aerobic conditions. Thus decarboxylation of ascorbic acid or related compounds cannot be the only source of the carbon dioxide production. The carbon dioxide production under anaerobic conditions was higher than would be expected from the pigment production, if these two were related. After 48 hours under anaerobic conditions, the mixtures still contained appreciable amounts of ascorbic acid.

The absorbance of the solution after it first reached a maximum decreased in experiments conducted at 50° C. under aerobic conditions. Reactions in the other experiments proceeded so slowly that no maximum was reached during the time the reaction was followed. Seaver and Kertesz (*19*) found maximum color production when ascorbic acid was heated in the presence of glycine and at times when ascorbic acid was heated alone. The reason for this is not known. Joslyn (*15*) more recently found that the concentration of ascorbic acid in browning systems determined whether or not the color production went through a maximum. At low concentrations of ascorbic acid the color increased continuously with time. The same relation was observed at high concentrations of ascorbic acid, but at intermediate levels the color production went through a maximum.

In sugar-glycine browning reaction considerable amounts of carboxyl carbon from glycine become associated with the brown pigment produced. This was not the case in the ascorbic acid–glycine–citric acid browning reaction studied. No carboxyl carbon was found associated with the pigment, and in the experiments with 1-C^{14} glycine very little labeling was found in anything except the original glycine. Most of the methylene carbon of glycine also remains in the original compound, although it was detected in at least three other compounds; of these the major one was the pigment. Formaldehyde production could not be detected, but as some carbon dioxide was derived from glycine the glycine was apparently degraded to a small degree.

The relation between the amount of carbon dioxide produced and the pigment production differs markedly at 37° and 50° C. This is in agreement with the findings of Joslyn that a change in reaction mechanism may occur in ascorbic acid systems between 30° and 50° C. (*15*). At lower temperatures the carbon dioxide and pigment production are linearly related. At higher temperatures a more complex relationship exists. Pigment may be formed at higher temperatures by a separate mechanism or course of reaction than at the lower temperatures, or a second pigment-producing reaction may be activated at the higher temperatures. This striking difference is shown in Figure 4.

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FOOD EMULSIFIERS

Effect of Nonionic Emulsifiers on Experimental Dietary Injury of the Liver in Rats

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The intestinal flora may influence experimental and clinical injury of the liver. Because it has been claimed that emulsifiers may change the intestinal flora, their effect on experimental hepatic injury was studied. Emulsifiers of the polyoxyethylene series, and monoglycerides in doses of 1% of total food intake, had no influence on development of experimental hepatic necrosis. Even in doses up to 10% no effect was noticed on experimental cirrhosis of the liver. Some emulsifiers in doses of 5 and 10% slightly retarded production of experimental hepatic necrosis. It is improbable that in the doses used in practice these emulsifiers have a deleterious effect on the liver.

SOME NONIONIC FOOD EMULSIFIERS, after prolonged ingestion, may produce changes in the intestinal flora (*14, 16*). Reduction in the numbers of the intestinal flora of rats has been reported after feeding of high levels (25%) of sorbitan and polyoxyethylene sorbitan esters of lauric, stearic, and oleic acids and of polyoxyethylene esters of stearic acids. This effect was thought to be causally related to the reduced growth rates of the experimental animals (*4*). On the other hand, Ely (*8*) reported that several surfactants given in small doses stimulated growth of chicks in 10- to 12-

week experiments. The growth promotion by surfactants has been compared with the much more reproducible and more intensive growth-stimulating effect of antibiotics in chicks, turkey poults, pigs, and rats (*3*, *15*, *17-21*).

In this metabolic reaction the intestinal flora plays a determining, although still unexplained, role. Measurements of interfacial tension in the presence of certain nonionic emulsifiers (*6*, *7*, *16*) make it very improbable that these substances ingested at reasonable dietary level materially change surface tension in the intestinal tract.

Antibiotics may not only promote growth in animals but also delay development of dietary massive necrosis of the liver in rats fed a diet low in vitamin E and with yeast as sole source of protein (*11*, *13*), as well as development of dietary cirrhosis of the liver (*11*, *12*). Here again the intestinal flora appears to be the determining pathogenetic factor (*11*).

