.1	—	89.6	—
4	2.4	60	29.2	—
11	—	7	—	—

AUTENRIETH (1919) administered methanol to an experimental subject and arrived at the following results for the additional excretion of formic acid:

1st and 2nd days administr. of 25 g methanol; additional exer.: 0 g formic acid
3rd - 4th - - - 25 g methanol; additional exer.: 1.313 g formic acid
5th - 6th - - - 0 g methanol; additional exer.: 0.714 g formic acid
7th - 8th - - - 30 g methanol; additional exer.: 3.164 g formic acid

AUTENRIETH's manner of dosing, however, completely conceals the real facts. The figures only show that increased formic acid excretion is seen after administration of methanol, but the characteristic course of the excretion does not appear sufficiently plainly from his figures. Furthermore, AUTENRIETH's conclusion that the excretion of formic acid after administration of 80 g methanol amounts to no more than 5% of the alcohol amount is mistaken, because formic acid excretion during the days following the administration of the last and largest amount of alcohol has not been estimated.

KLAUER (1938) reports 3 fatal cases of methanol poisoning and indicates a few values for the concentration of formic acid in blood and urine. The same 3 cases are found described in BANNICKE's Dissertation (1939). In the first case the formic acid concentration in blood was estimated at 1.5 mg %, in the second that in urine was estimated at 116 mg %, and in the third a mixture of the urine and the kidney brei was found to contain 24 mg % formic acid. Methanol in the blood could be demonstrated in one case only and even there only in "very small amounts".

A paper by ROE (1946), dealing with the clinical course and treatment of methanol poisoning, has some few indications of the formic acid concentrations in blood and urine. Thus, in 4 cases the blood was found to contain 3.5, 4.6, 7.2, and 8.8 mg % formic acid respectively, while the urine from the same 4 patients contained 5.2, 2.2, 3.2, and 1.8 mg % formic acid respectively. In 3 fatal cases of poisoning there were found 5.7, 17, and 19 mg % formic acid in the blood. It is surprising to see that in all except one case the stated concentrations of formic acid in the urine are much lower than those in the blood.

The figures mentioned in the present section have been calculated by workers having highly differing views, and probably also using different, more or less suitable methods for analysis. If any general principles are deducible from the above figures, they must be as follows.

Intake of methanol produces in humans an increased formic acid excretion, which reaches its maximum 1 or 2 days after the intake and is not ended till after 4 to 10 days. The maximum formic acid concentration in the urine seems to be proportional to the amount of methanol consumed (20 g: 32.5 mg %, 80 g: 126 mg %). The same is true of the total amount of formic acid excreted. Of 20 g methanol 1.6 % is excreted as formic acid, and of 80 g not less than 5 %. The figures indicating the formic acid concentration in blood range from 3.5 to 8.8 mg % for non-fatal cases of poisoning, whereas up to 19 mg % formic acid has been found in the blood in cases ending fatally.

4) the concentrations of formic acid found in the urine were normal. Consequently the concentrations of formic acid found in the blood samples from these patients (in no case exceeding 1 mg %) must be regarded as normal values. When methanol is estimated according to Bildsten, titrations under 0.10 ml n/200 thiosulphate, corresponding to about 10 mg % CH_3OH, lie, according to all experience, below the limits of accuracy for the method, so that the blood can then be said to be methanol-free.

In all the cases where an increase of formic acid concentration in the urine was found the concentration was highest between the 14th and 15th of January, i. e. formic acid excretion reached its climax before 3 days after the intake of methanol.

Finally *2 more cases of fatal methanol poisoning will be reported*, where estimations of methanol and formic acid were carried out on blood, as well as on liver and urine.

Case 10: V. H., aged 53, drank in the course of Dec. 1, 1946, a non-specified quantity of fluid from a bottle, which used to contain ethanol, but which had been filled with methanol by mistake. On chemical analysis the bottle proved to contain about 53 % of methanol in aqueous solution. In the evening the patient complained of impaired vision and headache. He went to bed and slept till 4 o'clock the next morning. He then again complained of headache and soon after became restless and confused. On admission to hospital, on Dec. 2, at 10.30 a. m., the patient was almost unconscious, yet he was able to count fingers and complain of pain in chest and abdomen. The respiration was of Kussmaul's type, and there was an acid smell from his mouth. The patient became increasingly unconscious. His condition quickly deteriorated, and death occurred on Dec. 2, 1946, at 12.30. A postmortem examination revealed nothing abnormal.

Case 11: O. R., aged 55, applied to an asylum on July 2, 1947, at 3.30 p. m. with a request for bed and board. He was not perceptibly under the influence of drink, but was stated to have drunk "some spirits that were coloured". That evening he seemed well. The following morning, at 9.30 a. m., when getting out of bed, he dropped down and was put to bed again. He was short of breath and had difficulty in speaking. One hour later vomiting and convulsions occurred, after which the patient was unconscious until death occurred at 11.30 a. m., July 3, 1947. The postmortem examination revealed nothing to account for the occurrence of death.

The poisoning was diagnosed in neither of these two cases, and treatment, in particular with sodium bicarbonate, was therefore

not instituted. It was the forensic analysis that disclosed the cause of death. The results of this analysis have been set out in Table 3.

Table 3.

The concentration of methanol and formic acid in blood, liver and urine from human beings dying of methanol poisoning.

Case No.	mg methanol in 100 g of			mg formic acid in 100 g of		
	blood	liver	urine	blood	liver	urine
10. V.H.	74	106	140	68.1	59.6	785
11 O.R.*)		- -	240	- -	98.6	475

The concentrations of methanol and formic acid found leave no doubt that the cause of death was methanol poisoning in both cases. The formic acid concentration in the blood was thus over 5 times higher than that in the blood from the three fatal cases previously mentioned and even higher than that found in dogs after administration of 3 g methanol per kilogram body weight.

Discussion.

After intake of methanol there occurs in human subjects an increase in formic acid excretion to nearly 100 times the normal. The excretion reaches its maximum from 1 to 3 days after the alcohol intake and is not ended till after ab. 5 days.

It has been maintained that poisoning with methyl chloride should also cause an increased formic acid excretion (Baker 1927 and Füiner 1943). Baker, however, only states that on analysing urine from subjects poisoned with methyl chloride he found formic acid in all cases, but he says nothing about the amount. Thus we do not know for certain whether he takes into consideration the fact that formic acid is a normal constituent of urine (according to Klauer (1938) normal formic acid excretion in humans may be up to 100 mg within 24 hours). Mc Nally (1937) analysed the urine from 11 subjects poisoned with methyl chloride and found formic acid in no more than 3 cases, and even there only in amounts ranging from 7 to 10 mg %, in other words, hardly more than the amounts normally excreted.

*) Analysis kindly performed by Amanuensis, cand. pharm. Frantz Hansen, Department of Pharmacology, University of Copenhagen.

Recent investigations by SMITH (1947) on a large number of animals (dogs and rabbits) show that inhalation of methyl chloride over a fairly long period involves neither the formation of methanol in the blood nor an increase of formic acid concentration in the urine.

If, therefore, forensic analysis reveals an essentially higher formic acid concentration in the urine than that normally present (maximally 10 mg %), the most likely diagnosis is one of methanol poisoning.

Summary.

1) After intake of small quantities of methanol (10 to 20 ml) the human subjects showed no methanol in the blood in the course of 48 hours, and the concentration of formic acid in the urine was normal (6.5 to 12.8 mg within 24 hours).

2) Following intake of large amounts of methanol (ab. 50 ml) this substance could be demonstrated in human blood (25 to 120 mg %) after 48 hours. Furthermore, formic acid could be demonstrated in the blood (2.6 to 7.6 mg %), as well as an increased excretion of formic acid in the urine (54 to 205 mg %, up to 2050 mg within 24 hours).

3) In 5 fatal cases of methanol poisoning methanol (74 to 110 mg %) and formic acid (9 to 68 mg %) could be demonstrated in the blood and methanol (140 to 240 mg %) and formic acid (216 to 785 mg %) in the urine. In addition 106 mg % methanol and 60 to 99 mg % formic acid were found in the liver.

4) Maximum excretion of formic acid proved to occur not later than the 2nd to the 3rd day after intake of the alcohol.

REFERENCES

Autenrieth, W.: *München. med. Wchnschr.* 1919, **31**, 862.
Baker, H. M.: *J. A. M. A.* 1927, **88**, 1137.
Baumeke, H. *Die Bedeutung der Leichenfäulnis für den Ameisensäure-Nachweis bei der forensischen Feststellung von Methylalkoholvergiftung.* Diss., Halle 1939.
Fühner, H.: *Toxikologie*, Leipzig 1943, pag. 127.
Harrop, G. and E. Benedict: *J. A. M. A.* 1920, **74**, 25.
Klauer, H.: *Dtsch. Ztschr. f. d. ges. gerichtl. Med.* 1938, **30**, 279.
Kobro, M.: *Acta pharmacol.* 1946, **2**, 95.
Lund, Alf: *Acta pharmacol.* 1948 a, **4**, 99.
Idem: *ibid.* 1948 b, **4**, 108.
Mc Nally, W.: *Toxicology*, Chicago 1937, pag. 436.
Pohl, J.: *Arch. f. exper. Path. u. Pharmacol.* 1893, **31**, 286.
Ree, O.: *Acta med. Scandinav.* 1946, Suppl. 182.
Smith, W.: *J. Indust. Hyg. Toxicol.* 1947, **29**, 185.

Acta pharmacol. 1948, 4, 99—107.

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Metabolism of Methanol and Formic Acid in Rabbits.

By

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Methanol (methyl alcohol), used extensively for many years as a solvent in the chemical industry, has in the course of time given rise to numerous cases of poisoning, both chrome due to inhalation of methanol fumes for some length of time, and acute due in most cases to drinking of methanol-containing liquors. A characteristic feature of this poisoning is the very considerable excretion of formic acid, which is an oxidation product of methanol.

It is well known from the literature that different animal species behave differently in excretion of formic acid after administration of methanol. Dogs, for instance — like humans — excrete large amounts of formic acid, whereas rabbits do not seem able to excrete anything like the same amounts.

The object of the present investigation and another of which the results will be published later, has been to study the reactions of different animal species to methanol ingestion and to point out similarities and dissimilarities that may contribute to an understanding of the problems concerned in the fate of methanol in the animal organism.

In the present and a subsequent paper the metabolism of methanol and formic acid in rabbits and dogs will be further discussed, while a third paper to be published later will deal with some investigations into the conditions in humans.

Methods of Analysis.

1. Determination of Formic Acid in Urine.

The isolation of formic acid from urine is generally carried out by distillation in steam after the urine has been acidified with phosphoric acid. By saturating the urine with sodium chloride the formic acid can be distilled off quantitatively when the collected distillate amounts to 7 to 8 times the urine volume. The formic acid concentration in the distillate is estimated according to FINCKE (1913), but the precipitated mercurous chloride is determined iodometrically according to RIESSER (1916).

Where particularly accurate results are desirable the formic acid is first separated from the constituents of the urine (carbohydrates), which by boiling with acid for some length of time can give rise to formic acid, by extraction with ether (DAKIN 1913; GROSSFELD and PAYFER 1939). This is followed by distillation in steam of an aqueous extract of the calcium hydroxide-containing residue.

2. Determination of Formic Acid in Blood.

DROLLER's method (1932) is applied. Droller indicates the error to be ± 6 per cent, but in my experience it is more likely to be twice as high. Where possible blood is collected in a sufficient amount to be submitted to the same treatment as urine after deproteinisation. In cases where so much blood is available that it represents 4 or 5 mg of formic acid, this method of determination gives more accurate results than the colorimetric.

3. Determination of Methanol in Urine.

10 ml of urine is acidified with sulphuric acid and distilled. Sodium hydroxide is added to the distillate, which is afterwards redistilled. Thus the alcohol becomes more concentrated and is obtained quantitatively when 4 ml of distillate is collected. Determination of methanol is performed next according to DÉNIGÉS, by using the technique indicated by ROSEMAN et al. (1946). The colour intensity is compared with standard colours, produced with known amounts of methanol, in Hellige's colorimeter.

4. Determination of Methanol in Blood.

Widmark's method is applied in the modification indicated by BILDSTEN (1924). Testing of the method on aqueous methanol solutions gives no bases for a change of the reduction constant (1.33) found by Bildsten.

Previous Investigations.

ASSER (1914) found that administration of 15 ml of methanol to rabbits gave total formic acid excretion of 1) 186 mg, 2) 199 mg, 3) not weighable, and 4) 62 mg (the last value even after a dose of 25 ml of methanol). In judging of these figures we must take into account the fact that the *normal* excretion of formic acid within a period of 5 days may amount to nearly 50 mg, so that the *additional* amount of excreted formic acid constitutes a very small percentage only of the amount of methanol. DAKIN (1913) found the daily normal excretion of formic acid in rabbits to be from 3 to 10 mg. Similarly KAPELLER-ADLER and LAUDA (1932) indicated the normal output to be 6 to 11 mg. These results having been arrived at by two essentially different methods of analysis, there is reason to suppose that the daily formic acid excretion in rabbits does not exceed c. 10 mg. Thus Asser's investigations have shown that administration of methanol to rabbits does not lead to an increase in the concentration of formic acid in the urine — or at least only to a very small extent.

Present Investigations.

1. Principles of Procedure.

Full-grown male rabbits, weighing from 3000 to 3500 g, were used. At the beginning of the experiment the bladder was evacuated by catheterisation, and the alcohol*), diluted with 3 to 4 parts of water, was administered by oesophageal tube. The animal was placed in a cage with a device for collecting urine, and care was taken, by frequent catheterisation and collection, that the urine was exposed to the smallest possible extent to free evaporation. The acidity of the urine was measured by glass electrode and valve potentiometer.

Blood was taken from the auricular veins, generally 10 ml, sometimes more.

*) *Methanol pro analysi, Merck*, was applied, containing 99.5—100% CH_3OH (d_{20} = 0.793).

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

In a preliminary experiment with a dose of 1.58 g Methanol per kilogram body weight, the total excretion of formic acid amounted to no more than 35 mg within 70 hours after the administration of alcohol — in other words hardly more than normal. In the following 2 experiments the dose was then raised to 2.38 g/kg. Within the experimental period the diet consisted exclusively of turnips, which gave the urine a slightly alkaline reaction (pH 8).

Table 1.

Rabbit No. 2360. Weight 3200 g. Oct. 5, 11 o'clock, 9.6 ml of methanol plus 30 ml of water perorally (2.38 g methanol/kg). Diet during experimental period: turnips.

Hours aft. admin. of methanol	Diuresis ml	Formic acid excret. mg/100 ml	Formic acid excret. mg total	Methanol excret. mg/100 ml	Methanol excret. mg total	pH
0	22	5.1	1.1	-	—	-
6	94	7.1	6.6	298	280	-
22.5	195	10.5	20.4	175	342	8.10
30.7	90	4.5	4.0	85	77	-
46.5	96	1.4	1.3	10	10	-
58	170	0.9	1.6	—	—	—
70.5	78	0.5	0.4	—	—	8.00
95	450	0.6	2.9	—	—	—

Total excretion: *38.1 mg formic acid* and *0.709 g methanol*

It appears from the example Table 1 that even large methanol doses were unable to elicit any increase of the formic acid excretion worth mentioning. In one of the experiments there was, indeed, observed a rise in the excretion with a maximum some 20 hours after the administration of alcohol, decreasing again the following day; but the total output of formic acid (38 to 64 mg) hardly exceeded what may be found in normal animals within the same space of time.

The course of the excretion of non-converted methanol accords with BERNHARD and GOLDBERG's (1933) observations that the methanol concentration in blood decreases steadily and that the blood is alcohol-free after 50 hours when the initial concentration is 150 mg per cent. Fig. 1, which graphical lyillustrates the values found for the methanol concentration in the urine, accords with these observations. The total amounts of methanol excreted

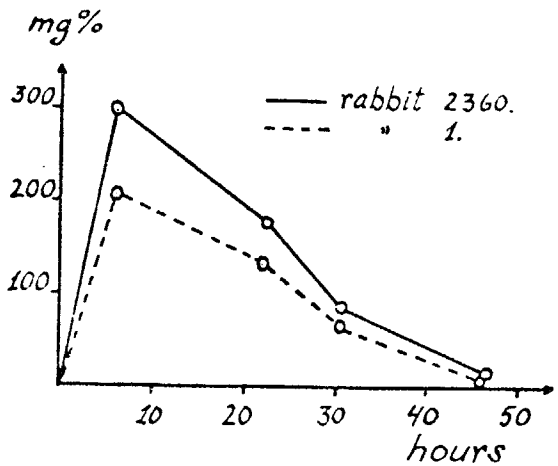

Fig. 1. *Excretion of methanol in the urine of rabbits after administration of 2.38 g methanol per kg body weight.* Abscissa: Number of hours after administration of methanol. Ordinate: mg methanol in 100 ml of urine.

in the two experiments amounted to 9.4 and 13.3 per cent respectively of the administered quantities.

The *p*H values found for the urine samples show that before the experiments the acid-base balance was situated on the alkaline side and had remained uninfluenced by the alcohol administered. The large alkali reserve of the rabbits could thus prevent the occurrence of the acidosis well known in methanol-poisoned humans. A shift in the acid-base balance of the blood of a rabbit might be conceived to involve also a change in the animal's reaction to methanol. The experiment indicated in Table 2 aims at an elucidation of this question. The same rabbit as was used in experiment No. 1 was now fed exclusively on oats and water,

on which diet the urine became faintly acid. The animal was then given 15 ml of methanol diluted with 75 ml of water, corresponding to 3.90 g/kg (the largest dose so far).

Table 2.

Rabbit No. 2360. Weight 3050 g. Nov. 4, 10 o'clock: 15 ml of methanol plus 75 ml of water perorally (3.90 g methanol/kg). Diet during experimental period: oats.

Hours aft. admin. of methanol	Diuresis ml	Formic acid excreted		Methanol in blood mg/100 g	pH
		mg/100 ml	mg total		
—	100	2.1	2.1	—	5.24
0	25	—	—	—	5.29
3	10	6.9	0.7	353	—
6.5	10	4.9	0.5	—	—
24	110	0.8	0.8	276	5.20
34	30	1.1	0.3	—	—
48	25	1.7	0.4	—	—

Total excretion: *2.7 mg formic acid.*

It appears from Table 2 that even a dose of 3.90 g methanol per kilogram body weight did not increase the formic acid concentration in the urine. On the contrary, the formic acid excretion was lower, the rabbit's acid-base balance having been shifted markedly in the acid direction.

In the last experiment, unlike the others on rabbits, there were observed signs of poisoning, indeed of a rather severe degree, beginning with incoordinated movements and gradually increasing to narcosis. Not till 20 hours after the administration of alcohol did the animal move about naturally again and begin to eat.

3. Excretion of Formic Acid after Administration of Formic Acid.

The experiments described above showed that administration of methanol to rabbits elicits no increase in the formic acid excretion — or only a very slight one. This may be due to the fact either that the rabbit organism is unable to oxidise methanol or that a

certain amount of the administered alcohol is catabolized, but that the catabolism is so complete (to CO_2) that the formic acid concentration in the urine is not increased.

In order to throw light on this problem experiments were made on the same two rabbits as had been used for the experiments on administration of methanol. One was kept on a turnip diet, while the other was given oats and water to reduce its alkali reserve. 10 ml of a 10 per cent sodium formate solution, corresponding to 676 mg formic acid, were injected subcutaneously into both animals. The formic acid concentrations in the blood and urine samples were estimated according to Droller's method and the extraction method respectively.

Table 3.

Rabbit No. 2360. Weight 3150 g. Oct. 16, 10 o'clock: subcutaneous injection of 10 ml 10% solution of sodium formate (~ 676 mg formic acid). Diet during the experimental period: turnips.

Hours aft. Injection	Diuresis ml	Formic acid excret.		Formic acid in blood mg/100 ml	pH
		mg/100 ml	mg total		
3	—	—	—	20	—
4	100	36.8	36.8	16	8.24
10	115	5.5	6.3	10	8.54
23	250	3.0	7.5	—	8.43
32	190	1.4	2.6	—	8.15
48	65	1.0	0.7	—	8.41

Total excretion: *53.9 mg formic acid.*

Tables 3 and 4 show plainly that rabbits are able to oxidise formic acid, so to speak quantitatively. They excrete none of the injected amounts, or only a few per cent, although the blood shows considerable formic acid concentration, amounting to 50 mg per cent one hour after the injection, to fall in the course of 10 hours to non-demonstrable values. The results obtained from the two experiments were nearly the same. This shows that a change in the acid-base balance has no noticeable influence on the animals' power of oxidising formic acid.

Table 4.

Rabbit No. 1. Weight 3300 g. Oct. 19, 11 o'clock: subcutaneous injection of 10 ml 10% solution of sodium formate (~ 676 mg formic acid). Diet during the experimental period: oats.

Hours aft. injection	Diuresis ml	Formic acid excreted		Formic acid in blood mg/100 ml	pH
		mg/100 ml	mg total		
—	60	3.2	2.2	—	7.58
—	15	—	—	—	6.21
0	40	—	—	—	5.67
1	—	—	—	48	—
3	10	81.3	8.1	21	5.48
9	10	17.9	1.8	< 10	5.07
26	50	3.8	1.9	—	5.46
46	55	2.1	1.1	—	5.02

Total excretion: *12.9 mg formic acid.*

Discussion.

Methanol-treated rabbits present no increase worth mentioning in the formic acid concentration of the urine, no matter whether the alkali reserve is large or small. This is due to the fact that rabbits are able to oxidise formic acid injected as sodium formate practically completely. This process seems independent of their acid-base balance. It is worth remarking, however, that the amount of *excreted* formic acid is larger in the animals with alkaline urine than in those with acid urine. The same is true of the excretion of salicylic acid in rabbits, as shown by SMITH et al. (1946) and by DALGAARD-MIKKELSEN in experiments carried out in this Department but not yet published.

It is thus to be supposed that a certain amount of the methanol administered to rabbits, as well as to dogs, is converted into formic acid, but that in rabbits the formic acid is oxidised so completely that there occurs no increase, or only a slight one, in formic acid excretion.

Summary.

1) After administration of methanol to rabbits c. 10 per cent is excreted unchanged in the urine.

2) There is found no increase in the formic acid excretion in the urine, or only a slight one.

3) It is shown that rabbits are able to oxidise formic acid almost completely, and that the power of oxidation is not influenced to any great extend by shifts in the acid-base balance.

4) Despite the small output of formic acid, the amount excreted is larger in alkalotic animals than in acidotic animals.

REFERENCES

Asser, E.: *Ztschr. f. exp. Path. u. Therap.* 1914, **15**, 322.
Bernhard, C. and L. Goldberg: *Skandinav. Arch. Physiol.* 1933, **67**, 117.
Bildsten, N.: *Biochem. Ztschr.* 1924, **146**, 361.
Dakin, H. D.: *J. Biol. Chem.* 1913, **14**, 344.
Droller, H.: *Ztschr. f. physiol. Chem.* 1932, **157**, 211.
Fincke, H.: *Biochem. Ztschr.* 1913, **51**, 253.
Grossfeld, J. and R. Payfer: *Ztschr. f. Unters. Lebensmitt.* 1939, **78**, 1.
Kupeller-Adler, R. and E. Lauda: *Biochem. Ztschr.* 1932, **258**, 429.
Riesser, O.: *Ztschr. f. physiol. Chem.* 1916, **96**, 360.
Roseman, R. et al.: *Chem. Abstr.* 1946, **40**, 1550 .
Smith, P., H. Gleason, C. G. Stoll and S. Ogorzalek: *J. Pharmacol.* 1946, **86**, 236.

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The Volatile Acids of Mutton Fat

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Recent investigations on ox perinephric fat revealed the presence of a consecutive series of 'odd and even' *normal* volatile fatty acids (VFA) ranging from C_2 to C_{10} (Hansen & McInnes, 1954). This present paper reports the identification of the same series of VFA together with formic, *iso*butyric, *iso*valeric and α-methylbutyric acids in the external carcass fat of sheep. Formic acid has not hitherto been reported as a constituent of natural fats, although it has been found in the venous and arterial blood of animals (Annison, 1954*b*). *Iso* acids with molecular weight lower than that of the C_{15} *iso* acid 13-methyltetradecanoic acid (Hansen, Shorland & Cooke, 1953) have not formerly been isolated from animal depot fats, except *iso*valeric acid, which was identified in the body and head oils of dolphins and porpoises (Lovern, 1934). Until the present no *anteiso* acid with a molecular weight lower than that of 10-methyldodecanoic acid (Hansen, Shorland & Cooke, 1954*a*) had been shown to occur in animal depot fats. In cow milk, however, all the even-numbered *normal* volatile acids (C_4–C_{12}) are present (Hilditch, 1947), as are a number of odd-numbered *normal*, *iso*, and *anteiso* acids of higher molecular weight (Shorland, Gerson & Hansen, 1955*a*, *b*; Hansen, Shorland & Cooke, 1954*b*; Hansen & Shorland, 1951.)

EXPERIMENTAL

In preliminary investigations on the VFA of mutton fat, the glycerides (24·0 kg.) from a sample of fat (G 99) were cold-saponified, acidified and steam-distilled, and the VFA were extracted from the distillate with ether. The ether extract was fractionated in a 60 cm. fractionating column packed with glass helices, and the fractions obtained were titrated with approximately 0·5 N-KOH with phenolphthalein as indicator, and the soap solutions taken to dryness. In order to regenerate the fatty acids the soaps were moistened with a little water, treated with an excess of $KHSO_4$ and 2 drops of 10 N-H_2SO_4 and extracted with chloroform containing 5% (v/v) *n*-butanol. An attempt was then made to use buffered partition chromatography as described by Moyle, Baldwin & Scarisbrick (1948), to separate and identify the saturated acids from C_2 to C_8. Quantitative results have not been provided for the acids isolated by this method, as they could not be completely eluted from the columns. In order to confirm the identification of these acids gas-liquid chromatography was employed, the soaps (denoted C) obtained by evaporation of the chloroform-butanol solutions from the buffered silica-gel columns being dissolved in water and made up to approximately 2 N solutions.

To obtain more exact information this work was continued with two further samples from the external carcass fat of sheep (K 7 comprising wethers, ewes and rams; K 40 wethers). In K 7 the fatty tissues were minced and heated with water on a water bath. After repeated washing with water, and drying to constant weight under vacuum at 100°, the extracted glycerides (wt. 9·784 kg., saponification equiv. 285·2, iodine value 86·4, acid value 0·56) were converted into soaps, acidified and steam-distilled, the distillate passing into receivers containing aqueous KOH. The distillate (20 l.) was taken to dryness on a water bath, and the soaps were redissolved in water (200 ml.). In order to remove the unsaponifiable material the solution was extracted with ether, after which the soaps were reacidified and steam-distilled. The distillate (7 l.) was collected as seven equal fractions, the first of which was titrated with 0·742 N-NaOH, evaporated to dryness on a water bath and then dissolved in water and made up to an approximately 4 N solution (denoted A). Owing to the presence of higher fatty acids the soaps obtained from the other six fractions could not be dissolved in water to give a 2 N solution. The soaps were redissolved in water (2 l.) containing $MgSO_4$ (50 g.), steam-distilled in the manner suggested by Friedemann (1938) to yield a distillate (1·5 l.) which was titrated as before, and the solution evaporated to dryness. The soaps were dissolved in water and made up to an approximately 2 N solution (denoted B). K 7 (A) and K 7 (B) required 20 and 32·2 ml. of 0·742 N-NaOH respectively. Samples (30 μl.) of each of the solutions (A), (B) and (C) were treated as prescribed by James & Martin (1952) for the micro-estimation of volatile acids isolated as their sodium or potassium soaps.

The sample K 40 (wt. 1·06 kg., saponification equiv. 289·1, iodine value 26·0, acid value 0·8) was treated in much the same manner, except that the soaps obtained by titrating the steam distillate (23 ml. of 0·466 N-NaOH) were dissolved in water and made up to 20 ml. in a standard flask. A portion (2 ml.) of this solution was then treated in a manner devised by one of us (McInnes, 1956). The free VFA obtained on acidifying the soaps with $KHSO_4$ were dissolved in ether and the anhydrous extract was made up to 10 ml. A portion (0·5 ml.) of the ethereal solution was then introduced into a modified gas-liquid chromatographic column. The ether used throughout the work was freshly distilled from a mixture of $FeSO_4$ and KOH.

Analysis of volatile fatty acids. The gas-liquid partition chromatography method of James & Martin (1952) was employed at a temperature of 137°, with Cellosolve (2-ethoxyethanol) in the heating jacket. The column length was 4 ft. and the liquid phase was silicone oil M.S. 550 (Hopkin and Williams Ltd.) containing 10% (w/w) of stearic

acid. The gaseous phase was N_2. The C_1–C_5 acids were checked by operating the column at a temperature of 100°; the column was loaded with three times the quantity of acids mentioned above in order that those present in small proportions could be estimated more accurately.

Synthetic mixtures of VFA (Eastman Kodak Co.) were used to check the retention volumes of the acids which were present in the external carcass fat of sheep. Unless stated otherwise, all percentages of VFA are expressed on a molecular basis.

RESULTS

By means of gas–liquid chromatography all the straight-chain VFA (both odd and even) from C_1 to C_{10}, together with *iso*butyric, *iso*valeric, α-methylbutyric and four unidentified acids, have been shown to be present in the external carcass fat of sheep. It can be seen from Table 1 that each acid is expressed as a percentage of the total concentration of VFA from C_1 to C_{10}. In K7, it is likely that the value for formic acid is too low since it has been the experience in this *Laboratory* that the method suggested by James & Martin (1952) for the application of fatty acids on to a 4 ft. chromatographic column results in a loss of formic acid from the column when a temperature of 150° is used, and is not safe for quantitative work even at a temperature of 100°. This has been overcome by the new procedure mentioned above (McInnes, 1956). Combined results for K7 (A) and K7 (B) are also given in Table 1.

In the work now reported VFA were first detected in the steam distillate of mutton fat by buffered-partition chromatography (Moyle *et al.* 1948). The individual acids isolated by this method were checked by the gas–liquid chromatographic procedure of James & Martin (1952), and it was found that the acids had not been eluted in the order described by Moyle *et al.* (1948). This was attributed to the gradual deterioration of the non-adsorptive properties of the silica gel, as it had been kept in an air-tight bottle for approximately 9 months. It appeared, however, that the method of Moyle *et al.* (1948) could be used satisfactorily if a check were kept on the properties of the silica gel by chromatographing standard solutions of acids alongside the unknown samples. A further advantage of the gas–liquid chromatogram of James & Martin (1952) was that the method provided for the identification of all the *normal* saturated VFA from C_1 to C_{10}, whereas the buffered columns were limited to a range of C_2–C_8. In addition, the above method enabled all the isomers up to *n*-valeric to be estimated.

The total weight of VFA as determined from the graphs obtained by the gas–liquid chromatographic apparatus are approximately 1·25 g. (K7) and 0·60 g. (K40). These are approximately 0·01 and 0·06 % by weight respectively of the total fatty acids.

Four unidentified acids with retention volumes relative to *n*-butyric acid of 2·5, 5·74, 9·68 and 17·00 at 137° were found. These values suggest that they may be isomers, or mixtures of isomers, of *n*-hexanoic, *n*-heptanoic, *n*-octanoic and *n*-nonanoic acids respectively. As the fatty acids were not hydrogenated before they were steam-distilled, the possibility that these four acids could be unsaturated cannot be overlooked.

The steam-distillation procedure which is adopted for preparing a volatile-acid fraction free from higher fatty acids is unsatisfactory; it is not possible to ensure that any given volatile acid has been completely separated from the higher fatty acids, and no procedure is known to the authors that will give exclusively any given fraction of volatile acids. Comparing the volatile acids in K7 (A) and K7 (B) in Table 1, it will be seen that fraction B contains all the formic acid together with a fair proportion of the acetic acid. This suggests that when fatty acids are steam-distilled, most of the acids from C_3 to C_9 pass over in a comparatively small volume of

Table 1. *Molecular percentages of C_1–C_{10} fatty acids from the external carcass fat of sheep*

	K7 (A)	K7 (B)	K7 (A) + K7 (B)	K40	G99 (C)
Formic	—	40·3	27·4	37·0	Present
Acetic	16·2	56·2	43·3	39·0	Present
Propionic	0·8	—	0·3	1·4	Present
*iso*Butyric	1·0	—	0·3	0·4	Present
n-Butyric	5·3	3·1	3·8	1·8	Present
*iso*Valeric / α-Methylbutyric	1·2	—	0·4	0·4	Present
n-Valeric	1·6	—	0·5	1·0	Present
Unknown	Trace	—	Trace	Trace	—
n-Hexanoic	29·0	—	9·3	9·9	Present
Unknown	2·8	—	0·9	0·4	Present
n-Heptanoic	4·4	—	1·4	1·7	Present
Unknown	2·4	—	0·8	0·7	—
n-Octanoic	11·6	—	3·7	2·1	Present
Unknown	4·8	—	1·5	0·4	—
n-Nonanoic	4·4	—	1·4	1·0	—
n-Decanoic	14·5	0·4	5·0	2·8	—

distillate, formic and acetic acids being steam-distilled with more difficulty.

Since the glycerides of K 7 and K 40 were dried under vacuum at 100° the lower VFA would have been removed had they been present as free fatty acids. Furthermore, although glycerides containing formic acid can be hydrolysed by boiling with water, it would appear that under the conditions used in this investigation hydrolysis does not occur to any appreciable extent, and the VFA identified in K 7, K 40 and G 99 are present as glycerides.

DISCUSSION

The occurrence of *n*-, *iso*- and *anteiso*-acids in the VFA from the external carcass fat of sheep, as shown by this work, is not unexpected, since it is known that acetic, propionic, *iso*butyric, *n*-butyric, *iso*valeric, α-methylbutyric and trace amounts of hexanoic acids are present in the rumen of sheep (Annison, 1954*a*). It is of interest, however, to find that there exist differences in the relative amounts of VFA in the carcass fat and the rumen. For example, *n*-hexanoic acid occurs to the extent of approximately 10% of the total VFA from the fat, although it is present only in trace amounts in the rumen. It can be seen from Table 1 that the even-carbon VFA (approximately 55% of total VFA) occur in the following descending order of molecular concentration: *n*-acetic, *n*-hexanoic, *n*-decanoic, *n*-butyric, *n*-octanoic, *iso*butyric. The odd-carbon acids, on the other hand, excluding formic acid, occur to the extent of less than 5% of the total VFA in mutton fat, the higher members *n*-heptanoic and *n*-nonanoic being present in the greater proportions. The work of Gray, Pilgrim, Rodda & Weller (1951) has shown that *n*-valeric acid can be synthesized from propionate in the rumen of sheep, and it is in accordance with modern views that the higher acids in this series are synthesized by the successive addition of acetate units to propionate.

The concentrations of the odd-carbon (0·35% approximately of VFA) and even-carbon (0·35% approximately of VFA) branched-chain acids *iso*-valeric, α-methylbutyric and *iso*butyric acids together account for only a very small proportion of the total VFA. Their presence in the depot fat, however, supports the suggestions of various workers (Velick & English, 1945; Weitzel & Lennert, 1951; El-Shazly, 1952; Shorland, 1953) that these acids are the precursors of the higher branched-chain acids found by this laboratory in animal depot fats in amounts greater than 1% by weight of the total fatty acids.

No explanation can be offered at present for the large proportion (approximately 37%) of formic acid in the VFA of mutton fat, although it is known to be present in the rumen of sheep (Gray, Pilgrim, Rodda & Weller, 1951, 1952) and the venous and arterial blood of animals (Annison, 1954*b*).

SUMMARY

1. By means of gas–liquid chromatography all the *normal* fatty acids, both odd and even, from C_2 to C_{10} have been shown to occur in the external carcass fat of sheep.

2. Two *iso* acids, *iso*butyric and *iso*valeric, and an *anteiso* acid, α-methylbutyric, as well as formic acid, have for the first time been shown to be components of an animal depot fat.

The authors are much indebted to Dr F. B. Shorland, Director of this Laboratory, for advice and helpful discussion.

REFERENCES

Annison, E. F. (1954*a*). *Biochem. J.* **57**, 400.
Annison, E. F. (1954*b*). *Biochem. J.* **58**, 670.
El-Shazly, K. (1952). *Biochem. J.* **51**, 647.
Friedemann, T. E. (1938). *J. biol. Chem.* **123**, 166.
Gray, F., V. Pilgrim, A. F., Rodda, H. J. & Weller, R. A. (1951). *Nature, Lond.*, **167**, 954.
Gray, F. V., Pilgrim, A. F., Rodda, H. J. & Weller, R. A. (1952). *J. exp. Biol.* **29**, 57.
Hansen, R. P. & McInnes, A. G. (1954). *Nature, Lond.*, **173**, 1093.
Hansen, R. P. & Shorland, F. B. (1951). *Biochem. J.* **50**, 207.
Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1953). *Biochem. J.* **53**, 374.
Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1954*a*). *Chem. & Ind.* p. 1229.
Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1954*b*). *Biochem. J.* **57**, 297.
Hilditch, T. P. (1947). *The Chemical Constitution of Natural Fats*, 2nd ed. revised. London: Chapman and Hall Ltd.
James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
Lovern, J. A. (1934). *Biochem. J.* **28**, 394.
McInnes, A. G. (1956). *Vapour Phase Chromatography, Symp. Inst. Petrol.* (in the Press).
Moyle, V., Baldwin, E. & Searisbrick, R. (1948). *Biochem. J.* **43**, 308.
Shorland, F. B. (1953). *J. Sci. Fd Agric.* **4**, 497.
Shorland, F. B., Gerson, T. & Hansen, R. P. (1955*a*). *Biochem. J.* **59**, 350.
Shorland, F. B., Gerson, T. & Hansen, R. P. (1955*b*). *Biochem. J.* **61**, 702.
Velick, S. F. & English, J. (1945). *J. biol. Chem.* **160**, 473.
Weitzel, G. & Lennert, K. (1951). *Hoppe-Seyl. Z.* **288**, 251.

The Effect of Nyloxin on the Pain of Arthritis

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For many years enthusiastic reports have appeared periodically on the use of snake venom for the alleviation of pain. Most of the earlier studies were in the French literature, but from time to time encouraging reports appeared in the United States.

In 1940 Steinbrocker *et al.*[1] reported that cobra venom appeared to alleviate the pain of arthritis when compared to the effects in the same patient of saline injections. They concluded that in some patients there seemed to be a therapeutic effect of the cobra venom. However, they reserved a final opinion because their series was admittedly too small. In 1943 Abbee and Maier[2] stated unequivocally that the use of venom in the treatment of arthritis was followed by a marked salutary effect and that only [illegible] patients in a total of 56 were not relieved.

One of the difficulties in using venom has been in titrating the potency. When cobra venom became available in a stable solution of formic and silicic acid,* further reports appeared which attested to its effectiveness. Hills and Firor[3] believed that this drug alleviated the pain of patients with malignant disease, and Lumpkin and Firor[4] believe that 80 per cent of their patients with "chronic arthritis" were relieved by an injection of Nyloxin.

Bryson,[5] who developed the formula of cobra venom with formic and silicic acid, reported that the overwhelming majority of his patients not only had marked relief of pain but also obtained a sense of well-being with better appetite and less physical and mental depression. Because of these encouraging reports in the treatment of osteoarthritis, a disease which can at times test the therapeutic ingenuity of the practitioner, we decided to study this preparation by the double blindfold technic.

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* Nyloxin is the trade name of the Hynson, Westcott and Dunning Pharmaceutical Co., Baltimore, Maryland, solution of cobra venom, formic and silicic acid. It is through the kindness of Dr. John Brewer, director of Biological Research, that adequate supplies of Nyloxin and the control substances were made available.

Methods

Patients who attended the Maimonides Hospital Arthritis Clinic and who fulfilled the criteria of the American Rheumatism Association for osteoarthritis were chosen for this study. Three colorless solutions labeled A, B, and C were employed. Solution A was formic acid, solution B the regular Nyloxin solution, and solution C formic and silicic acid. Silicic acid by itself could not be employed because it was not possible to put it into solution. The technic of Lumpkin and Firor[4] was followed. These injections began with 0.5 ml. bilaterally and were increased by 0.25 ml. at each weekly injection until a dose of 1.5 ml. was given in each deltoid area. In addition, 1 ml. of 1 per cent procaine was added to the injected material to alleviate the expected pain at the site of the injection.

The same nurses gave the injection every week, and the same physicians evaluated the results. Neither the nurses nor the physicians were aware of the contents of the various solutions. Pain was the only symptom which was evaluated. The patients were rated as "no change," "some relief," and "considerable relief." No patient who received less than eight weekly injections was considered in the final evaluation before the code was opened. In many instances the patient was rotated through two and, in some instances, three groups.

Results

Fifteen patients received solution A, formic acid; 14 patients received solution B, Nyloxin, and 16 patients received solution C, formic and silicic acid.

There were 29 patients who were evaluated. In 12 instances they were rotated through two groups, and in two instances they were rotated through the three groups. Table I shows the results in the three groups. It will be noted that in these groups the effect on relief of pain was similar. About two out of every three patients felt that the injections were helping them regard-

TABLE I.—TOTAL NUMBER OF PATIENTS RECEIVING EACH PREPARATION AND THE EFFECTS OF MEDICATION BY GROUPS

Effect	Formic Acid	Nyloxin	Formic and Silicic Acid
Some relief	7	6	7
Considerable relief	3	3	3
No relief	5	5	6
TOTAL	15	14	16

less of the nature of the material.

The cobra venom solution could not be recognized by the degree of the response of the patient in those patients who were rotated through either two or three groups. Some felt better with A or B or C; some felt better on all groups tested. Table I shows that there was no consistent pattern of response. Thus, from this study we cannot confirm the results of others who have claimed that Nyloxin has a place in the treatment of arthritis.

However, this study does affirm the extreme caution that must be exercised in assessing a supposed analgesic agent in patients with joint diseases. Furthermore, it emphasizes that the continued interest of the physician, the participation of the patient in a research project, and his desire to please the physician by responding to the drug may be some of the factors that are responsible for his positive reaction. The recognition of the positive reactor state by the physician should help him realize that many of his therapeutic triumphs are not the results of the medication but of his sympathetic management of the total patient. Such a realization will help him distinguish the pattern of response and help to ease his frustration when the next patient does not respond to the same medication.

Summary

1. Twenty-nine patients with osteoarthritis were treated by the double blind study method with Nyloxin, a cobra venom preparation, for periods of two to six months.

