DISCUSSION

The evidence given above makes it unlikely that the low enzymic activity of the extracts from aneurin-deficient animals is explained by a co-enzyme-like role of aneurin or cocarboxylase. It appears more probable that the lack of the vitamin exerts its effect indirectly, by reducing the food intake of the animals. This interpretation is similar to that given by Lu & Needham (1941) of their observations on the reduction of the adenylic deaminase activity in the breast muscle of aneurin-deficient pigeons. These observations are not without a more general interest for the understanding of metabolic changes during starvation: it is well known that the reduction of the weight and composition of different organs in starvation is not uniform and it seems as if there is also a sequence in the loss of activity of different enzymic systems. A general survey of the order in which different enzymes of importance for metabolism are reduced in activity during starvation has never been made.

The difference between our own results on the rat's kidney and those of Medes & Floyd (1942) has not been fully investigated. In the manometric experiment no reliable proof of the existence of a cysteic acid decarboxylase in the kidney was obtained, but it is possible that the difference in the methods employed may be responsible for our negative results.

SUMMARY

1. Extracts from the liver of rats deficient in aneurin showed marked decrease of cysteic acid decarboxylase activity. No increase in activity occurred after adding aneurin or cocarboxylase *in vitro*.

2. Feeding experiments suggest that the reduction of enzymic activity is due to restricted food intake and that the connexion with the aneurin deficiency is an indirect one.

3. No certain evidence of the presence of *l*-cysteic acid decarboxylase in extracts of rat's kidney has been obtained.

4. *dl*-Homocysteic acid was not decarboxylated by enzyme preparations from the dog or the rat, but it reduced the rate of decarboxylation of *l*-cysteic acid.

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Addendum added in proof 19 April 1945

Experiments (in collaboration with Dr Ruth Duthie) on enzyme preparations from dog's liver have recently shown that (a) removal of a precipitate formed at pH 4.5 does not lead to impairment of the enzymic activity, and (b) active acetone-dried preparations of the enzyme can be obtained.

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The Effect of Unsaturated Fatty Acids on *Lactobacillus helveticus* and other Gram-positive Micro-organisms

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Bauernfeind, Sotier & Boruff (1942) reported that certain fatty acids stimulated the growth of *Lactobacillus helveticus* in the presence of suboptimal amounts of riboflavin. Strong & Carpenter (1942) confirmed this effect with a number of fatty acids, but with linoleic acid they observed that, whereas

lower concentrations stimulated the production of lactic acid, higher concentrations had an inhibitory action. Both groups of workers made their tests with the original medium of Snell & Strong (1939), which has been used for the microbiological assay of riboflavin. It is evident, therefore, that the pre-

sence of certain fatty acids in extracts which are being assayed for riboflavin by this method may interfere with the accuracy of the result.

The response of *L. helveticus* towards different fatty acids appeared from the above observations to be complex, and we therefore decided to study the problem. The work has been extended to other Gram-positive bacteria, which have been found to react similarly to *L. helveticus* (Kodicek & Worden, 1944).

A. THE EFFECT OF UNSATURATED FATTY ACIDS ON *L. HELVETICUS*

METHODS

Strain of bacteria. An American strain of *L. helveticus* (from Snell & Strong's original strain no. 7469) was kindly supplied by Dr Barton-Wright.

Medium. Snell & Strong's (1939, 1941) riboflavin-free medium was modified by the incorporation of xylose, pantothenic acid and nicotinic acid, as suggested by Barton-Wright & Booth (1943). This constitutes the 'unextracted' medium, of which the final composition was therefore:

Photolyzed peptone*	200 ml.
Cystine	200 mg.
Salt solution 'A'	10 ml.
Salt solution 'B'	10 ml.
Glucose	20 g.
Xylose	2 g.
Calcium pantothenate	100 µg.
Nicotinic acid	100 µg.
Yeast extract (riboflavin-free)*	20 ml.
Water to	1000 ml.
Final pH	6.8
Toluene as preservative	

* Prepared as described by Snell & Strong (1939).