These observations and analogies made it desirable to test various nonionic emulsifiers, proposed for use in small concentrations as food additives, as to their possible effect on the development of experimental dietary injury of liver (necrosis, cirrhosis) and on the intestinal flora.

Experimental Method

The nonionic partial esters of sorbitan and/or polyoxyethylene with long-chain fatty acids, known as Tween, Myrj, and Span emulsifiers, were used as food additives. In a few experiments molecularly distilled monoglycerides, Myverol products, were included (*1*). The following emulsifiers were added as supplements in varying concentrations to the basal experimental diet.

Tween 60, polyoxyethylene (20) sorbitan monostearate
Tween 80, polyoxyethylene (20) sorbitan monooleate
Span 60, sorbitan monostearate
Myrj 45, polyoxyethylene (8) stearate
Myrj 52, polyoxyethylene (40) stearate
Myverol 18–40, monoglycerides prepared from lard
Myverol 18–85, monoglycerides prepared from cottonseed oil

The basal diet (*10*, *12*) for the production of dietary massive necrosis of the liver consisted of yeast (British type of baker's yeast) 18 parts; cornstarch 79; and salt mixture USP II, 3. Peanut oil, 6.25 ml., and cod liver oil, 1.25 ml., were added to each 100 grams of the mixture. The emulsifiers when used as supplements were substituted for cornstarch. The animals also received daily supplements of vitamins: 20 γ of thiamine, 25 γ of riboflavin, 20 γ of pyridoxine, 100 γ of calcium pantothenate, and 20 γ of vitamin K, dissolved in 1 ml. of water. Young rats (Carworth Farms or Sprague-Dawley) with a starting weight between 45 and 55 grams were used.

Dietary cirrhosis of the liver was produced with use of the experimental ration proposed by Copeland and Salmon (*5*). The basal diet consisted of methanol-extracted peanut meal (Session Oil Mills, Enterprise, Ala.) 30, casein (vitamin-test, General Biochemicals Corp., Chagrin Falls, Ohio) 6, sucrose 40, lard 18, salt mixture (USP No. 2) 4, cod liver oil 2, and niacin 10 mg. per kg. The emulsifiers when used as supplements were substituted for sucrose. As vitamin supplements the animals received the same mixture as those fed the necrogenic yeast diet, with the addition of tocopherol (3 mg. per week). Rats (Sprague-Dawley) with a starting average weight of about 95 grams were used.

The animals were kept in single metal cages with wide-meshed bottoms. Food intake and weight were regularly recorded. The food intake in the various groups was kept on approximately the same level, without direct paired feeding.

In a selected number of rats the effect of 5% Tween 60 and 5% Tween 80 in the diet on the bacteriological composition of the intestinal flora was studied in comparison with rats receiving the same basal experimental necrogenic yeast diet without supplements of emulsifiers.

For bacteriological studies rats were killed with ether after they had been on the experimental diet for 2, 4, 7, 14, 21, and 45 days. The abdominal cavity was opened, sections of the ileum (about 6 inches) and the cecum were removed aseptically, and the contents of each segment were squeezed into a dry sterile weighed glass vial.

In other studies fecal samples were collected within 2 hours after excretion and introduced into sterile glass vials. The weight of the sample was determined; 10 ml. of sterile saline solution and sterile glass beads were added to each vial. The samples were thoroughly shaken and tenfold serial dilutions were made. Inoculations were made from these dilutions into the appropriate tubes or Petri dishes required for each cultural procedure.

One milliliter of the original 10 ml. of suspension of intestinal material was evaporated with some alcohol on the steam bath on dried weighed aluminum foil dishes. The dishes containing the residue were dried overnight in vacuo over calcium chloride and weighed. This weight served as basis for calculation of data on the intestinal bacterial count. The data on the fecal material were based on the wet weight.

Media and Cultural Procedures. Dilution tube counts were used for determination of Coliform bacilli, *E. coli* and *Enterococci*. Tubes containing the appropriate selective medium were inoculated in triplicate with 0.5-ml. aliquots of the dilutions of the original sample and incubated for 5 days at the appropriate temperature. Calculations were based on the assumption that the highest dilution of the inoculum which caused growth in at least two of the triplicate tubes contained one organism.