2. Two out of every three patients in the control or test groups reacted favorably with relief of pain.

3. Nyloxin appears to have no better effect than an inert substance.

4. Psychogenic factors contributing greatly to the complaint of pain would appear to have operated in about 65 per cent of patients in this group with osteoarthritis.

References

1. Steinbrocker, O., McEachern, G., LaMotta, E. P., and Brooks, F.: J.A.M.A. 114: 318 (1940).
2. Albee, F. H., and Maier, E.: M. Rec. 156: 217 (1943).
3. Hills, R. G., and Firor, W. M.: Am. Surgeon 18: 875 (1952).
4. Lumpkin, W. R., and Firor, W. M.: *ibid.* 20: 752 (1954).
5. Bryson, K. D.: *ibid.* 20: 751 (1954).

FORMIC ACID

BA 22, 16563 (1948)

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16563. Miller, P. G., P. L. Zimmerman, and E. B. Oberg. (*Carnation Co. Res. Lab., Milwaukee, Wis.*) **Comparison of vacuum and steam distillation for determining the volatile acidity of evaporated milk.** *Jour. Dairy Sci.* 31(3): 189–198. 4 fig. 1948.—Vacuum distillation at constant volume and pressure was compared with steam distillation as a means of measuring formic and total volatile acids (expressed as formic) in heated milk. By vacuum distillation about 40 mg./l. of formic acid and 70 mg./l. of total volatile acids were found in reconstituted evaporated milk, while by steam distillation 32 and 134 mg./l. of formic and total volatile acids, respectively, were found. Both distillation procedures gave satisfactory recovery of formic acid added to evaporated milk. The difference between the procedures in volatile acids not formic obtained may have been due to the evolution of CO_2, splitting of glycerides of fatty acids, and/or the rate of distillation by the steam distillation procedure. Addition of formic acid to milk reduced the amt. of volatile acids not formic obtained by steam distillation.—*P. G. Miller.*

DETECTION

SOME ORGANIC ACIDS IN RAW AND HEATED SKIMMILK[1]

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Chromatographic analysis of fresh, raw, mixed-herd skimmilk heated at 100° C. for varying periods up to eight hr. revealed butyric, propionic, acetic, pyruvic, formic, lactic, and four unknown acid fractions—I, II, III, IV—of which I, II, and III eluted between propionic and acetic, and IV between formic and lactic. Of these ten acids, six were produced in large quantities, and their order of decreasing production was formic, acetic, I, II, III, and lactic. All ten acids were found in fresh, raw milk. Different skimmilks yielded variations in individual and in total acids. *Editor.*

It is known that high-temperature heat treatment of milk produces acids, but the specific ones involved, and the relative concentration of those formed under different conditions, have not been completely investigated. Furthermore, the application of sensitive and accurate chromatographic techniques for identifying and measuring individual organic acids is a means of elucidating some of these changes.

A review of earlier work on the acids in heated milk was given by Gould *(5)*. This and subsequent papers dealt with lactic and formic acids *(5, 6, 7)*. Results revealed lactic acid to constitute not more than five per cent of the total acidity (3 to 7 mg/100 gm.) and formic acid about 50% of the total acid produced by heating milk at 116° C. for 2.5 hours *(5)*.

Recent work of a preliminary nature, in which a silica gel chromatographic procedure was utilized, revealed the presence of a variety of acids, including pyruvic, acetic, butyric, and propionic in heated milk *(9, 10)*. Subsequent investigation demonstrated that other acids, in addition to those reported, were present as unrecognized components. Therefore, additional studies were conducted, using an improved column chromatographic technique, and the results are presented in this paper.

EXPERIMENTAL PROCEDURES

Source and treatment of samples. Fresh, raw skimmilk, obtained from mixed-herd milk during early-summer months, was placed in metal cans (approximately 500-ml. capacity), sealed, and heated at 100° C. in a constant temperature bath for varying periods of time up to eight hours. The cans were agitated constantly until they reached 100° C., then agitated every 30 minutes. In certain cases, 0.5% disodium phosphate was added just prior to heating. After heating, the samples were cooled rapidly in an ice bath and stored at from 2 to 5° C. until lyophilized.

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Lyophilization of the skimmilk samples was conducted in order to: (*a*) Preserve the samples and thereby prevent alteration of the relative acid concentrations, and (*b*) concentrate the acids of the sample to a point where they could be more satisfactorily measured by the chromatographic method. Care was taken to accomplish this lyophilization in a manner that would minimize any possible alteration of the acids present. Rapid freezing of the neutralized sample by rotation in a dry ice–ethanol bath (approximately −72° C.) preceded the drying process, which required an average of six hours to reduce the moisture content to five per cent.

Chromatographic analysis. The silica gel column chromatographic procedure used was adapted from that proposed by Bulen *et al.* (*1*). Two steps were found necessary: (*a*) a preliminary extraction, to remove the acids from the lyophilized skimmilk and render them suitable for chromatographic analysis, and (*b*) addition of the extracted acids to the standard survey column, and measurement of their respective concentrations.

Preparation of reagents: Reagents were prepared according to the method of Bulen *et al.* (*1*). The eluting solvents were prepared, to contain zero, one, five, ten, 15, and 50 per cent (v/v) *n*-butanol in chloroform.

Step I—Preliminary extraction. The acids were extracted from the lyophilized samples as follows: A 4- to 4.5-gm. sample, accurately weighed, was mixed in a mortar with 15 ml. distilled water and sufficient 2.5 *N* sulfuric acid added to lower the pH to 2. Thirty gm. of previously washed and dried silicic acid were added and the mixture was ground thoroughly. One hundred ml. of 50% butanol-chloroform solvent were then used to slurry the mixture, and to transfer it to a 35 mm. × 25 cm. chromatographic tube. An additional 400 ml. of the solvent was drawn through the column into a suction filter flask at the approximate rate of 100 ml. per five minutes. Fifty ml. of distilled water were added to the contents of the filter flask and the acid was neutralized with N/10 sodium hydroxide to the phenol red end-point. Excessive overneutralization was avoided, to prevent losses of pyruvic acid.

The neutralized acids and solvent were transferred to a separatory funnel, where they were shaken vigorously and permitted to stand until two distinct layers were formed. The water layer containing the sodium salts of the acids was drawn off and the butanol-chloroform layer was subjected to a second washing. Both water layers were combined, and evaporated to dryness under vacuum, at temperatures not exceeding 35° C.

Step II—Chromatographic analysis. The chromatographic column was prepared as described by Bulen *et al.* (*1*), with certain modifications. The column was prepared in two sections: the bottom section for separation and the top section for the sample. An 18 mm. diameter chromatographic tube was employed, to allow use of a larger amount of sample than was possible with Bulen's method.

The modified method for preparing the bottom section follows: A filter-paper disc was placed at the bottom of the chromatographic tube to support the column. Silicic acid (25 gm.) was mixed with 18 ml. 0.5 *N* sulfuric acid in a mortar and ground to a uniform mixture. The resulting free-flowing powder

was slurried with 60 to 70 ml. chloroform and added to the chromatographic tube. A slight pressure (nitrogen) was applied to the top of the column, to speed the removal of excess solvent, with care being used not to allow the solvent to fall below the level of the column. This procedure gave a uniformly packed column about 20 cm. in length.

The top section of the survey column was prepared by dissolving the acids obtained from the milk in 0.5 *N* sulfuric acid, acidifying to pH 2 with 2.5 *N* sulfuric acid, and grinding with 3 gm. of silicic acid. This dry mixture was transferred to the top of the column and slurried with a minimum amount of chloroform. A second filter paper and a glass wool plug were placed on top of the column, to prevent disturbance during addition of eluting solvents.

The remainder of 100 ml. chloroform (30 to 40 ml.) was added, using a special reservoir made from a 250-ml. separatory funnel. Completion of the chromatogram was then accomplished by adding in order, 85, 170, 65, and 200 ml. of one, five, ten, and 15 per cent butanol-chloroform mixtures, respectively. The eluted solvent, containing the individual acids, was collected in three-ml. fractions, using an automatic fraction collecter. Individual fractions were titrated to the phenol red end-point with 0.01 *N* sodium hydroxide in absolute alcohol, using a two-ml. capacity microburette graduated in 0.01-ml. volumes.

Brown color measurement. The method of Choi *et al.* (2), slightly modified, was used to measure the degree of color development in the heated samples as follows: Duplicate 25-ml. portions of each sample, taken just after heating, were added to 50-ml. test tubes, along with two ml. of a 10% suspension of trypsin, and the mixture was incubated at 45° C. for one hour. Two ml. of 50% trichloroacetic acid were then added and the mixture was allowed to stand for an additional 30 minutes. The contents of each tube were filtered and the filtrate examined for per cent light transmission with a Model B Beckman spectrophotometer, using a wave length of 500 millimicrons.

RESULTS

Preliminary application was made of the adopted chromatographic method, to determine the threshold volumes and per cent recoveries of the individual acids expected to be present in heated skimmilk. Aqueous 0.1 *M* solutions of butyric, propionic, acetic, pyruvic, formic, and lactic acids, and various combinations of these acids (two and three in different combinations), were added to lyophilized skimmilk (100 to 150 microequivalents of each) and the recovery determined. Results are shown (Table 1).

Recoveries for the different acids varied widely. Satisfactory recovery was obtained for butyric, propionic, and lactic (93 to 99%). Acetic acid was recovered less satisfactorily (86%); whereas, values for formic and pyruvic acids were poor: 59.2 and 51.8%, respectively. The recoveries were consistent within the experimental error of the method, and were not affected by the use of combinations of acids. In analyses of milk samples, appropriate corrections were made for expressing the concentration of acetic, formic, and pyruvic acids, so that all values could be reported on the basis of 100% recovery.

TABLE 1

Recovery values for acids added to lyophilized skimmilk[a]

Acid added	Threshold volume	Recovery Range	Recovery Average
	(*ml.*)	(%)	
Butyric	55	97.6- 97.7	97.6
Propionic	90	97.0-100.8	98.9
Acetic	235	80.5- 91.5	86.0
Pyruvic	285	45.2- 55.8	51.8
Formic	369	52.8- 69.6	59.2
Lactic	465	89.8- 96.8	93.3

[a] Mixtures of acids were added to milk prior to lyophilizing. Analysis of extracts from preliminary extraction revealed no loss of these acids during the lyophilization process.

An observation made in respect to the comparatively low recovery of pyruvic and formic was that the losses occurred during the concentration after the initial extraction. If they were added as pure acids to the second column, the recoveries were 80 to 90%. Excess alkali added during the first step of separating and concentration resulted in complete loss of these two acids.

Four replicate analyses for various acids in the milk sample used for determining recovery revealed results that agreed within ± five per cent of the average of all acids.

The threshold volumes for elution of each acid were constant when added as mixtures to water, or added alone or in mixtures to milk. A representative elution curve for a mixture of known acids, showing solvent changes and threshold volumes, is shown (Figure 1). The first elution peak was the higher fatty acids. Because of adsorption on the assay column of acids higher than C_8, this peak was comprised primarily of C_5 and C_6 acids. No further analysis of this group of acids was attempted, to obtain positive identification.

Based on these analyses, subsequent identification of acids in raw and heated milk was made from the threshold volume.

Acids in unheated skimmilk. Chromatographic analysis was made of raw skimmilk with and without added disodium phosphate and the results are presented (Table 2), with the acids listed in order of their elution.

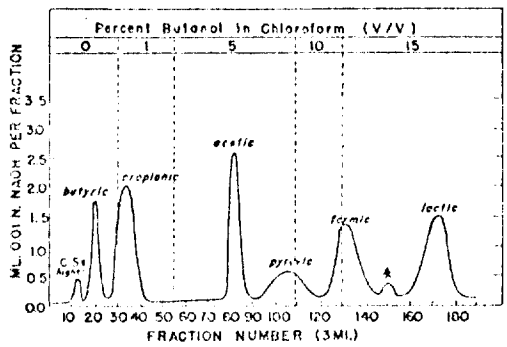

FIG. 1. Elution of known fatty acids from column. (* Indicates acid that always appeared when lactic acid was added.)

TABLE 2

Acids in unheated skimmilk with and without 0.5% added disodium phosphate (reported as microequivalents/100 ml.)*

Acids	Normal skimmilk		Skimmilk with 0.5% disodium phosphate	
	Range	Average	Range	Average
Butyric	1.8- 10.8	5.2	3.2- 8.4	5.8
Propionic	0.0- 4.6	2.2	0.0- 7.8	3.9
Unknown Group I	8.8- 42.6	29.6	13.2- 45.2	29.2
Acetic	19.2- 87.4	63.0	34.8- 89.4	57.6
Pyruvic	0.0- 28.6	12.4	0.4- 13.6	7.0
Formic	39.6-182.4	89.6	73.6-103.6	88.6
Unknown Group II	5.0- 87.6	31.0	8.6- 20.0	14.3
Lactic	38.2-115.6	63.0	21.2- 55.2	38.2
Total	114.2-385.6	296.0	155.0-334.2	244.6

* Four trials conducted with normal skimmilk; two trials with phosphate-treated skimmilk.

These results reveal the presence in raw skimmilk of measurable amounts of butyric, acetic, formic, and lactic acid. Pyruvic and propionic acids were also detected in small and variable quantities in at least two of the samples examined. Also, four unknown fractions were separated and labeled I, II, III, and IV. For the different trials, acetic acid varied from 19.2 to 87.4 μ eq./100 ml., formic from 39.6 to 182.4 μ eq./100 ml., and unknown fraction II from 5.0 to 87.6 μ eq./100 ml.

Results for the total acid concentrations for the unknown acids, acetic, pyruvic, and formic acids were slightly higher in the skimmilk containing added phosphate than in the corresponding normal skimmilk.

Acids in heated skimmilk. The heated skimmilk samples were chromatographed and results are presented (Table 3) as increases in concentration, as compared to the unheated milk. A typical chromatogram for heated milk is shown (Figure 2).

Acetic, formic, lactic, and unknown fraction I accounted for from 97 to 98% of the total acid produced by heating the normal and the phosphate-treated skimmilk. In contrast, the increase of butyric, propionic, and pyruvic acid due to six hours of heating of the milk did not average more than eight micro-

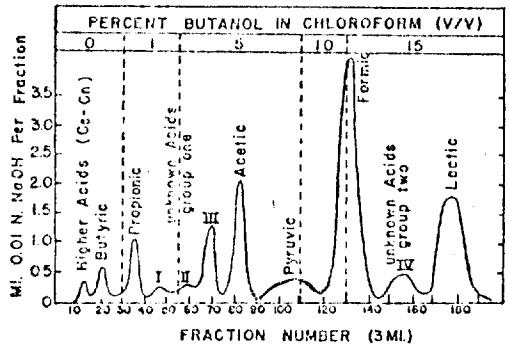

FIG. 2. Schedule of solvent addition and elution of acids from a heated milk sample.

TABLE 3

Increase in concentration of acids resulting from heat treatment of skimmilk[a] *(reported as microequivalents/100 ml.)*

Acids	Time of heating at 100° C. (*hr.*) 1	1.5	2	6	Average Range
		Normal skimmilk			
Butyric	0.2	−3.4	4.2	2.0	−4.8 to 7.0
Propionic	2.6	2.4	4.4	5.0	0.0 to 11.6
Unknown Group I	4.4	1.2	26.0	20.4	−11.4 to 51.8
Acetic	6.0	25.6	80.0	90.2	55.6 to 152.6
Pyruvic	3.4	−3.4	1.6	8.0	3.8 to 12
Formic	57.4	102.2	153.6	421.0	313.6 to 468
Unknown Group II	−7.4	−6.8	−2.6	−2.6	−7.2 to 1.2
Lactic	11.6	15.4	21.4	16.6	2.4 to 40.0
Total	78.2	133.2	288.2	560.6	361 to 588
		Phosphate-treated skimmilk			
Butyric	2.4	−1.6	3.4	15.2[b]	
Propionic	2.2	4.4	1.2	7.0	
Unknown Group I	2.2	33.0	60.0	54.4	
Acetic	21.6	81.0	102.2	267.6	
Pyruvic	8.4	−0.4	5.8	5.0	
Formic	129.4	325.4	415.4	2,068.6	
Unknown Group II	−3.6	2.8	−5.8	1.0	
Lactic	25.4	42.4	44.4	64.2	
Total	188.2	487.0	626.6	2,483.0	

[a] Three trials conducted with normal skimmilk and two with phosphate-treated skimmilk.
[b] Data for one determination only, owing to loss of sample.

equivalents per 100 ml., and accounted for only a small proportion of the total acid production in those samples containing disodium phosphate.

In the milk heated for six hours, 75.4 and 83.3% of the total acid production in normal and phosphate-treated skimmilk, respectively, was identified as formic acid. The heat production of formic, lactic, and acetic acid was greater in the phosphate-treated milk after six hours of heating than in the normal skimmilk. The phosphate samples exhibited acid concentrations that were two to three times those of the normal samples.

The variations in acid production obtained among the different trials is indicated (last column of Table 3) for the normal skimmilk. For the six hours of heating, the production varied from 313.6 to 468 μ eq./100 ml. for formic acid and from 55.6 to 152.6 μ eq./100 ml. for acetic acid for the three trials. For any particular experiment, the relationship between production of the major acids remained nearly constant, and those samples which exhibited the largest acetic acid production similarly showed a higher concentration of formic and unknown acids I, II, and III.

Identification of acids. For more positive identification, the three-ml. fractions for each acid component were collected from a raw milk sample and from a sample heated to 100° C. for two hours. An equivalent to 200 ml. of milk was chromatographed. Formic, acetic, propionic, and butyric acids were identified by the method of Ramsey and Patterson (12). The cerous salt of the formic acid fractions showed crystals identical to authentic cerous formate. The mercurous salts of the acetic, propionic, and active fractions showed the same crystal

structure as their respective known mercurous salts. Paper chromatographic analysis of the four components by the method of Reid and Lederer *(13)* showed single components with R_f values corresponding to those of the respective authentic compound. Chromatographic analysis of the residue after steam distillation (for nonvolatile organic acids) by the method of Jones *et al. (8)* showed no acid present in any of these fractions.

The pyruvic acid fraction was converted to its 2,4-dinitrophenylhydrazone and chromatographed by the method of El Hawary and Thompson *(3)*. The component characteristics were identical to the known pyruvic acid derivative.

The lactic acid fraction was steam-distilled. The distillate showed no volatile compounds by paper chromatography *(13)*. The residue showed a single component on paper chromatography with an R_f value the same as known lactic acid *(8)*.

Attempts to identify the so-called unknown acids were not successful. Organic acids which might be chromatographed in the same area as the unknown fractions were investigated. The threshold volume for these acids was found to be as follows:

Acid	Threshold fraction number
Alpha-ketoglutaric	70
Succinic	73
Fumaric	123
Beta-hydroxybutyric	80
Glutaric	75

None of these acids were eluted in the area of Unknowns I, II, or IV. Three of the acids, alpha-ketoglutaric, succinic, and glutaric, could be included in the Unknown III. Attempts to identify this compound were not successful. Acids I and II are presumably substituted fatty acids with more than three carbon atoms. Unknown IV appears to be closely associated with lactic acid, because commercial samples of lactic acid exhibit an acidic impurity with the same threshold volume.

Relationship of acid production to browning. An attempt was made to determine if a relationship existed between the browning reaction and the heating time at which the principal acids (formic and acetic) were produced. Results obtained are illustrated (Figure 3). On the basis of these observations, it appears that formation of browning upon heating coincides quite closely with the production of both acetic and formic acid.

DISCUSSION AND SUMMARY

Although a considerable amount of previous work has been conducted, to determine the acid production in milk or skimmilk as the result of heat treatment, the application of a chromatographic procedure for this purpose is relatively new. It appears that the improved sensitivity and accuracy of this newer procedure permits fuller elucidation of the changes occurring in milk as a result of high-temperature heat treatment, than has been possible heretofore.

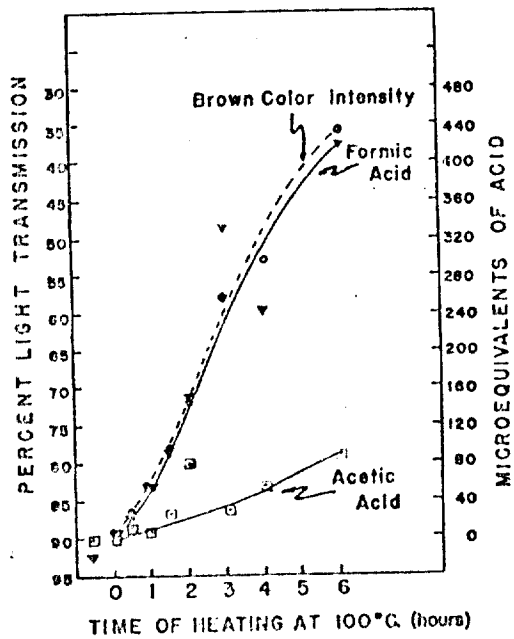

FIG. 3. Relation between production of browning and the increase of formic and acetic acids by heating normal skimmilk (reported as average of three trials).

Chromatographic analysis of fresh, raw, mixed-herd skimmilk revealed the presence of butyric, propionic, acetic, pyruvic, formic, lactic, and four unknown acids. The reliability of method was established by positive identification of the acids by crystallography or by paper chromatography. Unknown acid fractions I, II, and III eluted between propionic and acetic, and unknown acid fraction IV between formic and lactic. Unsuccessful attempts were made to identify the components of these unknown groups, by comparing their threshold volumes with those of other known pure acids thought likely to be present.

The distinct variation of individual and total acid concentration noted among the different samples of raw skimmilk indicates the need for further work, to establish the source and importance of these acids in the fresh-milk supply.

The chromatographic analysis revealed that of the ten acids studied, only six were produced in consistently large quantities. These acids, listed in the order of decreasing rate of production are: formic, acetic, unknown acids I, II, and III, and lactic. Results for formic and lactic acid compare relatively close to these reported by Gould (5) and Gould and Frantz (7), considering the wide difference in analytical procedures.

Somewhat different results for individual acid production were reported earlier by Kern (9) and Kern *et al.* (10), who used a buffered silica gel chromatographic column to measure the acids in skimmilk sterilized for bacterial cultures. In this work, the acids were eluted in the order of butyric, propionic, acetic, and pyruvic. It now appears that the fraction labeled butyric acid contained un-

known fractions I, II, and III, propionic acid contained unknown fraction IV, and pyruvic acid contained a considerable amount of formic acid. The last-named acid was not separated and identified in Kern's work.

The difference in the method of heating employed, whether the milk is in a sealed or open container, may influence the results obtained with these various acids. As an observation in this connection, an experiment was conducted in which milk was heated in sealed and unsealed containers at 121° C. The sealed milk exhibited a much greater production of the majority of the acids measured than the unsealed milk. Formic acid comprised a lower percentage of the total acid production in the latter, and some of the acids were in lower concentration after heating in the sealed can than in the original raw skimmilk. The need for additional investigations on this subject is indicated.

The relationship of individual acid production to browning in the heating of milk may yield information of value for eventually explaining the mechanism involved in browning. Patton and Flipse (*11*) isolated a colorless lactose-protein complex from heated milk which turned brown upon reheating, with concomitant disruption of the carbon bond of the lactose and of the protein. The noticeable production of formic acid just prior to the occurence of color production seems to substantiate the theory that lactose is sustaining a cleavage at the 1-2 carbon position. The simultaneous production of other acids (two or more carbon atoms) raises the possibility that additional cleavages of the lactose molecule are involved which also may be essential for the production of colored matter in milk.

REFERENCES

(1) Bulen, W. A., Varner, J. E., and Burrell, R. D. Separation of Organic Acids from Plant Tissues; Chromatographic Technique. *Anal. Chem.*, **24**: 187. 1952.

(2) Choi, R. P., Koneps, A. F., O'Malley, C. M., and Fairbanks, B. W. A Proposed Method for the Determination of Color of Dry Products of Milk. *J. Dairy Sci.*, **32**: 580. 1949.

(3) El Hawary, M. F. S., and Thompson, R. H. S. Estimation of Blood Keto Acids. *Biochem. J.*, **53**: 340. 1953.

(4) Gould, I. A. Lactic Acid in Dairy Products: Application of the Hillig Method. *J. Dairy Sci.*, **27**: 743. 1944.

(5) Gould, I. A. Lactic Acid in Dairy Products: The Effect of Heat on Total Acid and Lactic Acid Production and on Lactose Destruction. *J. Dairy Sci.*, **28**: 367. 1945.

(6) Gould, I. A. The Formation of Volatile Acids in Milk by High-Temperature Heat Treatment. *J. Dairy Sci.*, **28**: 379. 1945.

(7) Gould, I. A., and Frantz, E. S. Formic Acid Content of Milk Heated to High Temperatures as Determined by Distillation Procedure. *J. Dairy Sci.*, **29**: 27. 1946.

(8) Jones, A. R., Dawling, E. J., and Skraba, W. J. *Anal. Chem.*, **25**: 394. 1953.

(9) Kern, J. Bacteriological and Biochemical Studies of Italian Cheese Starter Organisms. M. S. thesis, The Ohio State University. 1953.

(10) Kern, J., Weiser, H. H., Harper, W. J., and Gould, I. A. Observations on Organic Acids Formed During the Heat Sterilization of Milk. *J. Dairy Sci.*, **37**: 904. 1954.

(11) Patton, S., and Flipse, R. J. Studies of Heated Milk. V. The Reaction of Lactose with Milk Protein as Shown by Lactose-1-C^{14}. *J. Dairy Sci.*, **36**: 766. 1953.

(12) Ramsey, I. L., and Patterson, W. I. *J. Assoc. Offic. Agr. Chemists*, **28**: 643. 1945.

(13) Reid, R. L., and Lederer, M. Paper Chromatography of Fatty Acids. *Biochem. J.*, **50**: 60. 1951.

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On the areas of the stomach of sheep that are sensitive to formic acid and histamine

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It was recently found by Neumark, Bondi & Volcani (1964) that the quantity of silage eaten by sheep was reduced when fermentative products such as formaldehyde, formic acid and histamine were present in the silage. The inference was drawn that these undesirable products were responsible for the consumption of such silages. It was also found by Neumark, unpublished work) that the introduction of solutions of formic acid and histamine via a stomach tube into the rumen of sheep depressed the quantity of food subsequently eaten. The taste of silages cannot be eliminated as a reason why sheep are reluctant to eat them when they contain formic acid and histamine, but even so, these experiments show that the substances have an effect in the stomach or systemically which are sufficient to depress appetite.

The experiments reported here were designed to show whether the effects of formic acid and histamine could be attributed to peripheral effects in the stomach or to systemic effects. They indicate that the area between the cardia and the fundic area of the abomasum is sensitive to formic acid and that the same, when it is effective, is so by the well-known systemic effects it produces.

EXPERIMENTAL

Four clun forest wethers (nos. 174, 265, 34 and 70) were used for all experiments. These animals were maintained on 350 g chopped hay with 100 g flaked oats which was given twice daily at 8 a.m. and 5 p.m. The animals under normal circumstances ate the food within 30 min of receiving it.

The average 'eating time' for sheep 174 and 265 was found to be 24 min and 23 min 40 s respectively, calculated from twenty-seven observations. The maximum 'eating time' was 27 min 30 s and the minimum was 21 min.

Sheep. Sheep 174 and 265 were both fitted with a chronic rumen cannula. Sheep 265, 70 and 34 were later fitted with abomasal cannulas.

Procedures. Solutions were given either directly into the rumen via the rumen cannula or by stomach tube (sheep 174 and 265). In initial experiments the stomach tube was passed into the rumen to an unknown depth. In order to standardize the procedure, radiographs were taken of the sheep with the stomach tube filled with a suspension of barium sulphate and the length of tube needed to place the orifice at the region of the cardia was measured. It was found that 72–73 cm of stomach tube, measured from the incisor teeth, was needed to reach the cardia.

In experiments in which solutions were run into the anterior rumen or reticulum via a rumen cannula, a flexible copper wire was introduced into the lumen of the dosing tube so that it could be passed forwards over the anterior pillar.

Solutions introduced directly into the abomasum were introduced through the abomasal cannula to the region of the omasal orifice or caudally into the pyloric part of the abomasum.

Intravenous infusions were made with sterile solutions from a 'Braun Constant flow' infusion pump through polyvinyl tubing inserted into the jugular vein. The duration of the infusions was 5 min. All infusions into the stomach or into the jugular vein were made immediately before the sheep were offered their usual meal at the usual time.

The effects of the infusions were measured by the time taken for sheep to consume the food they were offered and by noting any systemic effects on repirations or on behaviour. To measure the rate at which solutions introduced into the rumen via the cannula or stomach tube reached the abomasum, phenol red (0·8 g/l.) was added to the solutions.

Chemical. Analar grade formic acid was used (98–100%). Histamine was given as the di-HCl or di-H_3PO_4 salts. All values for histamine in the text are expressed as free histamine and not as the salt.

Analytical. The concentrations of phenol red in the abomasal or rumen liquor was determined by the method used by Hecker, Budtz-Olsen & Ostwald (1964). The coarse particles were removed by low-speed centrifugation and the supernatant fluid was spun at 30,000 rev/min for 30 min. A buffer solution (13·2 ml 0·1 M-NaOH + 0·05 M-Na_2HPO_4) was added —1 ml sample to 9 ml buffer—and the pH adjusted to 11. The optical density was compared with the optical density of an aliquot of the initial dose treated in the same way.

Formic and acetic acids in blood were estimated

by gas liquid chromatography according to Grey & Stevens (1966). The acids were isolated from blood by standard procedures as follows: 2 ml heparinized blood was added to 10 ml water and 1 ml of orthophosphoric acid, and filtered after standing. The filtrate was neutralized by 3 N-KOH to a faint pink colour with phenol phthalein. The solution was concentrated *in vacuo* to a small volume and the pH was then adjusted to 2·0–2·5 with 10 N-H_2SO_4 and steam distilled in the Markham apparatus. One hundred ml. were collected and titrated against 0·01 N-NaOH; excess NaOH was added and the distillate was evaporated to a small volume and finally dried over P_2O_5.

RESULTS

The infusion of formic acid and/or histamine into the rumen via a rumen cannula

There was no effect on the time taken by sheep 174 and 265 to consume their food when 1 l. of 0·2 M formic acid with or without histamine was introduced directly into the rumen. Both sheep were slightly affected and failed to consume all their food within their usual time when solutions of formic acid and histamine were introduced into the anterior part of the rumen, oral to the anterior pillar (Table 1). The introduction of saline in the same volume into the rumen in either the anterior or posterior regions was without effect.

The infusion of formic acid with and without histamine into the rumen via a stomach tube

The introduction of 1 l. 0·2 M formic acid with or without histamine, 1 l. of saline with histamine or 1 l. of saline alone gave variable results with sheep 265. Saline had no effect in the trials; formic acid alone had no or only a slight effect in five trials; histamine alone had a very slight effect on one trial; formic acid with histamine had variable effects in out of twelve trials, a pronounced response was

Table 1. *The effects of introducing formic acid with or without histamine into the rumen via a cannula on the 'eating time'*

Sheep no. 174			Sheep no. 265		
1 l. 0·2 M formic acid					
No. of experiment	Histamine (mg)	Response	No. of experiment	Histamine (mg)	Response
1	Without	No	1	360*	No
2	Without	No	2	545	No
3	650	No	3	650	No
Introduction into the anterior rumen					
4	651	Left a very small amount of food, after eating for 34 min; later cleaned up	4	651	Stopped eating after 34 min, left a small amount of food

* All figures for histamine in this and the following tables are calculated as free histamine.

Table 2. *The effects of introducing formic acid, histamine, saline or combinations of them into the rumen via a stomach tube on the 'eating time' of sheep* 265

No. of trials	Sheep 265, solution introduced	Effect on 'eating time'
Tube introduced to 72–73 cm		
3	1 l. saline	None
4	1 l. 0·2 M formic acid	None
5	1 l. 0·2 M formic acid + 400–660 mg histamine	Slight to none

Tube inserted to measured lengths

Length of tube inserted (cm)	Histamine included in 1 l. 0·2 M formic acid (mg)	Response
67	720	None
68	660	None
70	660	Slight
77	901	Very slight
73	651	Marked. Food left after eating for 42 min.

Tube introduced to an unknown length

No. of trials		
1	1 l. 0·2 M formic acid	Slight
2	1 l. 0·2 M formic acid + 360–541 mg histamine	Marked effect
1	541 mg histamine in 1 l. saline	Very slight

[illegible] in three trials, a slight response in seven trials and no response in two trials. Comparisons of the effects of formic acid with histamine introduced through different length of stomach tube gave a large response when 73 cm of tube were introduced and slight or no responses when more or less of this length of tube was introduced (Table 2).

The responses of sheep 174 were more pronounced than those of sheep 265 to formic acid alone or to histamine alone. Five trials with 1 l. of saline gave no response at all. Four trials with 1 l. of 0·2 M formic acid gave three positive responses and one slight response. Three trials with 1 l. of 0·2 M formic acid containing 360–541 mg histamine gave no response when the stomach tube was inserted to an unknown length, but in one further trial with 902 mg histamine, a strong response was obtained. In seven further trials with 1 l. 0·2 M formic acid containing 497–660 mg histamine when the stomach tube was inserted to a length of 72–73 cm, strong responses were obtained in every instance (Table 3).

Sodium formate (0·2 M) had no effect in two trials neither had acetic acid (0·2 M) in one trial. Acetic acid plus histamine however produced a marked effect (Table 3).

Variability of the responses do not give confidence in the specificity of any of the agents tested in delaying the consumption of food in these two animals. It is clear, however, that volume effects can be ignored as in no trial did 1 l. of saline as such alter the eating time of the animals. In general, formic acid plus histamine had a greater effect than either agent alone but variability was sufficient to suggest that the destination of the solutions administered might influence the response exhibited by the sheep.

Table 3. *The effects of introducing formic acid, histamine, saline and other agents into the rumen via a stomach tube on the 'eating time' of sheep* 174

No. of trials	Solution introduced	Effect on 'eating time'
5	1 l. saline	None
4	1 l. 0·2 M formic acid	Marked effect in 3 trials. Slight effect in 1 trial
4	1 l. 0·2 M formic acid + 360–541 mg histamine (3 trials) or 902 mg histamine (1 trial)	None at lower rate of dosage. Marked at high rate
	Uncontrolled length of tube used	
7	1 l. 0·2 M formic acid + 497–660 mg histamine (tube introduced to 72–75 cm)	Marked effect in all trials
1	1 l. 0·2 M formic acid plus 497 mg histamine (tube introduced to 67 cm)	None
1	1 l. 0·2 M acetic acid (tube introduced to 72 cm)	None
1	1 l. 0·25 M acetic acid plus 497 mg histamine (tube introduced to [illegible] cm)	Marked effect
2	1 l. 0·2 M sodium formate (tube introduced to 72 cm)	None

Table 4. *The rate of passage to the abomasum of solutions introduced into the rumen by fistula or stomach tube*

No. of trial	Introduction by rumen fistula or stomach tube	Sample collecting time*/min.	Optical density of sample	Optical density of phenol-red solution
1	Stomach tube, 72 cm length	5	0·137	0·326
		15	0·139	
2	Rumen fistula	5	0·009	2·100
		10	0·024	
		30	0·037	
3	Stomach tube, 72 cm length	Immediately after introduction	0·300	0·364
		7	0·300	
		30	0·254	
4	Stomach tube, 72 cm length	Immediately after introduction	0·266	0·550
		30	0·335	
		from the rumen†	0·010	

* Time which elapsed from introducing the solutions into the rumen till collecting a sample from the abomasum.
† A very short time after introducing the phenol-red solution, a sample was taken from the rumen.

The destination of 0·2 M solutions of formic acid given by stomach tube

The administration of solutions which included phenol red to sheep fitted with abomasal cannulas allowed an estimate to be made of the speed with which they passed to that organ. Table 4 sets out the optical density to phenol red of the abomasal and ruminal fluids after the administration of 1 l. solutions including phenol red when 72 cm of stomach tube was introduced. In trials 1, 3 and 4 the concentration of solution passing to the abomasum was considerable in sheep no. 174 compared to the concentration given. When the solution was introduced into the rumen via the rumen cannula the quantity of phenol red passing to the abomasum was small and it arrived there in a very diluted state. The responses in trials 1, 3 and 4 were roughly equivalent to the concentrations of phenol red arriving in the abomasum. The responses in trials 3 and 4 were greater than the response to trial 1, while no response at all was found in trial 2.

The responses to the infusion of formic acid and histamine into the abomasum

Solutions containing 75 and 94 mg of histamine increased the rate of respirations when introduced into the abomasum, feeding was slightly delayed. 40 ml. of solutions of 0·02 to 0·2 M formic acid had no effect and did nothing to potentiate the effect of histamine. 40 ml solutions of 0·15 M formic acid containing 121 mg of histamine depressed appetite for considerable periods when introduced into the fundus region of the abomasum, but had no influence upon breathing.

The effects of infusing formic acid and histamine intravenously

Formic acid by itself (10 ml. of 0·001 M to 0·004 M in saline) slowly infused into the jugular vein had no depressing effect on food consumption or on the time needed to finish a meal. The inclusion of 1 mg histamine in the infusion fluid had no effect but the infusion of 2 or more mg increased respirations and reduced the speed of eating. It was calculated that the infusion of 10 ml of 0·004 M formic acid should raise the concentration of formate in the blood by about 4·6 μg/ml of blood, assuming a blood volume of 4 l.

The concentration of formic acid attained in blood following its introduction into the rumen

The concentration of formic acid attained in the venous blood of sheep 174 after 1 l. 0·2 M formic acid containing 561 mg histamine was introduced by stomach tube into the rumen was 76·4 μg/ml after

Table 5. *The responses of sheep to infusions of formic acid, histamine or of both into the abomasum*

Sheep	Treatment	Response
	(Infused into pyloric region)	
174	0·016 M formic acid plus 42 mg histamine	None
174	0·026 M formic acid + 75 mg histamine	Rapid respiration. Finished food in 31 min.
174	94·5 mg histamine	Rapid respiration. Finished food in 35 min.
174	0·05 M formic acid	None
174	0·19 M formic acid	None
	(Infused into fundic region)	
265	0·15 M formic acid plus 120·6 mg histamine	Normal respiration. Stopped feeding after 6 min. 150 g left after 42 min. Feeding normally after 24
70		Normal respiration. Stopped eating after 9 min. and did not feed normally for 2 days
34		Normal respirations. Did not feed after infusion; food consumption depressed for several days

Table 6. *The response of food intake to infusion of formic acid and/or histamine into the jugular vein (sheep no. 265)*

No. of trial	Treatment*	Response
1	0·0005 M formic acid plus 1 mg histamine	Finished eating after 27 min; several very slight interruptions during eating
2	0·001 M formic acid	No
3	0·001 M formic acid 1 mg histamine	No
4	0·002 M formic acid 2 mg histamine	Respiration rate raised, eating speed lowered (31 min)
5	0·004 M formic acid 1 mg histamine	No
6	0·004 M formic acid	No (finished eating after 24 min.)
7	3·2 mg histamine	Respiration rate raised, finished eating after 32 min.

* Formic acid and/or histamine were dissolved in 10 c.c. and in 5 c.c. saline in trials 1–6 and 7 respectively

Table 7. *The concentration of acetic and formic acids in the jugular blood of sheep no. 174 before and after treatment**

No. of trial	Time of blood collection	Infusion by stomach tube: Formic acid (μg/ml blood)	Infusion by stomach tube: Acetic acid (μg/ml blood)	Infusion by rumen fistula: Formic acid (μg/ml blood)	Infusion by rumen fistula: Acetic acid (μg/ml blood)
1	Before treatment	14·0	43·7	5·9	29·0
2	6 min after treatment	—	—	16·9	27·0
3	30 min after treatment	76·4	68·7	8·9	28·7

* One litre 0·2 M formic acid solution, containing 861 mg histamine, was infused by stomach tube into the rumen of sheep no. 174, and 5 days later an equal solution, containing only 581 mg histamine, was introduced into the rumen of the sheep by rumen fistula.

30 min, This was a fivefold increase over the pre-dosing level. When the same solution, except for a reduced amount of histamine (581 mg), was introduced into the rumen via the rumen cannula the concentration of formic acid in the blood increased threefold within 6 min but was reduced to a value close to the pre-dosing level after 30 min.

DISCUSSION

The previous work of Neumark *et al.* (1954) has been confirmed in that solutions of formic acid and histamine introduced into the rumen via a stomach tube delayed the time taken by sheep to eat their food. The irregularity of the results was reduced by inserting the stomach tube to the region of the cardia only so that the [illegible] was in a favourable position to take a rapid passage to the abomasum. That this occurred was demonstrated by incorporating phenol red in the solution and obtaining the concentrations that appeared in the abomasal contents. These observations suggested that some [illegible] in the region of the oesophageal groove, [illegible] groove or abomasum was sensitive to these solutions.

Sheep 265 (Table 2) gave slight responses to solutions of formic acid alone but marked responses to formic acid solutions containing histamine; sheep 174 (Table 3), however, responded to solutions of formic acid alone but less regularly than when they also contained histamine. On the other hand, solutions of formic acid alone marked by phenol red which passed rapidly to the abomasum were effective in depressing the rate of food consumption (Table 4).

It is not surprising that the introduction of formic acid solutions into the rumen directly through the rumen cannula had no effect when it is considered how unstable this substance is in rumen contents. It is rapidly destroyed and provides a source of hydrogen for methane formation (Beijer, 1952).

A recent report of the Rowett Research Institute on the fate of histamine in the rumen indicates that about 90 % of histamine introduced into the rumen is destroyed by the time it reaches the duodenum and that absorption from the isolated rumen and reticulum, if it occurs, is very small. As the quantities given were large, ranging from about 4 to 9 mg/kg. body weight, localized effects of the sub-epithelial blood vessels could be expected when high concentrations are in contact with the epithelium even if only traces of it be absorbed. The marked respiratory effects that occurred when histamine containing solutions were introduced into the pyloric part of the abomasum are an indication that absorption occurred, presumably from the duodenum or upper intestine. Under these circumstances the secretion of gastric juice into the abomasum would also be stimulated but the effects of this on appetite cannot be predicted.