(Salt solution 'A' comprised K_2HPO_4 , 25 g.; KH_2PO_4 , 25 g.; water to 250 ml. Salt solution 'B' comprised $MgSO_4 \cdot 7H_2O$, 10 g.; NaCl, 0.5 g.; $FeSO_4 \cdot 7H_2O$, 0.5 g.; $MnSO_4 \cdot 4H_2O$, 0.5 g.; water to 250 ml.)

Chloroform-extracted medium. For the study of the effect of lipids, a fat-free medium was prepared by extracting the original liquid medium twice with chloroform. It was hoped that the action of lipids would be better defined when other lipid material had been removed. This 'extracted' medium was used throughout, although in some instances the experiments were duplicated with an unextracted medium.

Stock cultures. Stock cultures of *L. helveticus* were kept at 4°; stab cultures were taken at frequent intervals on to yeast-water-glucose agar.

Preparation of inoculum. A fresh inoculum was prepared from the stock culture for each set of determinations.

In the case of *L. helveticus* the inoculum was grown for 24 hr. in 5 ml. of the riboflavin-free medium, to which 0.5 µg. of riboflavin and saline to a total volume of 10 ml. had been added. The 24 hr. bacterial suspension was centrifuged and taken up in 50 ml. of sterile saline. A portion of the diluted bacterial suspension was compared nephelometrically with suitable standards, and if necessary adjusted so as to contain approximately 800 millions of bacteria per ml., no variations wider than 20–40% being permitted. When necessary, a fresh suspension from spare tubes was prepared to contain the required concentration of bacteria; 0.5 ml. of this concentration (*S*) of bacteria constituted the standard inoculum.

This standard procedure was found necessary in order to obtain comparative results and to interpret the effect of the substances under investigation; it

Fig. 1. Effect of different concentrations of inoculum (48 hours' growth). *S* = standard concentration of bacteria.

has eventually been adopted for the determination of riboflavin in biological materials (Kodicek & Worden, unpublished results).

The results obtained with different concentrations of bacteria are shown in Fig. 1. It will be seen that whereas concentrations half as strong or twice as strong as *S* did not influence the production of acid, a concentration ten times that of *S* yielded too high blanks, due probably to the riboflavin content of dead bacteria which could be utilized by fresh generations. A concentration 1/100 that of *S* gave poor growth and acid production.

Test procedure. In order to construct a standard reference curve, riboflavin was added to duplicate or triplicate tubes, containing 5 ml. of medium, in amounts equivalent to 0.03, 0.05, 0.1, 0.3 and 0.5 µg. respectively, while blanks not incorporating riboflavin were put up also. Similar concentrations of riboflavin were added to the set of tubes in which the effect of the substance under investigation was being studied. Saline was added to all tubes to make a volume of 9.5 ml., and after being autoclaved for

15 min. at 15 lb./sq. in., each tube was inoculated with 0.5 ml. of standardized bacterial suspension *S*. The final volume of medium + inoculum was 10 ml.

The growth of the organisms was then determined by estimating the amount of acid produced during incubation for 24, 48 or 72 hr. at 37°, the blank tubes also being incubated. The acid was titrated directly against 0.1 N-NaOH, with bromo-thymol-blue as internal indicator (Snell & Strong, 1939). It was found that the titration could readily be judged by direct visual comparison.

The results obtained after 24, 48 and 72 hr. incubation are shown in Fig. 2. There was a graded

Fig. 2. Effect of different times of incubation.

increase of maximal lactic acid production up to an equivalent of about 10 ml. of 0.1 N-NaOH. In our experiments in which various fatty acids or other lipids were added, the time of incubation influenced the results, as discussed later. For assays on biological material we have adopted a 72 hr. period of incubation.

RESULTS

Pilot experiments with the 'extracted' medium indicated that the removal of lipids did not appreciably affect growth or lactic acid production. The titration curves obtained were similar to those with the unextracted medium, although, in most cases, there was a slightly lower production of acid after 24 or 48 hr. Such differences were equalized after 72 hr.