EC broth (Difco) was used at 37° C. for dilution counts of Coliforms and at 45° C. for counts of *E. coli*. The last dilution of the EC medium showing gas production was streaked on EMB agar (Difco) for confirmation of *E. coli* or Coliform.

SF broth (Difco) was used at 37° C. for dilution counts of *Enterococci*. The last dilution was streaked on blood agar plate for confirmation that the growth was due to cocci.

Plate counts were used for the determination of total counts, yeast, and Clostridia count. Known aliquots of the appropriate dilutions of the sample were spread on agar plates. Duplicate plates were run on each dilution. The plates were incubated and the colonies counted. Counts on duplicate plates were averaged and this figure served as basis for calculation of the number of bacteria in the sample.

Tryptone yeast agar (Difco) was used for total count. Duplicate dishes were incubated aerobically and anaerobically at 37° C. for 48 hours. Potato dextrose agar (Difco) at pH 3.5 was used for yeast counts. Plates were incubated at room temperature for 4 to 5 days.

The plate technique was used for the determination of Clostridia. A known aliquot of the appropriate dilution of the sample was introduced on a sterile Petri dish and the agar warmed to about 40° C. was poured over the sample.

Nutrient agar (Difco), to which 1% bactodextrose (Difco), 1% sodium sulfite, and 0.04% ferric chloride were added, was used for Clostridia count. After anaerobic incubation for 18 to 24 hours, Clostridia grew out as characteristic black colonies. The colonies were counted and representative colonies were streaked on blood agar plates, and, if hemolytic, were inoculated into litmus milk for further identification.

Experimental Results

None of the emulsifiers tested affected development of dietary massive necrosis when added as 1% of the diet mixture. In this concentration they appeared to be without influence on the hepatic necrosis or the growth rate and general condition of the experimental animals. As shown in Table I, Tween 60, Myverol 18-40, and Myverol 18-85 mixed with the basal necrogenic diet at the 5 or 10% level significantly delayed development of liver necrosis in rats, with correspondingly prolonged survival time. In contrast, Tween 80 and Myrj 52 were completely ineffective, while Span 60 and

Myrj 45 showed a slight tendency toward delaying this acute form of hepatic injury. The gain in weight on the basal experimental necrogenic diet represents only a fraction of that expected in rats of similar age, fed a nutritionally complete diet. Span 60 and Myrj 45, mixed at the 5% level with the diet, caused an additional transitory retardation. At the end of the experimental period, the control animals gained on the average 11.6 ± 2.5 grams, the rats receiving Span 60 9.5 ± 2.4 grams, and those with Myrj 45 12.0 ± 1.5 grams. No such effect on growth was noticed with Tween 60, Tween 80, Myrj 52, and Myverol 18-85 fed at the 5% level. Myverol 18-40 slowed growth, but this retardation was not further accentuated when this emulsifier was fed at the 10% level.

The effect of Myverol 18-85 in delaying the development of liver necrosis in rats may reside in the fact that monoglycerides distilled from cottonseed oil (such as Myverol 18-85) contain vitamin E. The same explanation may not be applicable to the other emulsifiers, and in particular not to Myverol 18-40 or to Tween 60, which was free from vitamin E (Analysis by Food Research Laboratories, Long Island City, N. Y.).

The assumption that Tween 60 may act through modification of the intestinal flora was not borne out by direct bacteriological studies in which the intestinal flora was investigated in control animals and in rats receiving 5% supplements of Tween 60 and Tween 80. Minor quantitative changes in the composition of the intestinal flora were statistically not significant. A summary of the observations, based on the enumeration of various bacterial types in the ileum and cecum of rats kept on the necrogenic basal diet and supplemented with Tween 60 or Tween 80, appears in Table II.

In some instances, number of intestinal bacteria was reduced in the ileum in the presence of loose, watery intestinal content. In analogous studies of intestinal flora in rats fed the same experimental diet separately supplemented with aureomycin or penicillin, similar negative results were obtained (*9*). No significant modification of the flora was shown. As it seems clear that antibiotics modify the intestinal flora, it is evident that the technique used may not demonstrate all possible changes. It did in this case indicate, however, that no significant changes ascertainable by direct count occur.