Histamine is known to increase the permeability of capillaries (Miles & Miles, 1952; Sparrow & Wilhelm, 1957), but whether it effects the permeability of the keratinized epithelium covering the omasum or any other part of the first three compartments of the sheeps' stomach is unknown. An increased local blood supply, however, could favour absorption of short chain fatty acids. Histamine is also known to cause release of adrenaline in the cat (Feldberg, 1940) and adrenaline has been shown to increase the sensitivity of gastric sensory nerve endings (Paintal, 1954). The latter effect was found by Kay (1953) to offer a satisfactory explanation for increased parotid secretion, following an initial expulsion of parotid saliva consequent upon the injection of adrenaline in sheep. These experiments are quoted to show that it is not inconceivable that the depressing effect on appetite of formic acid solutions containing histamine is due to local irritation of nerve endings in the stomach caused by formic acid rapidly penetrating the epithelial layer. Acetic acid is readily absorbed from the rumen and there is no reason to suppose that formic acid is not absorbed from the ruminant stomach if it is in

contact with the epithelium. The acidifying effect of solutions of 0·2 M formic acid would also favour absorption of other short chain fatty acids present although their concentrations would be less than those of formic acid. The local action of histamine besides possibly assisting absorption may have a different effect. It should not be overlooked that in the skin of man the local effects of histamine are also associated with a sensation of itching (Emmelin & Feldberg, 1947), and if penetration of histamine occurred, it may have produced sensations that were inhibitory to eating.

Sodium formate did not depress appetite when given by stomach tube in a way in which it could be expected to pass rapidly to the abomasum although, once in the abomasum, it would be converted to formic acid owing to the acidity of the abomasal contents. This experiment suggests that the sensitive area may be in the omasum for when solutions of formic acid and histamine were introduced in the abomasum in an oral direction, some may have penetrated the omasum. It further seems that the omasum may not have a specific sensitivity to formic acid since 0·25 M acetic acid, given with histamine in the same manner, depressed food consumption.

The increase in concentration in the blood following the introduction of formic acid, through a stomach tube, is a further indication of rapid passage to the abomasum; the same volume of formic acid placed directly in the rumen via a cannula had a much smaller effect. The direct infusion into the blood of formic acid gave no indication that central effects were concerned.

In order to see if a direct indication of irritation to the epithelium in the oesophageal groove region, or the omasum could be obtained, sheep no. 174 was infused with 1 l. 0·2 M formic acid and the sheep was given an overdose of Nembutal (pentobarbitol) by rapid intravenous injection. The interior of the rumen, reticulum, omasum and abomasum was examined as rapidly as possible after death. The region of the omasal groove was slightly pinker than normal, although on histological examination no evidence of hyperaemia or of any other change from the normal was observed.

SUMMARY

1. The infusion into the rumen via a cannula of 1 l. of aqueous solutions containing formic acid and/or histamine had no influence on the time sheep took to eat their food offered immediately after the infusion.

2. The infusion of formic acid solutions containing histamine into the rumen via stomach tube had variable effects with the length of stomach tube introduced was random. When they were introduced through 72 cm of stomach tube (measured from the incisor teeth), so that the end of the tube was at the cardia, one sheep consistently refused food or ate only a little but another sheep was only occasionally affected in this way.

3. Solutions marked with phenol red passed rapidly to the abomasum when introduced into the region of the cardia via a stomach tube. Only insignificant quantities of phenol red were detected within the same time in the abomasum when the solution was introduced into the rumen via a rumen cannula.

4. Histamine, but not formic acid, depressed the rate of eating when introduced into the caudal part of the abomasum. Increased respiration indicated a systemic response.

5. Formic acid and histamine introduced into the oral part of the abomasum resulted in sheep refusing to eat, in some animals for several days afterwards. This treatment did not affect the rate of respiration after dosing.

6. Solutions of sodium formate introduced into the rumen at the level of the cardia through a stomach tube, passed rapidly to the abomasum but had no effect on the time the sheep devoted to eating.

7. Intravenous infusions of formic acid had no effect on eating behaviour, but infusions of histamine caused systemic effects and inhibited eating for a short time.

8. The introduction of a formic acid solution containing histamine into the rumen at the level of the cardia through a stomach tube caused a rise in increase in concentration of formic acid in the blood within 30 min. The introduction of an equivalent solution into the rumen via a rumen cannula caused a smaller, temporary increase of formate in the blood after 6 minutes but this was not sustained.

9. The evidence indicates that a rapid passage of the solutions used containing formic acid and histamine to the abomasum was needed before the eating behaviour of the sheep was affected.

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REFERENCES

Annual Report of the Rowett Research Institute (1966), no. 22, p. 31.

[illegible], N. H. (1952). Methane formation in the rumen of cattle. *Nature, Lond.* **170**, 576–7.

EXMELIN, N. & FELDBERG, W. (1947). The mechanism of the sting of the common nettle (*Utrica urens*). *J. Physiol.* **105**, 440–55.

FELDBERG, W. (1940). The action of bee venom, cobra venom and lysolecithin on the adrenal medulla. *J. Physiol.* **99**, 104–18.

GELY, T. C. & STEVENS, B. J. (1966). Analysis of the lower fatty acids from microbal fermentations. *Analyt. Chem.* **38**, 724–6.

KOLLER, J. F., BUDTZ-OLSEN, O. E. & OTTWALD, M. (1954). The rumen as a water store in sheep. *Aust. J. agric. Res.* **15**, 961–8.

KAY, R. N. B. (1958). The effects of stimulation of the sympathetic nerve and of adrenaline on the flow of parotid saliva in sheep. *J. Physiol.* **144**, 476–89.

MILES, A. A. & MILES, E. M. (1952). Vascular reactions to histamine, histamine liberator and leukotoxine in the skin of guinea-pigs. *J. Physiol.* **138**, 228–257.

NEUMARK, H., BONDI, A. & VOLCANI, R. (1964). Amines, aldehydes and keto-acids in silages and the effect on food intake by ruminants. *J. Sci. Fd Agric.* **15**, 487–492.

PAINTAL, A. S. (1954). The response of gastric stretch receptors and certain other abdominal and thoracic vagal receptors to some drugs. *J. Physiol.* **126**, 271–85.

SEARROW, E. M. & WILHELM, D. L. (1957). Species differences in susceptibility to capillary factors: histamine, 5-hydroxytryptamine and compound 48/80. *J. Physiol.* **137**, 51–65.

The Urinary Excretion of Formic Acid and Formiminoglutamic Acid in Folic Acid Deficiency*

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In previous investigations it was shown that in folic acid deficiency there is a large increase in the urinary excretion of formimino-L-glutamic acid (1-6). This increase has been explained by recent enzymatic studies demonstrating that the first step in the metabolism of formimino-L-glutamic acid is the transfer of the formimino group to tetrahydrofolic acid (7-9). Formimino-L-glutamic acid is an intermediate in histidine metabolism (10, 11), and the urinary excretion of this compound in folic acid deficiency can be increased by the administration of L-histidine (3). Experiments with N^{15}-labeled histidine demonstrated that the urinary formimino-L-glutamic acid was derived from histidine (3).

In the present work we have demonstrated that, in addition to formimino-L-glutamic acid, there is a large increase in the excretion of formic acid in the urine of folic acid-deficient rats; in contrast to the results with formiminoglutamic acid, this formic acid is not derived from histidine. Our current studies have been facilitated by the recent development of sensitive spectrophotometric methods for the determination of formic acid (12) and formiminoglutamic acid (13).

EXPERIMENTAL

Formic acid was assayed enzymatically (12) with tetrahydrofolic acid formylase,[1] which catalyzes the formation of 10-formyltetrahydrofolic acid in the presence of adenosine triphosphate, tetrahydrofolic acid, and formic acid. The 10-formyltetrahydrofolic acid formed was converted by acid to 5,10-methenyltetrahydrofolic acid, which was quantitatively determined by measuring its absorption at 350 mμ. Formimino-L-glutamic acid was measured in a similar manner by the formation of 10-formyltetrahydrofolic acid in the presence of formiminoglutamic acid transferase, cyclodeaminase, tetrahydrofolic acid, and phosphate buffer (7). Details on these procedures have been presented elsewhere (7, 12, 13).

Formiminoglutamic acid was synthesized as previously described (14). Isotopic histidine (imidazole-2-C^{14}) was synthesized by a modification of the method of Tesar and Rittenberg (15). Radioactivity determinations were carried out at infinite thinness by means of a gas flow counter.

The folic acid-deficient, succinylsulfathiazole-containing diet previously used (16) was modified as recommended by E. McDaniel;[2] it contained sucrose (710 gm.), casein (180 gm.), salt mixture (40 gm.), ferric citrate·5 H_2O (10 gm.), $CuSO_4$·5 H_2O (400 mg.), Crisco (Proctor and Gamble) (50 gm.), Natola (Parke-Davis) (2.5 ml.), choline chloride (2 gm.), folic acid-free vitamin mixture (5 gm.), and succinylsulfathiazole (10 gm.).

A total of 12 female rats (Sprague-Dawley strain) were divided into two equal groups at weaning ("deficient" and "supplemented") and placed on the succinylsulfathiazole-containing, folic acid-deficient diet. Each rat in the "supplemented" group received three times each week by pipette an oral supplement of 0.25 μmole of folic acid. The diet was fed *ad libitum* except as indicated below.

Urine collections were made each week in accordance with the following procedure. Each rat received 1 ml. of water by stomach tube and 5 ml. of 0.15 M NaCl by an intraperitoneal injection (to increase the urine volume); the urine was then collected for 7 hours in a metabolism cage. After an interval of 3 days another urinary collection was made for 7 hours from the same rats after the administration of 1 ml. of 0.05 M L-histidine by stomach tube and 5 ml. of 0.15 M NaCl by an intraperitoneal injection. After collection, the urine was stored at −20° until it was assayed.

Average values for the excretion of formiminoglutamic acid and of formic acid in the normal and the folic acid-deficient rats are presented in Fig. 1A. The excretion of formiminoglutamic acid (per 7 hour collection period) increased during the course of the deficiency from a normal value of 0.22 μmole (range 0 to 0.7 μmole) to 36.5 μmoles. After supplementation with folic acid, this excretion decreased to 0.07 μmole. The validity of the enzymatic assay for formiminoglutamic acid in these urines was confirmed by paper chromatography, by alkaline hydrolysis, and by colorimetric assay with the alkaline ferrocyanide-nitroprusside reagent (13, 17).

The increase in the excretion of formiminoglutamic acid was observed in the folic acid-deficient rats, even though the dietary intake (and consequently the histidine intake) was considerably less in these rats than in the folic acid-supplemented rats. On the 38th day, for example, the folic acid-deficient rats consumed an average of 3 gm. of food per rat (calculated histidine content (18), 100 μmoles per day), whereas the folic acid-supplemented rats consumed an average of 9 gm. of food per rat (calculated histidine content, 300 μmoles per day).

* A preliminary report on this work was presented by J. C. Rabinowitz at the Gordon Conference on Vitamins and Metabolism, August 12, 1957.

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[1] The preparations used in these experiments were supplied by Mr. W. E. Priver, Jr.

[2] Personal communication.

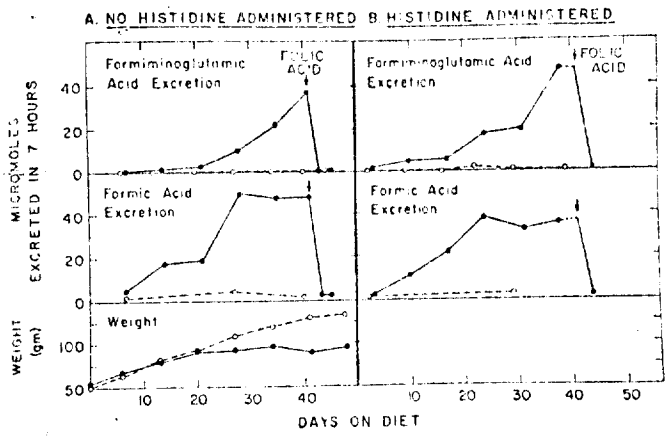

FIG. 1. Urinary excretion of formic acid and formiminoglutamic acid during the development of a folic acid deficiency. ●, rats on the deficient diet; ○, rats supplemented with folic acid. Each point represents the average value from a group of six rats. In the folic acid-deficient group, three rats died, on the 40th, 41st, and 42nd days, respectively; the subsequent points represent the excretion data and weights of the survivors.

A, no histidine administered. The range (micromoles) for formiminoglutamic acid excretion in folic acid-deficient rats was 0.09 to 0.6 (7th day), 0.6 to 2.2 (14th day), 1.0 to 3.1 (21st day), 3.6 to 16.5 (28th day), 12.0 to 29.8 (35th day), and 12.1 to 66.5 (41st day).

Beginning at the point represented by ↓, each rat in the "deficient" group received 0.25 μmole of folic acid by mouth each day. The dietary intake was restricted to that selected by the rat before supplementation.

B, the rats and experimental conditions the same, except for the administration of histidine (50 μmoles) by stomach tube to each rat immediately prior to the urinary collection period.

Excretion of formiminoglutamic acid in the folic acid-supplemented rats after the administration of histidine averaged 0.6 μmole, with a range of 0.06 to 3.0 μmoles. The range (micromoles) in the folic acid-deficient group was 0.1 to 3.0 (3rd day), 1.5 to 6.4 (10th day), 1.5 to 10.5 (17th day), 13.1 to 25.7 (21th day), 6.0 to 30.0 (31st day), and 8.9 to 76.3 (38th day).

The excretion of formic acid in folic acid-deficient rats rose from a normal value of 1.5 μmoles to a value of 49 μmoles per 7 hour collection period. The increase in the excretion of formic acid preceded the increase in the excretion of formiminoglutamic acid. After supplementation with folic acid the excretion of formic acid decreased to 1.5 μmoles. No decrease in either the formiminoglutamic or the formic acid level was found in comparable experiments after the administration of vitamin B_{12}. The assays for formic acid in representative urinary samples were confirmed by lactylation after acidification with H_2SO_4, and by recovery of the formic acid in the supernate.

The administration of histidine caused a large increase in the excretion of formiminoglutamic acid, but not of formic acid (Fig. 1*B*, Table I). The increase in excretion of formiminoglutamic acid was particularly marked in the folic acid-deficient group, even when only small quantities of histidine were administered; large increases in the excretion of formiminoglutamic acid (30 to 90 μmoles) were found in normal rats only when very large doses of histidine (400 μmoles) were administered. The increase in excretion of formiminoglutamic acid without any increase in excretion of the free formic acid indicated that the free formic acid found in the urine of folic acid-deficient rats did not arise from histidine or from formiminoglutamic acid. The origin of the formic acid from compounds other than histidine was demonstrated definitively by an experiment (Table II) in which 50 μmoles of L-histidine, labeled with C^{14} in position 2 of the imidazole ring (specific activity, 8900 c.p.m. per μmole), were administered by stomach tube to a folic acid-deficient rat. During the standard 7 hour collection period, 68.5 μmoles of formimino-L-glutamic acid were excreted, with a specific activity of 3300 c.p.m. per μmole. 49 μmoles of formic acid were excreted, with essentially no (less than 0.5 per cent) radioactivity.

DISCUSSION

Possible Sources of Urinary Formic Acid in Folic Acid Deficiency—The increased excretion of formiminoglutamic acid in folic acid-deficient rats can be explained by the demonstration that tetrahydrofolic acid is required for the further metabolism

TABLE I

Urinary excretion of formiminoglutamic and formic acids after histidine administration

Histidine administered	Folic acid-deficient rats*		Normal rats†	
	Formimino glutamic acid	Formic acid	Formimino glutamic acid	Formic acid
μmoles	*μmoles*	*μmoles*	*μmoles*	*μmoles*
0	5.3	36.5	0.05	1.87
100	24.7	31.0	0.53	1.97
200			0.64	2.06
400	180	39.8	89	2.69

* The diet and other conditions in the experiment were essentially the same as those described in Fig. 1. Six rats (Sprague-Dawley strain, average weight, 95 gm.) were used after they had received a folic acid-deficient diet for 23 days. Urine was then collected for 7 hours in order to obtain the data for formiminoglutamic and formic acid excretion in the absence of histidine administration. On the following day, three of these rats received 100 μmoles of histidine by stomach tube; the remaining three rats received 400 μmoles of histidine. The urine was again collected for 7 hours. All values are expressed as the average excretion per rat.

† Female rats (Sprague Dawley strain, average weight, 75 gm.) on a folic acid-supplemented stock diet. Each value represents the average excretion for three rats.

Additional data were obtained in two other experiments with normal rats. In one experiment, six weanling Sprague-Dawley female rats were fed a purified diet without succinylsulfathiazole; in addition, 0.25 μmole of folic acid was administered by oral pipette three times weekly. After 35 days (average weight, 140 gm.), the formiminoglutamic acid and formic acid in the urine, collected over a 7 hour period, averaged 0.33 μmole (range 0.01 to 0.27 μmole) and 1.89 μmoles, respectively. No data were obtained in this group after histidine administration.

In another experiment, five Sprague-Dawley weanling rats were fed the succinylsulfathiazole-containing diet, but received a folic acid supplement (0.25 μmole) by oral pipette once each week. After 48 days (average weight, 121 gm.), the rats were divided into three groups. Each group received either 0, 200, or 400 μmoles of histidine by stomach tube. The formiminoglutamic acid excretion over a 7 hour period averaged 0.21, 8.8, and 36.5 μmoles, respectively.

TABLE II

Distribution of C^{14} in urinary formiminoglutamic and formic acids after administration of histidine-imidazole-C^{14} to a folic acid-deficient rat

	μmoles	c.p.m.	Specific activity c.p.m./μmole
Histidine-C^{14} administered*	50	407,000	8100
Urinary formiminoglutamic acid†	68.5	226,000	3300
Urinary formic acid‡	49	< 2,000	< 40

* 50 μmoles of L-histidine, labeled with C^{14} in position 2 of the imidazole ring, were administered by stomach tube in a volume of 1 ml. to a folic acid-deficient rat (33rd day of deficient diet; weight of rat, 90 gm.). 5 ml. of isotonic saline were administered intraperitoneally. Urine was collected for 7 hours.

† The urine was assayed for formic acid enzymatically; this determination was confirmed by analysis of the distillate after addition of acid to the urine and lyophilization. The distillate, after neutralization, was also assayed for radioactivity.

Urinary formiminoglutamic acid was determined enzymatically. To determine the C^{14} content of the formiminoglutamic acid, an aliquot of urine was subjected to alkaline hydrolysis (13); after acidification, the formic acid was distilled (by lyophilization), and the neutralized distillate was assayed for radioactivity. These results were confirmed by similar assays after chromatography on Dowex 1 acetate.

of this compound *in vitro*. Thus, in folic acid deficiency, formiminoglutamic acid accumulates, and is excreted in the urine. The explanation for the increased excretion of formic acid in folic acid deficiency, on the other hand, is not so obvious; it can be postulated, however, that formic acid is constantly being formed in the normal animal, not only by pathways involving tetrahydrofolic acid, but by hydrolytic and oxidative reactions that do not require folic acid cofactors. In the normal animal, this formic acid is largely metabolized further, and only a small amount is excreted in the urine (19). In the folic acid-deficient animal, on the other hand, as will be discussed below, formic acid is metabolized poorly, and is excreted in the urine.

Possible reactions that might lead to formic acid without involving folic acid cofactors are suggested by the experiments of MacKenzie (20) and of Weinhouse and Friedmann (21). MacKenzie (20) demonstrated that urinary formic acid was increased after the administration of sarcosine. This study was extended by Weinhouse and Friedmann (21), who administered methionine-$C^{14}H_3$, choline-$C^{14}H_3$, and sarcosine-$C^{14}H_3$ together with large quantities of sodium formate in a "trapping" experiment *in vivo*. The radioactivity recovered in the formic acid excreted was markedly increased in folic acid-deficient rats. These studies are consistent with the fact that in various enzymatic studies folic acid cofactors have not been demonstrated as a requirement for the formation of formaldehyde from choline, dimethylglycine, or sarcosine (20), or in the oxidation of formaldehyde to formic acid (22).

The studies of Knox and Mehler (23, 24) on the enzymatic degradation of tryptophan to formic acid and kynurenine indicate another metabolic reaction in which formic acid might be formed without the participation of folic acid, since no folic acid cofactor has as yet been demonstrated for these enzymes. We have recently tested this postulation *in vivo*, and, as indicated in Table III, the administration of tryptophan led to an increase in the level of urinary formic acid. This increase was much greater in folic acid-deficient rats than in the folic acid-supplemented group.

Effect of Folic Acid Deficiency on Metabolism of Formic Acid in Vivo—The effect of folic acid deficiency on the metabolism of formic acid *in vivo* has been shown by Plaut *et al.* (25) and

TABLE III

Urinary excretion of formic acid after administration of tryptophan

L-tryptophan administered	Urinary excretion of formic acid	
	Folic acid-deficient rats*†	Normal rats‡
μmoles	μmoles	μmoles
0	38.6	1.9
200§	126	6.4
400§	182	38.0

* The diet and conditions in the experiment were essentially the same as those described in Fig. 1. Six rats (average weight, 100 gm.) were used after they had received a folic acid-deficient diet for 28 days. The experiment was repeated 3 days later, and the values presented represent the average of both experiments.

† The urinary samples were also analyzed for formiminoglutamic acid. After 0, 200, and 400 μmoles of tryptophan, the urine contained 12.5, 20.1, and 10.5 μmoles of formiminoglutamic acid, respectively.

‡ The normal diet used in these experiments was essentially the same as the folic acid-deficient diet, except for the omission of succinylsulfathiazole. Each rat received an oral supplement of 0.25 μmole of folic acid three times weekly. Each figure represents the average value from a group of three to six rats.

§ Administered as a 0.2 M suspension in 2 per cent gum arabic.

TABLE IV

Urinary formate-C^{14} after administration of formate-C^{14} intraperitoneally

Formate-C^{14}* administered	Counts recovered in urinary formate†	
	Folic acid-deficient rats‡	Folic acid-supplemented rats§
μmoles	%	%
1	38	1.5
10	53	1
100	63	8
600		45

* Each rat received 800,000 c.p.m. of sodium formate-C^{14}, diluted with sufficient nonradioactive sodium formate (0.1 M solution) to give the dose indicated. The volume was adjusted to 6 ml. with 0.15 M NaCl and injected intraperitoneally. Each figure represents the average value from a group of three to six rats.

† Per cent of administered counts.

‡ The diet and urinary collection period (7 hours) were the same as those described in Fig. 1. The rats (average weight, 100 gm.) had been fed the succinylsulfathiazole-containing diet for 31 days before this experiment was carried out.

§ The diet for this group was the same as that used in the folic acid-deficient group, except for the omission of succinylsulfathiazole. Each rat received 0.25 μmole of folic acid orally three times weekly. The average weight at the time of the experiment was 95 gm.

Friedmann *et al.* (26), who demonstrated a marked decrease in the respiratory $C^{14}O_2$ excreted after the administration of formic acid-C^{14} to folic acid-deficient rats. Friedmann *et al.* (26) were unable, however, to demonstrate an effect of folic acid deficiency on the urinary excretion of formic acid-C^{14}. This was presumably because of the large amount of formic acid (2000 μmoles) administered in their experiments; with smaller doses we have been able to show a marked increase in the urinary formic acid-C^{14} of folic acid-deficient rats (Table IV).

The data presented in this paper on the accumulation of formic acid in folic acid-deficient rats and on the rapid metabolism of administered formic acid-C^{14} in the presence of folic acid indicate a large turnover of formic acid *in vivo* in normal rats.

SUMMARY

The urinary excretion of formiminoglutamic acid and of formic acid was quantitatively followed during the development of a folic acid deficiency in rats. In addition to the previously reported increase in the excretion of formiminoglutamic acid, there was a marked increase in the excretion of formic acid.

The role of histidine as a precursor of formiminoglutamic acid was confirmed. Histidine, however, was not a precursor of the urinary formic acid in the folic acid-deficient rats. The administration of tryptophan, on the other hand, resulted in an increased excretion of formic acid, with essentially no change in the excretion of formiminoglutamic acid.

Formic acid can be produced from tryptophan and other precursors by metabolic reactions that do not require folic acid. In the absence of folic acid, the formic acid is metabolized poorly and is excreted in the urine.

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REFERENCES

1. Bakerman, H. A., Silverman, M., and Daft, F. S., *J. Biol. Chem.*, **188**, 117 (1951).
2. Silverman, M., Gardiner, R. C., and Bakerman, H. A., *J. Biol. Chem.*, **194**, 815 (1952).
3. Tabor, H., Silverman, M., Mehler, A. H., Daft, F. S., and Bauer, H., *J. Am. Chem. Soc.*, **75**, 756 (1953).
4. Seegmiller, J. E., Silverman, M., Tabor, H., and Mehler, A. H., *J. Am. Chem. Soc.*, **76**, 6205 (1954).
5. Brequist, H. P., *J. Am. Chem. Soc.*, **78**, 6205 (1956).
6. Luebby, A. L., *Clinical Research Proc.*, **5**, 8 (1957).
7. Tabor, H., and Rabinowitz, J. C., *J. Am. Chem. Soc.*, **78**, 5705 (1956).
8. Miller, A., and Waelsch, H., *Arch. Biochem. Biophys.*, **63**, 263 (1956).
9. Miller, A., and Waelsch, H., *J. Biol. Chem.*, **228**, 397 (1957).
10. Borek, B. A., and Waelsch, H., *J. Biol. Chem.*, **205**, 459 (1953).
11. Tabor, H., and Mehler, A. H., *J. Biol. Chem.*, **210**, 559 (1954).
12. Rabinowitz, J. C., and Pricer, W. E., Jr., *J. Biol. Chem.*, **229**, 321 (1957).
13. Tabor, H., and Wyngarden, L., *J. Clin. Invest.*, **37**, 824 (1958).
14. Tabor, H., and Rabinowitz, J. C., *Biochem. Preparations*, **5**, 100 (1957).
15. Tesar, C., and Rittenberg, D., *J. Biol. Chem.*, **170**, 35 (1947).
16. Kornberg, A., Tabor, H., and Sebrell, W. H., *Am. J. Physiol.*, **142**, 604 (1944).
17. Rabinowitz, J. C., and Pricer, W. E., Jr., *J. Biol. Chem.*, **222**, 537 (1956).
18. Block, R. J., and Bolling, D., *The amino acid composition of proteins and foods*, 2nd edition, Charles C. Thomas, Springfield, Illinois, 1951.
19. Dakin, H. D., Janney, N. W., and Wakeman, A. J., *J. Biol. Chem.*, **14**, 341 (1913).
20. MacKenzie, C. G., In W. D. McElroy, and B. Glass, (Editors) *Amino acid metabolism*, Johns Hopkins Press, Baltimore, 1955, p. 684.
21. Weinhouse, S., and Friedmann, B., *J. Biol. Chem.*, **210**, 423 (1954).
22. Stritmatter, P., and Ball, E. G., *J. Biol. Chem.*, **213**, 445 (1955).
23. Knox, W. E., and Mehler, A. H., *J. Biol. Chem.*, **187**, 419 (1950).
24. Mehler, A. H., and Knox, W. E., *J. Biol. Chem.*, **187**, 431 (1950).
25. Plaut, G. W. E., Betheil, J. J., and Lardy, H. A., *J. Biol. Chem.*, **184**, 795 (1950).
26. Friedmann, B., Nakada, H. I., and Weinhouse, S., *J. Biol. Chem.*, **210**, 413 (1954).

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Formate Oxidation by Erythrocytes[1]

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Introduction

Recent investigations on the metabolism of formate in various organisms have established its function as a precursor in the biosynthesis of serine, methionine, cystathionine, and purines (1–4). It has also been demonstrated that formate is oxidized by animal tissues to carbon dioxide both *in vivo* and *in vitro* (5–7). *In vitro* studies on the metabolism of formate by nucleated frog erythrocytes in this laboratory showed that only a small fraction of formate-C^{14} was incorporated into erythrocytes and that a larger portion of the formate was oxidized to carbon dioxide. The addition of specific sulfhydryl compounds markedly enhanced this oxidation. Similar data were obtained from experiments with dog, human, and chicken erythrocytes. The details of formate oxidation by hemolyzates of erythrocytes from frog, dog, human, and chicken are reported here, and a mechanism for this oxidation is presented.

Experimental

Venous blood was collected from normal human subjects by means of heparinized syringes. Dogs were anesthetized with pentobarbital (30 mg./kg.) before blood was withdrawn. Bullfrogs, *Rana catesbeiana* (purchased from Southern Biological Supply Co. Inc., New Orleans, La.), were pithed with a sharp probe and the heart exposed through an incision in the ventral abdominal wall. Approximately 0.1 ml. of heparin was injected into the ventricle, permitted to circulate for about 1 min., then blood was withdrawn. In this manner, 8–12 ml. of blood was obtained from a single bullfrog. Chickens weighing 3–4 lb. were bought in the open market. They were decapitated and the blood was collected in a beaker

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containing heparin. All blood samples were placed in ice-cooled graduated cylinders until used. After centrifugation the erythrocytes were suspended in ice-cold isotonic saline and recentrifuged. This was repeated three times. Hemolyzates were prepared by shaking a suspension of the washed erythrocytes in distilled water containing glass beads, and then dialyzed against 0.05 *M* phosphate buffer, pH 7.4, at 5-7°C. for 3 hr. with one or two changes of buffer.

The incubation vessels were 33-ml. bottles, into which a small test tube (12 × 37 mm.) containing 0.2 ml. of 20% potassium hydroxide and a folded filter paper (Whatman #52) was inserted to trap carbon dioxide. All reagents were dissolved or diluted with an appropriate amount of phosphate buffer to approximate a final buffer concentration of 0.05 *M*. Incubation mixtures consisted of 0.2 ml. of formate-C^{14} (10 μM), 0.2 ml. of each additive, and sufficient 0.05 *M* phosphate buffer to bring the volume to 1 ml. Immediately prior to incubation, 1 ml. of the desired hemolyzate in 0.05 *M* phosphate buffer was added, the vessels were flushed with oxygen, and then quickly stoppered with serum bottle stoppers. They were placed in a Dubnoff metabolic shaker for 4 hr. at 25°C. for frog, and 37°C. for dog, human, and chicken hemolyzates.

At the end of the experiment, 0.5 ml. of 20% trichloroacetic acid was injected with a hypodermic syringe, and the vessels were refrigerated overnight. Subsequently, the collected carbon dioxide was precipitated as barium carbonate, then plated on aluminum planchets (5 sq. cm. area), and the radioactivity determined with a Geiger-Müller tube. Corrections for self-absorption and background were made. All counts were made to within ±5% error. The counts are reported as relative specific activity (R.S.A.)[2] per milligram dry weight of hemolyzates in the tables and as direct counts in the graphs.

Paper chromatography of DL homocysteine, DL-homocystine, glutathione, and oxidized glutathione[3] was carried out by means of the circular plate technique (8). The solvent consisted of *n*-propanol-formic acid-diethylene glycol-water in proportion of 70:10:3:20 by volume. Ninhydrin was used to locate the position of these compounds on paper. The R_f values for homocysteine, homocystine, glutathione, and oxidized glutathione were 0.76, 0.49, 0.60, and 0.20, respectively.

Reduced glutathione, DL-homocysteine, DL-homocystine, L-cysteine, hypoxanthine, L-leucine, D-leucine, L-methionine, and D-methionine were purchased from Nutritional Biochemicals Corporation. Tyramine was purchased from Eastman Organic Chemicals, and catalase was purchased from Worthington Biochemical Corporation. Thiomalate and mercaptoethanol were obtained from Brothers Chemical Company, and ergothionine from California Foundation for Biochemical Research. Formate-C^{14} was purchased from the Isotopes Specialties Company.

[2] $$R.S.A. = \frac{\text{Total activity of } BaC^{14}O_3 \times 100}{\text{Total activity of added formate-}C^{14}}$$

[3] Prepared by adding diluted hydrogen peroxide to an aqueous solution of glutathione in equal molar quantities. The mixture was allowed to evaporate to dryness over $CaCl_2$ *in vacuo*. Oxidized glutathione was recovered and identified by paper chromatography.

Results

Preliminary experiments with frog and dog washed erythrocytes suspended in isotonic saline showed upon incubation with formate-C^{14} approximately 1% of the added formate oxidized to carbon dioxide/mg. dry weight of cells. Similar data were obtained with lysed cells.

In an effort to determine whether oxidases, such as D- and L-amino acid oxidases, amine oxidase, or hypoxanthine oxidase, were involved, L-leucine, D-leucine, L-methionine, tyramine, hypoxanthine, homocysteine, and glutathione were tested. Only the latter two compounds increased the formate oxidation. Hypoxanthine, with or without sulfhydryl compounds, did not exert any influence on this reaction (Table I).

In order to determine whether the enhanced oxidation of formate was specific with respect to these sulfur-containing compounds, cysteine, thiomalic acid, ergothionine, mercaptoethanol, D-methionine, cystine,

TABLE I

Influence of Various Additives on Formate Oxidation

Hemolyzates from frog erythrocytes. See text for details on preparation, sequence of addition, and conditions of incubation.

Expt.	Additives	$\frac{\text{R.S.A.}}{\text{mg. dry wt.}} \times 10^{-3}$		
		Boiled	Fresh	Fresh dialyzed
I	None	6.3	176	88.7
	DL-Homocysteine, 40 μM	13.5	644	805
	Glutathione, 20 μM	11.5	531	871
	L-Leucine, 20 μM	4.8	95.7	97.6
	D-Leucine, 20 μM	7.4	124	95.3
	L-Methionine, 20 μM	3.8	97.0	114
	D-Methionine, 20 μM	5.2	309	201
	Tyramine, 20 μM	1.3	79.8	130
II	None	9.4	74.3	
	Hypoxanthine, 10 μM	18.7	53.1	
	DL-Homocysteine, 40 μM	8.6	444	
	DL-Homocysteine, 40 μM + hypoxanthine, 10 μM	—	396	
	Glutathione, 20 μM	6.0	310	
	Glutathione, 20 μM + hypoxanthine, 10 μM	—	317	

TABLE II

Effect of Sulfur Compounds on Formate Oxidation

Hemolyzates from human erythrocytes. See text for details on preparation, sequence of addition, and conditions of incubation.

Expt.	Additives	R.S.A. / mg. dry wt. × 10^{-3}	
		Boiled	Fresh dialyzed
I	None	17.7	35.8
	DL-Homocysteine, 40 μM	17.5	599
	DL-Homocystine, 40 μM	18.9	59.7
	Glutathione, 20 μM	12.2	374
	Oxid. glutathione, 20 μM	15.1	59.8
	L-Cysteine, 20 μM	0.8	434
	L-Cystine, 20 μM	2.0	68.1
II	None		26.5
	DL-Homocysteine, 40 μM		237
	Glutathione, 20 μM		232
	Thiomalate, 20 μM		91.6
	Ergothionine, 20 μM		23.3
	Mercaptoethanol, 20 μM		16.8
	D-Methionine, 20 μM		33.0

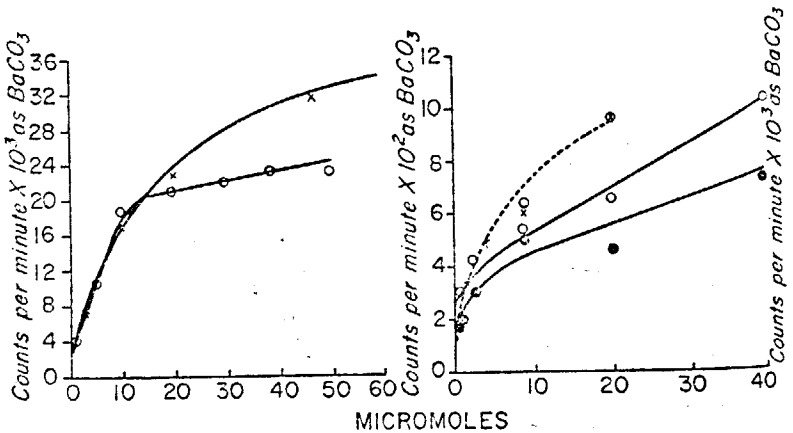

FIG. 1. Effect of varying concentrations of homocysteine (O) and glutathione (X) on formate oxidation by hemolyzates of human erythrocytes.

FIG. 2. Effect of varying concentrations of homocysteine (O - - - - -) and glutathione (X - - - - -) on formate oxidation by frog hemolyzates; solid lines (———) indicate the effect of homocysteine (O) and glutathione (●) on formate oxidation by chicken hemolyzates.

homocystine, and oxidized glutathione were tested. Table II, Expt. I, shows that the oxidized sulfhydryls were practically without effect, and in Expt. II it is noted that the other sulfur-containing compounds did not appreciably influence formate oxidation in comparison to the effect produced by homocysteine, glutathione, and cysteine.

Essentially similar results were observed on hemolyzates from nucleated erythrocytes. Oxidized glutathione, DL-homocystine and L-cystine with added diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) had no significant effect. Moreover, the

TABLE III

Influence of Inhibitors on the Oxidation of Formate by Hemolyzates

See text for details on preparation, sequence of addition, and conditions of incubation.

Source of erythrocytes	Inhibitors	No additions		DL-Homocysteine, 40 μM		Glutathione, 20 μM	
		R.S.A.* × 10^{-3}	Inhib.	R.S.A.* × 10^{-3}	Inhib.	R.S.A.* × 10^{-3}	Inhib.
			%		%		%
Frog	No additions	42.1	0	344	0	364	0
	Ethanol, 20 μM	15.6	63	64.6	81	44.2	88
	$NaNO_2$, 20 μM	23.4	44	56.8	83	47.6	87
	NaN_3, 5 μM	17.0	60	5.9	98	9.2	97
Dog	No additions	9.8	0	21.7	0	18.7	0
	Ethanol, 20 μM	9.8	0	10.6	51	7.0	63
	$NaNO_2$, 20 μM	4.7	52	5.5	75	3.7	80
	NaN_3, 5 μM	7.2	27	3.9	82	3.6	81
Human	No additions	22.8	0	237	0	232	0
	NaN_3, 5 μM	1.8	92	1.3	100	1.6	100
	KCN, 5 μM	17.3	24	177	25	160	31
	KCN, 20 μM	6.4	72	20.2	91	49.5	79
	Boiled hemolyzate	1.0		0.8		0.9	
Chicken[a]	No additions			13.6	0	9.4	0
	Ethanol, 20 μM			5.8	57	4.1	56
	$NaNO_2$, 20 μM			3.8	72	4.3	54
	NaN_3, 20 μM			3.5	74	1.9	80
	KCN, 20 μM			5.2	62	5.7	39

* mg. dry wt.

[a] In these experiments, homocysteine concentration was 20 μM.

presence of DPN or TPN, with or without the sulfhydryl, did not influence the oxidation of formate by the hemolyzates of frog or human erythrocytes.

To ascertain whether this enhanced oxidation was enzymatically catalyzed, boiled erythrocytes and hemolyzates were incubated as controls. Although some non-enzymatic oxidation was noted, the oxidation of formate in the presence of sulfhydryl compounds was much greater with fresh hemolyzed frog and human red cells, as is indicated in Tables I and II. Paper chromatograms of the media, after incubation with either glutathione or homocysteine, showed mainly the presence of oxidized glutathione and homocystine with only trace amounts of the reduced forms of these compounds.

Increasing concentrations of DL-homocysteine and of glutathione also increased the amount of formate oxidized. Figures 1 and 2 show the results of these experiments.

Since formate oxidation by a catalase–peroxide complex has been indicated (12), various inhibitors were tested on the formate-oxidizing system. Addition of sodium azide and sodium nitrite effectively inhibited formate oxidation even in the presence of sulfhydryl compounds. Ethanol had no effect on the action of the hemolyzate alone, but it did decrease formate oxidation when glutathione or homocysteine was present (Table III). To test the influence of catalase on formate oxidation, catalase was added to boiled hemolyzates and to the aqueous extracts

TABLE IV

Effect of Sulfhydryls and Catalase on Formate Oxidation

Hemolyzates from human red cells. See text for details on preparation, sequence of addition, and conditions of incubation.

Additives	R.S.A. × 10^{-3}/mg. dry wt.		
	Boiled	Extracts from boiled	Fresh dialyzed
No additions	2.8	42.0	15.7
Catalase[a]	22.4	249	
DL-Homocysteine, 100 μM	3.1	64.3	212
DL-Homocysteine, 100 μM plus catalase[a]	207	6,350	
Glutathione, 50 μM	2.7	18.4	317
Glutathione, 50 μM plus catalase[a]	621	10,800	

[a] Added 0.2 ml. catalase solution containing 3000 units of catalase.

from boiled hemolyzates from the four species studied. Table IV shows the results with human erythrocytes, and this illustrates the type of data obtained. In the presence of homocysteine and glutathione, catalase effectively increased the formate oxidation.

Discussion

In confirmation of earlier reports (7, 12–15) it has been demonstrated that formate oxidation to carbon dioxide by various types of erythrocytes is due to catalase activity. The findings that hemolyzates from dog and chicken erythrocytes have a lower formate oxidizing activity than that of the frog and human, is consistent with the observation that the former species have a lower catalase content (11).

Since catalase oxidation of formate requires hydrogen peroxide, systems capable of producing hydrogen peroxide, such as xanthine oxidase, D- and L-amino acid oxidase, and amine oxidase, were investigated in hemolyzates by means of specific substrates. However, these systems were found to be either inactive or were entirely absent.

Previous investigators have demonstrated that hydrogen peroxide is produced non-enzymatically by the oxidation of sulfhydryl compounds such as cysteine and glutathione, under aerobic conditions (9). However, none of these reports have considered these sulfhydryl compounds as a source of peroxide for catalatic oxidation of formate in erythrocytes.

In the absence of enzymic systems for peroxide formation, this investigation demonstrated that sulfhydryl compounds, in the presence of erythrocyte catalase, markedly enhanced formate oxidation. While a direct stoichiometric relationship was not observed over all the concentrations of homocysteine and glutathione studied, the yield of carbon dioxide was increased by increasing concentrations of added sulfhydryl compounds.

Thus, the role of sulfhydryl compounds in formate oxidation in erythrocytes may be explained by the following sequence of reactions which account for the observed data:

1. $2RSH + O_2 \rightarrow R{-}S{-}S{-}R + H_2O_2$
2. $\text{Catalase} + H_2O_2 \rightarrow \text{catalase-}H_2O_2\text{-complex}$
3. $\text{Catalase-}H_2O_2\text{-complex} + HCOOH \rightarrow CO_2 + 2H_2O + \text{catalase}$

Over-all: $2RSH + HCOOH + O_2 \rightarrow R{-}S{-}S{-}R + CO_2 + 2H_2O$

Reaction 1 is non-enzymic, and this accounts for the observed oxidation of glutathione, homocysteine, and the formation of hydrogen peroxide.