Effect of saturated long-chain fatty acids. Addition of 160 μg. of stearic or palmitic acid to 10 ml. of unextracted or of 'extracted' medium caused an increase in acid production from the first 24 hr. of incubation (Fig. 3). Caprylic and caproic acids showed little or no effect.

Inhibiting effect of certain unsaturated long-chain fatty acids. Oleic acid, linoleic acid and linolenic acid, their sodium salts and methyl esters were used.

The acids were prepared from the corresponding methyl esters supplied by Prof. T. P. Hilditch, who also provided us with the following information: 'The iodine values of methyl oleate were 84.5, of methyl linoleate 173, and of methyl linolenate 256. The methyl linoleate was therefore pure (within the limits of experimental error of iodine value determination); the methyl linolenate was almost pure and the methyl oleate still contained a very small proportion of methyl stearate but was probably entirely free from linoleate.'

Fig. 3. Effect of stearic acid in ordinary medium (48 hours' incubation).

Other samples investigated were: oleic acid redistilled (The British Drug Houses, Ltd., iodine number 100), linoleic acid (Kahlbaum, iodine number 202), and technical linoleic acid (The British Drug Houses, Ltd.). The acids were added in 75% ethanol or chloroform to the 'extracted' medium in amounts ranging from 8 to 160 μg./10 ml. of final medium. The small amounts of ethanol or chloroform had no effect on the bacteria, and were presumably volatilized during autoclaving.

Oleic, linoleic and linolenic acids, and also their sodium salts, inhibited growth and acid production (Table 1). This effect, which was contrary to that of stearic and palmitic acids, depended upon: the concentration of bacteria, the amount and nature of the acid added, the time of incubation, and the presence of other lipids in the medium. The methyl esters of oleic, linoleic and linolenic acids were without effect.

When *oleic acid* or its salts, prepared from the methyl ester (iodine number 84.5), was added (160 μg./10 ml.) to the 'extracted' medium, there was complete inhibition of growth and acid production over a period of 24 hr., and a partial inhibition for 48 hr.; after 72 hr., however, the acid production was almost the same as in control samples without added oleic acid. Lower concentrations of oleic acid (80 and 40 μg./10 ml.) caused a graded response during the first 48 hr. of incubation. Oleic acid in a concentration of 8 μg./10 ml. was without effect. When the oleic acid was increased to 320 μg./10 ml.

of medium, the inhibition of growth lasted for 48 hr. and even after 72 hr. there was a partial inhibition of growth. Oleic acid was ineffective in the unextracted medium, due to the presence, as was ascertained later, of other lipids.

A much more pronounced effect was obtained with *linoleic acid* or *sodium linoleate*, which in concentrations of 160 $\mu\text{g.}/10\text{ ml.}$ inhibited growth completely for 72 hr. (and longer) in both the unextracted

growth completely for 72 hr. Unlike oleic acid, linoleic acid inhibited growth in unextracted medium and also in glucose broth when added at the level of 160 $\mu\text{g.}/100\text{ ml.}$ Similar results were obtained when less pure oleic and linoleic acids were used (iodine number 100 and 202, respectively). Solutions of both oleic and linoleic acids lost their activity on storage.

The activity of *sodium linolenate* was very similar to that of linoleic acid and of sodium linoleate (Fig. 5). There was a complete inhibition of growth and acid production in 'extracted' medium in concentrations of 160 and 320 $\mu\text{g.}/10\text{ ml.}$ for 72 hr. A concentration of 80 $\mu\text{g.}/10\text{ ml.}$ was more effective than linoleic acid in inhibiting growth and acid production (completely for 48 hr., and to a great extent even for 72 hr.). Sodium linolenate in amounts of 40 $\mu\text{g.}$, on the other hand, depressed growth and acid production only partially for 24 hr., and very slightly over a period of 48 hr. In unextracted medium sodium linolenate was as effective as sodium linoleate.