Table III summarizes the results of one experiment in which the effect of various nonionic emulsifiers (Tween 60, Span 60, Myrj 45, Myverol 18-40, and Myverol 18-85) on dietary cirrhosis of the liver was investigated. All groups of animals received 7 to 8 grams per day, of the basal cirrhosis-producing diet supplemented from the start with emulsifiers. At the end of 120 days all rats were sacrificed. The salient gross and histological findings on the liver and kidney and the changes in weight from the start to the end of the experiment are included in Table III.

In all groups very severe cirrhosis was found. The incidence of necrotizing nephrosis was equally high throughout. No significant effect of the emulsifiers in the doses used has been demonstrated. If anything, the monoglycerides aggravate both hepatic and renal changes. The smallest increase in weight was observed in the group of rats receiving 2% Myverol 18-40. However, the fact that 10% of Myverol 18-40 was less deleterious in weight increase, seems to indicate that the findings with 2% supplement were more chance than real. No specifically harmful effect was

Table I. Effect on Course of Dietary Liver Necrosis in Rats

Group	No. of Rats	Survival Time, Days, Av.	Weight Gain during First 4 Weeks, G. Av.	Food Intake (Av. G./Day) during 1–4 Weeks	Cause of Death
A. Controls[a]	10	54.6 ± 9.3[b]	6.8 ± 1.8[b]	5.8 ± 1.7[b]	Liver necrosis (10)
B. Tween 60,[a] 5%	9 1	103 ± 7.8 12	17.5 ± 1.6	6.8 ± 0.3	Liver necrosis Inanition
C. Tween 80,[a] 5%	7 3	54 ± 5.5 11	13.3 ± 3.3	6.7 ± 0.2	Liver necrosis Inanition
D. Controls[b]	9 1	68.6 ± 19.4 200	30.0 ± 2.1 43.0	7.2 ± 0.4	Liver necrosis
E. Myrj 52,[c] 5%	9 1	48.5 ± 4.4 200	23.0 ± 2.0 29	7.2 ± 0.1	Liver necrosis
A. Controls[c]	9 1	40 ± 2.7[b] Alive 200 days	10.7 ± 1.9[b] 3	6.0 ± 0.2[b]	Liver necrosis
B. Span 60,[c] 5%	9 2	52 ± 4.1 19.5	1.4 ± 2.4	6.2 ± 0.2	Liver necrosis Inanition
C. Myrj 45,[c] 5%	9 1	50 ± 2.0 21	2.6 ± 1.6	6.4 ± 0.1	Liver necrosis Inanition
D. Myverol 18-85,[c] 5%	8 4 1	64.5 ± 9.7 16 Alive 200 days	7.7 ± 1.5 0.0	6.5 ± 0.1	Liver necrosis Inanition
A. Controls[c]	7 2 1	28.4 ± 1.9[b] 29.0 Alive 200 days	13.1 ± 1.7[b] −16 −4	6.5 ± 0.2[b]	Liver necrosis Inanition
C. Tween 60,[c] 5%	10 1	59.8 ± 5.5 27.0	9.6 ± 1.0	7.3 ± 0.2	Liver necrosis Inanition
D. Tween 60,[c] 10%	8 4	73.3 ± 8.4 17.0	1.2 ± 2.5	7.2 ± 0.2	Liver necrosis Inanition
F. Myverol 18-40,[c] 5%	9 2	75.4 ± 16.4 15	5.2 ± 4.3	7.7 ± 0.2	Liver necrosis Inanition
G. Myverol 18-40,[c] 10%	9 2 1	67.5 ± 17.0 17 Alive 200 days	6.8 ± 2.8 24.0	6.8 ± 0.2	Liver necrosis Inanition

[a] Sprague-Dawley males.
[b] Standard error.
[c] Carsworth Farm males.