Reactions 2 and 3 represent the action of erythrocyte catalase as the peroxide-complexing enzyme responsible for the oxidation of formate (14).

To test this set of reactions experimentally, boiled hemolyzates and extracts from boiled hemolyzates were incubated with and without added catalase, glutathione, and homocysteine, respectively, in the presence of formate-C^{14}. The results show that glutathione and homocysteine are non-enzymatically oxidized aerobically and thus form hydrogen peroxide which, in combination with added catalase, oxidized formate.

The presence of glutathione and catalase in erythrocytes, and the inactivity or absence of enzymic systems for peroxide formation, definitely points to the above mechanism for formate oxidation in erythrocytes of the species studied.

The present investigation established that nucleated and enucleated erythrocytes oxidize formate in an identical manner differing only in the extent of oxidation.

Summary

Frog, dog, human, and chicken erythrocytes aerobically oxidize formate to carbon dioxide by the mediation of catalase-peroxide complex. The peroxide is supplied from a non-enzymic oxidation of certain sulfhydryl compounds: glutathione, homocysteine, and cysteine.

References

1. Sakami, W., *J. Biol. Chem.* **176**, 995 (1948).
2. Berg, P., *J. Biol. Chem.* **205**, 145 (1953).
3. Sonne, J. C., Buchanan, J. M., and Delluva, A. M., *J. Biol. Chem.* **173**, 69 (1948).
4. Greenberg, G. R., *J. Biol. Chem.* **190**, 611 (1951).
5. Plant, G. W. E., Bethell, J., and Lardy, H. A., *J. Biol. Chem.* **184**, 795 (1950).
6. Mathews, M. B., and Vennesland, B., *J. Biol. Chem.* **186**, 667 (1950).
7. Nakada, H. I., and Weinhouse, S., *Arch. Biochem. and Biophys.* **42**, 264 (1953).
8. Rappoport, D. A., Calvert, C., Loeffler, R. K., and Gast, J. H., *Anal. Chem.* **27**, 820 (1955).
9. Lyman, C. M., and Barron, E. S. G., *J. Biol. Chem.* **121**, 275 (1937).
10. Barron, E. S. G., *Advances in Enzymol.* **11**, 205 (1951).
11. Richardson, M., Huddleson, I. F., Bethea, R., and Trustdorf, M., *Arch. Biochem. and Biophys.* **42**, 114, 124 (1953).
12. Batelli, F. S., *Compt. rend.* **135**, 651 (1904).
13. Batelli, F. B., and Stern, L., *Biochem. Z.* **13**, 59 (1908).

14. Chance, B., "The Mechanism of Enzyme Action" (W. D. McElroy and B. Glass, eds.), p. 402. Johns Hopkins Press, Baltimore, 1954.
15. Rappoport, D. A., Oro, J., and Collins, V. P., *Federation Proc.* **14**, 381 (1955).
16. Keilin, D., and Hartree, E. F., *Biochem. J.* **60**, 310 (1955).
17. Keilin, D., and Hartree, E. F., *Biochem. J.* **39**, 148 (1945).
18. Harrison, D. C., *Biochem. J.* **18**, 1000 (1924).
19. Michaelis, L., *J. Biol. Chem.* **84**, 777 (1929).

RESEARCH ON THE TOXICITY OF METHYL ALCOHOL

IV. Critical Summary Of Research Aimed At A More Rational Estimate and Interpretation Of The Mode Of Action Of Methyl Alcohol

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Source: Archivio di farmacologia sperimentale 56: 364-71, Aug. 1, 1933.

I. Results Of Personal Experiments

In our previous reports, we have made a comparative study of 3 points of the action exerted by methyl alcohol, formaldehyde and formic acid (1). We shall now summarize briefly the results obtained.

1. In the frog heart isolated with Straub's cannula, we have reached the following conclusions: the isolated frog heart is finally arrested in an irreversible manner under the action of 0.00434 g.-mole/l of formic acid and 0.03333 g.-mole/l formaldehyde. On the other hand, the heart arrest with methyl alcohol takes place at 6.25 g.-mole/l, and the heart action resumes after washing with Ringer's solution.

2. Methyl alcohol dilates the vessels of the posterior limbs of the frog, prepared by the Lawen-Trendelenburg method, much better than equimolecular doses of formaldehyde and formic acid. The latter two compounds (and the acid to a greater extent than the aldehyde), at dosages greater than those causing dilation, display a considerable vasoconstricting power, which grows with further increase in dosage. On the other hand, the vasodilating action of methyl alcohol is still clearly noticeable

at a dose over 4000 times greater than the minimum dose of formaldehyde capable of exerting an appreciable vasoconstriction, and more than 7000 times greater than the dose of formic acid producing the same effect.

3. The minimum long-term lethal dose of formic acid in the rabbit, when given intravenously, is much greater than the corresponding dose of formaldehyde, and the latter, in turn, is much higher than the dose of methyl alcohol. The duration of life of animals receiving the minimum long-term lethal dose of methyl alcohol is much longer than the survival time of animals receiving a corresponding dose of formaldehyde and formic acid. The latter two compounds do not cause lesions of the eye-ground (fundus oculi) observed with methyl alcohol.

The symptoms observed, however, are quite different: The paralysis causing death from methyl alcohol is preceded by a long series of violent convulsions, which are completely absent with formaldehyde and formic acid, having a constant depressive action.

In conclusion, all these experiments lead to a unique and certain result, namely, that among the 3 compounds being compared, formic acid is more toxic than formaldehyde and the latter in turn is more toxic than methyl alcohol, and that the intoxication pattern of methyl alcohol can never be reproduced with formaldehyde or formic acid.

II. Results Of Experiments Preceding Our Own And Current Opinions On The Toxicity of Methyl Alcohol

Let us now summarize briefly the current opinions on the toxic action of methyl alcohol.

Pohl has observed that when methyl alcohol is introduced into the organism, it undergoes a slow oxidation to formic

acid (2). This fact was confirmed by Harnack (3), who believed that this was the reason for the specific toxicity of methanol; he thought that its conversion to formic acid, especially in regard to the site of the organism where this conversion takes place, may have fatal consequences, since formic acid is the most toxic of the acids in its homologous series. Methyl alcohol, when introduced into the organism, would be attracted in a special manner by definite parts of the central nervous system, by nervous elements of the retina, the optic nerve and its centers: at these points, a slow oxidation would take place, capable of converting methyl alcohol to formic acid, which would cause disastrous injuries to these parts by exerting an irritant and paralyzing effect, thus explaining the blindness, cramps and paralysis observed in animals and man. In this manner, the fact that symptoms of methyl alcohol intoxication are different from those observed with ethyl alcohol would become understandable.

The long time during which methyl alcohol remains in tissues and blood was demonstrated by Völtz and Dietrich (4). These authors, after giving a fixed dose of methyl alcohol to dogs, determined during a certain time the amount eliminated with expired air and the amount converted to formic acid which passed into the urine; they noted that only part of the methyl alcohol is eliminated in 48 hours, since after the animals were killed and the amount of alcohol present in tissues was determined, they still found 37% methyl alcohol present. They concluded, therefore, that repeated introduction of large amounts of methyl alcohol into higher animals results in its accumulation, as well as of its oxidation product, formic acid, in tissues and thus causes the death of the animals.

It must also be noted that among the most dreadful symptoms of methyl alcohol poisoning are those affecting the visual organs, which have been studied in detail in man by Lewin (5).

This author states that suddenly and without any warning signs, preceded or accompanied by general disturbances such as weakness, headaches, dizziness, stomach and intestinal pains, sweating, general weakness and drowsiness and rarely delirium, the following conditions are generally observed with the same intensity in both eyes after an incubation stage of 24-48 hours: muco-purulent conjunctivitis, eye pupil rigidity, accomodation disturbances, restriction of the visual field, scotoma, severe visual disturbances and, finally, total blindness. Alterations of the refractive media were never observed.

Ophthalmoscopic examination revealed white or slightly pale papillae, which were recognizable only in their external half; the retina vessels were generally dialated and sometimes narrow. In one case, atrophy of the optic nerve was found, accompanied by a slight dialation and twisting of the retinal veins, while the arteries were narrowed.

Treatment (cure) of intoxicated persons, if death can be avoided, rarely succeeds in achieving improvement and extremely rarely in a complete cure of ocular symptoms; on the contrary, a progressive deterioration is regularly noted, after an initial improvement of blindness, which leads to atrophy of the optic nerve.

In summary, according to information presently available, methyl alcohol causes blindness in man and animals (b) and may also lead to death. The toxic action, according to Harnack, is due not to the alcohol but to formic acid, its conversion product. Völtz and Dietrich also basically accept this theory by admitting that not only alcohol, but also its oxidation products, accumulate in tissues and kill the animals.

III. Deductions Derived From A Comparison Of Our Own Results With Those Of Other Authors

Let us now note if our studies can confirm, in some way or another, those reviewed above or whether they lead to other

conclusions. Our studies establish a series of rather distinct facts:

1. The major toxicity of formic acid and formaldehyde, in comparison to that of methyl alcohol, both on the isolated heart, its blood vessels and the whole animal.

2. The much shorter duration of survival of animals receiving the minimum lethal long-term dose of the first two compounds, given intravenously.

3. The constantly depressive symptoms produced by formaldehyde and formic acid, as compared to the long convulsive period produced by methyl alcohol.

4. The absence of lesions in the eyeground in animals given sublethal doses of formaldehyde and formic acid.

All these facts unanimously testify against the hypothesis that the toxicity of methyl alcohol is due to its oxidation products and particularly formic acid. In fact, the major toxicity and the rapidity of action of this acid lead us to believe that this compound does not have a cumulative action and that therefore it cannot be conceivably present in the organism until a toxic dose is reached. This induction is strengthened by two well-known facts: first, by the observation of Pohl (2) that formic acid, as it is gradually formed after administration of methyl alcohol, is eliminated with the urine; and second, from the now old concept that only a small portion of formic acid is oxidized in the animal organism and that the unconsumed portion is rapidly eliminated with the urine (7).

Added to all this is the observation now made by us that the symptoms of formic acid poisoning are completely different from those of methyl alcohol and that formic acid does not produce any symptoms affecting vision, even at dosages rather close to the minimum lethal dose.

Thus, a number of valid arguments are available to us, which make it possible to exclude the assumption that formic acid, formed in the organism by oxidation of methyl alcohol, may be the cause of the toxicity of the latter.

The same reasoning can be applied to formaldehyde, which is partially oxidized to formic acid and acts as such, but which is probably active also in part as its own molecule. Its toxicity greater than that of methyl alcohol, the different symptomatology and the absence of visual symptoms lead us to the same conclusion.

This whole set of facts justifies our affirmation that the toxicity of methyl alcohol is due to its own molecule. The fact that 37% of the methyl alcohol is found unmodified in the organism after 48 hours (we all know that such analyses can never be perfect and that losses of material are high) shows that the drug remains a long time unmodified in the organism and proves that it stays long in contact with all organs having a more delicate function and structure. Now, one of the most certain facts that we know about the activity of drugs is that this activity increases the longer the drugs are in contact with the elements upon which they act.

Let us imagine in our case a blood which is becoming an aqueous-alcoholic diluent (humectant), that stays in contact for many hours with nerve elements. It is clear that a washing of the organ is performed by this diluent, and that cellular substances are selected which are soluble and can enter into this diluent; it is also clear that this dissolving action will be the more effective and damaging to cellular elements the longer this washing process goes on. After 48 hours, in a dog to which Völtz and Dietrich administered a nonlethal dose of methyl alcohol, 37% of the alcohol was still present! Can we imagine what happens when the dose given is lethal or very close to a lethal one and the amount of drug remaining in the organs and in the blood is even greater?

If Sabbatani (8) was able to predict so brilliantly and with certainty the altered chemical composition of blood under the effect of ethyl alcohol (which breaks down so rapidly and so completely), if Ducceschi (9), in perfect agreement with Sabbatani, found such a significant cholesteremia in acute and chronic ethanol poisoning, if Reicher (10) and Cattoretti (11) were able to demonstrate a lipemia due to the effect of chloroform, it is not illogical in our case to conceive an action even more severe and deep-rooted. Moreover, it is a well known fact, used in the pharmaceutical, histological and industrial field, that the effectiveness of alcohols as solvents is related to the duration of their use.

However, along with this solvent action, we are also concerned with other types of action, of a chemical-physical and chemical nature. The chemical-physical actions include the rise in osmotic pressure effected by these compounds (8), the decrease in the dissociation of electrolytes (12), and the action on protein substances, an indicator of which is the modification of viscosity (13). It is noted, for example, that the action of ethyl alcohol on the viscosity of blood serum increases with an increase of the time during which alcohol exerts its action (14). We can therefore admit that this set of facts can also be confirmed for methyl alcohol, with all the conceivable consequences for the cellular activity. Another well-known fact is the property of methyl alcohol to fractionally precipitate serum protiens (15), upon which it thus exerts a selective power which ethyl alcohol does not have. This concept assumes a rather great importance when it is related to the prolonged time during which alcohol remains in contact with blood and tissues.

Finally, if we add to all these modifications of a chemical-physical nature also the chemical action of the molecule, we are

faced with a complex pattern which can give us a faint idea of all that can happen in the cells of the more delicate and noble organs in the animal organism under the effect of methyl alcohol. In addition, we have to point out the modifications, unknown to us, which the alcohol molecule can effect during its long contact with various organs.

In conclusion, we can state the following. The highly specific and demonstrated property of methyl alcohol to remain unchanged for a long time in tissues and blood provides the large number of modifications known to us and those, perhaps just as important, unknown to us; the demonstrated property of formic acid, which is just as important, to quickly abandon the organism and the speed of its action, as compared to that of methyl alcohol, thus prompt us to exclude the possibility that the toxicity of this alcohol is due to formic acid, its conversion product. The statement of Simon that, although the pattern of methyl alcohol poisoning is not the same as that of formic acid poisoning, we cannot attribute the toxicity of the former to the latter (16), is fully demonstrated in these experiments.

IV. Conclusions

1. The toxicity of methyl alcohol, which, as noted, follows a rather slow course, must be attributed to the molecule of the drug remaining as such for a long time in the organism, causing rather complex modifications of a chemical and chemical-physical nature, the severity of which is the greater the longer the alcohol remains in the tissues and in the blood.

2. Formaldehyde and formic acid display a rapid and violent pharmacological action, which has no similarity whatsoever with the toxic action of methyl alcohol and which never is the same as the action of the latter.

For these reasons, it appears impossible to still support today the statement that the toxicity of methyl alcohol is due to its oxidation products in the animal organism.

Summary

Numerous experiments carried out by the author on the isolated frog heart, on posterior limbs of the frog, and on the minimum long-term lethal dose by intravenous injection into rabbits prompt him to reach the unanimous conclusion that formic acid and formaldehyde are rather more toxic than methyl alcohol, that their action is much more rapid than that of the alcohol, that the pharmacological pattern of formic acid and formaldehyde is never the same, at any moment and in anyway, as that of methyl alcohol poisoning; also, that the toxicity of this alcohol must therefore be attributed to its prolonged presence in an unmodified state in the animal organism, where it is capable of producing various and severe modifications of a chemical and chemical-physical nature in the organs and in the blood.

Bibliography

1. Sammartino U. Research on the toxicity of methyl alcohol. I. Comparative action of methyl alcohol, formaldehyde and formic acid on the isolated frog heart. II. Comparative action of methyl alcohol, formaldehyde and formic acid on posterior limb vessels of the frog. III. Minimum long-term lethal dose, by intravenous injection, of methyl alcohol, formaldehyde and formic acid, and their relative symptomatology - see this Archivio, Vol. 56, No. 8

2. Pohl I.: Archiv. f. experimentelle Pathologie u. Pharmak. XXXI, 1893, 281.
3. Harnack E.: Deutsche medizinische Wochenschrift, XXXVIII, 1912, 358.
4. Völtz e Dietrich: Biochemische Zeitschrift, XL, 1912, 15.
5. Lewin L.: Medizinische Klinik, I. 1912, 95.
6. Noe M.: Biochimica e Terapia Sperimentale, Anno XVI, 1929, 355.
7. Schotten C.: Zeitschrift f. Physiol. Chemie, VII. 1882-83, 375; Grehant e Quinquad: Arch. de Physiol., IX (3ª serie), 1887, 197; Pohl I.: l. c. al n. (2).
8. Sabbatani L.: Archivio di Fisiologia, VII, 1909, 49.
9. Ducceschi V.: Archivio di Fisiologia, XIII, 1915, 147; XVI, 1918, 117 e 231; Ducceschi e Barilari: Id. id., XIV, 1916, 21.
10. Reicher: Zeit. f. Klin. Med., LXV, 1908.
11. Cattoretti F.: Giornale della R. Accademia di Medicina di Torino, Anno 77, 1914, 208.
12. Simon e Buglia: Rendiconti. della R. Accademia dei Lincei, XVI (1° Semestre), 1907, 418.
13. Simon I.: Archivio di Fisiologia, VI, 1908, 479.
14. Simon I.: Archivio di Fisiologia, IV, 1907-908, 594.
15. Simon I.: Archivio di Fisiologia, IV, 1907-908, 402.
16. Simon I.: Farmacologia, Utet, 1930, pag. 521.

RICERCHE SULLA TOSSICITA' DELL'ALCOOL METILICO.
IV. RIASSUNTO CRITICO DELLE RICERCHE IN ARGOMENTO ED INTERPRETAZIONE PIU' RAZIONALE DEL MODO DI AGIRE DELL'ALCOOL METILICO.

Prof. U. Sammartino

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I. – Risultato delle esperienze personali.

Nelle note precedenti abbiamo studiato comparativamente tre punti dell'azione dell'alcool metilico, dell'aldeide formica, dell'acido formico (1).

Riassumiamo ora brevemente i risultati ottenuti.

I. Sul cuore di rana isolato con cannula *Straub* siamo giunti alle seguenti conclusioni:

Il cuore di rana isolato si arresta definitivamente in modo irreversibile con gr. mol. 0,00434 per litro di acido formico, con gr. molecole 0,03333 di aldeide formica. Invece con alcool metilico l'arresto si ha con gr. mol. 6,25 per litro ed il cuore si riprende lavando con *Ringer*.

II. L'alcool metilico dilata i vasi del treno posteriore della rana preparata alla *Lawen-Trendelenburg* assai meglio che dosi equimolecolari di aldeide formica e di acido formico. Questi ultimi due farmaci (e più l'acido che l'aldeide) con dosi più elevate delle dilatatrici spiegano un notevole potere vasocostrittore, che si esagera col crescere ulteriore delle dosi. Invece l'azione vasodilatatrice dell'alcool metilico è ancora chiaramente rilevabile con una dose che è oltre *quattromila* volte maggiore della dose più piccola di aldeide formica atta a produrre una vasocostrizione apprezzabile ed oltre *settemila* volte superiore alla dose di acido formico che produce lo stesso effetto.

III. La dose minima letale lontana per via endovenosa dell'acido formico nel coniglio è assai più elevata della dose corrispondente di aldeide formica: questa ultima dose alla sua volta è molto superiore a quella di alcool metilico. La durata in vita degli animali trattati con la dose minima letale lontana di alcool metilico è assai superiore alla sopravvivenza degli animali che abbiano ricevuto la dose corrispondente di aldeide formica e di acido formico. Con questi due farmaci manca ogni lesione del fondo dell'occhio osservata invece per l'alcool metilico.

La sintomatologia infine è molto diversa: la paralisi che dà la morte con alcool metilico è preceduta da una lunga serie di violente convulsioni, che mancano completamente con l'aldeide formica e con l'acido formico, ad azione costantemente depressiva.

In conclusione tutte queste esperienze portano ad un risultato unico e sicuro: che, fra i tre farmaci posti a confronto, l'acido formico è più tossico dell'aldeide formica e questa alla sua volta più dell'alcool metilico e che mai nè con l'aldeide formica, nè con l'acido formico si riuscì a riprodurre il quadro dell'intossicazione da alcool metilico.

II. – Risultati delle esperienze che precedettero le nostre ed opinioni correnti sulla tossicità dell'alcool metilico.

Riepiloghiamo ora brevemente le opinioni concernenti l'azione tossica dell'alcool metilico.

Il *Pohl* aveva osservato che, introducendo nell'organismo dell'alcool metilico, vi subisse un'ossidazione lenta fino ad acido formico (2). Questo fatto venne confermato dall'*Harnack*, (3) il quale credette di trovarvi la ragione della tossicità particolare dell'alcool. Egli pensa che la trasformazione di questo in acido formico, in rapporto specialmente al punto dell'organismo in cui essa avviene, può essere funesta nelle sue conseguenze, essendo questo acido il più tossico di tutti gli acidi della sua serie omologa. L'alcool metilico, introdotto nell'organismo, verrebbe attirato in speciale modo da determinate parti del sistema nervoso centrale, da elementi nervosi della retina, del nervo ottico e dai suoi centri: in questi punti avverrebbe una lenta ossida-

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zione atta a trasformare l'alcool metilico in acido formico, il quale lederebbe queste parti in modo disastroso, irritandole e paralizzandole: si spiegherebbero così la cecità, i crampi e le paralisi che si osservano negli animali e nell'uomo. Così diventerebbe pure comprensibile il fatto che le manifestazioni della intossicazione da alcool metilico si differenziano da quelle dell'alcool etilico.

La lunga permanenza dell'alcool metilico nei tessuti e nel sangue fu dimostrata da *Völtz* e *Dietrich* (4). Questi AA., somministrata a cani una dose fissa di alcool metilico, determinarono per un certo tempo la quantità che viene eliminata con l'aria espirata e quella che passa nelle urine trasformata in acido formico e videro che in 48 ore se ne elimina solo una parte, perchè, uccisi gli animali e dosando l'alcool metilico esistente nei tessuti, ne trovarono ancora il 37 %. Conclusero perciò che, introducendo ripetutamente negli animali superiori grandi quantità di alcool metilico, si ha nei tessuti l'accumulo del farmaco e del suo prodotto di ossidazione, l'acido formico, e ne consegue per questo la morte degli animali.

È poi noto che fra i più temibili sintomi dell'avvelenamento da alcool metilico sono quelli a carico degli organi della visione studiati nell'uomo minutamente da *Lewin* (5). Egli dice che, improvvisamente senza segni premonitori, preceduti od accompagnati da disturbi generali (fiacchezza, cefalea, vertigini, dolori di stomaco e d'intestino, sudore, debolezza generale, sonnolenza, raramente delirio) si presentano generalmente in entrambi gli occhi con la stessa intensità, dopo uno stadio di incubazione di 24-48 ore: congiuntivite muco-purulenta, rigidità della pupilla, disturbi di accomodazione, limitazione del campo visivo, scotomi, gravi disturbi visivi e, perfino, una completa cecità. Non rilevò mai alterazioni dei mezzi rifrangenti.

All'esame oftalmoscopico le papille erano bianche o leggermente pallide, ovvero riconoscibili solamente nella loro metà esterna; i vasi della retina generalmente erano dilatati, qualche volta ristretti. In un caso trovò atrofia del nervo ottico, accompagnata da leggera dilatazione ed attorcigliamento delle vene della retina mentre le arterie erano ristrette.

La cura degli individui intossicati, se riuscì a scongiurare la morte, raramente valse ad ottenere un miglioramento e rarissimamente una guarigione completa dei sintomi oculari, ma invece, regolarmente, dopo un primo miglioramento della cecità si ebbe un peggioramento progressivo che condusse all'atrofia del nervo ottico.

Riassumendo: l'alcool metilico ,secondo quanto oggi si ammette, provoca la cecità nell'uomo e negli animali (6) e può produrre anche la morte. L'azione tossica, secondo *Harnack*, sarebbe dovuta non all'alcool, ma all'acido formico, suo prodotto di trasformazione: anche *Völtz* e *Dietrich* accettano in fondo questa teoria quando ammettono che non solo l'alcool, ma i suoi prodotti di ossidazione si accumulino nei tessuti ed uccidano gli animali.

III. — Deduzioni che scaturiscono dal confronto fra i nostri risultati e quelli degli altri Autori.

Osserviamo ora se le nostre ricerche possono in qualche modo confermare queste vedute o se, invece, portino ad altre conclusioni.

Esse stabiliscono una serie di fatti assai distinti:

1° La maggiore tossicità dell'acido formico e dell'aldeide formica rispetto all'alcool metilico sia sul cuore isolato, sia sui vasi, sia sull'animale intiero;

2° La molto minore durata della sopravvivenza dell'animale al quale si somministrino i primi due farmaci nella dose minima letale lontana per via endovenosa;

3° La sintomatologia costantemente depressiva prodotta dall'aldeide formica e dall'acido formico in confronto al lungo periodo convulsivo che l'alcool metilico produce;

4° La mancanza di lesioni del fondo dell'occhio negli animali trattati con dosi sub-letali di aldeide formica e di acido formico.

* * *

Tutti questi fatti concordemente depongono contro l'ipotesi che la tossicità dell'alcool metilico sia dovuta ai suoi prodotti di ossidazione e particolarmente all'acido formico. Infatti la maggiore tossicità e la rapidità dell'azione di questo acido fanno pensare che il farmaco sia sfornito di azione cumulativa e che possa perciò immagazzinarsi nell'organismo fino a raggiungervi la dose tossica. Questa induzione è avvalorata da due fatti

ben noti: anzitutto dalla constatazione di *Pohl* (2) che l'acido formico a mano a mano che si forma dopo somministrazione di alcool metilico viene eliminato con le urine; in secondo luogo dalla nozione ormai antica che l'acido formico solo in piccola parte viene ossidato nell'organismo animale e che la parte non bruciata si elimina rapidamente con le urine (7).

A tutto questo si aggiunga la constatazione fatta ora da noi che la sintomatologia dell'avvelenamento da acido formico è completamente diversa dalla sintomatologia dell'avvelenamento da alcool metilico e che l'acido non produce alcun sintomo a carico della visione, anche con dosi assai vicine alla minima letale.

Noi dunque possediamo una serie di validi argomenti atti a farci escludere che l'acido formico che si forma nell'organismo per ossidazione dell'alcool metilico possa ritenersi causa della tossicità di questo.

Gli stessi ragionamenti potremmo ripetere per l'aldeide formica, che si ossida in parte ad acido formico e che agisce come tale, ma che probabilmente è attiva anche in parte per la sua molecola. La tossicità sua maggiore dell'alcool metilico, la sintomatologia diversa, l'assenza di sintomi visivi ci portano alla stessa conclusione.

* * *

Tutto questo complesso di fatti giustifica la nostra affermazione che la tossicità dell'alcool metilico sia dovuta alla sua molecola. Il fatto che dopo 48 ore se ne trova il 37 % immodificato nell'organismo (ognuno sa che analisi di questo genere non possono essere mai perfette e che molte sono le perdite di materiale) ci dimostra che il farmaco permane a lungo *immodificato* nell'organismo, ci dà la prova che sta molto tempo in contatto con tutti gli organi a funzione ed a struttura più delicata.

Ora uno dei fatti più sicuri che noi conosciamo sull'attività dei farmaci è che questa diventa maggiore quanto più a lungo essi stanno in contatto con gli elementi su cui agiscono.

Immaginiamo nel caso nostro un sangue che sia diventato un mestruo idro-alcoolico il quale venga in contatto per molte e molte ore con elementi nervosi. È chiaro che pratica un lavaggio dell'organo; è chiaro che scioglie delle sostanze cellulari che sono solubili nel mestruo e che vi passano; è chiaro che questa azione solvente sarà tanto più efficace e tanto più dannosa

per gli elementi cellulari quanto più a lungo il lavaggio dura. Dopo 48 ore, nel cane cui era stata somministrata da *Völtz* e *Dietrich* una dose *non letale* di alcool metilico, ne permaneva il 37 %! Si immagini quello che avviene quando la dose somministrata *è letale* o molto vicina ad essa e la quantità di farmaco che permane negli organi e nel sangue è ancora più elevata!

Se *Sabbatani* (8) poteva brillantemente prevedere con sicurezza la mutata composizione chimica del sangue per azione dell'alcool etilico (che pure brucia tanto rapidamente e quasi completamente); se *Duceschi* (9), in perfetto accordo con le vedute di *Sabbatani*, trovava una colesterinemia tanto notevole nell'avvelenamento acuto da alcool etilico e nel cronico, se *Reicher* (10), *Cattoretti* (11) potevano dimostrare una lipemia per effetto della cloroformizzazione, non è illogico nel caso nostro pensare ad un'azione ancora più grave e profonda. D'altra parte che gli alcooli agiscano come solventi tanto meglio quanto più dura la loro azione è un fatto noto e sfruttato nell'arte farmaceutica, nell'istologia, nell'industria.

Ma, a lato di quest'azione solvente, abbiamo una serie di altre azioni: chimico-fisiche e chimiche. Tra le chimico-fisiche l'aumento della pressione osmotica che essi determinano (8), la diminuzione della dissociazione degli elettroliti (12), l'azione sulle sostanze proteiche di cui è indice la modificazione della viscosità (13). È noto, ad es., che l'azione dell'alcool etilico sulla viscosità del siero di sangue cresce col crescere del tempo durante il quale agisce l'alcool (14). Si può perciò ammettere che questo complesso di fatti si verifichi anche per l'alcool metilico, con tutte le conseguenze sulle attività cellulari che si possono intravedere. Un altro fatto che si conosce bene è la proprietà posseduta dall'alcool metilico di precipitare frazionatamente le proteine del siero (15), sulle quali avrebbe dunque un potere di scelta che l'alcool etilico non ha. Questa nozione acquista un'importanza assai grande, quando la si mette in relazione col lungo permanere dell'alcool in contatto del sangue e dei tessuti.

Aggiungiamo finalmente a tutte queste modificazioni d'ordine chimico-fisico l'azione chimica della molecola, e noi avremo sotto gli occhi un quadro complesso che può darci un'idea pallida di quello che può avvenire nelle cellule degli organi più delicati e più nobili dell'organismo animale per effetto dell'alcool metilico. A lato di esse dobbiamo porre le modificazioni a noi

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ignote che la molecola dell'alcool nel suo lungo contatto può portare.

In conclusione si può affermare quanto segue. La particolarissima, dimostrata, proprietà dell'alcool metilico di rimanere a lungo immodificato nei tessuti e nel sangue ove apporta la massa di modificazioni a noi note e quelle, forse altrettanto importanti, ignote, la non meno importante dimostrata proprietà dell'acido formico di abbandonare rapidamente l'organismo e la rapidità della sua azione in confronto di quella dell'alcool metilico, ci portano concordemente ad escludere che la tossicità del detto alcool sia dovuta all'acido formico, suo prodotto di trasformazione. L'affermazione di *Simon* che, non riproducendo l'avvelenamento da acido formico il quadro dell'avvelenamento di alcool metilico, non si può attribuire al primo la tossicità del secondo (16), trova in queste esperienze piena dimostrazione.

IV. — Conclusioni.

1. La tossicità dell'alcool metilico la quale, com'è noto, si svolge con una certa lentezza, si deve attribuire alla molecola del farmaco che rimane a lungo come tale nell'organismo producendovi modificazioni d'ordine chimico e chimico-fisico assai complesse e tanto più gravi quanto più dura la sua permanenza nei tessuti e nel sangue.

2. L'aldeide formica e l'acido formico spiegano un'azione farmacologica che è rapida, violenta e che non ha alcun punto di contatto con l'azione tossica dell'alcool metilico, di cui non riproducono mai il quadro dell'azione.

Per queste ragioni non pare che si possa oggi sostenere ancora che la tossicità dell'alcool metilico sia dovuta ai suoi prodotti di ossidazione nell'organismo animale.

RIASSUNTO

Le numerose esperienze fatte dall'A. sul cuore isolato di rana, sui vasi del treno posteriore di rana, sulla dose minima letale lontana per via endovenosa nel coniglio, lo portano concordemente a concludere che l'acido formico e l'aldeide formica sono assai più tossici dell'alcool metilico; che la loro azione è molto più rapida di quella dell'alcool; che il quadro farmacologico da essi prodotto non riproduce in alcun momento ed in modo alcuno il quadro dell'intossicazione da alcool metilico; che la tossicità di quest'alcool si deve perciò attribuire al fatto che permane a lungo immodificato nell'organismo animale ove ha campo di produrre svariate e gravi modificazioni d'ordine chimico e chimico-fisico negli organi e nel sangue.

LETTERATURA

(1) Sammartino U.: *Ricerche sulla tossicità dell'alcool metilico.* — I. *Azione comparata dell'alcool metilico, dell'aldeide formica, dell'acido formico sul cuore isolato di rana.* II. *Azione comparata dell'alcool metilico, dell'aldeide formica, dell'acido formico sui vasi del treno posteriore della rana.* III. *Dose minima letale lontana per via endovenosa dell'alcool metilico dell'aldeide formica dell'acido formico e sintomatologia relativa.* vedi questo Archivio, rispettivamente Vol. LVI fasc. 8.

(2) Pohl I.: Archiv. f. experimentelle Pathologie u. Pharmak. XXXI, 1893, 281.

(3) Harnack E.: Deutsche medizinische Wochenschrift, XXXVIII, 1912, 358.

(4) Völtz e Dietrich: Biochemische Zeitschrift, XL, 1912, 15.

(5) Lewin L.: Medizinische Klinik, I, 1912, 95.

(6) Noè M.: Biochimica e Terapia Sperimentale, Anno XVI, 1929, 355.

(7) Schotten C.: Zeitschrift f. Physiol. Chemie, VII, 1882-83, 375; Grèhant e Quinquaud: Arch. de Physiol., IX (3ª serie), 1887, 197; Pohl I.: l. c. al n. (2).

(8) Sabbatani L.: Archivio di Fisiologia, VII, 1909, 49.

(9) Ducceschi V.: Archivio di Fisiologia, XIII, 1915, 147; XVI, 1918, 117 e 231; Ducceschi e Barilari: Id. id., XIV, 1916, 21.

(10) Reicher: Zeit. f. Klin. Med., LXV, 1908.

(11) Cattoretti F.: Giornale della R. Accademia di Medicina di Torino, Anno 77, 1914, 208.

(12) Simon e Buglia: Rendiconti della R. Accademia dei Lincei, XVI (1° Semestre), 1907, 418.

(13) Simon I.: Archivio di Fisiologia, VI, 1908, 479.

(14) Simon I.: Archivio di Fisiologia, IV, 1907-908, 594.

(15) Simon I.: Archivio di Fisiologia, IV, 1907-908, 402.

(16) Simon I.: Farmacologia, Utet, 1930, pag. 521.

FORMIC ACID

FS + TA 1, 11 J 996 (1969)

150

11 J 996
[Modified steam distillation method for the determination of formic acid in fruit syrups.]
Sarudi, I., Jr.
Konzerv-es Paprikaipar 1969 (2) 32-34 (1969) [6 ref. Hu, de, ru] [Minosegvizsgalo Intezet, Szekesfehervar, Hungary]

DETECTION

Steam distillation of fruit syrup samples is carried out in a modified micro-Parnas-Wagner apparatus originally used for micro-N determinations. A 10-g sample is washed with distilled water in the apparatus, the condenser of which is fitted with a 250 ml fractionation flask. 2 g $CaCO_3$ and 100 ml distilled water are transferred to this flask. The condenser tube is extended so as to end 1-2 mm above flask bottom. During distillation (for 1 h), heating is controlled to maintain the initial vol. of suspension in the receiver flask. The procedure is the same as for N determination, but no water is circulated in the condenser. Using this method the formic acid contents of strawberry, blackberry, wild pear and raspberry syrups, were found to be 0.80, 1.41, 0.65 and 1.00-1.63 g/kg, respectively. On adding 10.7-36.6 mg formic acid to 10 g samples of the above syrup types, differences between added and recovered formic acid contents ranged from -1.1 to +0.7 mg. IF

FORMIC ACID

CA 49, 1987c (1955)

284

Content of formic acid in fruit and fruit products. B. A. J. Sedláček and St. Procházka (Ústav hyg., Prague). *Průmysl Potravin* 5, 491-3(1954).—Several methods of estg. formic acid (I) were compared and over 150 tabulated results are reported. Content of I in 29 kinds of fruit never exceeded 2.5 mg. %, except in peaches (3.34 mg. %). Content of I in 29 fruit products (both in cans and glass containers) differs only slightly from that in the fresh fruit and never exceeds 4 mg. %, except in the peach compote (4.02 mg. %). Packing material and storage do not affect substantially the content of I and it is not raised by technological processes either. L. J. Urbánek

DETECTION

JOURNAL OF THE A.O.A.C. (Vol. 46, No. 3, 1963)

Quantitative Determination of Formic, Acetic, Propionic, and Butyric Acids by Gas Chromatography

By RUTH N. SHELLEY, HAROLD SALWIN, and WILLIAM HORWITZ (Division of Food, Food and Drug Administration, Washington 25, D.C.)

Volatile acids were separated from food samples by a steam distillation according to AOAC methods, neutralized, and evaporated to dryness. The acids were then liberated with an equivalent amount of 0.5*N* dichloroacetic acid in acetone. Three microliters of the acetone solution containing from 0.1 to 1.0 mg of each acid per ml was injected directly into the column of the gas chromatograph. Experimental conditions were: Six-foot glass column; support, Anakrom 110/120 mesh; substrate, 10.5% ethyleneglycol adipate and 1.75% phosphoric acid; column temperature, 100°C; Sr^{90} detector temperature, 170°C; vaporizer temperature, 200°C; detector voltage, 1400 volts; argon flow rate, 50 ml per minute.

Peak heights and responses relative to methyl enanthate (heptanoate) as an internal standard were used to calculate concentrations of the individual acids. Recoveries from known mixtures were 95–105% for lower concentrations of the acids and 97–101% for higher concentrations. Results of analyses of frozen whole eggs and of frozen fish fillets agreed with results by AOAC methods.

Volatile acids are important as indexes of decomposition in many foods and as constituents of food flavors. The characteristic increase of the volatile acids content during decomposition of foods provides a means for classifying the different stages of the decomposition process (1, 2). Separation, identification, and quantitation of the volatile acids also offers valuable information on the composition of food flavors.

According to AOAC methods (3), volatile acids are isolated from food products by steam distillation. Acetic acid and the higher homologs are separated by column chromatography and determined by titration of the eluted fractions. Formic acid is analyzed separately by reaction with mercuric chloride. The application of quantitative gas chromatography was investigated for the following advantages: A single procedure for all of the volatile acids, improved specificity and sensitivity, and greater speed. For regulatory purposes, the chromatograms should provide useful confirmation of the results obtained by the AOAC methods.

Experimental

This paper outlines the method which was adopted and the several approaches taken in developing it. During the development of this method the gas chromatography of the free acids and of their methyl, ethyl, and benzyl esters was explored. The methyl and ethyl esters were easily chromatographed on column supports coated with various polyesters at temperatures below 100°. However, quantitative recovery of the esters from the esterified mixture was difficult because of their high volatility and appreciable solubility in water. In addition, methyl alcohol and ethyl alcohol interfered with the chromatographic separation because the retention times of the alcohols were similar to those of the corresponding esters. Molecular sieves were used for removing ethyl alcohol but with limited success. These unique problems are not encountered in the gas chromatography of methyl esters of the higher fatty acids which have high boiling points and are insoluble in water. Alcohol and volatile solvents can be removed without appreciable loss of these esters. In order to work with higher boiling esters, benzyl esters of C_1 to C_4 acids were prepared and chromatographed on polyester columns at 175°. The chromatograms were satisfactory.

but incomplete esterification and the formation of polymeric byproducts limited the usefulness of this approach.

Because of the difficulties encountered with esterification procedures, methods involving gas chromatography of the free acids were investigated. Ether solutions of the C_1 to C_4 acids were chromatographed on Chromosorb column support coated with dimer acid-silicone oil 550. The instrument used was a Perkin-Elmer Model 154-B Fractometer equipped with filament detector, metal injection port, and metal column. Figure 1 shows that the formic acid was completely lost. Similar results were obtained with other column substrates, each containing a small percentage of nonvolatile organic acid. A Research Specialties gas chromatograph equipped with ionization detector, metal injection port, and metal column was also used. Sensitivity was improved, but the formic acid could not be detected, and the chromatograms were similar to Fig. 1. The metal system probably catalyzed the decomposition of formic acid to carbon dioxide and hydrogen.

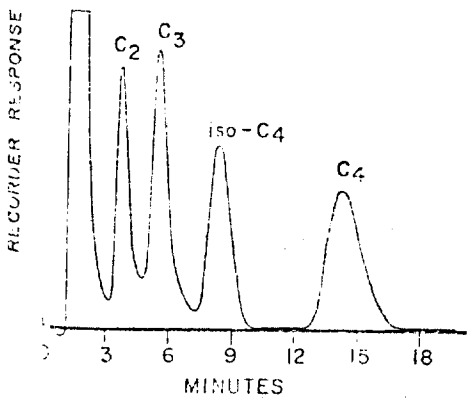


Fig. 1—Chromatogram of C_2–C_4 Acids. C_2, Acetic; C_3, Propionic; Iso-C_4, Isobutyric; and C_4, Butyric Acid.
Instrument: Perkin Elmer 154B, Katharometer Detector. Current: 280 ma; Column 6′ × ¼″, 25% Dimer Acid-Silicone Oil 550 on Chromosorb W, 60 mesh; Carrier Gas: Helium; Flow Rate: 20 ml/min.; Chart Speed: 20 inch/hr; Attenuation: X3; Column Temp.: 123°C.

The metal injection port of the Fractometer was replaced with the Perkin-Elmer glass dipper injection system. Figure 2 shows that formic acid was separated and

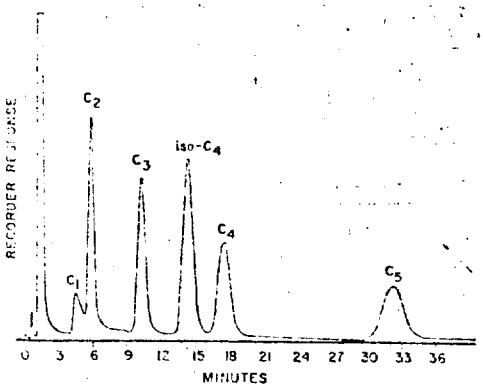


Fig. 2—Chromatogram of C_1–C_5 Acids. C_1, Formic; C_2, Acetic; C_3, Propionic; C_4, Butyric; C_5, Valeric Acid.
Instrument: Perkin Elmer 154B, Katharometer Detector, with glass microdipper injection system. Current: 290 ma; Column: 6′ × ¼″, 25% Didecylphthalate, 2% Sebacic Acid on Chromosorb W, 60 mesh. Carrier Gas: Helium; Flow Rate: 40 ml/min.; Chart Speed: 20 inch/hr; Attenuation X32; Column Temp.: 136°C.

detected in a mixture of C_1 to C_5 acids which contained equal amounts of formic and acetic acids. However, the small peak for formic acid demonstrates that the recovery of formic acid was not complete. The protection afforded by the glass injection system at the point of highest temperature accounted for the partial recovery of formic acid.