Fig. 4. Effect of different amounts of linoleic acid in chloroform-extracted medium (48 hours' incubation).

and the 'extracted' media. With the 'extracted' medium, a graded response was demonstrated with lesser concentrations of linoleic acid. Thus 80 $\mu\text{g.}/10\text{ ml.}$ inhibited growth completely for 24 hr., and inhibited it to a large extent for 48 hr., while 40 $\mu\text{g.}/10\text{ ml.}$ inhibited growth completely for 24 hr., and depressed it for 48 hr. (Fig. 4). A concentration of 8 $\mu\text{g.}/10\text{ ml.}$ was without effect. When the inoculum

Fig. 5. Effect of different amounts of sodium linolenate in chloroform-extracted medium (48 hours' incubation).

Reversal of the inhibiting effect of unsaturated fatty acids

It was found that the addition of the residue, obtained after evaporation *in vacuo* of the chloroform-extract, reversed the effect of oleic acid in 'extracted' medium. The addition of 160 $\mu\text{g.}$ of

Fig. 6. Effect of different amounts of lecithin in presence of linoleic acid (72 hours' incubation).

lecithin or cholesterol/10 ml. of 'extracted' medium similarly reversed the effect of 160 $\mu\text{g.}$ of oleic or linoleic acid, and lesser concentrations of either compound were effective against oleic acid. In the case of linoleic acid, there was a graded response to decreasing concentrations of lecithin (Fig. 6) or of cholesterol.

was reduced from about 400 to about 200 millions of bacteria, 80 $\mu\text{g.}$ of linoleic acid per 10 ml. inhibited

Table 1. *Effect on acid-production by L. helveticus of various compounds added to: (A) 'extracted' medium; (B) 'extracted' medium+linoleic acid (160 µg./10 ml.)*

Substance added (160 µg./10 ml.)	Acid production (72 hr.) in medium	
	A	B
Caproic acid	+	
Caprylic acid	+	
Palmitic acid	++	
Stearic acid	++	±
Oleic acid	0*	
Sodium oleate	0*	
Methyl oleate	+	
Linoleic acid	0	
Sodium linoleate	0	
Methyl linoleate	+	
Sodium linolenate	0	
Methyl linolenate	+	
Lecithin	++	++
Cholesterol	++	+
Lumisterol	+	+
Ergosterol	+	0
Calciferol	+	+
Vitamin A acetate	+	0
Vitamin E	+	+
Vitamin E acetate	+	+
2-Methyl-1:4-naphthoquinone	+	0
Aneurin	+	0
Pyridoxin	+	0
p-Aminobenzoic acid	+	0
Biotin concentrate	+	0
Inositol	+	0
Choline chloride	+	0
Ascorbic acid	+	0
Glutathione	+	0
Methionine	+	0
Acetylcholine		0
Saponin	+	0
Yeast nucleic acid	+	0
Allantoin	+	0
Sodium taurocholate	+	0
3:4-Benzpyrene	+	0
1:2:5:6-Dibenzanthracene	+	0
Methyl cholanthrene	+	0
Butter yellow	+	0
Insulin	+	0
Adrenaline	+	0
Thyroxine	+	0
Oestrone	+	±
Stilboestrol	0	0
Hexoestrol	0	0
Dienoestrol	0	0
Dienoestrol dipropionate	+	0
Dienoestrol dibenzoate	+	0
Dihydroxymaleic acid	+	±
Sodium fumarate	+	±
Oxaloacetic acid	+	±
Malic acid	+	±
Calcium chloride (710 µg.)	+	+

* Inhibition up to 24 hr.; production of acid later became normal.

Effects in medium B show reversal of, or failure to reverse, inhibition by linoleic acid.

++ = increased production of lactic acid.

+ = normal production of lactic acid.

± = reduced production of lactic acid.

0 = no production of lactic acid.

Blank = not tested.