Table II. Effect of Dietary Supplements of Tween 60 and Tween 80 on Intestinal Flora of Rats on a Necrogenic Diet

Bacterial Type	Effect of 5% Tween 60	Effect of 5% Tween 80
Total count	No effect	No effect
Coliform	Possible increase in ileum No effect in cecum or in feces	Possible increase in ileum No effect in cecum or in feces
E. coli	Same as with Coliform	Same as with Coliform
Enterococci	Same as with Coliform	Same as with Coliform
Clostridia	Possible increase in incidence	No effect
Yeasts	No effect	No effect

Table III. Effect of Emulsifiers on Dietary Cirrhosis of Liver

Group	Supplement	Weight, G. Before	Weight, G. After	Polyserositis[a] 0	Polyserositis[a] +	Cirrhosis 0	Cirrhosis +	Cirrhosis ++ to +++	Cirrhosis ++++	Kidney 0	Kidney Necr. Nephr.
I	...	94 ± 2.4[b]	195 ± 4.0	8	2	1	0	5	4	3	7
II	2% Tween 60	94.5 ± 2.2	195 ± 4.0	9	1	0	2	3	5	3	7
III	10% Tween 60	94 ± 1.8	202 ± 2.75	9	1	0	4	3	3	6	4
IV	2% Span 60	94 ± 1.7	193 ± 5.6	9	1	1	1	2	6	5	5
V	10% Span 60	94.5 ± 1.8	182 ± 3.9	9	1	0	0	2	8	3	7
VI	2% Myrj 45[c]	93 ± 1.6	189 ± 4.6	9	0	1	0	5	3	1	8
VII	10% Myrj 45[c]	97 ± 1.6	184 ± 2.4	9	0	0	0	3	6	1	8
VIII	2% Myverol 18-40	94 ± 1.5	179 ± 5.15	4	6	0	0	3	7	0	10
IX	10% Myverol 18-40	94 ± 1.4	197 ± 5.8	7	3	0	1	1	8	1	9
X	2% Myverol 18-85[c]	95 ± 3.8	194 ± 4.6	8	1	0	0	1	8	0	9
XI	10% Myverol 18-85	95 ± 1.75	202 ± 7.3	7	3	0	0	3	7	1	9

Duration of experiment, 120 days

[a] Free fluid in serous cavities.
[b] Standard error.
[c] One rat in each experiment died early.

induced by the nonionic emulsifiers used in this experiment.

In an additional experiment all groups of animals received the basal cirrhosis-producing diet without supplements of emulsifiers. At the end of 4 weeks, at which time it was assumed the basal ration must have produced at least fatty liver, emulsifiers (Tween 60, Span 60, Myrj 45) in the concentration of 2 and 10% were added to the ration of various groups; one control group remained without such supplement. No significant effect of the emulsifiers in the doses used was demonstrated. A slight, but statistically significant, retardation of growth occurred in rats receiving 10% Span 60 or Myrj 45.

Discussion

The absence of any discernible effect on the development of dietary hepatic necrosis following the feeding of 1% concentrations of emulsifiers is consistent with the assumption that these products do not affect the intestinal flora at this concentration. At a level of 5%, Myrj 45 and Span 60 slightly delayed development of hepatic necrosis. Tween 60 and Myverol 18-40 have significantly prolonged the survival time of rats, when added at 5 or 10% level to the necrogenic basal diet. In contrast, Tween 80 and Myrj 52 have been found ineffective even at 5% level in the diet. All these emulsifiers are practically free from vitamin E. No adequate explanation can be offered for their varying effect on the development of experimental hepatic necrosis. It may be assumed that all are hydrolyzed by intestinal lipase to their component fatty acid and corresponding polyhydric alcohol moieties, with resultant loss of surface activity. Recently, Beveridge (*2*) called attention to the effect of dietary fat on the development of experimental dietary hepatic necrosis in rats and related it to changes in the intestinal flora. In contrast to the authors' selected observations, in Beveridge's studies fat enhanced the development of hepatic necrosis. This discrepancy may be due to difference in the chemical nature of the dietary fats used.

The beneficial effect of Myverol 18-85 has to be related to its high vitamin E content.

In long-term experiments (150 days) no difference was observed in the intensity of cirrhotic changes between groups of rats whose basal cirrhosis-producing diet was supplemented after 4 weeks with Span 60 or Myrj 45 or Tween 60 or Myverol 18-40 or Myverol 18-85.

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