Because of the small percentages of volatile acids encountered in our food decomposition studies, it was necessary to have greater sensitivity than that provided by the filament detector. Since hydrogen-flame detectors do not respond to formic acid, the work was continued with a Research Specialties gas chromatograph equipped with a strontium-90 detector. It was necessary to modify the instrument in order to have the advantage of an all-glass injection and column system. The instrument was originally equipped with a horizontal metal injection port leading into a 6′ stainless steel column by means of 1/16″ stainless steel tubing. The metal housing at the top of the column oven was replaced by a modified housing which accommodated a Barber-Colman type 6′ glass column. The flash heater was disconnected from the metal injection port, connected firmly to the injection zone of the glass column, and supported by the metal housing

The gas connections were rearranged to permit bypassing the metal injection port and injecting directly into the column. Formic acid and the other acids were then chromatographed satisfactorily.

Hunter, *et al.* (4) reported a method for the gas chromatography of free acids from acetic to capric acid on a diethyleneglycol adipate column. They started with the sodium salts and liberated the acids with dichloroacetic acid in acetone. The free acids were soluble in acetone but the sodium dichloroacetate was insoluble. Liberation of the acids in a nonaqueous medium and simplicity of operation were distinct advantages of their procedure. It was therefore adopted in principle, but for our application it was necessary to make the following changes: (*a*) The concentration of dichloroacetic acid was reduced from 7*N* to 0.5*N* in order to permit more accurate acidification of the sodium salts. By avoiding a large excess of dichloroacetic acid, the stability of the final solution was improved. (*b*) Phosphoric acid was included in the column substrate to achieve quantitative recovery of formic acid as well as of other volatile acids. (*c*) An internal standard or marker of known concentration was added to each solution as a reference for calculating acid concentrations. (*d*) The column temperature was lowered from 120° to 100° to insure good resolution of the peaks for the solvent, internal standard, formic acid, and acetic acid.

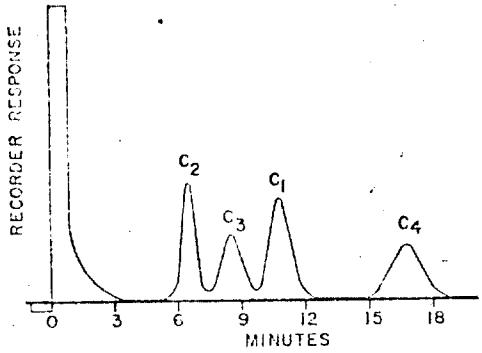


Fig. 3—Chromatogram of C_1–C_4 Acids. C_2, Acetic; C_3, Propionic; C_1, Formic; and C_4, Butyric Acid. Instrument: Modified Research Specialties Gas Chromatograph, Ionization Detector Si-90. High Voltage: 1430 volts. Column 6' × ½", glass; 12% Carbowax 1540, 5% H_3PO_4 on Anakrom A, 100 mesh. Carrier Gas: Argon; Flow Rate: 65 ml/min.; Chart Speed: 30 inch/hr; Attenuation: X10; Detector Oven Temp.: 170°C; Injection Zone Temp.: 200°C; Column Temp.: 103°C.

Figure 3 shows a chromatogram obtained with a Carbowax-phosphoric acid column. The order of peak emergence was acetic, propionic, formic, and butyric acids. Isobutyric acid had the same retention time as propionic acid. A normal order of emergence and better resolution were obtained with a column coated with ethyleneglycol adipate-phosphoric acid (Figs. 4 and 5).

METHOD

Reagents

(a) *Acetone.*—Redistilled.

(b) *Dichloroacetic acid (DCA) solution (0.5N).*—Redistill and weigh 0.645 g DCA into a 10 ml volumetric flask, and dilute to 10 ml with acetone. Prepare daily.

(c) *Methyl enanthate.*—Weigh 250 ml anhydrous methanol in a 500 ml flask, cool in an icebath, and transfer to hood. Bubble BF_3 slowly through a glass tube into the methanol until 31.3 g BF_3 is taken up. BF_3 must be passing through the glass tube before the tube is placed in methanol (5). Weigh 7 g enanthic acid (heptanoic acid) (b.p. 223°) into an erlenmeyer and add 70 ml of the 12.5% by weight BF_3-methanol solution. Boil 3 minutes on steam bath. Cool, and transfer to a 750 ml separator containing 210 ml H_2O and 140 ml petroleum ether. Shake 3 minutes. Remove lower layer. Add 10 ml 5% Na_2CO_3 solution to separator and shake. Remove lower layer. Add 20 ml H_2O to separator, shake, and draw off H_2O; repeat once. Filter petroleum ether extracts through funnel with glass wool plug covered with Na_2SO_4 into erlenmeyer. Evaporate the solvent to 40 ml on steam bath; complete solvent removal in N stream. Distill residual methyl enanthate, collecting the fraction boiling at 172–172.5°. Store in tightly stoppered glass vial in refrigerator.

(d) *Methyl enanthate (ME) internal standard solution.*—Dilute about 0.35 g methyl enanthate, weighed to nearest mg, to 100 ml with acetone.

(e) *Dilute methyl enanthate (dil. ME) internal standard.*—Dilute 1 ml ME internal standard solution to 10 ml with acetone.

(f) *Standard acid solutions.*—Formic, acetic, propionic, and butyric acids, 0.05*N*. Check purity of stock solutions of acids initially by gas chromatography. Prepare acid dilution

for calibration by pipeting the calculated volume of each acid into a separate 200 ml volumetric flask (0.39 ml formic, 0.57 ml acetic, 0.75 ml propionic, and 0.92 ml butyric acid). Dilute to mark with acetone. Pipet a 20 ml aliquot of acid dilution into an erlenmeyer, add 20 ml water and 2 drops phenolphthalein, and determine the acid content by titrating with 0.05*N* NaOH.

$$\text{mg acid/ml standard soln} = \text{ml } 0.05N \text{ NaOH} \times F/20,$$

where F = weight of acid equivalent to 1 ml 0.05*N* NaOH: 2.30 mg formic, 3.00 mg acetic, 3.70 mg propionic, and 4.40 mg butyric acid.

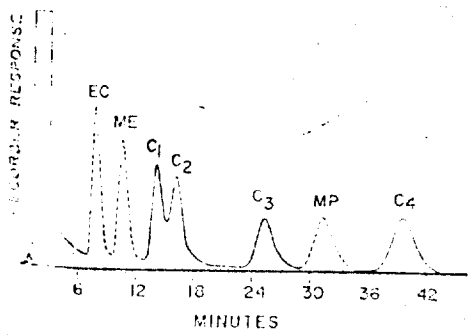
Apparatus

(a) *Gas chromatograph.*—Use gas chromatograph equipped with an argon ionization detector and an all-glass injection and column system.

Parameters for modified Research Specialties gas chromatograph:

Column temperature 100°, detector temperature 170°, injection zone temperature 200°, argon flow rate 50 ml/min., high voltage 1400 volts, attenuation × 5, and 6′ glass column.

Optimum conditions for the gas chromatographic separation are obtained with the peaks for solvent, internal standard, and formic acid are completely resolved. Standard and acid peaks are sharp, although formic and acetic acid peaks are not completely resolved. Conditions vary to some degree from instrument to instrument and should be experimentally re-established.



Chromatogram of C_1–C_4 Acids and Internal Standards. C_1, Formic; C_2, Acetic; C_3, Propionic; C_4, Butyric Acid; EC (Ethyl Caproate), ME (Methyl Caprate), and MP (Methyl Palmitate) Internal Standards. Modified Research Specialties Gas Chromatograph, Ionization Detector Sr-90. High Voltage: 1400 volts. Column: 6′ × ¼″, glass, 10.5% Ethylene Glycol Adipate, 1.75% H_3PO_4 on Anakron ABS, 80–120 mesh. Carrier Gas: Argon; Flow Rate: 50 ml/min; Chart Speed: 20 inch/hr; Attenuation: X5; Detector Oven Temp.: 170°C; Injection Zone Temp.: 200°C; Column Temp.: 100°C

(b) *Preparation of column packing.*—Weigh 1.3 g of ethylene glycol adipate (Applied Science Laboratories, State College, Pa.), into a 150 ml beaker and dissolve by stirring in 70 ml acetone. Into second beaker weigh 0.26 g phosphoric acid and dissolve in 30 ml acetone. Combine both solutions. Weigh 12.5 g Anakron ABS (Analabs) into 500 ml round-bottom flask, add combined acetone solutions, and evaporate in a rotating evaporator at reduced pressure in water bath at 35° (will pack one 6′ column).

Calibration

Pipet into same 5 ml volumetric flask a volume of each standard acid solution and a volume of ME internal standard, as below:

Individual Acid Concn in Sample, mg	*C_1–C_4 Acid Std Soln (0.05N), ml*	*Vol. of Internal Std Added, ml*	*Concn*
less than 1	0.25–0.5	1.0	(c)
1.0–2.5	0.5–1.0	0.5	(d)
2.0–5.0	1.0–2.0	1.0	(d)

*See footnote.

Dilute to mark with acetone and mix. Repeat, using varying volumes of acids to provide a range of concentration for each acid (see Table I). Inject 3 μl aliquots into gas chromatograph. Measure peak heights on chromatograms and calculate *R* value for each acid:

$R_A = h_A c_{ME}/c_A h_{ME}$, where h_A and h_{ME} are the peak heights of the acid and methyl enanthate, respectively, and c_A and c_{ME} are the corresponding concentrations (mg per 5 ml acetone solution).

Determination

Quantitatively neutralize the steam distillate of the volatile acids obtained by AOAC method 18.019 with 0.01*N* NaOH and phenolphthalein as indicator. Add a known excess of base (1 ml 0.1*N* NaOH). If color is discharged, repeat as below. Concentrate to about 15 ml on steam bath in air stream. If color is discharged, add 0.5 ml more 0.1*N* NaOH, transfer the solution to a 50 ml beaker, and evaporate to dryness in air stream. Loosen the salts with a micro spatula and transfer carefully through a funnel (1.5 cm diameter) into

a tube-type, glass-stoppered volumetric flask (see table, last column, for volume).

0.01N NaOH for Neutralization of Steam Distillate (Before Adding Excess), ml	Volatile Acid Content as Acetic Acid, mg	Vol. of Internal Std Added, ml	Conc'n[b]	Total Vol. After Dilu, ml
1.0–1.5	0.6–0.9	1.0 (dil)	(c)	2.0
1.5–3.0	0.9–1.8	0.5	(d)	5.0
3.0–6.0	1.8–3.6	1.0[a]	(d)	5.0
6.0–8.0	3.6–4.8	1.0	(d)	5.0
9.0–12.0	5.4–7.2	2.0	(d)	10.0

[a] For fish samples, add 1 ml ME internal standard; for egg samples, add only 0.5 ml (because the volatile acids are more evenly distributed between formic and acetic acids).
[b] See *Reagents*.

Add to beaker, rinsing walls, volume of 0.5N DCA equivalent to total NaOH added to distillate. Transfer through funnel into the volumetric flask. Repeat the rinsing with 0.5 ml or more acetone, and add to volumetric flask. Swirl the flask to disperse the salts and to liberate the volatile acids. Pipet a volume of dilute ME standard or ME standard (as specified for acid content of sample in table above) to volumetric flask and mix. Centrifuge 3 min. at low speed in the glass-stoppered tube to separate the precipitated sodium dichloroacetate. Inject 3 μl of clear solution in gas chromatograph. Measure peak heights and calculate acid concentrations with R values established in calibration. $c_X = h_X c_{ME}/R_X h_{ME}$.

To calculate mg of each acid in weight of sample used for distillation, divide by fraction recovered in distillation. Typical recoveries for first 200 ml distillate are: Formic acid 0.405, acetic acid 0.57, propionic acid 0.81, and butyric acid 0.92 (6).

Results and Discussion

Internal Standard.—In order to obtain quantitative results it was necessary to correct for variations in volume of sample injected, and for variations in the sensitivity of the detector from run to run. With known amount of marker compound or internal standard added to each acetone solution of acids it was possible to correct for such variations.

Figure 4 shows the positions of three esters which can be used as standards: Ethyl caproate (EC), methyl enanthate (ME) and methyl pelargonate (MP). Methyl octanoate interferes with the acetic acid peak. Because of the emphasis on form

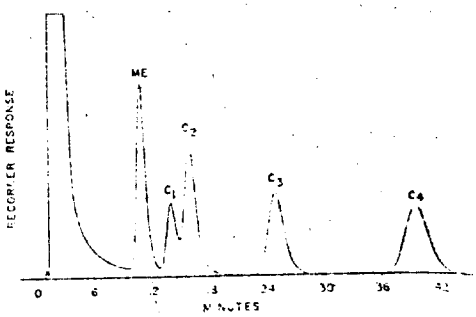


Fig. 5—Chromatogram of C_1–C_4 Acids and Methyl Enanthate. ME (Methyl Enanthate), C_1, Formic; C_2, Acetic; C_3, Propionic; and C_4, Butyric Acid. Instrument: Modified Research Specialties Gas Chromatograph, Ionization Detector Sr-90. High Voltage: 1400 volts. Column: 6' × ¼", glass, 10.5% Ethylene Glycol Adipate, 1.75% H_3PO_4 on Anakrom ABS, 110/120 mesh. Carrier Gas: Argon; Flow Rate: 50 ml/min.; Chart Speed: 20 inch/hr; Attenuation: X5; Detector Oven Temp.: 170°C; Injection Zone Temp.: 200°C; Column Temp.: 100°C.

Table 1. Response values of volatile acids relative to methyl enanthate

Range of Acid Concn in Sample, mg	R Value: Low	High	Range	Average R Value
Formic Acid				
0.204–1.633	0.668	0.723	0.055	0.678
1.633–2.449	0.668	0.675	0.007	0.671
2.449–4.496	0.672	0.675	0.003	0.673
Acetic Acid				
0.615–1.230	0.633	0.674	0.041	0.659
1.230–2.460	0.633	0.645	0.012	0.647
2.460–4.200	0.645	0.652	0.007	0.648
Propionic Acid				
0.645–1.289	0.326	0.342	0.016	0.338
1.289–2.578	0.326	0.337	0.011	0.335
2.578–4.921	0.337	0.339	0.002	0.338
Butyric Acid				
2.368–4.700	0.285	0.291	0.006	0.289

and acetic acids as primary indexes of decomposition in certain foods, methyl enanthate was the preferred standard. Methyl pelargonate may have some advantage if butyric acid is the acid of principal interest.

Calibration.—Bartlet and Smith (7) described a method for correcting peak heights of partially resolved peaks. Their method assumes a Gaussian distribution and estimates the peak-height correction from the peak separation in units of σ. In our chromatograms (Figs. 4 and 5) the distance between the formic acid and acetic acid peaks was approximately 2.8 σ, which corresponded to a peak-height correction of approximately 2%. This correction was disregarded; it was compensated by the internal standard.

The detector response (S) is defined as the peak height (h) per unit concentration. Concentration (c) is expressed in terms of mg of compound in total volume of the acetone solution. For the methyl enanthate standard (ME),

$$S_{ME} = h_{ME}/c_{ME} \qquad (1)$$

For each volatile acid (A) in the mixture of standard acids,

$$S_A = h_A/c_A \qquad (2)$$

For each acid, relative response (R) is defined as

$$R_A = S_A/S_{ME} = (h_A \times c_{ME})/(c_A \times h_{ME}) \qquad (3)$$

Having established R values for each acid, unknown concentrations were calculated by substituting the R values in equation 3:

$$c_A = (h_A \times c_{ME})/(R_A \times h_{ME}) \qquad (4)$$

Table 2. Recovery of volatile acids

Acid	mg in Sample	Equivalent mg/100 g Food: Fish AOAC 18.015	Equivalent mg/100 g Food: Liquid Egg AOAC 16.035	mg Found	% Recovery
Formic	0.204	2.02	1.47	0.214	105
Acetic	0.655	4.32	3.15	0.636	97
Propionic	0.645	3.48	2.32	0.631	98
Formic	0.198	4.03	2.94	0.429	105
Acetic	0.615	4.32	3.15	0.581	95
Propionic	0.645	3.48	2.32	0.648	101
Formic	0.817	8.06	5.88	0.819	100
Acetic	1.230	8.63	6.30	1.258	102
Propionic	1.289	6.37	4.64	1.289	100
Formic	1.633	16.13	11.76	1.643	101
Acetic	2.460	17.26	12.59	2.504	102
Propionic	2.578	12.73	9.28	2.578	100
Butyric	2.368	10.29	7.50	2.273	96
Formic	2.248	22.20	16.18	2.242	98
Acetic	4.200	29.47	21.49	4.204	100
Propionic	4.921	24.30	17.72	4.921	100
Butyric	4.700	20.44	14.90	4.595	97
Formic	2.149	24.19	17.63	2.149	100
Acetic	2.460	17.26	12.59	2.462	100
Propionic	2.578	12.73	9.28	2.578	100
Formic	4.496	44.40	32.37	4.505	100
Acetic	4.200	29.47	21.49	4.238	101
Propionic	4.920	24.30	17.72	4.900	100

Table 3. Comparison of volatile acid determination by gas chromatography and by AOAC methods 16.305 and 18.015 (3)

Sample	AOAC	Gas Chromatography: Individual Results*				Av.
		Formic Acid, mg/100 g				
Egg						
1	0					trace
2	0	0.51	0.58	0.56	0.55	0.6
3	11.5	10.8	10.9	11.7	11.3	11.2
4	9.7	9.99	9.99	10.0		10.0
5	0	1.04	1.13	1.07	1.11	1.1
6	35.7	31.2	31.3	31.1	31.2	31.2
Fish						
1	0	0.66	0.70	0.73		0.7
2	0	0.68	0.63	0.63	0.72	0.7
3	trace	1.28	1.16	1.00		1.1
4	5.0	3.90	3.83	3.95	3.99	3.9
5	7.6	3.29	3.26			3.3
6	5.5	4.46	4.56	4.44		4.5
		Acetic Acid, mg/100 g				
Egg						
1	0	1.64	1.63	1.65		1.6
2	0	2.03	2.03	2.06	2.04	2.0
3	19.0	18.2	18.2	18.1	18.3	18.3
4	15.7	15.4	15.7	15.7	15.3	15.8
5	9.4	7.17	6.92	7.04	7.17	7.1
6	37.8	34.6	35.7	35.0	34.6	34.8
Fish						
1	5.5	4.24	4.24	4.29	4.28	4.3
2	6.8	3.31	3.30	3.40	3.34	3.3
3	10.3	7.63	8.45	7.95	7.69	7.9
4	7.6	6.43	6.45	6.36	6.51	6.4
5	42.4	37.8	38.8	40.0	40.3	39.2
6	105	101	102	102	102	102.5

* Replicates of the same solution.

In equations *1* and *2*, a linear relationship between peak height and concentration was assumed for any single run, and any intercept correction at zero concentration was ignored. The relative response term (R_A) from equation *3* was a constant for each acid, and it corrected for differences in the response of the detector to the various acids. The reciprocal detector response for the standard (c_{ME}/h_{VE}) in equation *4* corrected for variations in volume of sample injected and for observed variations in instrument sensitivity between runs. Areas can be substituted for peak heights in equations *1* to *4*. However, with the internal standard method, peak heights proved to be satisfactory for calculating acid concentrations and were used for simplicity.

Results.—Table 1 shows the experimental R values for the C_1 to C_4 volatile acids over a wide range of concentrations. Each value is the average of six determinations. The largest variation (range 0.055, or 8% of the R value) occurred with the lowest concen-

tration of acid. Errors in measuring peak heights and any intercept correction in equation 2 would be more important at low concentrations. The average value for each acid was nearly constant over the entire range of concentrations tested.

Table 2 shows recovery data for mixtures of acids of known concentration. The data are presented in increasing order of formic acid concentration. Columns 3 and 4 show the calculated equivalent concentrations of the acids if present in fish and in eggs. These values were derived from the figures in column 2 by applying the appropriate computations in AOAC methods 18.015 and 16.035 (3). The results in column 5 are the averages of 4 determinations on each sample. The recoveries were 95–105% for the lower concentrations of acids and 97–101% for the higher concentrations.

Samples of frozen whole egg and of frozen fish fillets were analyzed for volatile acids by the gas chromatographic method and by AOAC methods 16.035 and 18.015 (3). Table 3 shows the agreement in results by the two methods and also demonstrates the greater sensitivity of the gas chromatographic method for the lowest concentrations of acids.

Acknowledgment

The assistance of Cynthia Campbell in analyzing the fish and egg samples by AOAC methods is gratefully acknowledged.

REFERENCES

(1) Hillig, F., Shelton, L. R., and Loughrey, J. H., *This Journal*, **45**, 721 (1962).
(2) Hillig, F., Dunnigan, A. P., Horwitz, W., and Warden, L. L., *ibid.*, **43**, 108 (1960).
(3) *Official Methods of Analysis*, 9th Ed., Association of Official Agricultural Chemists, Washington, D.C., 1960, sec. 16.035 and 18.015.
(4) Hunter, I. R., Ng, H., and Pence, J. W., *Anal. Chem.*, **32**, 1757 (1960).
(5) Metcalfe, L. D., and Schmitz, A. A., *ibid.*, **33**, 363 (1961).
(6) Clark, E. P., and Hillig, F., *This Journal*, **21**, 681 (1938).
(7) Bartlet, J. C., and Smith, D. M., *Can. J. Chem.*, **38**, 2057 (1960).

This paper was presented at the Seventy-sixth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 15–17, 1962, at Washington, D.C.

Stecher, P.G. Editor. 1968
The Merck Index, An Encyclopedia of Chemicals and Drugs, 8th Edition
Merck and Company, Inc., Rahway, New Jersey

Effect of Vitamin B_{12} and Folic Acid on the Metabolism of Formiminoglutamate, Formate, and Propionate in the Rat

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ABSTRACT The effects of deficiencies of vitamin B_{12} on excretion of formiminoglutamate (FIGLU), formate, and propionate were investigated. The excretion of FIGLU was increased in vitamin B_{12} deficiency and was higher with a 30% soy protein diet than with a 70% soy protein diet, although the vitamin B_{12} deficiency was more severe with the diet at higher protein level. Excretion of endogenous formate was increased in both folic acid and vitamin B_{12} deficiencies and roughly paralleled that of FIGLU. Excretion of injected ^{14}C-formate increased in folic acid deficiency but was less affected by vitamin B_{12} deficiency with a soy protein diet. The excretion of a test dose of propionate was increased in vitamin B_{12} deficiency and developed more rapidly with the 30% than with the 20% soy protein diet. The feeding of either 20 or 40 g/kg calcium propionate produced a growth depression, but this was not appreciably diminished by vitamin B_{12}. Calcium propionate did not increase FIGLU excretion.

Deficiencies of both folic acid and vitamin B_{12} increase the excretion of formiminoglutamic acid (FIGLU) in rats (1-3) and chicks (4), although folic acid alone is known to be directly involved in the enzymatic breakdown of FIGLU to glutamic acid. The excretion of FIGLU has been used as a diagnostic test for clinical folic acid deficiency (5-7), but its usefulness is somewhat diminished because in some instances FIGLU excretion also occurs in the vitamin B_{12} deficiency of pernicious anemia (8). In order to throw light on the mechanism of action of vitamin B_{12} in affecting FIGLU metabolism, a study was made on the effect of various deficiencies which induce vitamin B_{12} deficiency. These include elevation of dietary protein level and the feeding of high levels of propionic acid (9, 10). Since the metabolism of propionate is known to be altered in vitamin B_{12} deficiency in the rat (11) and the ruminant (12), the excretion of a test dose of propionate was studied as a possible parameter for assessing vitamin B_{12} deficiency. The effect of vitamin B_{12} deficiency on the metabolism of formate was also studied since the excretion of formate is increased in folic acid deficiency (2).

METHODS

Groups of 8 weanling male rats (2 rats/cage) were fed the experimental diets at 28 days of age. The composition of the basal diets is shown in table 1. A 20% soy protein diet and a 70% soy protein diet were used to produce the vitamin B_{12} deficiency. The 30% soy protein diet contained 0.27% methionine which is lower than the methionine requirement (in the presence of cystine) (13) of 0.4% for growth in the rat. No supplementary methionine was used because supplementary methionine is known to decrease FIGLU excretion (2-4) and it was deemed desirable to keep FIGLU excretion as high as possible in order to accentuate differences produced by dietary changes. The 30% soy protein diet was only marginally deficient with respect to amino acids as evidenced by the fact that growth with this diet, supplemented with folic acid and vitamin B_{12}, was 80% of that with a casein gelatin diet.

The basal diet was supplemented with 20 or 40 g/kg of calcium propionate with the objective of further increasing the need for vitamin B_{12}. The calcium content of all diets was kept constant by adjustment of the calcium carbonate used in the salt mixture to compensate for the calcium furnished by the calcium propionate. Since

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TABLE 1
Composition of basal diets

	30% Soy protein diet	70% Soy protein diet	20% Casein diet
	g/kg	*g/kg*	*g/kg*
Glucose monohydrate [1]	639	239	645
Soy protein [2]	300	700	—
Crude casein	—	—	200
Gelatin	—	—	80
Calcium carbonate	20	20	20
Salts [3]	20	20	20
Vitamin mixture [4]	10	10	10
Vitamins A, D, E, in corn oil [5]	10	10	10
Choline chloride	1	1	1
Cystine	—	—	4
Sulfaguxidine	—	—	10
Methionine content (calculated)	2.7 [6]	6.3	6.1
Histidine content (calculated)	7.0 [6]	16.5	4.5

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] 2101 C.I. Assay Protein, Archer Daniels-Midland, Minneapolis.
[3] Composition of salt mixture: (in grams) $CaCO_3$, 228; K_2HPO_4, 100; KH_2PO_4, 100; NaCl, 170; boric acid, 300; Al_2SO_4, 50; ferric citrate·3H_2O, 35; $MnSO_4$·4H_2O, 10; $CuSO_4$·5H_2O, 1; zinc [illegible] H_2O, 1; K_2SO_4, 50; [illegible], 0.05; [illegible] $CoCl_2$, 0.05; [illegible] CrO_3, 0.02.
[4] The vitamin mixture furnished the following amounts/kg of diet: (in milligrams) riboflavin, 10; Ca pantothenate, 50; niacin, 50; pyridoxine, 10; thiamine, 10; and biotin, 0.2.
[5] Vitamins A, D, and E were supplied in the following amounts dissolved in 10 g of corn oil/kg of diet: vitamin A, 17,500 units; vitamin D, 2,000 units; and vitamin E, 70 units.
[6] Amino acid composition data supplied by manufacturer.

the soy protein was contaminated with appreciable amounts of folic acid, a casein diet low in folic acid was used for inducing folic acid deficiency for comparative purposes.

Metabolic tests were carried out in metabolism cages using 2 animals per group. The ^{14}C formate excretion tests were made by giving about 1 μc of ^{14}C-formate intraperitoneally and measuring in a liquid scintillation counter the total radioactivity of the urine for the next 22-hour period using the solvent and phosphor mixture of Bray (14). The excretion of ^{14}C-propionate was measured by administering 5 μc of propionate intraperitoneally and collecting the urine for the next 22 hours. The "^{14}C-propionic acid" in the urine was measured by extracting 2.0 ml of urine plus 0.2 ml of 5 N hydrochloric acid with 5 ml of ether. Under these conditions 85% of the propionic acid goes into the ether layer. One milliliter of the ether solution was counted in a liquid scintillation counter.

Formic acid was measured chemically by the method of Grant (15) in which formic acid is reduced to formaldehyde by reduction with magnesium metal and the resulting formaldehyde estimated with chromotropic acid. Formic acid was first recovered by distillation from the frozen state of 3 ml of urine which had been acidified to pH 1 to 2 with hydrochloric acid. One-half milliliter of the distillate was placed in a test tube in an ice bath and a coil of 10 cm of magnesium strip added. Preliminary washing of the magnesium with dilute hydrochloric acid was necessary to remove corrosion products which increased the blank. One drop of concentrated hydrochloric acid was added every 2 minutes until 10 drops had been added, the tube being shaken after each drop. Two minutes after the addition of the last drop, the solution was transferred to a 15-ml centrifuge tube with a capillary pipette, leaving the pieces of undissolved magnesium behind. One-and-a-half milliliters of chromotropic acid solution were added. This was prepared fresh every week by dissolving 0.6 g chromotropic acid in 20 ml water and adding 180 ml concentrated sulfuric acid. The tube was covered with a glass marble and placed in a boiling water bath for 30 minutes protected from light. The reaction mixture was stored overnight at 4° to facilitate removal of the precipitate which formed slowly. The solution was centrifuged, and

[illegible] Dr. Stokes Ballard [illegible] the [illegible] of [illegible] Medical Products.

the supernatant removed and read in a spectrophotometer at 570 mμ. In this procedure 0.2 μmole of formic acid gives an optical density of about 0.3 in a 1-cm cell. Each sample was corrected for the efficiency of formic acid distillation from recovery of added ^{14}C-formate.

Formiminoglutamic acid was measured by the enzymatic method of Tabor and Wyndgarden (16).

RESULTS

The addition of 20 or 40 g of calcium propionate markedly depressed growth with the 30% soy protein diets (table 2). However, it does not appear that the growth depression is due primarily to an increased vitamin B_{12} requirement, since the growth increment due to vitamin B_{12} supplementation was the same in the absence or presence of calcium propionate (table 2). This is in contrast with the observation that the addition of propionic acid (9) or sodium propionate (10) to a casein diet would increase the response to vitamin B_{12}. The growth depression produced by calcium propionate was much less with the 70% than with the 30% protein diet.

The excretion of FIGLU with the different types of diets is indicated in table 3. An increase in FIGLU excretion occurred in the absence of vitamin B_{12} with the soy protein diets and in the absence of folic acid with the casein diet. The increased FIGLU excretion developed rapidly with the 30% soy protein diet, rising to 125 μmole in 4 days from a baseline value of 2 to 10 μmole for weanling rats fed a commercial stock diet.

The excretion rate on the 30% soy protein diet without vitamin B_{12} continued to increase with time, reaching a maximum after 29 days. In the 30% soy protein diet supplemented with vitamin B_{12}, there was an appreciable excretion of FIGLU (about 50 μmole/day) during the first 2 weeks which was about 10 times higher than that with the casein diet supplemented with both vitamin B_{12} and folic acid. The FIGLU excretion with the 30% soy protein diet with vitamin B_{12} decreased with time to a level of 1 μmole by the 56th day. The higher FIGLU excretions with the 30% soy protein diet even in the presence of both folic acid and vitamin B_{12} may be due to the lower methionine content of this diet (0.27%) than in the casein diet (0.51%), and also to its slightly higher content of histidine (0.7% in the 30% soy protein diet and 0.45% in the casein diet) which serves as the precursor of FIGLU. This initial rise in FIGLU concentration with the 30% soy protein with vitamin B_{12} and folic acid, followed by a decrease to low levels as the animals grew older, has been observed in other similar experiments. This may represent a decreased requirement for methionine in the larger animal.

By 29 days the FIGLU excretion with the unsupplemented 30% soy protein diet had reached a maximum indicating that the maximal degree of vitamin B_{12} deficiency had been reached as measured by this biochemical criteria. At this time

TABLE 2

Effect of calcium propionate on growth and the supplementary effect of vitamin B_{12}

Basal diet [1]	Ca propionate/kg diet	Avg body wt and survivors at 56 days: Without vitamin B_{12}	Avg body wt and survivors at 56 days: With vitamin B_{12}	Gain due to vitamin B_{12}
	g	g	g	g
30% Soy protein	—	162(7) [3]	212(8)	50
30% Soy protein	20	112(8)	176(8)	64
30% Soy protein	40	105(8)	87(8)	−18
70% Soy protein	—	224(8)	326(8)	102
70% Soy protein	20	216(8)	302(8)	86
70% Soy protein	40	194(8)	302(8)	108

[1] [illegible] supplemented with 2 mg folic acid/kg diet.
[2] [illegible] Kodak.
[3] Numbers in parentheses indicate number of survivors.

TABLE 3

Effect of vitamin B_{12} and calcium propionate on excretion of formiminoglutamic acid (FIGLU)

Group no.	Basal diet	Supplement/kg diet: Vitamin B_{12}	Supplement/kg diet: Folate	Supplement/kg diet: Ca propionate	FIGLU excretion/kg body wt/day: 4 Days	FIGLU excretion/kg body wt/day: 15 Days	FIGLU excretion/kg body wt/day: 29 Days	FIGLU excretion/kg body wt/day: 56 Days	Avg body wt: 29 Days	Avg body wt: 56 Days
		μg	mg	g	μmole	μmole	μmole	μmole	g	g
1	30% Soy protein	—	2	—	125	172	400	221	103 [1]	162 [1]
2	30% Soy protein	—	2	20		79			83	112
3	30% Soy protein	—	2	40		30			71	105
4	30% Soy protein	30	2	—	68	51	11	1	123	212
5	30% Soy protein	30	2	20		35			105	176
6	30% Soy protein	30	2	40		7			61	87
7	70% Soy protein	—	2	—	1	45		9	142	224
8	70% Soy protein	—	2	20		48			121	216
9	70% Soy protein	—	2	40		58			105	194
10	70% Soy protein	30	2	—	1	4		2	217	326
11	70% Soy protein	30	2	20		4			193	302
12	70% Soy protein	30	2	40		8			194	302
13	20% Casein	30	—	—		181	450	480	136	184 [1]
14	20% Casein	30	2	—		5	1	2	175	262

[1] Seven survivors.

TABLE 4

Effect of vitamin B_{12}, folic acid and protein levels on metabolism of formate, formiminoglutamic (FIGLU) acid and propionate

Group no.	Basal diet	Supplement/kg diet: Vitamin B_{12}	Supplement/kg diet: Folate	Avg body wt at 56 days	^{14}C-propionate excretion	^{14}C-Formate excretion	Endogenous FIGLU excretion/kg body wt/day	Endogenous formate excretion/kg body wt/day
		μg	mg	g	%	%	μmole	μmole
1	30% Soy protein	—	2	162 [1]	3.10	7.6	221	730
4	30% Soy protein	30	2	212	0.28	3.5	1	32
9	70% Soy protein	—	2	224	11.40	8.3	9	125
12	70% Soy protein	30	2	326	0.44	7.6	2	26
15	20% Casein	30	—	184	0.27	18.7	480	1810
16	20% Casein	30	2	262	0.28	4.3	2	23

[1] Seven survivors.

there was a 20% difference in growth between the vitamin B_{12}-deficient animals and those given the supplement, whereas at 56 days there was a 30% difference. The addition of calcium propionate decreased FIGLU excretion rather than increasing it, so there is no evidence as measured by FIGLU excretion that calcium propionate accentuates vitamin B_{12} deficiency.

With the 70% soy protein diet the growth rate was approximately 50% higher than with the 30% soy protein diet. Vitamin B_{12} deficiency was, however, more marked as the growth response due to vitamin B_{12} was 52% with the 70% protein diet at 29 days, as compared with a 20% response with the 30% soy protein diet. Despite the more acute vitamin B_{12} deficiency with the 70% soy protein diet, the excretion of FIGLU was much lower than with the 30% diet. This lower excretion may be a reflection of the higher methionine content (0.7%) in the 70% soy protein diet, since the addition of methionine greatly reduces FIGLU excretion in rats in both vitamin B_{12} and folic acid deficiencies (3). The 70% soy protein diet contains the same amount of methionine (0.70%) as the folic acid-deficient casein diet.

The excretion of endogenous formate and the excretion of a test dose of ^{14}C-formate was measured and the results are shown in table 4. The excretion of endogenous formate approximately paralleled that of FIGLU, both being increased in folic acid deficiency with the casein diet and in vitamin B_{12} deficiency with the 30 and 70% soy protein diets. With the casein diet the excretion of ^{14}C-formate was increased fourfold by folic acid deficiency, whereas with the 70% soy protein diet it was not affected by vitamin B_{12}, and with the 30% soy protein diet it was increased 2 times by vitamin B_{12} deficiency.

The effect of deficiencies of vitamin B_{12} and folic acid on the excretion of a test dose of propionate is shown in table 5. The excretion was measured over a 22-hour period following injection of the test dose. Excretion during the first 22-hour period was essentially complete since the excretion during the second and third days was about 2 and 1%, respectively, of that

TABLE 5

Effect of vitamin B_{12}, folic acid, and protein level on excretion of an intraperitoneal dose of tritiated propionic acid

Group no.	Basal diet	Supplement to basal diet: Vitamin B_{12}	Supplement to basal diet: Folate	Excretion of ^{3}H-propionate: 14 Days	Excretion of ^{3}H-propionate: 28 Days	Excretion of ^{3}H-propionate: 56 Days	Avg body wt: 14 Days	Avg body wt: 28 Days	Avg body wt: 56 Days
		μg	mg	%	%	%	g	g	g
1	30% Soy protein	—	2	0.35	1.03	3.10	71	103 [1]	162 [1]
2	30% Soy protein	30	2	0.21	0.27	0.28	73	123	212
5	70% Soy protein	—	2	5.70	11.20	11.40	88	142	221
6	70% Soy protein	30	2	0.37	0.37	0.44	105	217	326
15	20% Casein	30	—			0.27	83	136	181
16	20% Casein	30	2			0.28	97	175	262

[1] Seven survivors.

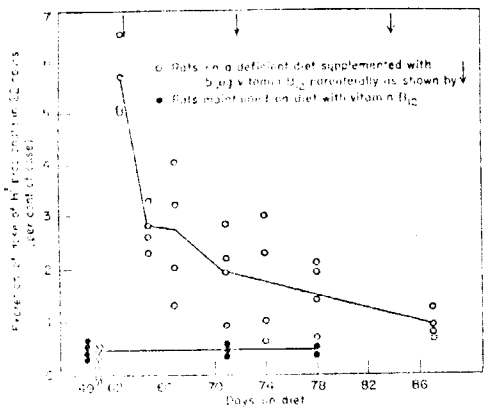

Fig. 1 Effect of vitamin B_{12} in reducing excretion of a parenteral dose of ^{3}H propionate.

amount excreted on the first day. The results showed that the excretion of a test dose of propionate was increased in vitamin B_{12} deficiency but not in folic acid deficiency. With the 30% soy protein diet there was no difference at 14 days between diets containing, and those not containing, vitamin B_{12}. At 28 days the excretion of propionate in the absence of vitamin B_{12} had increased threefold and at 56 days had increased tenfold. With the 70% soy protein diet the excretion of propionate in the absence of vitamin B_{12} had increased to 15 times that of the supplemented group and by 28 days to 30 times.

When vitamin B_{12} was administered, the excretion of administered propionate decreased slowly, requiring 4 weeks to return to normal, as shown in figure 1. Four rats that had been fed the 30% soy protein diet, vitamin B_{12}-deficient, for 62 days were given 5 μg vitamin B_{12} intraperitoneally. Their excretion of administered propionate was 5.7% prior to the administration of vitamin B_{12}, and this decreased to 2.75% 2 days after giving vitamin B_{12}, and to 1.8% 8 days later. The excretion still remained 3 times higher than normal after 15 days, and 24 days were required for complete normalization of propionate excretion.

DISCUSSION

Although vitamin B_{12} is not directly involved in the metabolism of either FIGLU or formate, it can influence the excretion rates of both. The excretion of formate roughly parallels that of FIGLU and is five to ten times as high. It appears that the excretion of formate offers little advantage over FIGLU as a diagnostic aid for the differentiation between vitamin B_{12} and folic acid deficiency. Although formate and FIGLU excretions parallel each other in the conditions studied here, their metabolic origins are different. The formate excreted in folic acid deficiency is not derived from breakdown of FIGLU but may be derived in part from tryptophan, since the feeding of tryptophan markedly increases the excretion of formate with a folic acid-deficient diet (2).

The similarity of effect of vitamin B_{12} and folic acid on the excretion of both formate and FIGLU suggests that the effect of vitamin B_{12} may be an indirect one exerted by modification of folic acid metabolism. This finds support in the effect of vitamin B_{12} in decreasing the proportion of 5-methyl tetrahydrofolic acid (5-methyl FH_4) in tissues. Herbert and Zalusky (17) have observed an increase in the proportion of 5-methyl FH_4 relative to other forms of folate in blood in pernicious anemia. The administration of vitamin B_{12} decreases the relative proportion of 5-methyl FH_4 and increases the proportion of other forms of folate. An increase in the relative proportion of 5-methyl FH_4 has also been observed in livers of vitamin B_{12}-deficient rats, and this can be decreased by giving either vitamin B_{12} or methionine (18). It has been suggested by Herbert and Zalusky (17) that 5-methyl FH_4 is not available for certain folic acid coenzyme functions such as metabolism of FIGLU until the methyl group has been removed by the transfer of the methyl group to homocysteine to give methionine by means of a vitamin B_{12}-dependent reaction. The methyl group of methionine is readily oxidized (19) (8 to 50% in 6 hours) in a reaction not dependent on folic acid. Thus the transfer of the methyl group of 5-methyl FH_4 to homocysteine and subsequent oxidation to carbon dioxide may represent a mechanism for regenerating FH_4 and making it available for metabolism of FIGLU. Such a mechanism is suggested by the observation that feeding methionine decreases the pro-

[illegible] of a methyl [illegible] in the liver (18), and also markedly decreases FIGLU excretion in both folic acid and vitamin B_{12} deficiencies (7, 14).

The failure of vitamin B_{12} to prevent the growth depression produced by 2 and 4% of calcium propionate is at variance with the observations of Hoque and Elliot (10) that 3 and 6% sodium propionate produced a growth depression with a casein-sucrose diet which could be partially prevented by vitamin B_{12}. Gammon and Aronoff (20) have observed an increased mortality produced by feeding 10% tripropionin with a casein-sucrose vitamin B_{12}-deficient diet. Since a soy protein diet was used in the experiments reported here, the possibility exists that the differences in response may be in part due to the difference in the basal diet used.