The reversal could be brought about not only by simultaneous addition of lecithin or cholesterol but also by addition of these compounds after growth had already been inhibited by linoleic acid for 48 hr. This was shown by adding a sterile suspension of lecithin or cholesterol to the 48 hr. cultures containing 160 µg. of linoleic acid/10 ml. and the usual concentrations of riboflavin. Growth subsequently occurred, indicating that the effect of linoleic acid was bacteriostatic rather than bactericidal.

Both lecithin and cholesterol are surface-active, and the study was therefore extended to other surface-active substances and other compounds. The substances studied, with the effects obtained, are recorded in Table 1.

The inhibitory effect of linoleic acid was reversed not only by lecithin and cholesterol (and to a limited extent by stearic acid), but also by lumisterol, calciferol (but not ergosterol), α -tocopherol and α -tocopherol acetate. Dihydroxymaleic acid, sodium fumarate, oxaloacetic acid and malic acid produced a certain degree of reversal, but to a much less extent than the first-mentioned compounds. Dihydroxymaleic acid, oxaloacetic acid and sodium fumarate, with one double bond, did not show the same bacteriostatic effect as the long-chain unsaturated fatty acids. Calcium chloride in a concentration of 710 µg./10 ml. likewise reversed almost completely the inhibitory action of linoleic acid in 'extracted' medium.

The acid production by *L. helveticus*, with or without added linoleic acid, was not affected by a number of other compounds tested (see Table 1). Butter-yellow (dimethylazobenzene) was of interest in view of its connexion with linoleic acid during destructive oxidation (György & Tomarelli, 1943) but was inactive. Oestrone in a concentration of 160 µg./10 ml. showed some reversing effect, but only in slight degree and only on the third day of incubation.

The bacteriostatic effect of synthetic oestrogens

In confirmation of the observations of Faulkner (1943) and Brownlee, Copp, Duffin & Tonkin (1943) on other Gram-positive bacteria, we found that both diethyl stilboestrol and hexoestrol, in amounts of 160 µg./10 ml., inhibited the growth of *L. helveticus*. The action of these compounds could not be reversed by the addition of lecithin or cholesterol. Dienoestrol (4:4-dihydroxy- γ : δ -diphenyl- β : δ -hexadiene) was also inhibitory. Its dibenzoate and dipropionate esters did not stop growth, which indicates the importance of the two hydroxyl groups for the inhibition.

B. THE EFFECT OF UNSATURATED FATTY ACIDS ON OTHER BACTERIA

So far as possible, the methods adopted were identical with those described for *L. helveticus*.

Strains of bacteria. These were obtained from, and classified by, Dr Marjory Stephenson or our colleagues at the Institute of Animal Pathology, Cambridge.

Stock cultures were maintained on ordinary laboratory media, and were usually transferred to yeast-water glucose agar before use.

With *Streptococcus agalactiae*, *Staphylococcus albus*, *Bacillus anthracis*, *Listeria monocytogenes* and *Erysipelothrix rhusiopathiae*, linoleic acid exerted an inhibitory effect similar to that in the case of *L. helveticus*. The effect was not constant in the case of diphtheroid organisms. With Gram-negative bacteria (*Esch. coli*, *Proteus vulgaris*), linoleic acid was without inhibitory effect (Table 2).

depression of growth was obtained only after the addition of at least 320 µg. of linoleic acid/10 ml. of medium (Table 2).

DISCUSSION

Linolenic acid, linoleic acid, and to a lesser degree oleic acid, exert an inhibitory effect on the growth and acid-production of *L. helveticus* and other Gram-positive bacteria. The effect is similar to that reported by Strong & Carpenter (1942) with linoleic acid, and is of a bacteriostatic nature, since it can be reversed after as long as 48 hr. by the addition of lecithin or cholesterol. Baker, Harrison & Miller (1941) noted that phospholipids could reverse the inhibitory effect of synthetic detergents, but in their experiments the reversal could be effected only when the phospholipid was added prior to, or simultaneously with, the detergent. The effect of linolenic seems to be greater than that of linoleic acid. The