The increase in propionate excretion following injection of a test dose in vitamin B_{12} deficiency is in accord with the known role of vitamin B_{12} in isomerization of methylmalonyl CoA to succinyl CoA in *Propionibacterium shermanii* (21), *Ochromonas malhamensis* (22), rat liver (11) and sheep liver (12). A decreased clearance rate of propionic acid from blood of vitamin B_{12} deficient sheep (12) has been observed. Methylmalonic acid has been observed in increased concentration in the urine of vitamin B_{12} deficient rats (23) and in patients with pernicious anemia (24-26).

LITERATURE CITED

1. Silverman, M., and A. J. Pitney 1958 Dietary methionine and the excretion of formiminoglutamic acid by the rat. J. Biol. Chem., 233: 1179.
2. Rabinowitz, J. C., and H. Tabor 1958 The urinary excretion of formic acid and formiminoglutamic acid in folic acid deficiency. J. Biol. Chem., 233: 252.
3. Brown, D. D., O. L. Silva, R. C. Gardiner and M. Silverman 1959 Metabolism of formiminoglutamic acid by vitamin B_{12} and folic acid-deficient rats fed excess methionine. J. Biol. Chem., 235: 2953.
4. Spivey Fox, M. R., and W. J. Ludwig 1961 Excretion of formiminoglutamic acid as an index of vitamin B_{12}, folic acid, and methionine deficiencies. Proc. Soc. Exp. Biol. Med., 106: 703.
5. Bronisch, H. P. 1956 Evidence for the excretion of formiminoglutamic acid following folic acid antagonist therapy in acute leukemia. J. Am. Chem. Soc., 78: 6295.
6. Lemley, A. L., J. M. Cooperman and D. N. Teller 1959 Histochemical metabolic test to distinguish folic acid deficiency from vitamin B_{12} in megaloblastic anemias. Proc. Soc. Exp. Biol. Med., 101: 350.
7. Lubby, A. L., J. M. Cooperman and D. N. Teller 1959 Urinary excretion of formiminoglutamic acid. Application to diagnosis of clinical folic acid deficiency. Am. J. Clin. Nutrition 7: 247.
8. Zalusky, R., and V. Herbert 1961 Failure of formiminoglutamic [illegible] in megaloblastic anemia [illegible] in a patient with functional folic acid deficiency. Am. Soc. Clin. Investigation 40: 1091.
9. Hartman, A. M., and L. P. Dryden 1962 Vitamin B_{12} and the metabolism of fatty acids. J. Dairy Sci., 45: 641.
10. Hoque, D. E., and J. M. Elliot 1961 Effect of propionate on the dietary vitamin B_{12}, biotin and folic acid requirement of the rat. J. Nutrition, 73: 171.
11. Garmani, G. S. P., Shabry and B. C. Johnson 1960 Function of vitamin B_{12} in methylmalonate metabolism. Biochem. Biophys. Acta, 37: 127.
12. Marston, H. R., S. H. Allen and R. M. Smith 1961 Primary metabolic defect superveening on vitamin B_{12} deficiency in the sheep. Nature 190: 1025.
13. National Research Council Committee on Animal Nutrition 1962 Nutritional requirements of laboratory animals, pub. 930. National Academy of Sciences — National Research Council, Washington, D. C.
14. Beer, G. A. 1960 A simple effect of liquid scintillator for counting a gamma emitter in a liquid scintillation counter. Analyt. Biochem., 1: 209.
15. Grant, W. M. 1943 Colorimetric microdetermination of formic acid based on reduction to formaldehyde. Analyt. Chem., 21: 337.
16. Tabor, H., and L. Wyndenden 1951 A method for the determination of formiminoglutamic acid in urine. J. Clin. Invest., 37: 824.
17. Herbert, V., and R. Zalusky 1962 Interrelationship of vitamin B_{12} and folic acid metabolism in folic acid clearance studies. J. Clin. Invest. 41: 1263.
18. Noronha, J. M., and M. Silverman 1962 In: Vitamin B_{12} and Intrinsic Factor, ed., H. C. Heinrich, chapt. 10. Ferdinand Enke, Verlag, Stuttgart.
19. Weinhouse, S., and B. Friedman 1951 A study of formate production in normal and folic acid-deficient rats. J. Biol. Chem., 210: 423.
20. Gammon, S. D., and B. W. Aronoff 1963 Toxicity of tripropionin in rats fed a vitamin B_{12}-deficient diet. Proc. Soc. Exp. Biol. Med., 114: 600.
21. Stadtman, E. R., P. Overath, H. Eggerer and F. Lynen 1960 The role of biotin and vitamin B_{12} coenzyme in propionate metabolism. Biochem. Biophys. Res. Comm., 2: 1.
22. Arnstein, H. R. V., and A. M. White 1962 The function of vitamin B_{12} in the metabolism of propionate by the protozoan *Ochromonas malhamensis*. Biochem. J., 83: 264.
23. Barness, L. A., D. G. Young and R. Nocho 1963 Methylmalonate excretion in vitamin B_{12} deficiency. Science, 140: 76.
24. White, A. M. 1962 Vitamin B_{12} deficiency and the excretion of methylmalonic acid by the human. Biochem. J., 84: 41P.
25. Cox, E. O., and A. M. White 1962 Methylmalonic acid excretion an index of vitamin B_{12} deficiency. Lancet 2: 853.
26. Barness, L. A., D. G. Young, W. J. Mellman, S. B. Kahn and W. J. Williams 1963 Methylmalonate excretion in a patient with pernicious anemia. Case report. New England J. Med., 268: 141.

FORMIC ACID

CA 73, 33870u (1970)

426

33570u Contents of methanol and formic acid in some Yugoslav wines. Tomic-Nedeljkovic, M. (Yugoslavia). *Arh. farm.* 1970, 19(4), 225-8 (Croat). The content of MeOH in 23 Yugoslav white and red wines varies between 86.4 and 425 mg/l. wine; the MeOH content of the red wines is higher. The formic acid content of the wines varies between 51 and 337 mg/l.

J. J. Pohl

DETECTION

U.S. Department of Commerce. 1972
U.S. Foreign Trade Imports. Commodity by Country
U.S. Department of Commerce, Washington, D.C.
FT 135

FORMIC ACID

CA 69, 34651c (1968)

110

34651c **Formic acid in wines.** Usseglio-Tomasset, Luciano (Sta. Enol. Sper., Asti, Italy). *Accad. Ital. Vite Vino, Siena, Atti* 1967, 19, 229-42 (Ital). To det. the amt. of formic acid formed during the fermentation of wine, the wine was distd. in vacuo. The distillate (5 ml.) was placed in a 20-ml. flask which was placed in an ice bath; 150 mg. Mg turnings and 0.1 ml. concd. HCl were added with shaking, followed by 12.5 ml. of chromotropic acid cooled with ice. A standard curve was prepd. from solns. contg. 0.5-4.0 ml. formic acid. Controls were run to det. the amt. of H_2CO present in the distillate before redn. of the formic acid and the amt. of formic acid formed from the sugar present in the wine during distn.; formic acid is formed during distn. only when large amts. of reducing sugars are present. Both dry and sweet wines contain the same degree of formic acid. There is no relation between the content of volatile acids and formic acid. The amt. of formic acid in dry wines is 1-4 mg./l. The formic acid content of sweet wines is greater than that of dry wines. The formic acid content of sweet wines increases with the time of storage. After a slight initial increase, the formic acid decreases during fermentation. The must contains the same degree of formic acid as does the wine. Heat treatment of sweet wines probably increases the formic acid content.

Felix Saunders

Detection

The metabolism of formic acid in sheep

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Small amounts of formic acid can be detected in the steam-volatile fatty acids of rumen liquor, but the work of Annison (1954) shows that the formate in the peripheral circulation of ruminants is probably not derived from this source. Blood concentrations of formate, unlike those of acetate, do not rise during feeding, and fall but slightly during a 48 h fast. In this regard Annison & Pennington (1954) showed that formate was not metabolized by rumen epithelium. Experiments in vitro, notably those of Lewis (1951), Beijer (1952), Doetsch, Robinson, Brown & Shaw (1953) and [illegible] & Hungate (1955) have shown that formate added to rumen liquor is [illegible] to give carbon dioxide and methane. Short-term experiments with goats [illegible] rumen fistulas by Matsumoto (1961) showed that the rate of disappearance of [illegible] was constant, was unaffected by formate concentration and was associated [illegible] increase in the concentration of CH_4 and a decrease in the concentration of CO_2 in the gas phase of the rumen. Such changes, however, do not permit establishment of the relative amounts of CH_4 and CO_2 produced, because CO_2 diffuses from the rumen more rapidly than does CH_4.

Reid (1962) and Bensadoun, Palladines & Reid (1962), however, concluded, on the basis of differences in the concentration of formic acid as between rumen liquor and [illegible], that formic acid may constitute a sizeable proportion of the total steam-volatile acid absorbed. Reid (1962) also reported, but not in detail, that when, in four energy balance trials, formic acid was infused into the rumens of cattle receiving a basal diet of hay, heat production was reduced by an amount equivalent to 92% of the heat of combustion of the formic acid given, and that the formic acid increased the efficiency of energy utilization of the whole diet by 18%.

These conclusions by Reid that considerable amounts of formic acid are absorbed from the rumen do not agree with those of Annison (1954), nor is an effect of formic acid on metabolism consonant with what is known of its rapid breakdown by rumen microorganisms in vitro. The experiments now presented were undertaken to measure quantitatively the effect of formic acid on the metabolism of the sheep.

EXPERIMENTAL

Preliminary experiments were made to find the amounts of formic acid which could be used continuously without depressing the pH of the rumen by more than [illegible] unit. The maximum amount was 1·5 moles/day, which supplied 98 kcal/day. The

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basal diets supplied about 2000 kcal metabolizable energy, so that formic acid could be given to supply about 5% of the total metabolizable energy.

Animals and diets. Five castrated male sheep were used. Four of these sheep (C, R, B and F) had permanent rumen fistulas and one, sheep E, was intact. They were given diets of dried grass containing from 13·4 to 18·8% crude protein on a dry-matter basis.

Five experiments, two with sheep B and one with each of sheep C, R, and B, were made in which formic acid was given by ruminal infusion and the respiratory exchange was measured. In the experiments with sheep C, R and B, after an initial period of 1–2 weeks during which metabolism was measured, increasing amounts of formic acid were infused over a period of 3–5 days until 1·5 moles/day were being infused at constant rates. The total period of infusion lasted 12–17 days, and metabolism was measured continuously during that time. Formic acid infusion was then stopped and measurements continued for 9–14 days. With sheep B, after an initial period of 7 days, formic acid was infused at a rate of 1·0 mole/day for 12 days. The infusion was then stopped for 16 days, measurements being made on the last 9 days. The infusion of formic acid at the rate of 1·0 mole/day was then repeated and continued for 11 days, measurements being made on the last 8 days. The total volume infused was 4 l./day.

Three experiments similar to those above were made with sheep C, B and E kept in respiration chambers in which 0·5 mole sodium formate in 2·0 l. sterile water was infused intravenously through an indwelling catheter.

Two experiments were made in which sheep B and F with rumen fistulas were given constant food for 6 weeks. In the first 2 and last 2 weeks 4 l. saline were infused into the rumen each day. In the 3rd and 4th weeks 1·5 moles formic acid and 4 l. water were infused each day. Faeces were collected over the last 8 days of each 2-week period.

In four short-term experiments with two sheep (B and F) with rumen fistulas, 0·75–2·0 moles sodium formate were given by rapid (30–40 min) infusion into the rumen. The gaseous exchange was measured at 2 h intervals after the infusion began and continued for 8 h. Four control experiments were made with these sheep in which no formate was given.

Analytical and calorimetric methods. The respiration chambers were those of Wainman & Blaxter (1958) and chemical methods those of Graham, Blaxter & Armstrong (1958). Steam-volatile fatty acids were determined by steam distillation by the method of Annison (1954) and separated by gas chromatography (James & Martin, 1952).

RESULTS

Effect of infusion of formic acid or of sodium formate on the apparent digestibility of the basal diet. Table 1 summarizes determinations of the apparent digestibility of the diet before, during and after formic acid infusions. None of the infusions resulted in any significant depression of the digestibility or the energy of the basal diet. [illegible] digestibility of the N of the diet was also not significantly affected.

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CH_4 production. The records of CH_4 production showed that the day-to-day variation under constant conditions varied slightly from experiment to experiment between the range ±0·6–±0·7 l./day (standard deviation). This corresponds to a coefficient of variation of ±1·8–±2·1%. Fig. 1 shows the results for sheep R and B when 1·5 moles formic acid were infused daily into the rumen, and Table 2 gives the mean results of all experiments in which formic acid was infused into the rumen. The results apply to the last 4 or 5 days of the infusion period. When formic acid was infused into the rumen, CH_4 production increased. The mean increase in these experiments,

Table 1. *Mean apparent digestibility (%) of dietary energy when 1·5 moles formic acid were infused into the rumens or when 0·5 mole sodium formate was infused into the jugular veins of sheep*

Infusion	No. of experiments and code letters of sheep	Mean apparent digestibility of dietary energy (%) Before infusion	During infusion	After infusion	SE of mean
1·5 moles formic acid (rumen)	5 (C, R, B, E and F)	69·4	67·9	68·8	±0·7
1·0 moles formic acid (rumen)	2 (B)	68·6	68·3	67·5	±1·3
0·5 mole sodium formate (vein)	3 (C, B, E)	72·9	73·5	72·9	±1·0

Table 2. *Effect of infusing 1·5 moles or 1·0 mole formic acid into the rumen or 0·5 mole sodium formate into the jugular vein on CH_4 production by sheep*

Sheep	Amount of formic acid or sodium formate infused (moles/24 h)	CH_4 production (l./24 h) Before infusion	During infusion	After infusion	Increase during infusion	SE of increase
C	1·5 (rumen)	28·3	37·3	27·2	9·36	±0·35
R	1·5 (rumen)	32·8	42·1	33·2	9·10	±0·39
B	1·5 (rumen)	31·1	37·1	30·8	6·17	±0·35
Mean		30·7	38·8	30·4	8·21	±1·02*
B	1·0 (rumen)	—	34·0	30·2	3·74	±0·37
B	1·0 (rumen)	30·2	34·5	—	4·30	±0·43
C	0·5 (intravenous)	28·0	28·8	28·0	0·82	±0·51
B	0·5 (intravenous)	34·0	35·2	34·1	1·16	±0·51
E	0·5 (intravenous)	29·1†	32·4	33·1	−0·70	±0·69
Mean (intravenous)		31·7	32·1	31·7	0·43	±0·57*

* Between-experiment standard error; others are within-experiment standard errors.
† This value was not used in the analysis because it was thought that the sheep was not fully accustomed to the respiration apparatus during the initial period.

which was 5·1 l. CH_4/mole formic acid, was highly significant statistically. Sheep C and R produced 6·2 and 6·1 l. CH_4/mole, but sheep B produced about 4·0 l. CH_4/mole formic acid in each experiment. When the result of all the experiments in which formic acid was infused into the rumen were calculated as CH_4 production per mole formic acid given, CH_4 production was found to have increased by 4·9 ± 0·52 l./mole formic acid.

When sodium formate was given by intravenous infusion, no significant change

occurred in CH_4 production (Table 2). This is evident in Fig. 2 which shows the observations made with sheep B.

CO_2 production and O_2 consumption. Fig. 1 also shows the CO_2 and O_2 consumption of sheep R and B when given 1·5 moles formic acid by infusion into the rumen each

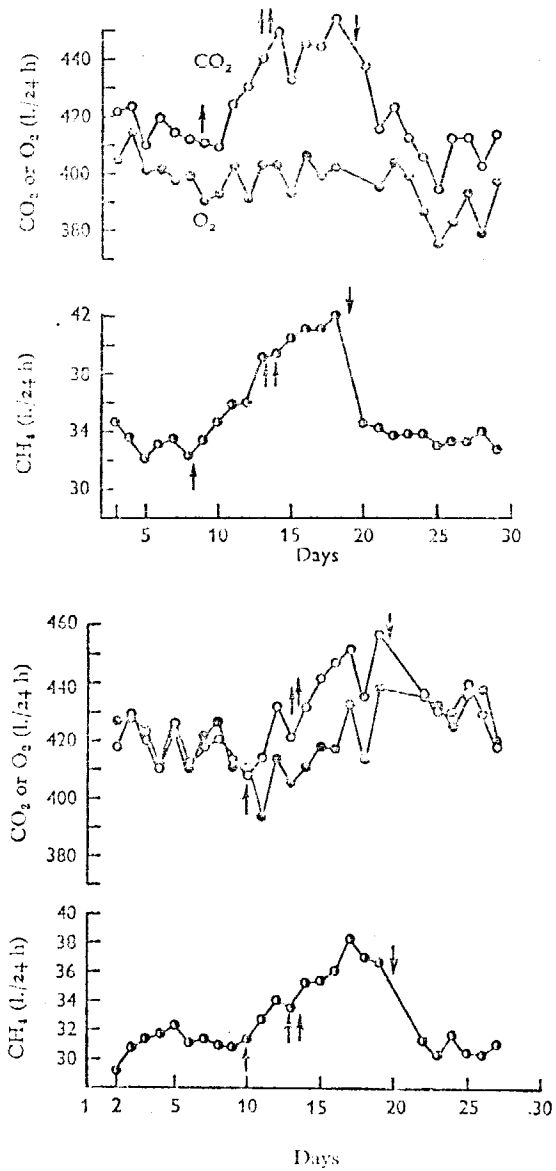

Fig. 1. O_2 consumption, CO_2 production and CH_4 production of sheep R (upper) and of sheep B (lower) when 1·5 moles formic acid/24 h were infused into the rumen: ●—●, O_2 consumption; ○—○, CO_2 production; ↓—↓, CH_4 production. The single arrow ↑ indicates commencement of infusion at the rate of 1·5 mole/24 h, the full amount of 1·5 moles [illegible]

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day. An increase in CO_2 production occurred with both sheep. With sheep B, O_2 consumption rose during the experimental period and remained elevated during the first period. With sheep R a slight increase occurred throughout the experiment. Lastly, was the effect of intravenous infusion of 0·5 mole formate/day on the gaseous exchange of sheep B. No effect on O_2 consumption or on CO_2 production was detected. The responses to infusion in all the experiments were computed as the difference between the mean values during the last 4–5 days of infusion and the mean values obtained in the last 4–5 days of the two control periods, and these values are given in Table 3.

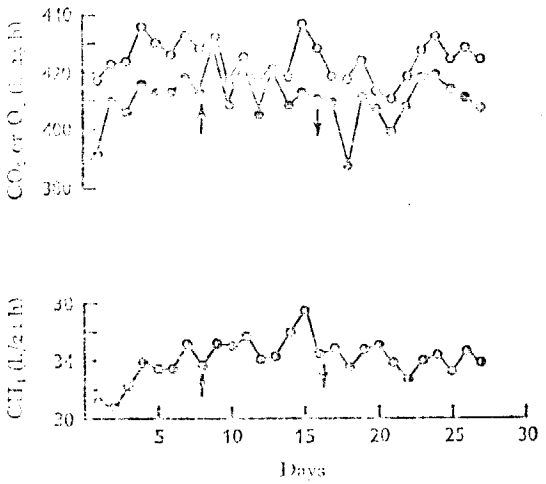

Fig. 2. O_2 consumption, CO_2 production and CH_4 production of sheep B when 0·5 mole sodium formate/24 h was infused into the jugular vein: ⊗—⊗, O_2 consumption; ○—○, CO_2 production; ⊗—⊗, CH_4 production. The arrows indicate the beginning and end of the infusion.

Steam-volatile acids in blood and urine. No increase in the concentration of steam-volatile acids in peripheral blood was noted in any experiment in which formic acid was infused into the rumen. No increases in the amount of steam-volatile acids in urine were detected in these experiments.

When sheep B was given 0·5 mole sodium formate/day by intravenous infusion, the total steam-volatile acids in the urine increased from 37 to 95 m-equiv./day, and when sheep C was given the same amount of formate the excretion of steam-volatile acids rose from 45 to 55 m-equiv./day. Determinations were not made with sheep E. From its behaviour on chromatography, the additional steam-volatile acid was probably formic acid but an absolute identification was not made.

Short-term experiments. When sodium formate was rapidly infused into the rumen of sheep, immediate increases in CH_4 production occurred. With the exception of sheep F given 2·0 moles sodium formate, by 4–6 h CH_4 production had fallen to that noted in control periods. The results given in Table 4 show the increases in CH_4 production that occurred during 8 h. According to Carroll & Hungate (1955) bacterial dissimilation of formic acid results in the formation of 5·6 l. CH_4 and 16·8 l.

CO_2/mole formic acid with no consumption of oxygen. The results in Table 4 show that CH_4 production was 90, 73, 75 and 59% of the theoretical when 0·75, 1·0, 1·5 and 2·0 moles sodium formate were infused. In contrast to the results of the long-term infusions, in only one instance was CO_2 production elevated during the 8 h of infusion. In this sheep the increase was small, however, and the CO_2 production over the whole 24 h was depressed. O_2 consumption was depressed in each experiment. These results suggest that rapid infusion of formate depressed the total metabolism of the sheep, probably owing to an effect on the rumen flora rather than on the animal itself.

Table 3. *Effect of infusion of 1·5 moles or 1·0 mole formic acid into the rumen and mean effect of injecting 0·5 mole sodium formate into the jugular vein on O_2 consumption and CO_2 production of sheep*

Sheep	Amount of formic acid or formate infused (moles/24 h)	O_2 consumption (l./24 h)				CO_2 production (l./24 h)			
		Before infusion	During infusion	After infusion	Increase during infusion	Before infusion	During infusion	After infusion	Increase during infusion
C	1·5 (rumen)	386·9	408·8	384·8	+23·0	390·9	435·5	399·4	42·3
E	1·5 (rumen)	399·4	400·1	387·1	+6·8	413·4	443·9	407·1	33·4
E	1·5 (rumen)	416·1	424·2	430·2	+1·0	414·3	436·8	420·5	24·9
B	1·0 (rumen)	—	389·3	389·1	+0·1	—	413·8	401·5	12·3
B	1·0 (rumen)	389·1	391·5	—	+2·4	401·5	414·0	—	12·5
Mean of three experiments in which 0·5 mole sodium formate was infused into the jugular vein		414·7	414·7	413·6	+0·54±6·5	420·7	419·9	419·6	−0·3±5·2

Table 4. *Changes in 8 h of the gaseous exchange of sheep given 0·75, 1·0, 1·5 or 2·0 moles sodium formate as an intraruminal infusion during 30 min periods*

Sheep	Amount of sodium formate infused (moles)	Increase or decrease in 8 h		
		CH_4 production (l.)	CO_2 production (l.)	O_2 consumption (l.)
B	0·75	3·8	−4·6	−14·9
B	1·50	6·1	−14·6	−22·4
F	1·00	4·2	+2·9	−1·3
F	2·00	6·6	−11·6	−9·5

DISCUSSION

The studies of Carroll & Hungate (1955) showed that the dissimilation of methane by methanogenic bacteria of the rumen takes place in two stages:

$$HCOOH \rightarrow CO_2 + H_2, \quad (1)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O, \quad (2)$$

so that

$$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O.$$

Reaction 2 was shown to be considerably faster than reaction 1, which largely accounts for the fact that free hydrogen is very rarely detected in the rumen. It would be expected, therefore, that bacterial dissimilation of 1 mole of formic acid would ...

in the formation of 0·25 mole methane and 0·75 mole CO_2; that is, 5·6 l. methane and 16·8 l. CO_2/mole formic acid. If formic acid were completely oxidized to CO_2 and water in the body, 0·5 mole O_2 would be consumed and 1·0 mole CO_2 produced/mole formic acid.

When small amounts of formic acid were given by intravenous infusion, no significant changes in the respiratory exchange were noted, but a small increase in urinary excretion of a steam-volatile acid occurred. These results indicate that formic acid if absorbed in small amounts is unlikely to cause large changes in metabolism.

The mean values obtained in the long-term experiments in which formic acid was infused into the rumen at constant rates for several days were 4·9 ± 0·5 l. CH_4 and 18·1 ± 2·8 l. CO_2/mole formic acid given. These mean values do not differ significantly from the values expected from a bacterial dissimilation of formic acid according to the above equations. The consumption, however, rose by a mean of 4·6 ± 2·8 l./mole formic acid given, whereas bacterial dissimilation of formic acid involves no consumption of O_2. This mean increase was not statistically significant because variation from animal to animal was large. It is evident from the results for individual sheep that, when an increase in O_2 consumption was noted, CO_2 production was greater than that to be expected from the increase in CH_4 production on the basis that three times as much CO_2 as CH_4 is produced when formic acid is broken down by rumen bacteria. This suggests that the individual high values arose from period-to-period variations in metabolism, unconnected with formic acid infusion. These variations, which reached a maximum of 15 l. O_2, represent maximal variations in metabolism of only 3·5%.

The depression of metabolism noted when sodium formate was introduced into the rumen in single large doses contrasts markedly with the results found in the long-term experiments. It seems probable, but details are not available, that Reid's (1962) results, suggesting that formic acid improves energy utilization by cattle, were based on the rapid infusion of formate. In the long-term studies with continuous infusion of formic acid, the results above show that, in the living animal, formic acid is quantitatively converted into CO_2 and CH_4 in agreement with studies with rumen micro-organisms. This necessarily means that very little is absorbed.

SUMMARY

1. In five experiments with sheep receiving diets of dried grass, formic acid was infused into the rumen at a constant rate for periods of up to 17 days. Methane production increased by 4·9 ± 0·5 l./mole and carbon dioxide production by 18·1 ± 2·8 l./mole. There was no significant change in oxygen consumption.

2. Intravenous infusion of 0·5 mole sodium formate did not significantly affect CH_4 production, CO_2 production or O_2 consumption but an increase occurred in the urinary excretion of steam-volatile fatty acids.

3. Rapid infusion into the rumen of up to 2·0 moles of sodium formate resulted in relatively smaller increases in CH_4 production with depressions of O_2 consumption and in most experiments with depressions of CO_2 production.

4. It is concluded that formic acid when supplied to the animal at slow constant rates is rapidly dissimilated by rumen bacteria to CH_4 and CO_2 in amounts which agree with those found in in vitro studies with methanogenic organisms. Rapid infusion, however, depresses the total metabolism, probably by interference with micro-organisms other than those concerned with methanogenesis.

REFERENCES

Annison, E. F. (1954). *Biochem. J.* 58, 670.
Annison, E. F. & Pennington, R. J. (1954). *Biochem. J.* 57, 685.
Baker, W. H. (1952). *Nature, Lond.*, 170, 576.
Bergman, A., Lakshmi, O. L. & Reid, J. T. (1962). *J. Dairy Sci.* 45, 1203.
Carroll, E. J. & Hungate, R. E. (1955). *Archs Biochem.* 56, 525.
Lewis, R. N., Robinson, R. Q., Brown, R. E. & Shaw, J. C. (1953). *J. Dairy Sci.* 36, 825.
Cochran, N. McC., Blaxter, K. L. & Armstrong, D. G. (1958). *Publs Eur. Ass. Anim. Prod.* no. 8, p. 117.
Lewis, A. T. & Martin, A. J. P. (1952). *Biochem. J.* 50, 679.
Lewis, D. (1951). *Biochem. J.* 49, 149.
Masumoto, T. (1961). *Tohoku J. agric. Res.* 12, 3.
Reid, J. T. (1962). In *Dedication Ceremony of Frank B. Morrison Hall and Symposium Papers on Animal Nutrition's Contributions to Modern Animal Agriculture*, p. 54. Ithaca, NY: Cornell University Agricultural Experiment Station.
Wainman, F. W. & Blaxter, K. L. (1958). *Publs Eur. Ass. Anim. Prod.* no. 8, p. 85.

Preservation Efficiency and Dairy Heifer Response from Unwilted Formic and Wilted Untreated Silages

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Abstract

Four experiments were planned directly to compare unwilted formic acid treated forage with untreated forage wilted to about 35% dry matter and stored in conventional tower silos. In two experiments forage was wilted from about 19 to 35% dry matter. A third attained similar wilting starting from 32% dry matter forage, but in the fourth weather prevented any wilting. In each experiment digestibility was determined with ad libitum and maintenance feeding in 4 x 4 Latin square trials while ad libitum feed intake and growth were measured concurrently in continuous growth trials of dairy heifers. In the three experiments where wilting occurred, daily gains were slightly more on two of three formic silages while energy digestibilities and intakes were slightly better on all three untreated silages. Energy recoveries were larger on the two untreated silages which were ensiled at about 35% dry matter than on formic silage, but wilting to 47% dry matter depressed energy recovery considerably below formic silage. Gain per metric ton of dry matter ensiled was slightly more for untreated silages ensiled at about 35% dry matter than for formic silages, but when ensiled at 47% dry matter it dropped to two-thirds that of formic silage. When untreated forage could not be wilted, the silage contained 1.9% butyric acid in dry matter and 29% of nitrogen as ammonia. Animals consumed this forage at essentially maintenance, and energy digestibility was depressed to 80% of formic silage. Gain per metric ton of forage dry matter was depressed to about 10% of values usual with good forages. Well made wilted silage without treatment was equivalent or slightly better than unwilted formic silage. Practical problems of either inadequate wilting or over wilting resulted in markedly less gain per unit of forage dry matter ensiled.

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Introduction

Alternatives for forage preservation are haymaking, ensiling wilted forage, or ensiling direct cut forage. Ensiling forage wilted to about 35 or 40% dry matter is widely recommended and practiced in the United States. When properly made, wilted silage is excellent feed, but one survey (5) found 27% of these silages with heat damage. Such heat damage is indicative of excessive wilting for the ensiling conditions, large storage losses, and reduced feeding value. A recent paper (6) considered forage harvesting alternatives with emphasis on silages and particularly formic acid silages. Unwilted forage ensiled with formic acid has produced larger gains than hay or untreated unwilted silage and daily milk productions equal to or larger than hay, wilted silage or untreated unwilted silage.

These experiments were planned to make direct comparisons of storage recovery, intake, digestibility, and growth from feeding silages from formic acid treated unwilted forage and untreated forage wilted to about 35% dry matter ensiled in conventional tower silos. These conditions were met in only two of four experiments. Since the problems of under or over wilting also occur in practical silage making, these four results are presented as indicative of what can occur when wilting is the goal.

Experimental Procedure

First growth forage consisting primarily of Williamsburg alfalfa (*Medicago sativa*) was cut on May 8 to 9, 1969, for Experiment 1. First growth mixed forage consisting of Williamsburg alfalfa in bud stage and Potomac orchardgrass (*Dactylis glomerata*), fully headed but not flowering, was cut on May 18 to 19, 1970, for Experiment 2. Fourth harvest regrowth of alfalfa, orchardgrass mixed forage was cut on October 12 to 13, 1970, for Experiment 3. Third harvest regrowth commercial

orchardgrass was cut on September 2 to 3, 1969, for Experiment 4. The forage for each experiment was harvested from common areas. They were stored in tower silos that were 3.05 x 12.2 m for Experiments 1, 2, and 4 and 4.06 x 12.2 m for Experiment 3. Additions at the field chopper of .49, .52 and .48 (w/w) of 90% formic acid solution were in Experiments 1, 2, and 4 while .82% of a 69% formic and 4% acetic acid aqueous solution were used in Experiment 3. Silages were stored 28, 29, 97, and 128 days prior to initial feeding.

All fresh forage and silage were weighed and sampled. Fresh forage samples from each load were composited into four samples for chemical analysis except for wilted silage in Experiment 3 which had three samples. Daily silage samples were composited over approximately 10 days for dry matter analysis. All other analyses were on the four samples composited during the digestion trials except for both silages in Experiment 4 where a fifth sample represented silage fed after the digestion trial.

In each experiment Holstein heifers were fed silage only at calculated maintenance and ad libitum in 4 x 4 Latin square designs balanced for residual effects to measure digestibility and nitrogen balance. Each period consisted of at least 10 days preliminary and 7 days total collection of excreta. In Experiments 1 and 4 all animals were fistulated with the weights of three animals between 706 and 822 kg and one animal between 299 and 441 kg. The animals were not fistulated and had weights of 298 to 327 kg for Experiment 2 and 337 to 356 kg for Experiment 3. Continuous growth trials were conducted simultaneously with digestion trials. Silage was fed ad libitum as the sole ration to Holstein heifers paired on body weight. Animal weights at the start of Experiments 1 to 4 were 89 to 319, 98 to 336, 156 to 337, and 258 to 272 kg.

All chemical analyses were on wet samples by the methods used previously (7). Data from the four experiments were combined for statistical analyses. The recovery data were analyzed as a two way analysis with one observation per cell which gave experiment, treatment, and treatment by experiment mean squares. The composition data on the removed silage and ensiled forages, where appropriate, were analyzed as two way analysis with more than one observation per cell which gave experiment, treatment, experiment by treatment, and error mean squares. Digestibility and nitrogen utilization data were analyzed as combined Latin squares which gave experiment, treatment, treatment by experiment, animal within experiment, periods within experiment, and error mean squares. Growth data were analyzed as combined two way analysis with one observation per cell which gave experiment, treatment, treatment by experiment, pair within experiment, and error mean square. All treatment effects were tested with treatment by experiment interaction mean square rather than residual error mean square.

Results and Discussion

Overall average compositional data on the two fresh forages (forage) and two silages are in Table 1. Three orthogonal tests compared 1) formic versus untreated forages, 2) forages versus silages of both types and 3) formic versus untreated silages. Among forages, formic forages unexpectedly contained

TABLE 1. Least squares means and standard errors for fresh forage and silage composition.

Criteria	Fresh forage		Silage		SE of a mean[a]
	Formic	Untreated	Formic	Untreated	
n	16	15	17	17	
Energy (kcal/g)	4.47	4.44	4.72	4.72	.067g
Cellulose (% DM)	28.3	28.9	32.0	31.2	.62g
Hemicellulose (% DM)	14.2	14.9	14.8	11.8	.46b
Lignin (% DM)	5.96	6.27	6.97	7.32	.195g
Sugar (% DM)	7.33	5.06	3.21	1.81	1.086g
Nitrogen (% DM)	2.67	2.83	2.81	2.91	.036f
Hot water insoluble N (% N)	62.8	52.9	47.0	35.5	1.86b
Ammonia N (% N)	1.85	4.66	6.78	14.74	2.68f
pH	4.11	5.38	4.23	4.78	.123d

[a]Standard error of a mean is for n = 17. Statistical significance is for difference between formic and untreated silages: b, P<.005; d, P<.025; f, P<.10 and g, P>.10. Other statistical tests are in text.

TABLE 2. Least squares treatment within experiment means and standard errors for fresh forage and silage composition.

Source	n	Dry matter[b]	Energy	Cellulose	Hemi-cellulose	Lignin	Sugar	Nitrogen	Hot water insoluble nitrogen	Ammonia nitrogen	pH
		(%)	(kcal/g)	(% DM)					(% N)		
Experiment 1											
Formic forage	4	18.9	4.28	27.5	9.6	5.59	11.28	3.06	61.4	1.4	4.31
Untreated forage	4	35.1	4.34	28.2	9.8	6.00	6.39	3.23	50.9	3.0	5.56
Formic silage	4	25.1	4.67	31.5	8.8	7.63	2.15	3.24	41.0	8.1	4.34
Untreated silage	4	35.0	4.58	29.5	6.7	6.94	1.43	3.34	28.1	11.9	4.52
Experiment 2											
Formic forage	4	19.6	4.54	28.6	12.7	5.65	8.14	2.79	54.4	1.9	4.39
Untreated forage	4	38.9	4.51	29.2	15.6	5.80	6.34	2.88	48.8	3.3	5.47
Formic silage	4	25.1	4.88	35.4	14.3	6.82	1.51	2.98	35.6	10.5	4.46
Untreated silage	4	39.1	4.70	31.9	12.6	6.60	2.09	2.96	30.1	9.6	4.56
Experiment 3											
Formic forage	4	31.7	4.53	26.1	14.0	7.11	4.74	2.69	63.7	1.9	4.46
Untreated forage	3	47.2	4.43	25.3	12.9	7.14	5.06	2.84	56.4	2.9	5.35
Formic silage	4	35.7	4.53	26.8	14.8	6.98	7.35	2.63	50.9	4.0	4.20
Untreated silage	4	51.7	4.51	28.4	16.0	8.04	3.69	2.91	40.8	8.0	4.93
Experiment 4											
Formic forage	4	23.4	4.51	30.8	20.5	5.50	5.17	2.13	71.8	2.1	4.49
Untreated forage	4	24.7	4.48	32.9	21.4	6.16	2.44	2.36	55.5	9.4	5.16
Formic silage	5	24.8	4.81	34.4	21.5	7.04	1.84	2.41	60.3	4.6	3.91
Untreated silage	5	23.9	5.10	34.9	17.8	7.73	.11	2.42	42.9	29.4	5.12
SE of a mean[1]		—	.051b	.82d	1.02g	.225b	.467b	.087g	1.95b	.79b	.134b

[b] Weighted oven dry matter.
[1] Standard error of a mean is for n = 4. Statistical significance is for experiment by treatment interaction: b, P<.005; d, P<.025 and g, P>.10.

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less ($P<.025$) nitrogen. A larger ($P<.01$) hot water insoluble nitrogen fraction and lower ($P<.001$) pH in formic forages resulted from immediate acid addition. Silage oven dry matter contained less hemicellulose ($P<.025$) and sugar ($P<.01$) but more energy ($P<.005$), cellulose ($P<.005$), lignin ($P<.001$) and nitrogen ($P<.025$) than forage. Nitrogen in silage contained smaller ($P<.001$) hot water insoluble and larger ($P<.025$) ammoniacal fractions than that in forage. The pH was lower ($P<.025$) in silages than forages. Formic silage had a higher overall average hemicellulose concentration than untreated silage although ensiled forages were not different. The nitrogen concentration in untreated silage was greater than formic silage by about the same differential as between forages. The hot-water insoluble nitrogen fraction was greater in formic silage than untreated silage, but 10 of the 12 percentage units difference were already present at ensiling. The ammoniacal nitrogen fraction was less in formic silage than untreated silage, but 3 of the 8 percentage units difference were already present at ensiling. The pH of formic silage was less than untreated silage, but the forage differential of one pH unit was reduced to about one half unit.

The chemical compositions within each experiment are in Table 2. The problems of attaining the desired wilting are indicated by the weighted dry matter percentages. Planned wilting to about 35% DM was attained in Experiments 1 and 2. In Experiment 3 standing forage was relatively dry and wilting increased dry matter to 47%. In Experiment 4 no wilting occurred due to poor weather conditions. These differences in dry matter percentage of untreated forage at ensiling caused variable responses from untreated silage which were expressed as experiment by treatment interaction in the analysis of variance. No interaction occurred for hemicellulose or nitrogen. In Experiments 1 and 2 when wilting was successfully done, the compositional differences were small with untreated silage dry matter containing slightly less energy, cellulose, and lignin than formic silage. Also the fraction of nitrogen insoluble in hot water was less. Sugar and ammoniacal nitrogen were inconsistent. In Experiment 3, when forage was wilted to 47% DM, the relation between the two silages changed with wilted silage containing more cellulose and lignin than formic silage. This untreated silage showed no evidence of heat damage with nitrogen in acid detergent fiber at .26% of DM as compared to .22 in the formic silage. The high sugar concentrations in these silages were checked, and no explanation for them is known. In Experiment 4, when wilting was unsuccessfully tried, the untreated forage already had considerable nitrogen change as indicated by smaller hot water insoluble and greater ammonia nitrogen fractions. This untreated silage had very poor chemical quality with pH above 5, total N containing 29% ammoniacal nitrogen, about 18 percentage units less hot water insoluble N than formic silage and a high energy content in oven dry matter.

Data on the recovery of ensiled forage is in Table 3. Hemicellulose recovery, which was the only significant difference between overall means, was nearly 25 percentage units greater with formic than with untreated silage. The average recovery of energy of both silages was about 90%. Energy recovery of properly wilted untreated silage was larger than formic silage but either the drier untreated silage or

TABLE 3. Percent recovery of ensiled forage from tower silos.

Constituent	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Mean		SE of a mean[b]
	Formic	Untreated	Formic	Untreated	Formic	Untreated	Formic	Untreated	Formic	Untreated	
Dry matter	80.0	95.9	78.6	84.6	95.8	76.6	89.6	78.4	86.0	83.9	5.6g
Energy	87.2	101.4	84.1	88.5	96.3	78.2	95.8	89.6	90.8	89.4	4.9g
Cellulose	91.4	99.0	96.8	92.0	99.1	85.6	100.7	82.4	97.0	89.8	4.0g
Hemicellulose	70.3	61.2	85.2	68.8	99.3	57.9	95.2	67.9	88.2	64.0	4.8e
Lignin	104.1	112.4	91.7	95.9	98.9	85.9	116.4	98.0	102.8	98.0	4.7g
Nitrogen	85.0	100.0	83.8	87.4	95.6	79.1	101.9	81.6	91.6	87.0	5.9g
Sugar	15.3	24.3	15.6	27.5	147.9	53.9	30.6	2.9	52.4	23.4	17.2g
Hot water insoluble N	56.7	54.8	57.9	53.9	78.3	56.3	85.5	61.5	69.1	56.6	4.3g
Ammoniacal N	473	378	419	255	196	219	225	254	336	277	37.8g

[b] Statistical significance of mean differences between formic and untreated silages: e, $P<.05$ and g, $P>.10$.

TABLE 4. Organic acid concentration in silages.

Criteria	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Overall means		SE of a mean[a]
	Formic	Untreated	Formic	Untreated	Formic	Untreated	Formic	Untreated	Formic	Untreated	
No. samples	4	4	4	4	4	4	5	5	..	..	..
Acetic (% DM)	5.29	7.42	2.95	4.14	1.06	1.62	5.57	0.31	3.72	3.38	1.24g
Lactic (% DM)	4.94	4.13	3.79	1.52	.70	.73	1.22	2.57	2.66	2.24	.55g
Propionic (% DM)	.08	.02	.16	.07	.01	.07	.06	.76	.08	.23	.14g
Butyric (% DM)	.06	0	.17	.06	0	.04	.13	1.65	.08	.59	.33g
Formic (% DM)	.56	.10	.28	.18	.79	.63	.99	.15	.66	.13	.12e
Total (% DM)	10.96	11.67	7.35	5.98	2.56	2.54	7.97	5.06	7.21	6.46	.19g

[a] Standard error of a mean and statistical significance is for overall means: e, $P<.05$ and g, $P>.10$.

and with no wilting gave less recovery.

Organic acid concentrations are in Table 4. Only formic acid, which had been added, differed between overall averages formic and untreated silages. Although the treatment by experiment interaction for total acids was not significant, those for all individual acids were significant ($P<.05$). Within experiments properly wilted silages produced more lactic and less acetic acid than formic silages but total acids were inconsistent. The more drier forages of Experiment 3 produced less than half the total acids of Experiments 1 and 2. These silages contained equal total acids composed of about equal acetic but less lactic acid and more formic acid in formic silages. When wilting was unsuccessful, untreated silage had about two-thirds total acids, less than one-tenth lactic, about double acetic and over 10 times more butyric acids than formic silage. The retention of formic acid applied at the field chopper as based on samples obtained as the forage entered the blower was 88, 80, 53, and 84% i.e., drier forage of Experiment 3 retained less. The formic acid concentration in silage dry matter was 24, 12, 44 and 5% of that added in the field.

Overall means for digestibility and nitrogen utilization of the silages are in Table 5. No significant differences were observed for dry matter, energy, cellulose, lignin or fecal soluble. Hemicellulose was about eight percentage units more digestible in formic than untreated silage. This resulted from the higher hemicellulose retained in formic silage since the hemicellulose concentration in fecal dry matter was still greater on formic silage (12.9 versus 12.0%). In previous experiments, hemicellulose digestibility was greater in unwilted formic silage than in hay or unwilted silage without treatment (7). Although the higher nitrogen digestibility in formic silage in this series of experiments resulted from the low value for the unsuccessfully wilted, untreated silage of Experiment 4, direct cut formic silage had higher nitrogen digestibility than wilted formic silage in two other experiments (1). Nearly twice as much nitrogen was retained when formic silage was fed but the difference was not significant.

Means for digestibilities and nitrogen utilization within experiments are in Table 6. No significant interaction occurred in hemicellulose digestion. Dry matter, energy, and nitrogen digestibilities, nitrogen retentions and fecal soluble excretion were similar for formic and untreated silage within Experiments 1, 2, and 3. Cellulose digestibility was similar in the first two experiments, but untreated silage cellulose was more digestible in Experiment 3. The untreated and unsuccessfully wilted silage in Experiment 4 differed by having depressed dry matter, energy, cellulose, and nitrogen digestibilities. Nitrogen retention was

TABLE 5. Least squares treatment means and standard error for digestibility and nitrogen utilization.

Criteria	Formic	Untreated	SE of a mean[a]
N	32	32	
Dry matter (%)	59.9	58.8	1.12g
Energy (%)	60.1	59.6	.79g
Cellulose (%)	66.0	65.3	1.19g
Hemicellulose (%)	62.1	53.9	1.27h
Lignin (%)	10.5	9.4	3.40g
Nitrogen (% digested)	66.2	63.2	1.02f
Nitrogen (g retained/day)	21.2	12.9	5.36g
Fecal soluble (g/100 g feed DM)	19.1	19.0	.38g

[a] Statistical significance: h, $P<.005$; f, $P<.10$ and g, $P>.10$.

TABLE 6. Least squares treatment within experiment means and standard errors for digestibility and nitrogen utilization.

	n	Dry matter	Energy	Cellulose	Hemicellulose	Lignin	Nitrogen digested	Nitrogen retained	Fecal solubles
		(%)						(g/day)	(g/100 g feed DM)
Experiment 1									
Formic ad lib	4	62.2	63.9	64.8	50.1	5.5	71.3	41.7	16.1
Formic maintenance	4	64.2	65.6	66.5	50.8	8.8	72.1	10.6	14.9
Untreated ad lib	4	64.5	65.9	65.8	35.6	14.9	72.6	46.2	15.8
Untreated maintenance	4	65.8	67.5	66.9	41.8	11.7	75.2	12.0	14.8
Experiment 2									
Formic ad lib	4	62.5	62.5	70.5	61.9	16.6	70.4	33.0	16.2
Formic maintenance	4	63.8	63.4	70.8	66.2	12.8	71.4	9.7	15.1
Untreated ad lib	4	63.2	62.7	69.1	60.8	17.1	66.8	20.9	17.0
Untreated maintenance	4	65.2	64.4	71.0	67.0	18.7	68.5	21.8	16.1
Experiment 3									
Formic ad lib	4	56.0	55.0	60.6	62.4	6.5	58.4	26.1	21.6
Formic maintenance	4	57.0	55.7	60.2	57.6	3.9	61.4	− 2.8	19.4
Untreated ad lib	4	58.2	56.4	65.6	44.6	16.6	57.4	29.8	20.0
Untreated maintenance	4	58.4	57.6	66.4	51.4	16.6	59.4	6.5	20.4
Experiment 4									
Formic ad lib	4	56.6	57.0	67.2	73.6	16.0	61.9	36.8	20.8
Formic maintenance	4	56.8	57.5	67.3	74.0	14.2	63.0	14.8	20.4
Untreated ad lib	4	46.6	50.7	58.2	62.8	− 9.7	51.8	−16.6	24.4
Untreated maintenance	4	48.3	51.3	59.5	67.1	−10.5	53.8	−17.0	23.6
SE of a mean[h]		1.31b	1.47c	1.74b	3.26g	5.78d	1.70d	7.08b	.56b

[h] Statistical significance is for experiment by treatment interaction: b, P<.005; d, P<.025 and g, P>.10.

negative, and fecal solubles increased on this silage. Although lignin digestibility was positive for all other silages, it was negative for this silage. The reasons for the digestibility depression of this silage are not apparent since total nitrogen was equal and nitrogen in acid detergent fiber was .30% of dry matter for untreated compared to .26 in formic silage.

The overall average heifer growth and ad libitum forage intakes are in Table 7. Average feed intakes and partial feed conversions after subtracting 145 kcal DE/kg$^{.75}$ for maintenance were not very different. Daily gains and gain per metric ton of dry matter stored were about 50% greater on the formic silage than on untreated silage, but these differences were not significant because of the large interactions. The relative gain per acre using formic silage would be increased some more by the extra harvesting loss from wilting (4).

TABLE 7. Least squares treatment means and standard errors of ad libitum forage intake and heifer growth.

Criteria	Formic	Untreated	SE of a mean[h]
No. animals	49	49	..
Daily gain (g)	803	548	142.4g
Daily gain (g/kg$^{.75}$)	12.1	8.6	1.88g
Daily DM consumed (% BW)	2.32	2.33	.076g
Daily Mcal DE consumed	17.3	16.8	.77g
Daily kcal DE/kg$^{.75}$	262	258	10.1g
Daily Mcal for growth	7.6	7.2	.74g
Mcal DE for growth/kg gain	10.4	11.8	.76g
Gain/metric ton DM stored (kg)	113	72	23.7g

[h] Statistical significance: g, P>.10.

Heifer gains and ad libitum feed intakes for silages within experiments are in Table 8. All silage by experiment interactions except partial feed conversion were highly significant. The properly wilted untreated silage of Experiments 1 and 2 produced daily gain, intakes, partial feed conversions and gains per unit of stored forage equivalent to formic silage. However, the excessively wilted silage of Experiment 3 had similar feed intake but reduced daily gain and consequently required more feed per unit of gain and less gain per unit of stored forage than formic silage. The untreated silage of Experiment 4, which was not wilted, gave essentially no gain as a result of very poor feed intake. This silage was subsequently supplemented for 62 days with .150 kg air dry ground corn per kilogram wet silage. Gains were 997 g/day on this ration versus 783 g/day on unsupplemented formic silage.

These and other Beltsville experiments indicate the nutritional value of formic acid in a hay crop silage program. Adding formic acid to unwilted forage produces silages equivalent to well made wilted silage with almost complete assurance against poor silage. Excluding one comparison with sudan-sorghum hybrid forage where animals were nitrogen deficient, the average daily gain on 10 unwilted formic silages fed without supplemental concentrates has been 748 g (7, 8). The average recovery of energy of seven unselected unwilted formic forages in tower silos has been 91% (7). Experiment 2 is the only one of 11 comparisons of unwilted formic silages with alternate forage preservation methods (7, 8) in which the alternate method produced greater gains and better feed conversion. In other direct comparisons of wilted silage with and without formic acid, formic acid increased recovery from both conventional (1) and horizontal (3) silos and increased heifer gain (2). Less advantage in daily milk production has been shown for formic silages fed with concentrates to milking cows (1, 2).

Properly wilting and ensiling is an excellent method of preserving forage. When wilting is excessive, high storage losses, heat damage, and consequent reduced protein digestibility occur. When wilting is inadequate, problems of high storage losses and poor intake occur. A good ensiling plan would be to wilt forage enough to avoid seepage when weather permits but avoid excessive wilting. During poor wilting weather, the addition of formic acid would allow continuous harvesting with the resultant silage equivalent to good wilted silage. In fact, addition of formic acid to wilted forage at ensiling has reduced storage losses and improved gains in our experiments, but the relative effect may be slightly less than on unwilted forage.

Acknowledgment

The authors wish to thank Union Carbide

TABLE 8. Least squares treatment within experiment means and standard errors of ad libitum forage intake and heifer growth.

	Experiment 1		Experiment 2		Experiment 3		Experiment 4		
Criteria	Formic	Untreated	Formic	Untreated	Formic	Untreated	Formic	Untreated	SE of a mean[h]
Days	72	72	69	69	84	84	36	36	..
No. animals	15	15	14	14	10	10	10	10	..
Daily gain (g)	763	653	570	651	951	824	949	64	49.0a
Daily gain (g/kg$^{.75}$)	12.7	10.8	9.6	11.0	13.5	11.7	12.4	.9	.74a
Daily DM consumed (% BW)	2.58	2.66	2.35	2.54	2.57	2.64	1.80	1.46	.054a
Daily Meal DE consumed	18.5	18.9	16.2	16.9	18.6	19.6	16.0	12.0	.41a
Daily kcal DE/kg$^{.75}$	300	311	276	286	263	276	209	160	5.7a
Daily Meal DE for growth	9.7	10.2	7.7	8.3	8.3	9.3	4.9	1.1	.35a
Meal DE for growth/kg gain	12.5	15.5	14.8	13.4	8.7	11.6	5.4	6.6	1.54g
Gain/metric ton DM stored (kg)	98	100	84	96	122	82	146	11	..

[h] Standard error of a mean is for n = 10. Statistical significance is for experiment by treatment interaction: a, P<.001 and g, P>.10.

Corporation for supplying the 90% formic acid and Celanese Chemical Company for supplying the formic-acetic acid mixture. B. T. Weinland, Biometrical Services, and R. H. Miller, Animal Science Research Division, are acknowledged for their help communicating with the computer.

References

(1) Derbyshire, J. C., and C. H. Gordon. 1972. Formic acid as a silage preservative for milking cows. Unpublished results.

(2) Derbyshire, J. C., D. R. Waldo, and C. H. Gordon. 1971. Dairy cattle performance on formic acid silages. J. Dairy Sci., 54:805.

(3) Derbyshire, J. C., D. R. Waldo, and C. H. Gordon. 1971. Dairy cattle performance on formic acid silages. DCRB Mimeo 71-12.

(4) Gordon, C. H., R. D. Holdren, and J. C. Derbyshire. 1969. Field losses in harvesting wilted forage. Agronomy J., 61:924.

(5) Pierson, D. C., R. D. Goodrich, J. C. Meiske, and J. C. Linn. 1971. Influence of heat damage on haylage quality. J. Animal Sci., 33:296.

(6) Waldo, D. R., and J. C. Derbyshire. 1971. The feeding value of hay crop silage. Technological Papers Presented at International Silage Research Conf., p. 141, Washington, D. C.

(7) Waldo, D. R., J. E. Keys, Jr., L. W. Smith, and C. H. Gordon. 1971. Effect of formic acid on recovery, intake, digestibility, and growth from unwilted silage. J. Dairy Sci., 54:77.

(8) Waldo, D. R., L. W. Smith, R. W. Miller, and L. A. Moore. 1969. Growth, intake, and digestibility from formic acid silage versus hay. J. Dairy Sci., 52:1609.

FORMIC ACID

C.A. 56, 5173g, (1962)

389

The natural formic acid content of fruit juices. Erich-Walter. *Alkohol Ind.* 1961, No. 15; 591-2.—Data are presented for 15 cherry juices preserved with 15 vol. % alc. and for Maraschino juice. Untreated juice showed 4.18-9.36 mg. formic acid/100 cc. juice and that preserved with alc. 4.80-11.01. R. J. Allgeier

DETECTION

JOURNAL OF CHROMATOGRAPHY 63

CHROM. 4902

GAS CHROMATOGRAPHIC SEPARATION AND DETECTION OF C_1 TO C_3 MONOCARBOXYLIC ACIDS AS THE *p*-SUBSTITUTED BENZYL ESTERS

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SUMMARY

A rapid, sensitive gas chromatographic method for the separation and detection of microgram and submicrogram quantities of formic, acetic and propionic acids is presented. The procedure involves reaction of microliter volumes of an ethanolic carboxylic acid solution mixture down to the 0.1 $\mu g/\mu l$ concentration level, with an appropriate *p*-substituted benzyl halide in a sealed capillary melting point tube at 110°. The esters formed in the reaction mixture are then isothermally eluted from a general-purpose OV-17 glass column.

INTRODUCTION

Separation and/or detection of the lower monocarboxylic acids in foods and in chemical, biological and ecological media has been achieved using spot tests, paper and thin-layer chromatography[1-5], column partition chromatography[6,7] and by gas chromatography as the free acids or after derivatization under a variety of experimental conditions[8-14].

Color tests are often subject to interference and are therefore of limited utility. Column, paper and thin-layer chromatographic techniques are virtually indispensable to the chemist but the lower volatile fatty acids sometimes demand assiduous sample separation and control of conditions, and may require relatively long development times for optimum separation. Though excellent results are reported in the literature for most of the gas chromatographic methods for the lower carboxylic acids, many require specialized columns which are not always convenient for those control laboratories which routinely process a wide diversity of chemical substances.

Therefore, in this paper we describe a sensitive method for the rapid separation and detection of microgram and submicrogram quantities of formic, acetic and propionic acids by isothermal gas chromatography on an all-purpose 5% OV-17 on Gas-Chrom Q column as their *p*-methylbenzyl, *p*-bromobenzyl or *p*-nitrobenzyl esters.

EXPERIMENTAL

Materials

The carboxylic acids used were of reagent grade quality.

The following *p*-substituted benzyl halides were used: *p*-nitrobenzyl iodide, K & K (95-99%); *p*-bromobenzyl bromide, K & K (95-99%); *p*-methylbenzyl bromide, Eastman (highest purity); *p*-nitrobenzyl bromide, Eastman (highest purity).

p-Nitrobenzyl, *p*-methylbenzyl and *p*-bromobenzyl esters of formic, acetic and propionic acids were obtained commercially where available (*p*-nitrobenzyl acetate, K & K, 95-99%; *p*-methylbenzyl acetate, K & K, 95-99%) or synthesized according to the procedures described below.

Synthesis of p-substituted benzyl propionate and acetate reference materials. Propionic acid or acetic acid (3 g) dissolved in water (5 ml) was neutralized to a phenolphthalein end-point with 20% aqueous sodium hydroxide solution. A few drops of dilute hydrochloric acid were added so that the final solution was faintly acid to litmus paper. To this was added the appropriate *p*-substituted benzyl bromide (1 g) dissolved in alcohol (25 ml) and the mixture was heated under reflux for 3 h. The mixture was evaporated to half volume and then diluted with water. After extraction into ether, the organic layer was dried over anhydrous sodium sulfate, the solvent removed and the residual oil distilled *in vacuo*.

Synthesis of p-substituted benzyl formate reference materials. The *p*-substituted benzyl alcohol (1 g) was heated under reflux in formic acid (20 ml) containing concentrated HCl (three drops) for 2 h. The mixture was cooled, diluted with ether and shaken with several portions of 3% sodium bicarbonate solution. The organic layer was dried over anhydrous sodium sulfate, the ether removed under a flow of dry nitrogen, leaving an oil which on vacuum distillation yielded the desired product. The halide may also be used, but the yields appeared to be considerably lower.

p-Bromobenzyl formate was synthesized according to the above procedure using *p*-bromobenzyl bromide.

Reference solutions

All reference esters were made up to a concentration of 1 μg/μl in ethanol.

Reagent solutions

The reagent solutions were prepared as follows: Individual carboxylic acid solutions were made up to a concentration of 1 μg/μl in ethanol. An ethanolic mixture of all three carboxylic acids was made up so as to contain 1 μg/μl of each component. Solutions of potassium hydroxide, *p*-nitrobenzyl iodide, *p*-methylbenzyl bromide, and *p*-bromobenzyl bromide each contained 3 μg per μl ethanol.

Micro sample preparation and gas chromatography

The carboxylic acid solution (10 μl) was transferred by means of a Hamilton microsyringe into a capillary melting point tube along with potassium hydroxide in ethanol solution (3 μl) and the appropriate *p*-substituted benzyl halide solution (5 μl) described above (in the case of solution 2, 15 μl were used). The tube was flame sealed and incubated at 110° for 1 h.

After cooling, 2 μl of the reaction mixture were injected into a gas chromato-

graph* equipped with a flame ionization detector unit and fitted with a 5% OV-17 on Gas-Chrom Q (100–120 mesh) glass column (4 ft. × $\frac{1}{8}$ in. I.D.) preconditioned at 280° for 24 h. Column and injection port temperatures were, respectively: for the *p*-methylbenzyl series 100° and 225°, for the *p*-bromobenzyl series 120° and 240°, and for the *p*-nitrobenzyl series 145° and 265°. Nitrogen flow was 80 ml/min, hydrogen flow 50 ml/min, column inlet pressure 8 p.s.i.g., and attenuation × 100.

RESULTS AND DISCUSSION

In order to have an adequate method for the identification of lower molecular weight carboxylic acids by gas chromatography, it was desirable to have at least three different derivatives of each acid. The *p*-substituted benzyl esters offered the most advantages as they are readily prepared and are amenable to gas chromatography.

Reference material preparation

When preparing the reference materials, not all of the ester derivatives were commercially available, hence the appropriate ones were synthesized as described. The feasibility of the synthesis routes was established by gas chromatographic comparison of the pure (95–99%) commercial ester—where available—with the vacuum distillate of the same ester prepared by laboratory synthesis. In addition, IR spectral traces were used to confirm the ester linkage by the presence of the carbonyl stretching frequency band near 1740 cm^{-1}. The correct structure for each of the remaining synthesized esters was then inferred *a posteriori* from these data. The gas chromatograms of the esters synthesized for this investigation did indicate that small quantities of impurities (*e.g.* the corresponding alcohol) were present after a single vacuum distillation but further purification did not appear to be warranted.

Micro sample preparation

In the simulated microanalysis of each carboxylic acid with each *p*-substituted benzyl halide, injection of the reaction mixture was subsequently followed by injection of the ethanolic solution of the reference ester and then finally by injection of a 1:1 mixture of the first two (see Fig. 1, chromatograms a, b and c). In every case, gas chromatography of the reaction mixture afforded a peak of identical retention time to that of the reference ester thus confirming the formation of the expected ester in the capillary tube. In a given series, ester retention times increased with molecular weight of the carboxylic acid such that a mixture of the three acids could be easily resolved after the derivatization step. Fig. 1, chromatogram d, clearly demonstrates a typical separation of a formic, acetic and propionic acid mixture after benzylation, in this instance effected with *p*-bromobenzyl bromide and ethanolic KOH at 110°. Table I gives the retention times, calculated from the point of injection, for each ester and for other relevant compounds in a series. The reaction mixtures gave, in addition to a symmetrical ester peak, a sharp secondary "reaction artifact" peak presumably resulting from isomerization or decomposition of the thermally unstable *p*-substituted benzyl halides but the nature of the product was not investigated. Ethanolic solutions of the halides can, however, be stored at 0° for a week without undergoing appreciable

* Research Specialities Co. "600 series". The FID unit was Model 660.

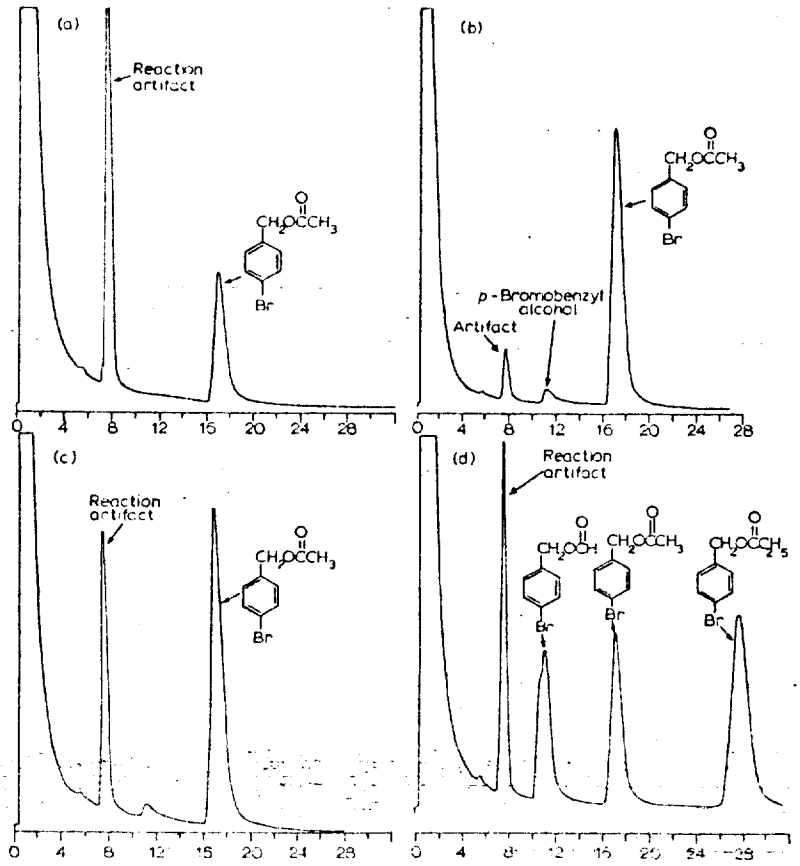

Fig. 1. (a) Reaction mixture (micro preparation using acetic acid solution). (b) Reference standard solution. (c) Reaction mixture of (a) + reference standard of (b) 1:1. (d) Reaction mixture (micro preparation using three-component acid solution).

TABLE I

RETENTION TIMES (min) OF *p*-SUBSTITUTED BENZYL FORMATE, ACETATE AND PROPIONATE ESTERS AND OTHER RELEVANT RELATED COMPOUNDS

	p-Methyl-benzyl (column temp. 100°)	*p-Bromo-benzyl (column temp. 120°)*	*p-Nitro-benzyl (column temp. 145°)*
Formate	8.4	10.9	11.0
Acetate	15.1	17.0	15.3
Propionate	26.3	27.4	22.8
Alcohol	6.6	10.2	12.6
Halide	9.3	12.7	25.5
Artifact	5.7	7.5	7.2

degradation. The presence of some unreacted *p*-nitrobenzyl iodide and its hydrolysis product *p*-nitrobenzyl alcohol was noted in the reaction mixtures of the *p*-nitrobenzyl esters. The iodide was found to be superior to the bromide in this series giving a cleaner reaction mixture and generally enhancing ester formation. In each series, the equilibrium constant for the esterification reaction appeared to be smallest for formic acid, this being suggested by the relative peak heights of the formate, acetate and propionate esters. Optimum results were realised with *p*-bromobenzyl derivatization; peaks were sharp and symmetrical and, aside from the "reaction artifact", the formation of the ester in the capillary tube was attented by no additional observable side reactions except in the case of the formate where, under the stated conditions, a small shoulder peak suggested the presence of some of the corresponding alcohol (see Fig. 1, chromatogram d).

With the appropriate attenuation setting, as little as 10 ng of reference ester were detected. However, in the micro preparation, detection of the carboxylic acid was not usually successful with sample solution concentrations lower than 0.1 $\mu g/\mu l$. The presence of appreciable amounts of water in the reaction mixture promoted virtually complete hydrolysis of the halide to the benzyl alcohol so that aqueous carboxylic acid solutions at the 1 $\mu g/\mu l$ level afforded only a very small ester peak. Nevertheless, detection of the acid was still possible when aqueous solutions of this concentration were diluted tenfold with ethanol and treated as described.

Though a number of chromatographic methods have already been proposed for the detection and separation of C_1 to C_3 monocarboxylic acids in various media such as in foods, in cigarette smoke or as pollutants in river waters, the present method might prove useful for such determinations carried out by control laboratories which, of necessity, employ general-purpose columns in their day to day operation.

REFERENCES

1 B. Pszonka, *Przegląd Papier.*, 23 (1967) 6; *C.A.*, 67 (1967) 23039s.
2 J. Wierzchowski and H. Witkowski, *Mitt. Gebiete Lebensm. Hyg.*, 57 (1966) 101; *C.A.*, 66 (1967) 1811m.
3 B. E. Kuvaev and N. S. Ivanitov, *Zavodsk. Lab.*, 33 (1967) 1382; *C.A.*, 68 (1968) 74914b.
4 . Dittman, *J. Chromatog.*, 31 (1968) 497.
5 S. Pszonka, *Chem. Anal. (Warszaw.)*, 13 (1968) 83; *C.A.*, 68 (1968) 96922e.
6 . O. Turgel and T. V. Kashanova, *Gidrolizn. i Lesokhim. Prom.*, 14 (1961) 16; *C.A.*, 55 (1961) 17374c.
7 . G. Stradomskaya and I. A. Goncharova, *Gidrokhim. Materialy*, 39 (1965) 169; *C.A.*, 65 (1966) 4869g.
8 T. Oakley, L. Weissbecker and F. E. Resnick, *Anal. Chem.*, 37 (1965) 380.
9 G. Nickelly, *Anal. Chem.*, 39 (1967) 2244.
10 . N. Sokolov and A. K. Kozlenko, *Nefekhimiya*, 5 (1965) 609; *C.A.*, 64 (1966) 2724h.
11 . Kaplanova and J. Janák, *Mikrochim. Ichnoanal. Acta*, 1966, 119; *C.A.*, 65 (1966) 5966d.
12 E. Frauenfori, *Z. Anal. Chem.*, 221 (1966) 305; *C.A.*, 66 (1967) 20201k.
13 . I. Nemtseva, T. S. Kishkinova and A. D. Semenov, *Gidrokhim. Materialy*, 41 (1966) 129; *C.A.*, 67 (1967) 93851z.
14 . K. Lough, R. S. Reid, M. McKeay and F. M. Black, *J. Sci. Food Agr.*, 18 (1967) 214.

The Effect of Methanol, Formaldehyde and Formic Acid on Tissue Respiration[1]

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Introduction

In the living organism methanol is oxidized to formaldehyde and on to formic acid (1,2). Formaldehyde has been demonstrated in the tissues of animals poisoned with methanol (1,3), and formic acid is found in the urine of animals and of man after the ingestion of methanol (4). It has been generally accepted that in methanol poisoning formic acid accumulated in the tissues and produced the clinical symptoms of acidosis, retinal damage, and formic acid in the urine. However, other investigators (4) have stressed the possibility that methanol, formaldehyde, or formic acid directly inhibits tissue metabolism and that the acidosis so characteristic of methanol poisoning is a metabolic acidosis rather than due to the accumulation of formate ions. Harrop and Benedict (5) found a large amount of lactate in the urine following methanol poisoning. Roe (4) gives an extensive review of the literature on methanol poisoning. There has been no systematic investigation of the effect of methanol, formaldehyde, and formic acid on tissue respiration. Warburg (6) reported that 5.0 *M* methanol inhibited avian red blood cell respiration by 50%. The object of the present investigation has been to compare the effect of methanol, formaldehyde, and formic acid on several important enzyme systems to determine which compound had the greatest potential as an enzymatic inhibitor in methanol poisoning.

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Experimental

Conventional Warburg manometric methods have been used to determine the effect of methanol, formaldehyde, and formic acid on the following enzyme systems: (*a*) the fermentation and oxidation of glucose by baker's yeast, (*b*) the oxidation of glucose by rat liver slices, and (*c*) the oxidation of succinate and of ascorbate by rat liver homogenates. The yeast cells were used because they have a high rate of both anaerobic fermentation and aerobic oxidation. It is also possible to use intact cells, and results are easily reproduced. The effect of the compounds on the oxidation of ascorbate, succinate, and glucose was investigated because these substrates require increasingly complex enzymatic chains for catalysis (7,8). Thus, differential inhibition of metabolism might be used as a basis for establishing the site of inhibition by the compounds. Fresh, washed, baker's yeast (Fleischmann's) was used for the yeast experiments. Rats were sacrificed by concussion. The tissues were immediately removed, prepared, and suspended in the proper medium in Warburg vessels. The rat tissues were equilibrated with the substrate and inhibitor in the vessels for 10 min. before the original reading was taken and the cocks were closed. Yeast cells were equilibrated in the same manner for 20 min. before the first readings were made. The manometers were then read at 10-min. intervals for 60 min. Liver slices were suspended in Krebs-Ringer phosphate (8). Tissue homogenates (8) prepared in a glass grinder were used for the oxidation of ascorbate and succinate. Additions to the Warburg vessels, and temperature and gas phases for each experimental condition are given in the legends for Table II. Standard errors and tests for significance were calculated by Fisher's (10) method for small samples.

Results and Discussion

In the first series of experiments the effect of a range of concentrations of methanol, formaldehyde, and formic acid on each enzyme system was determined. In these experiments duplicate control vessels with no inhibitor were run simultaneously with increasing concentrations of one of the compounds in a series of experimental vessels. Results were calculated as per cent difference of experimental vessels from control vessels. Figure 1, which is representative of all the experiments, shows the effect of increasing concentrations of methanol, formaldehyde, and formic acid on the oxidation of succinate by liver homogenate. These data show that formaldehyde is about 10 times as potent as formic acid and over 100 times as potent as methanol as an inhibitor of the succinoxidase system.

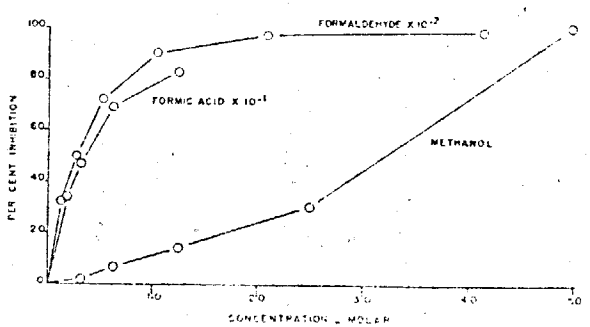

Fig. 1. The inhibition of the oxidation of succinate by rat liver homogenate by increasing concentrations of methanol, formaldehyde, and formic acid.

TABLE I

Concentration of Methanol, Formaldehyde and Formic Acid Required to Produce 50% Inhibition of Tissue Respiration

Tissue	Substrate and activity measured	Concentration Methanol *M*	Formaldehyde *M*	Formic acid *M*
Yeast	Glucose oxidation	>1.5	0.003	0.32
Yeast	Glucose fermentation	>1.0	0.003	0.16
Liver slices	Glucose oxidation	>1.0	0.010	0.50
Liver homogenate	Succinate oxidation	3.2	0.003	0.04
Liver homogenate	Ascorbate oxidation	>1.5	0.010	0.07

The results of all the experiments in which a range of concentrations of methanol, formaldehyde, and formic acid was used on the various enzyme systems are shown in Table I. The values given were obtained by plotting per cent inhibition against inhibitor, as shown in Fig. 1 for the succinoxidase system, and selecting the concentration which produced 50% inhibition. In all cases the order of inhibition was formaldehyde > formic acid > methanol. Such high concentrations of methanol and formic acid are required to produce inhibition that their action is probably due to osmotic effects rather than to any specificity for enzymes. When it was found that concentrations of methanol in the range of 1.5 *M* (5%) did not inhibit the enzyme systems, greater concentrations were not used because such concentrations were considered beyond the range of physiological significance insofar as methanol poisoning is concerned. All the above determinations with a range of concentrations of each compound were made at different times with different tissue preparations. In order to eliminate any differences due to tissue preparations or other experimental conditions, triplicate analyses were made with aliquots of a single tissue preparation in control vessels and in vessels containing methanol, formaldehyde, and formic acid at a final concentration of 0.005 *M*. Each experiment was repeated

once and the means with standard errors for the six determinations are given in Table II. These results show that at a final concentration of 0.005 *M*, formaldehyde produces from 25 to 67% inhibition of the various enzyme systems, whereas methanol and formic acid produce no significant inhibition. The fact that oxidation of succinate by liver homogenate is always inhibited more than ascorbate indicates that succinic dehydrogenase or some unknown factor (7) is more sensitive to formaldehyde than the cytochrome-cytochrome oxidase system.

These experiments have shown that formaldehyde is a much more potent inhibitor of cellular respiration than either formic acid or methanol. This is considered to be indirect evidence that formaldehyde

TABLE II

The Comparative Inhibition of Tissue Respiration by 0.005 M Methanol, Formaldehyde and Formic Acid

Tissue[a] and determination	Substrate	Control mm.³ gas, mean ± s.e.	Change from control: Methanol %	Formaldehyde %	Formic acid %
Yeast, $Q_{CO_2}^{N_2}$ (wet weight)	Glucose	79.0 ± 2.4	+0.09	−56.6[b]	+10.1
Yeast, $Q_{O_2}^{air}$ (wet weight)	Glucose	29.3 ± 0.5	−9.9	−67.3[b]	−5.1[c]
Liver slices, $Q_{O_2}^{O_2}$ (wet weight)	Glucose	1.47 ± 0.07	−6.7	−39.8[b]	−8.2
Liver homogenate $Q_{O_2}^{air}$ (wet weight)	Succinate	25.6 ± 0.5	−6.6	−60.5[b]	−10.3[c]
Liver homogenate $Q_{O_2}^{air}$ (wet weight)	Ascorbate	15.7 ± 1.4	−1.3	−24.8[b]	−3.2

[a] Additions to Warburg vessels:

(*a*) Five milligrams of yeast was suspended in 2 ml. of 0.02 *M* KH_2PO_4 (pH 4.8) to which was added 1 ml. of 3% glucose. Temperature 30°C.

(*b*) Liver slices were suspended in Krebs-Ringer phosphate containing 0.3% glucose. Temperature 37°C.

(*c*) With minor modifications the homogenate method as described by Potter (8) was used for the oxidation of succinate and ascorbate.

(*d*) The final volume in all vessels was 3.0 ml. plus 0.2 ml. 20% KOH in center well for oxygen uptake measurements. Formic acid was adjusted to the pH of the suspension media before adding it to the vessels.

[b] Statistical analysis of the data gave *P* values of less than 0.001 for these changes; this indicates the inhibition is highly significant.

[c] *P* values for these changes were less than 0.05 which indicates they are probably significant changes. *P* values for all the other changes were greater than 0.05, and hence are not significant.

rather than methanol or formic acid is the toxic agent in methanol poisoning. An excellent correlation can be made between the *in vitro* toxicity of these compounds and their potency as enzymatic inhibitors. Assuming equal distribution through 80% of the body mass, the concentration of methanol, formaldehyde, and formic acid following the administration of the minimal fatal dose in rabbits (9) would be 0.3, 0.004, and 0.06 *M*, respectively. These concentrations of formaldehyde and formic acid are almost identical to those which produce 50% inhibition of the oxidation of succinate (Table I).

Summary

1. The effect of methanol, formaldehyde, and formic acid on the following enzyme systems has been determined: (*a*) the oxidation and fermentation of glucose by yeast, (*b*) the oxidation of glucose by rat liver slices, and (*c*) the oxidation of ascorbate and succinate by rat liver homogenates.

2. The order of inhibition was formaldehyde > formic acid > methanol. Formaldehyde was a very potent inhibitor, whereas the other compounds were comparatively weak enzymatic inhibitors.

3. The possible relationship of these enzymatic inhibitors to methanol poisoning is discussed.

References

1. Pohl, J., *Arch. exptl. Path.* **31**, 2 (1893).
2. Krantz, J. C., and Carr, C. J., The Pharmacologic Principles of Medical Practice. Williams and Wilkins, Baltimore, 1949.
3. Keeser, E., *Arch. exptl. Path. Pharmakol.* **160**, 687 (1931).
4. Roe, O., *Acta Med. Scand. Suppl.* No. 182, (1946).
5. Harrop, G. A., and Benedict, E. W., *J. Am. Med. Assoc.* **74**, 25 (1920).
6. Warburg, O., Heavy Metal Prosthetic Groups and Enzyme Action. Clarendon Press, Oxford, 1949.
7. Slater, E. C., *Nature* **161**, 405 (1948).
8. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Techniques and Tissue Metabolism. Burgess, Minneapolis, 1949.
9. Sollman, T., and Hanzlik, P. J., Fundamentals of Experimental Pharmacology. Stacey, Inc., San Francisco, 1939.
10. Fisher, R. A., Statistical Methods for Research Workers, p. 121. Oliver and Boyd, Edinburgh, 1948.

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SYNTHESIS OF PURINES AND NUCLEIC ACIDS AND CATABOLISM OF PURINES BY RAT LIVER AND HEPATOMAS*†

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INTRODUCTION

PREVIOUS studies in several laboratories have shown that there is some correlation between certain enzyme activities measured *in vitro* and the growth rates of hepatomas.[1-15] Although *in vitro* studies, which are performed under artificial conditions, are useful and valuable in trying to detect differences between normal tissues and neoplastic tissues and differences between tumors of various growth rates, it is desirable to have direct information concerning *in vivo* metabolism. The techniques used in the present study permit one to obtain data on the *in vivo* flow of substrates into and through the pools of soluble components of the tissues and into the nucleic acids. Comparison of these data with data obtained in *in vitro* experiments and correlation of all of these data with the growth rates of adult liver and several rat hepatomas yield information which is pertinent to growth control mechanisms.

MATERIALS AND METHODS

Hepatomas

The rat hepatomas that were used in these experiments are listed in the order of decreasing rates of growth: Novikoff,[16] Morris 3683,[15] generation 228 or 251; Morris 8647, generation 6; Morris 7288-C, generation 8 or 17; Morris 5123 t.c., generation 4; H-35 t.c., generation 5; 5123-A,[17]

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† The following abbreviations are used in this report: Hx, hypoxanthine; HxR, inosine; IMP, inosinic acid; Ad, adenine; AdR, adenosine; AMP, adenylic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; NAD (or DPN), nicotinamide adenine dinucleotide; GMP, guanosine monophosphate; GTP, guanosine triphosphate; Xa, xanthine; XaR, xanthosine; U.A., uric acid; All., allantoin; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; L.A., lactic acid; Ser., serine; Gluc., glucose; Asp. A., aspartic acid; G.A., glutamic acid; Ala., alanine.

generation 203; 5123-C,[17] generation 25 or 35; and Reuber H-35,[18] generation 10. The Novikoff hepatoma was grown intraperitoneally, and the other tumors were grown either intramuscularly or subcutaneously. Morris 8647 is a hemangioendothelioma that was obtained following feeding of the carcinogen diethylnitrosamine. Morris 7288-C was initiated by feeding N,N'-2,7-fluorenylene-bis(2,2,2-trifluoroacetamide). Morris 5123 t.c. and H-35 t.c. were obtained by clonal isolation of cultured cells from Morris 5123-C and Reuber H-35, and these derived lines grew somewhat more rapidly than the parent lines.

Radioactive Compounds

The following radioactive compounds having the indicated specific activities (microcuries per milligram) were obtained from commercial sources and used in these experiments: sodium formate-C^{14}, 37.8, 52.7, or 55; adenine-8-C^{14}, 10.5, 11.0, 21.7, or 84.5; hypoxanthine-8-C^{14}, 24.8, 30.6, or 37.8; adenosine-8-C^{14} monophosphate (5'), 0.48 or 1.9; inosine-8-C^{14} monophosphate (5'), 0.52, 1.01, or 1.4; and inosine-8-C^{14} monophosphate (5'), barium salt, 0.28. The barium salt of the IMP was converted to the sodium salt by adding an equivalent amount of sodium sulfate to aqueous suspensions of the barium salt and removing the barium sulfate by centrifugation; the resulting supernatant solution was used in the experiments.

In vivo *Experiments*

Rats (two or three animals per group) bearing subcutaneously or intramuscularly implanted hepatomas were given intraperitoneal injections of sodium formate-C^{14} at dosages of 100 μc per rat, 200 μc per rat, or 1 μc per g of body weight, or adenine-8-C^{14} at dosages of 50 μc per rat or 0.3 μc per g of body weight. (The data given in the tables have been corrected for differences in specific activities and in dosages.) Two hours later the animals were killed by carbon dioxide asphyxiation, and the livers and tumors were excised, and, after rapid removal of necrotic and extraneous tissue, placed in ice-cold beakers. The tissues were homogenized and used for the preparation of aqueous alcoholic extracts, which were subsequently used for two-dimensional chromatography and radioautography.[19] The radioactive areas of the chromatograms were cut from the paper and assayed for radioactivity by means of a Tri-Carb liquid scintillation spectrometer. Radioactive compounds were identified by their positions on the two-dimensional chromatograms, and in some instances the identification was confirmed by rechromatography one-dimensionally in parallel with known compounds. Following the extraction with hot alcohol, the tissues were washed successively two times each with water, cold 5 per cent trichloroacetic acid, water, absolute ethanol, and ether and allowed

to dry at room temperature. Purines of RNA and of DNA were isolated and assayed for radioactivity by a previously described procedure (Procedure 1 of the reference).[20]

Determination of in vitro *Catabolic Activity of Hepatomas*

The procedures used for the experiments with minces and with sonicates of freshly excised tumors grossly freed of necrotic tissue have been described previously.[21,22] The minces, prepared by free-hand cutting with knives, were suspended in Krebs–Ringer phosphate buffer containing ATP, glucose, and the radioactive substrate (1–2 μc per g of wet tissue), and the mixture was incubated for 4½ hr at 37°C in a Dubnoff shaking incubator in an atmosphere of oxygen. The sonicates were prepared by homogenizing the tissues with a Virtis Model 27 homogenizer, suspending the homogenate in Krebs–Ringer phosphate buffer containing ATP and glucose, and then subjecting the resulting suspension to sonic vibrations by means of Raytheon 9-KC Magnetostriction Oscillator. After the addition of labeled substrates (1–2 μc per g of wet tissues), the sonicates were incubated for 60 min in the Dubnoff incubator under oxygen at 37°C. Alcoholic extracts of the minces and the sonicates were prepared and utilized for the preparation of paper chromatograms and radioautograms. The radioactive areas of the chromatograms were cut out and assayed for radioactivity by means of a Tri-Carb liquid scintillation spectrometer.

Determination of in vitro *Catabolic Activity of Regenerating Liver*

Partial hepatectomies were performed on 33 Holtzman male rats weighing 350–400 g using the procedure of Higgins and Anderson,[23] and approximately 70–75 per cent of each liver was removed. Laparotomies with massaging of the livers were performed on 28 similar rats. At selected intervals during the following 28 days, groups of three hepatectomized rats and three sham-operated rats were killed by carbon dioxide asphyxiation, and the livers were excised, weighed, and used for the preparation of minces or sonicates as described above. The minces and sonicates were utilized for determinations of catabolic activity as described above for the hepatomas.

RESULTS

In vivo *Metabolism of Formate-C^{14}*

Figure 1 shows the specific activity of the adenine of RNA and of DNA of the livers and hepatomas of animals that received intraperitoneal injections of sodium formate-C^{14}. The hepatomas are arranged in the order of decreasing rates of growth. The values given for the liver are the average values obtained for livers of all of the host animals. The specific activities

TABLE 1

Distribution of C^{14} Among the Components of Extracts of Hepatomas and Liver After Injection of Formate-C^{14}

	Per cent of total radioactivity								
	Novikoff	3683	8647	7288-C	5123 t.c.	H-35 t.c.	5123-C	H-35	Liver
No. of experiments	1	2	1	3	2	2	1	2	14
No. of tumors	1	2	1	6	3	2	1	3	—
Lactic acid	12	3	6	5	—	3	2	3	3 (±2)
Malic acid	1	—	—	1	—	1	1	—	1 (±1)
Glucose	1	1	2	2	6	9	16	9	21 (±8)
Serine	20	7	9	9	—	3	—	2	7 (±5)
Alanine	7	2	4	2	—	1	3	1	2 (±3)
Cysteine (?)	1	—	—	4	7	3	9	14	15 (±9)
Glutamine	—	1	—	1	—	1	1	1	4 (±3)
Aspartic acid	1	1	—	1	1	1	1	1	2 (±1)
Glutamic acid (+ uric acid)	3	3	2	4	2	4	4	3	2 (±1)
Hypoxanthine	8	14	20	14	11	16	11	11	4 (±3)
Xanthine	2	6	11	11	10	14	16	17	5 (±3)
Adenine	2	2	—	—	—	—	—	—	—
Inosine	6	14	19	19	19	20	20	13	12 (±6)
Xanthosine	—	—	—	1	—	—	—	—	—
Adenosine	—	3	—	2	16	—	—	1	1 (±2)
AMP	18	20	4	5	6	1	2	2	4 (±3)
ADP	3	3	1	4	2	2	1	2	2 (±1)
ATP	1	2	1	2	2	1	1	2	2 (±1)
NAD	—	—	—	—	1	—	—	—	—
IMP	2	4	12	6	1	1	—	—	1 (±1)
GMP	6	8	6	3	3	2	—	—	1 (±1)
GTP	1	—	—	—	—	1	—	—	—
Allantoin	1	2	—	3	7	9	9	10	5 (±3)
Urea	4	4	4	3	2	7	7	7	7 (±5)
Unknowns	3	4	—	—	1	2	—	—	1 (±2)
Total activity, cpm	25,800	15,800	4310	16,400	17,800	14,500	14,300	12,900	12,900

Note: The values in parentheses are standard deviations.

of both the RNA-adenine and DNA-adenine of each of the hepatomas were greater than those for the livers, and the specific activities were higher for the rapidly growing tumors than for the slowly growing tumors. Similar values were obtained for the RNA-guanine and the DNA-guanine, but they are not shown in the figure.

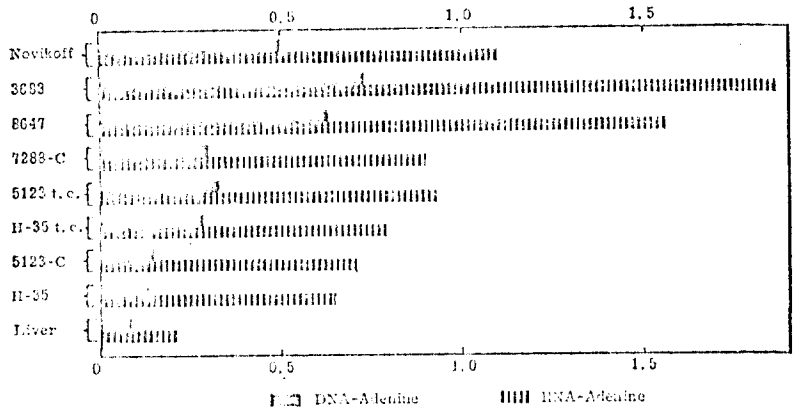

FIG. 1

In vivo incorporation of formate-C^{14} into the adenine of nucleic acids of hepatomas and liver.

Figure 2 shows photographs of the radioautograms for the extracts of Hepatoma 3683, Reuber H-35 hepatoma, and host liver of the animals that received injections of sodium formate-C^{14}. A number of radioactive compounds are detected, and some quantitative differences in the radioautograms can be detected by visual observation. The pattern for the slowly growing H-35 hepatoma is more like that of host liver than is the pattern of 3683, and the patterns for the other hepatomas included in this study are intermediate between those of 3683 and H-35. Table 1 contains the results of radioassays of the radioactive components of the extracts, and shows the total radioactivity assayed on the chromatogram and the distribution of the activity among the components of the extract. The identity of the compound listed as cysteine is doubtful, but it appears to be a sulfur-containing amino acid or peptide. The low total activity obtained for 8647 is perhaps due to the high fluid content of the tumor and the corresponding lower amount of tissue per unit of weight. In order to facilitate the assimilation and interpretation of the data, certain of the compounds were grouped together as shown in Table 2. The liver contained more radioactive glucose than the hepatomas, and the more slowly growing tumors contained more radioactive glucose than the rapidly growing

TABLE 2

Distribution of C^{14} Among Components of Extracts of Hepatomas and Liver After Injection of Formate-C^{14}

	Per cent of total radioactivity								
	Novikoff	3683	8647	7288-C	5123 t.c.	H-35 t.c.	5123-C	H-35	Liver
Glucose	1	1	2	2	6	9	16	9	21
Carboxylic acids	13	3	6	6	—	4	3	3	4
Amino acids	32	14	15	21	10	13	18	22	32
Purines and related compounds	50	78	74	70	78	67	60	58	37
Nucleotides	31	37	24	20	15	8	4	6	10
Xanthine + xanthosine + allantoin	3	8	11	15	17	23	25	27	10

tumors. These results are consistent with the results of other investigators who have studied glycolysis and gluconeogenesis in hepatomas.[6,14] With the exception of the Novikoff hepatoma, the quantities of radioactive carboxylic acids in the hepatomas were similar to those in the livers, but the livers contained somewhat more radioactive amino acids. On the other hand, the tumors contained larger quantities of radioactive purines and related compounds (nucleosides, nucleotides, and allantoin). A breakdown of the last category shows that the rapidly growing tumors contained larger quantities of nucleotides and smaller quantities of catabolic products (xanthine, xanthosine, and allantoin) than the slowly growing tumors. These results indicate that extensive purine synthesis is occurring in all of the tumors but that more extensive purine catabolism is occurring in the slowly growing tumors, and the decreased availability of labeled nucleotides corresponds with the decreased incorporation of C^{14} into the nucleic acids. On the other hand, the low incorporation of formate-C^{14} into the nucleic acids of liver appears to be due to a low rate of *de novo* synthesis of purine nucleotides rather than to extensive catabolism.

In vivo *Metabolism of Adenine-8-C^{14}*

Figure 3 shows the specific activities of the RNA-adenine and the DNA-adenine after injection of adenine-8-C^{14} into the tumor-bearing animals. As was the case with formate-C^{14}, more C^{14} was fixed in the nucleic acids of the tumors than of the liver of the host rats, but there was less correlation between the extent of incorporation of C^{14} from adenine-8-C^{14} and the rate of growth of the tumor. Table 3 contains data that show the total activity

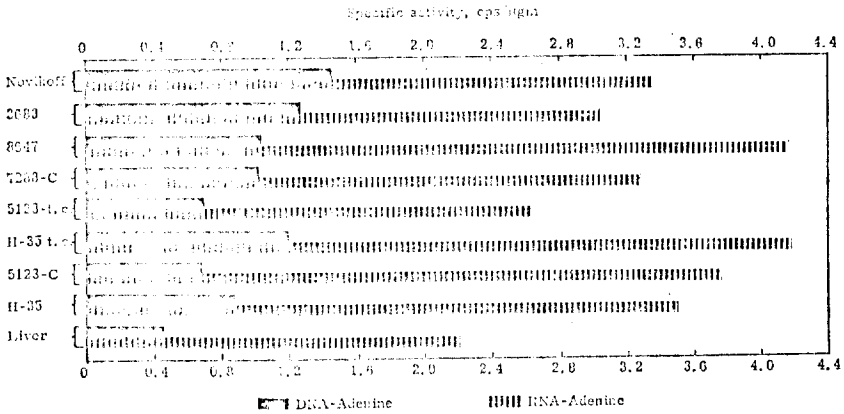

FIG. 3

In vivo incorporation of adenine-8-C^{14} into the adenine of nucleic acids of hepatomas and liver.

TABLE 3

Distribution of C^{14} Among the Components of Extracts of Hepatomas and Liver After Injection of Adenine-8-C^{14}

	Per cent of total radioactivity								
	Novikoff	3683	8647	7288-C	5123 t.c.	H-35 t.c.	5123-C	H-35	Liver
No. of experiments	1	2	1	2	1	1	1	1	14
No. of tumors	1	2	1	4	1	1	2	2	—
Adenine	1	1	—	1	2	1	1	1	1
Hypoxanthine	9	28	42	22	20	22	18	17	15
Xanthine	7	6	12	11	9	13	13	13	8
Adenosine	6	2	—	2	21	2	5	4	3
Inosine	30	26	31	30	31	38	40	31	40
Xanthosine	1	1	—	1	1	—	1	2	2
AMP	16	20	3	7	1	4	7	7	11
ADP	5	2	1	2	1	1	3	1	3
ATP	3	2	1	1	—	1	1	1	1
IMP	3	4	3	15	—	2	1	1	2
GMP	1	1	—	1	—	1	—	1	—
GTP	1	1	—	—	—	—	—	—	—
NAD	1	—	—	1	1	1	1	1	1
Unknowns	3	3	—	1	5	—	—	1	1
Uric acid	2	3	6	3	1	1	3	1	2
Allantoin	14	4	2	4	8	13	8	21	13
Total activity, cpm	49,800	—	11,600	38,100	40,400	30,200	39,800	24,500	40,300

of the radioactive compounds present on the chromatograms of the extracts and the distribution of this activity among the various components. Again the low total activity for 8647 might be due to the high fluid content of the tumor. In order to better assess the extents of anabolism and of catabolism the values for some of the compounds were combined, as shown in Table 4. There were perhaps more labeled nucleotides in the rapidly growing tumors than in the slowly growing tumors, but apparently no larger quantities of catabolic products were formed in the liver and in the slowly growing tumors than in the rapidly growing tumors.

Anabolism and Catabolism of Purines by Minced Tissues

Figure 4 shows photographs of radioautograms for extracts of tissues incubated with adenine-8-C^{14}. The extract for 3683 contained more radioactive nucleotides and less allantoin than that for H-35. The accumulation of uric acid in the 3683 in contrast to H-35 indicates that the rapidly growing tumor has less uricase activity than the slowly growing tumor. The pattern for H-35 is more similar to that of the liver than is that of the 3683. The lower density of the spots of the radioautogram for H-35 in comparison to those for 3683 and the liver are due to the fact that the adenine-8-C^{14} used in the experiment with H-35 had a lower specific activity than that used in the experiment with 3683 and liver. The spot marked "Unk." is a contaminant of unknown identity that was present in the substrate and that was carried along with the extracts. (The radioactivity of this spot was excluded in calculating the data of Table 5.) The patterns for the other hepatomas were intermediate between those for 3683 and H-35.

Figure 5 shows photographs of radioautograms that were obtained for extracts of tissues incubated with hypoxanthine-8-C^{14}. Whereas considerable quantities of nucleotides were formed by 3683, almost all of the substrate was converted to allantoin by the H-35 and the liver. The larger amounts of hypoxanthine, xanthine, and uric acid in the extract of 3683 indicates that this tumor had lower activities of xanthine oxidase and uricase than the H-35 and the liver. The patterns for the hepatomas of intermediate growth rates were correspondingly intermediate between those of 3683 and H-35.

Table 5 shows the distribution of C^{14} among the components of extracts of the tumor minces following incubation with adenine-8-C^{14}, and Table 6 shows the results of a similar experiment in which the labeled substrate was hypoxanthine-8-C^{14}. As has been shown previously,[21] both anabolism and catabolism of purines occur when purines are incubated with minced tissues. For the purpose at hand, catabolic products are defined as those products resulting from one or more oxidative transformations of the labeled substrate. In some instances anabolic processes may precede the catabolic process; for example, adenine might be converted to adenosine

TABLE 4

Distribution of C^{14} Among Anabolic and Catabolic Products in Hepatomas and Liver After Injection of Adenine-8-C^{14}

	Per cent of total radioactivity								
	Novikoff	3683	8647	7288-C	5123 t.c.	H-35 t.c.	5123-C	H-35	Liver
Nucleotides	30	30	8	26	3	10	13	12	18
Xanthine + xanthosine + uric acid + allantoin	24	14	20	19	19	27	25	37	25

or adenosine monophosphate prior to deamination and conversion to hypoxanthine, which would then be subject to the first oxidative catabolic step. Xanthine, xanthosine, uric acid, and allantoin are the catabolic products of adenine and hypoxanthine that are commonly found in these experiments. The anabolic products and catabolic products are grouped in Table 7. With both substrates, considerably more anabolism and considerably less catabolism occurred with the minces of the rapidly growing hepatomas than with the minces of the slowly growing tumors or the host livers.

TABLE 5

Distribution of C^{14} Among the Components of Extracts of Hepatomas and Liver After Incubation of Minces with Adenine-8-C^{14}

	Per cent of total radioactivity						
	Novikoff	3683	7288-C	5123 t.c.	5123-A	H-35	Liver
Adenine	1	1	1	2	22	12	5
Hypoxanthine	7	4	3	3	4	8	2
Xanthine	5	6	2	3	2	5	3
Adenosine	6	6	5	25	22	13	14
Inosine	10	4	4	3	8	5	5
Xanthosine	—	1	—	—	—	—	—
AMP	26	18	18	21	6	13	26
ADP	23	26	12	7	1	2	4
ATP	12	13	3	2	—	1	1
IMP	1	4	4	10	2	3	7
NAD	2	5	5	2	1	2	4
Unknowns	—	3	10	1	4	—	4
Uric acid	4	9	11	—	5	1	—
Allantoin	3	1	20	21	23	36	25
Total activity, cpm	—	25,000	28,000	26,300	4580	21,400	26,700

Catabolism of Purine Ribonucleotides by Sonicates of Tissues

Sonicates are used for the study of the catabolism of purine ribonucleotides in order to eliminate the question of permeability of whole cells to ribonucleotides. Table 8 contains data that show the distribution of the radioactivity among the compounds detected on the chromatograms of the tissue extracts. In this table the data are presented as cumulative values to facilitate recognition of the steps in the cumulation at which the values for various tumors become similar and thus pinpoint the enzymatic differences in the tumors that are responsible for the different extents of over-all

TABLE 6

Distribution of C^{14} Among the Components of Extracts of Hepatomas and Liver After Incubation of Minces with Hypoxanthine-8-C^{14}

	Per cent of total radioactivity								
	Novikoff	3683	8647	7288-C	5123 t.c.	H-35 t.c.	5123-A	H-35	Liver
Hypoxanthine	5	9	36	—	1	2	1	1	1
Xanthine	20	29	48	2	5	8	—	1	1
Adenosine	3	2	—	2	2	—	—	1	—
Inosine	3	2	2	1	1	—	—	—	—
Xanthosine	—	3	2	—	1	2	—	—	—
AMP	13	6	—	5	2	1	—	1	2
ADP	9	8	—	2	—	—	—	—	—
ATP	4	3	—	1	—	—	—	—	—
IMP	1	2	—	2	1	—	—	—	—
NAD	1	3	—	2	—	—	—	—	—
Unknowns	—	1	—	—	—	—	—	—	—
Uric acid	24	29	10	15	—	1	—	—	1
Allantoin	17	3	2	67	87	86	99	95	93
Total activity, cpm	—	18,100	7500	16,400	15,000	13,500	4170	16,800	11,300

TABLE 7

Distribution of C^{14} Among Anabolic Products and Catabolic Products in Extracts of Hepatomas and Liver After Incubation with Adenine-8-C^{14} or Hypoxanthine-8-C^{14}

	Per cent of total radioactivity								
	Novikoff	3683	8647	7288-C	5123 t.c.	H-35 t.c.	5123-A	H-35	Liver
Precursor: Adenine-8-C^{14}									
Anabolic products*	80	76	—	51	70	—	40	39	61
Catabolic products†	12	17	—	33	24	—	30	42	28
Precursor: Hypoxanthine-8-C^{14}									
Anabolic products*	32	26	2	15	6	1	0	2	2
Catabolic products†	61	64	62	84	93	97	99	96	95

* Anabolic products include adenosine, AMP, ADP, ATP, NAD, inosine, and IMP.
† Catabolic products include xanthine, xanthosine, uric acid, and allantoin.

TABLE 8

Distribution of Radioactivity Among the Compounds Derived from Labeled Purine Ribonucleotides During Incubation with Sonicates of Hepatomas

Substrate	Compound	Per cent of total radioactivity							
		Novikoff	3683	8647	7288-C	5123 t.c.	H-35 t.c.	5123-A	H-35
AMP-8-C^{14}(5')	All.	1	0	—	14	25	—	54	19
	All. + U.A.	4	3	—	27	26	—	55	19
	All. + U.A. + Xa	22	21	—	60	50	—	74	33
	All. + U.A. + Xa + XaR	22	21	—	62	52	—	76	34
	All. + U.A. + Xa + XaR + Hx	68	77	—	88	88	—	92	79
	All. + U.A. + Xa + XaR + Hx + HxR	99	100	—	100	99	—	100	98
	All. + U.A. + Xa + XaR + Hx + HxR + Ad	99	100	—	100	100	—	100	99
	All. + U.A. + Xa + XaR + Hx + HxR + Ad + AdR	99	100	—	100	100	—	100	99
	All. + U.A. + Xa + XaR + Hx + HxR + Ad + AdR + AMP	99	100	—	100	100	—	100	99
IMP-8-C^{14}(5')	All.	0	0	0	5	7	42	46	14
	All. + U.A.	2	2	1	9	7	42	47	14
	All. + U.A. + Xa	18	18	6	24	27	67	64	24
	All. + U.A. + Xa + XaR	18	18	6	25	28	68	66	25
	All. + U.A. + Xa + XaR + Hx	64	75	61	63	64	93	84	65
	All. + U.A. + Xa + XaR + Hx + HxR	100	100	98	100	98	100	100	100
	All. + U.A. + Xa + XaR + Hx + HxR + IMP	100	100	99	100	99	100	100	100

catabolism of the purine ribonucleotides. It is evident that complete dephosphorylation of the adenosine-8-C^{14} monophosphate and of the inosine-8-C^{14} monophosphate occurred with all of the hepatomas during the 60-min period of incubation. Complete deamination of the adenine moiety of the adenosine-8-C^{14} monophosphate also occurred, but there is no evidence to show whether the deamination occurred at the level of the ribonucleotide or the ribonucleoside. Examination of the data by the method previously described[22] leads to the conclusion that differences in the levels of xanthine oxidase activity in the tumors accounts for the major portions of the differences in over-all catabolism. Less scission of inosine occurred in the homogenates of the rapidly growing tumors than in the homogenates of the slowly growing tumors, but this might be partially the result of the accumulation of the hypoxanthine in the homogenates of rapidly growing tumors,[24] with resulting inhibition of inosine phosphorylase. In agreement with the data obtained with minces (Tables 5 and 6) there is also evidence of less uricase activity in the rapidly growing tumors. With the exception of data for H-35, there is rather good correlation between the extent of over-all catabolism and rate of growth of the tumor.

Catabolism of Purines and Purine Ribonucleotides by Minces and Sonicates of Regenerating Liver

Section A of Fig. 6 shows the weights of the livers of the laparotomized and partially hepatectomized animals during the course of the experiment. Since there was some increase in the weights of the animals during the course of the experiment, the weights of the livers are calculated on the basis of 100 g of body weight. On this basis there was no significant change in the weights of the livers of the laparotomized animals during this period. The regenerating livers had grown to their maximum size by day 11 after partial hepatectomy, but this maximum size was not as great as that of the livers of the laparotomized animals. Section B of Fig. 6 shows the extents of catabolism of adenine-8-C^{14} and hypoxanthine-8-C^{14} by minces and of adenosine-8-C^{14} monophosphate and inosine-8-C^{14} monophosphate by sonicates. With the exception of the first day in the experiment with inosine-8-C^{14} monophosphate, as much, or more, catabolism occurred with the regenerating liver as with the liver of the laparotomized rats, and there appeared to be no correlation between the rate of growth of the liver and the catabolic activity. The apparently greater catabolic activity obtained in the experiment with adenosine-8-C^{14} monophosphate for the livers of both the laparotomized and partially hepatectomized animals during the latter half of the period of the experiment is probably anomalous, and no explanation for this anomaly is offered. Nevertheless, the data show that the extents of catabolism by livers from the two sets of animals were similar.

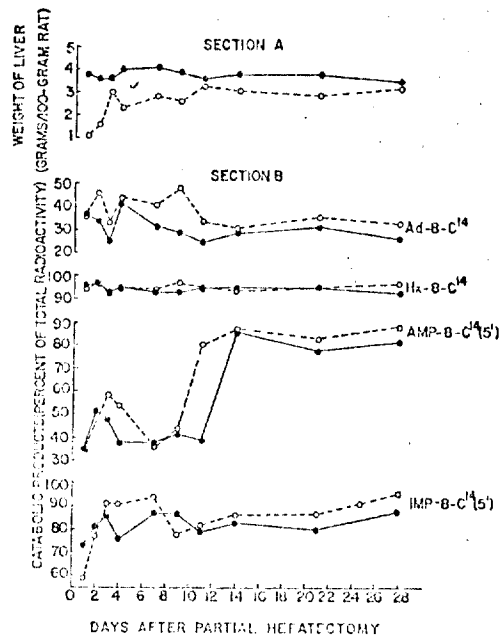

FIG. 6

Section A: Weights of regenerating liver and liver of laparotomized rats. Section B: Catabolism of purines by minces and of purine ribonucleotides by sonicates of regenerating liver and liver of laparotomized rats. O---O, Regenerating liver; O--O, Liver of laparotomized rats.

DISCUSSION

The correlation between the rate of incorporation of radioactive precursors into DNA and the rate of cellular proliferation of a tissue has been recognized for a number of years.[29] For example, less incorporation of certain labeled precursors into nucleic acids occurs in adult liver than in spleen, intestine, bone marrow, and thymus. Furthermore, less incorporation occurs in adult liver than in embryonic or young liver, or regenerating liver. The results of the present study show that there is less *in vivo* incorporation of formate-C^{14} and adenine-8-C^{14} into the DNA and RNA of host adult liver than into any of several transplantable hepatomas. There is good correlation between the extent of incorporation of formate-C^{14} into the purines of both the DNA and RNA and the rates of growth of the hepatomas (Fig. 1). There is a lower degree of correlation for the incorporation of adenine-8-C^{14} into the DNA's, and there is little or no correlation

for the incorporation of adenine-8-C^{14} into the RNA's (Fig. 3). The difference in the extent of incorporation of formate-C^{14} and adenine-C^{14} into the RNA's of the tumors is not too surprising, since it has been known for some time that even in normal adult liver, adenine is incorporated into the RNA much more extensively than formate.[25] These facts indicate that control mechanisms similar to those functioning in adult liver might be functioning to various degrees in hepatomas having various rates of growth. Thus the present study might shed some light upon metabolic processes operating in normal liver and regenerating liver as well as in hepatomas.

Perhaps the results can best be interpreted with the aid of Fig. 7, which serves as a simplified flowsheet of the metabolism of formate, adenine, and hypoxanthine. Although single arrows are used to connect consecutive compounds, the actual conversion may involve several individual steps, and in order to simplify the chart a number of intermediates and interconversions of compounds are omitted.

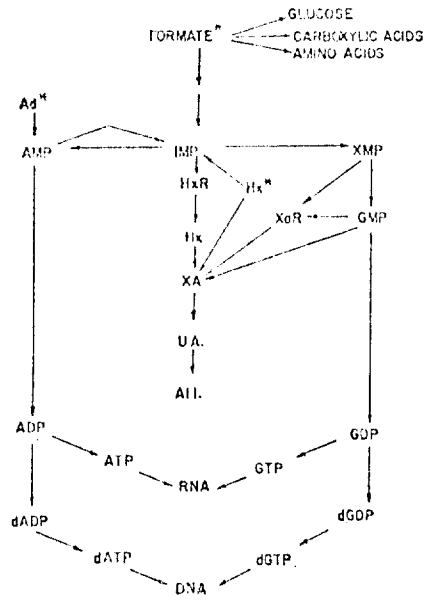

FIG. 7

A simplified flowsheet of the metabolism of formate, adenine, and hypoxanthine.

Formate can be metabolized in a number of ways including its incorporation into glucose, carboxylic acids, amino acids, and nucleic acids. As the data of Tables 1 and 2 show, the adult liver utilized formate for the synthesis of glucose more extensively than the hepatomas, and the slowly growing hepatomas utilized it more extensively for the synthesis of glucose

than the rapidly growing hepatomas. The liver and the hepatomas utilized similar quantities of formate for the synthesis of amino acids, but the liver and slowly growing hepatomas used it chiefly for the synthesis of an unidentified sulfur-containing amino acid or peptide, while the rapidly growing tumors used it chiefly for the synthesis of serine. With the exception of the Novikoff hepatoma, all of the tissues, including liver, contained about the same quantities of radioactive carboxylic acids. The data of Fig. 1 and of Table 2 show that the hepatomas incorporated more formate-C^{14} into purines and related compounds than the liver. When the soluble purines and related compounds are separated into nucleotides, which are potential building blocks for the synthesis of nucleic acids, and catabolic products (xanthine, xanthosine, and allantoin), which are probably not available for nucleic acid synthesis, the quantities of radioactive nucleotides are greatest for the rapidly growing tumors and progressively less for the more slowly growing tumors, and conversely the quantities of radioactive catabolic products are lowest for the rapidly growing tumors and progressively greater for the more slowly growing tumors (Table 2). These data indicate that both the rates of turnover of soluble nucleotides and the rates of catabolism of nucleotides can be correlated with the rates of growth of the tumors. One might speculate that the rate of catabolism of purine ribonucleotides partially controls the availability of the ribonucleotides for the synthesis of polynucleotides and thus helps control the rate of growth. The data (Table 4) obtained for the metabolism of adenine-8-C^{14} would not substantiate such a suggestion, however, because these data do not indicate any correlation of rates of growth with either rates of turnover of soluble nucleotides or rates of catabolism of nucleotides.

The results of the *in vitro* experiments give some additional information concerning the anabolic and catabolic capacities of these tumors. The data of Table 8 show that under the conditions used in these experiments there are larger quantities of anabolic products of adenine-8-C^{14} and of hypoxanthine-8-C^{14} and smaller quantities of radioactive catabolic products of these substrates in the extracts of the rapidly growing tumors than in the extracts of the slowly growing tumors. The correlation of extents of anabolism and catabolism with rates of growth are good. The data for the *in vitro* metabolism of adenine-8-C^{14} by host liver would lead one to predict that adenine nucleotides would be available for nucleic acid synthesis. The more extensive catabolism of hypoxanthine-8-C^{14} than of adenine-8-C^{14} might be expected from the positions of these compounds in the metabolic pathways. The more extensive catabolic activity of the slowly growing tumors is also shown by the data obtained in the experiments with sonicates. The lower extent of catabolism of inosine-8-C^{14} monophosphate (5′) by sonicates than of hypoxanthine-8-C^{14} by minces suggests that in the experiments with the minces much of the hypoxanthine-8-C^{14} was probably

degraded before it could be converted to IMP. Thus, these data would permit the interpretation that the rates of catabolism of purine ribonucleotides might partially determine the rate of growth. Such an interpretation would be consistent with the observation of Pomales *et al.* that the administration of an inhibitor of xanthine oxidase (4-hydroxypyrazolo-(3,4-d) pyrimidine) to mice bearing Adenocarcinoma 755/51-3D resulted in increased incorporation of C^{14} from hypoxanthine-8-C^{14} into the RNA of the liver and of the tumor and into the DNA of the tumor (no data were given for the DNA of the liver).[26]

If the rate of growth is determined by the rate of catabolism, then one would predict that following partial hepatectomy the catabolic activity of the residual liver would decrease to permit regeneration of the tissue and that as regeneration approached completion, the catabolic activity would increase to the level of normal liver. In contrast to the results obtained with the hepatomas there seems to be no correlation between the rate of growth and the purine-catabolic activity of regenerating rat liver; no decrease in catabolic activity was found for the regenerating liver (Fig. 6). Greenstein and co-workers[27] found that the xanthine dehydrogenase activity of regenerating liver was low for the first two days after partial hepatectomy but that by the third day it had risen to the normal level. DeLamirande* found a decrease in xanthine oxidase activity in regenerating liver at 12 hr after partial hepatectomy but by 24 hr (and up to 72 hr) after the surgery the xanthine oxidase activity of the regenerating liver was similar to that of livers of laparotomized animals. Feigelson and co-workers[28] found that regenerating mouse liver had increased xanthine oxidase activity compared to the livers of laparotomized mice whether the activity was expressed upon the basis of wet weight of tissue, nitrogen content of tissue, or cellularity. In contrast to these results, Canellakis and co-workers[29] found that regenerating rat liver had a lower capacity for degrading uracil and thymine than normal rat liver, and Stevens and Stocken[30] observed an increased activity of thymidine phosphorylase in regenerating liver.

One might now attempt to bring together these seemingly contradictory results to see if a reasonable explanation can be suggested. The adult liver carries out less *de novo* synthesis of purine ribonucleotides than the hepatomas and utilizes the formate-C^{14} more extensively in other metabolic areas. Therefore, fewer ribonucleotides are available to serve as building blocks for either RNA or DNA. On the other hand, the hepatomas synthesize purine ribonucleotides *de novo* rather extensively, and the availability of these nucleotides for the formation of RNA and DNA is partially determined by the rate of their catabolism, which probably begins as soon as IMP is formed. The slowly growing tumors, which have greater

* G. deLamirande, personal communication.

catabolic activity than the more rapidly growing tumors, would thus have smaller quantities of building blocks available for the synthesis of both RNA and DNA than the rapidly growing tumors.

Adenine-8-C^{14} would enter the metabolic sequence at a point beyond the locus at which the control mechanism of the liver prevents the *de novo* synthesis of ribonucleotides and beyond IMP, which would be the first compound along the *de novo* synthetic pathway that would be subjected to the catabolic enzymes. Hence, some of the radioactive AMP might flow along the pathways to nucleic acids before it is all deaminated and further degraded. Thus adenine-8-C^{14} should be incorporated into both the RNA and the DNA of the livers and of the hepatomas more extensively than formate-C^{14}, and there would be little or no correlation between the extent of incorporation and the rate of growth of the tissue or tumor. However, since there is limited incorporation of the adenine-8-C^{14} into the DNA and there is some correlation between the extent of incorporation into DNA and the growth rate of the tissue or tumor, there must be an auxiliary control mechanism that limits the conversion of ribonucleotides to DNA. The nature and locus of this control is not presently known, but it might involve the conversion of purine ribonucleotides to deoxyribonucleotides, the phosphorylation of these to the higher phosphate, the DNA polymerase reaction, or the availability of pyrimidine deoxyriboside triphosphates.

It therefore appears that at least three loci of control of nucleic acid synthesis by liver and hepatomas are indicated by these experimental results: (a) extent of *de novo* synthesis of purine ribonucleotides; (b) extent of catabolism of purine nucleotides; and (c) extent of utilization of ribonucleotides for subsequent formation of DNA. Perhaps adult liver limits the extent of *de novo* synthesis of purine ribonucleotides by some control mechanism and this limitation in conjuction with significant purine-catabolizing activity and regulated DNA polymerase activity determines the rate of cellular proliferation. In regenerating liver the control mechanism might permit more extensive *de novo* synthesis[31,32] and greater DNA polymerase activity[33] without extensive alteration of the capacity for purine catabolism, and growth of tissue would result; thus, growth would result from increased anabolic activity rather than decreased catabolic activity. Controlled decrease of anabolic activity would then accompany, and perhaps partially cause, cessation of regeneration. On the other hand, it appears that extensive *de novo* synthesis of purine ribonucleotides occurs in all of the hepatomas examined in the present study and that the rates of growth might be determined, at least partially, by the extents of catabolism of purine nucleotides and the activity of DNA polymerase. Deficiency of control of *de novo* synthesis would thus permit the continuous flow of substrate into the pools of soluble nucleotides and thence into nucleic acids.

It is recognized that an organism or cell functions as a co-ordinated whole and that interaction and interdependence of all parts (physical and chemical) determine the manner of functioning. Therefore, it is hazardous to identify any specific metabolic or physiological lesion as the prime cause of malfunctioning or abnormality. Nevertheless, an understanding of the whole is dependent upon knowledge of the parts, and it was the purpose of the present investigation to examine one of these parts and to try to correlate the functioning of this part with the functioning of the whole. It is not intended to imply in this report that the metabolic lesions described herein are the only or necessarily the most important ones.

SUMMARY

Studies of the *in vivo* metabolism of formate-C^{14} and adenine-8-C^{14} by hepatomas and host liver, of the *in vitro* metabolism of adenine-8-C^{14} and hypoxanthine-8-C^{14} by minced hepatomas, host liver, and regenerating liver, and of the *in vitro* catabolism of adenosine-8-C^{14} monophosphate (5′) and inosine-8-C^{14} monophosphate (5′) by sonicates of hepatomas, host liver and regenerating liver have been carried out. The quantities of C^{14} derived from the labeled substrate under the described conditions and present in a number of low molecular weight compounds and nucleic acids were determined. As a result of these studies it is suggested that control of cellular proliferation of liver and regenerating liver might be partially mediated through control of *de novo* synthesis of purine ribonucleotides and of DNA polymerase activity, while in hepatomas a faulty control of *de novo* synthesis permits purine ribonucleotides to be formed continuously and the levels of activity of catabolic enzymes and of DNA synthetase determine the rates of synthesis of nucleic acids and subsequent cell division.

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REFERENCES

1. V. R. Potter, H. C. Pitot, T. Ono and H. P. Morris, The comparative enzymology and cell origin of rat hepatomas. I. Doxycytidylate deaminase and thymine degradation, *Cancer Research* **20**, 1255–1261 (1960).
2. H. C. Pitot, The comparative enzymology and cell origin of rat hepatomas. II. Glutamate dehydrogenase, choline oxidase, and glucose-6-phosphatase, *Cancer Research* **20**, 1262–1268 (1960).

3. H. C. PITOT, C. PERAINO, R. H. BOTTOMLEY and H. P. MORRIS, The comparative enzymology and cell origin of rat hepatomas. III. Some enzymes of amino acid metabolism, *Cancer Research* **23**, 135–142 (1963).
4. T. ONO, D. G. R. BLAIR, V. R. POTTER and H. P. MORRIS, The comparative enzymology and cell origin of rat hepatomas. IV. Pyrimidine metabolism in minimal deviation tumors, *Cancer Research* **23**, 240–249 (1963).
5. F. MALEY and G. F. MALEY, Nucleotide interconversions. IV. Activities of deoxycytidylate deaminase and thymidylate synthetase in normal rat liver and hepatomas, *Cancer Research* **21**, 1421–1426 (1961).
6. G. WEBER, G. BANERJEE and H. P. MORRIS, Comparative biochemistry of hepatomas. I. Carbohydrate enzymes in Morris Hepatoma 5123, *Cancer Research* **21**, 933–937 (1961).
7. G. WEBER, H. P. MORRIS, W. C. LOVE and J. ASHMORE, Comparative biochemistry of hepatomas. II. Isotope studies of carbohydrate metabolism in Morris Hepatoma 5123, *Cancer Research* **21**, 1406–1411 (1961).
8. G. WEBER and H. P. MORRIS, Comparative biochemistry of hepatomas. III. Carbohydrate enzymes in liver tumors of different growth rates, *Cancer Research* **23**, 987–994 (1963).
9. M. J. SWENNY, J. ASHMORE, H. P. MORRIS and G. WEBER, Comparative biochemistry of hepatomas. IV. Isotope studies of glucose and fructose metabolism in liver tumors of different growth rates, *Cancer Research* **23**, 995–1002 (1963).
10. J. ASHMORE, M. J. SWENNY, H. P. MORRIS and G. WEBER, Change from liver-type to muscle-type fructose metabolism in hepatomas, *Biochim. Biophys. Acta* **71**, 451–453 (1963).
11. D. E. KIZER and S.-E. CHAN, The effect of hepatocarcinogenesis upon 5-hydroxytryptophan decarboxylase and serotonin deaminase, *Cancer Research* **21**, 489–495 (1961).
12. H. M. DYER, P. M. GULLINO, B. J. ENSFIELD and H. P. MORRIS, Transaminase activities of liver tumors and serum, *Cancer Research* **21**, 1522–1531 (1961).
13. J. S. ROTH, B. SHID and H. P. MORRIS, Some observations on the deamination of deoxynucleotides and deoxynucleosides by normal rat liver and hepatomas, *Cancer Research* **23**, 454–461 (1963).
14. J. C. ELWOOD, Y.-C. LEE, V. J. CRISTOFALO, S. WEINHOUSE and H. P. MORRIS, Glucose utilization in homogenates of the Morris Hepatoma 5123 and related tumors, *Cancer Research* **23**, 909–913 (1963).
15. G. P. WHEELER, J. A. ALEXANDER, A. S. DODSON, S. D. BRIGGS and H. P. MORRIS, Searches for exploitable biochemical differences between normal and cancer cells. IX. Anabolism and catabolism of purines by hepatomas 5123 and H-35, *Cancer Research* **22**, 769–778 (1962).
16. A. B. NOVIKOFF, A transplantable rat liver tumor induced by 4-dimethylaminoazobenzene, *Cancer Research* **17**, 1010–1027 (1957).
17. H. P. MORRIS, Some growth, morphological, and biochemical characteristics of Hepatoma 5123 and other new transplantable hepatomas, *Progr. Exp. Tumor Research* **3**, 370–411 (1963).
18. M. D. REUBER, A transplantable bile-secreting hepatocellular carcinoma in the rat, *J. Nat. Cancer Inst.* **26**, 891–899 (1961).
19. G. P. WHEELER and J. A. ALEXANDER, Searches for exploitable biochemical differences between normal and cancer cells. VI. Metabolism of purines *in vivo*, *Cancer Research* **21**, 390–398 (1961).
20. L. L. BENNETT, Jr., H. E. SKIPPER, C. C. STOCK and C. P. RHOADS, Searches for exploitable biochemical differences between normal and cancer cells. I. Nucleic acid purine metabolism in animal neoplasms, *Cancer Research* **15**, 485–491 (1955).
21. G. P. WHEELER and J. A. ALEXANDER, Searches for exploitable biochemical differences between normal and cancer cells. VII. Anabolism and catabolism of purines by minced tissues, *Cancer Research* **21**, 399–406 (1961).
22. G. P. WHEELER and J. A. ALEXANDER, Searches for exploitable biochemical differences between normal and cancer cells. VIII. Catabolism of purines and purine nucleotides by sonicates, *Cancer Research* **21**, 407–421 (1961).

23. G. N. Higgins and R. M. Anderson, Experimental pathology of the liver: restoration of the liver of the white rat following partial surgical removal, *Arch. Path.* **12**, 186–202 (1931).
24. M. Friedkin and H. Kalckar, Nucleoside phosphorylases, *The Enzymes* (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), Second edition, Vol. 5, pp. 237–255, Academic Press, New York, 1961.
25. G. B. Brown and P. M. Roll, Biosynthesis of nucleic acids, *The Nucleic Acids* (E. Chargaff and J. N. Davidson, eds.) Vol. 2, pp. 341–392, Academic Press, New York, 1955.
26. R. Pomales, S. Bieber, R. Friedman, and G. H. Hitchings, Augmentation of the incorporation of hypoxanthine into nucleic acids by the administration of an inhibitor of xanthine oxidase, *Biochim. Biophys. Acta* **72**, 119–120 (1963).
27. J. P. Greenstein, W. V. Jenrette and J. White, The relative activity of xanthine dehydrogenase, catalase, and amylase in normal and cancerous hepatic tissues of the rat, *J. Nat. Cancer Inst.* **2**, 17–22 (1941).
28. M. Fligelson, P. Fligelson and P. R. Gross, Xanthine oxidase activity in regenerating liver, *J. Gen. Physiol.* **41**, 233–242 (1957).
29. E. S. Canellakis, J. J. Jaffe, R. Mantsavinos and J. S. Krakow, Pyrimidine metabolism. IV. A comparison of normal and regenerating rat liver, *J. Biol. Chem.* **234**, 2096–2099 (1959).
30. L. Stevens and L. A. Stocken, Studies on enzymes involved in nucleic acid metabolism in foetal, young, and regenerating rat liver, *Biochem. J.* **87**, 12–15 (1963).
31. J. C. Nixon and S. H. Zbarsky, The incorporation of formate-C^{14} into the nucleic acids of rats with regenerating liver and Novikoff hepatoma, *Can. J. Biochem. and Physiol.* **37**, 1405–1416 (1959).
32. A. Sibatani, Incorporation of ^{14}C-formate and (2-^{14}C)-glycine into nucleic acid bases of the rat liver *in vivo* and *in vitro*, *Biochim. Biophys. Acta* **25**, 592–599 (1957).
33. F. J. Bollum and V. R. Potter, Nucleic acid metabolism in regenerating rat liver. VI. Soluble enzymes which convert thymidine to thymidine phosphates and DNA, *Cancer Research* **19**, 561–565 (1959).