Table 2. *Effect of linoleic acid on various micro-organisms*

(All quantities 160 µg./10 ml. medium, unless otherwise stated)

	Extinction*	0.1 N-acid formed (72 hr.)
<i>Strep. agalactiae</i>		
Ordinary medium	—	1.7
Ordinary medium + linoleic acid	—	0
Ordinary medium + linoleic acid + cholesterol	—	0
'Extracted' medium	2.2	1.4
'Extracted' medium + stearic acid	2.4	1.5
'Extracted' medium + lecithin	2.7	1.6
'Extracted' medium + cholesterol	—	1.5
'Extracted' medium + linoleic acid	0.3	0
'Extracted' medium + linoleic acid (80 µg.)	—	0
'Extracted' medium + linoleic acid (40 µg.)	—	1.8
'Extracted' medium + linoleic acid + lecithin	—	0
'Extracted' medium + linoleic acid + cholesterol	—	0
'Extracted' medium + linoleic acid (80 µg.) + cholesterol	—	0.7
'Extracted' medium + linoleic acid (40 µg.) + cholesterol	—	2.0
<i>Staph. albus</i>		
Ordinary medium	3.0	3.4
Ordinary medium + linoleic acid (320 µg.)	—	1.5
'Extracted' medium	2.4	3.5
'Extracted' medium + linoleic acid	2.2	1.9
'Extracted' medium + linoleic acid (320 µg.)	—	1.5
'Extracted' medium + linoleic acid (320 µg.) + cholesterol	—	2.5
<i>Esch. coli</i>		
'Extracted' medium	1.8	1.2
'Extracted' medium + linoleic acid	2.4	3.1
<i>Proteus vulgaris</i>		
'Extracted' medium	1.5	1.0
'Extracted' medium + linoleic acid	2.0	1.9

Definite inhibitory effect with *B. anthracis*, *Listeria monocytogenes*, *Erysipelothrix rhusiopathiae*; doubtful effect with diphtheroids.

* Extinction = readings on step-photometer.

— = not tested.

The effect of linoleic acid on *Strep. agalactiae* was marked, and could be reversed by the addition of cholesterol only in the case of lower concentrations of acid. *Staphylococcus albus* (field and laboratory strains) appeared less sensitive, and a significant

inhibitory influence appears to be correlated with the degree of unsaturation, but the methyl esters of these acids exert no inhibitory action.

There are at least two possible explanations of the inhibitory action of the unsaturated fatty acids.

First, it may be a direct chemical action upon the metabolism of the bacteria, or upon the availability of some metabolite present in the medium, although we have no evidence of this. The effect has been limited to fatty acids with unsaturated double bonds. The possibility has to be considered that the inhibitory action of linoleic and linolenic acids is in some way connected with the fact that H_2O_2 is being formed in increasing amounts, or is not being removed from the medium (Birkinshaw & Raistrick, 1943). We have, however, found no evidence of H_2O_2 formation in either the unextracted or 'extracted' medium, or in the presence of linoleic acid, or linoleic acid + lecithin.

A second possible explanation postulates a physicochemical mechanism. If the unsaturated fatty acid were to form a monolayer around the bacteria it might exert its effect in at least three different ways, viz. (a) by changing the permeability of the adjacent surfaces, or (b) by exerting some chemical influence, or (c) by altering the surface tension and so interfering with bacterial division. The nature of the monolayer formed by an unsaturated fatty acid is different from that of the monolayer formed by a saturated fatty acid (Adam, 1941). Molecules of unsaturated fatty acids tend to arrange themselves with bends at the double bonds, while saturated fatty acid molecules tend to lie as parallel straight chains. The surface area covered by a monolayer of 160 μ g. of linoleic acid is of the order of $1.75 \times 10^{11} \mu^2$, while the approximate surface area of the 'lactobacilli in 0.5 ml. of inoculum is of the order of $3.3 \times 10^{10} \mu^2$, taking 400 millions/ml. as the average concentration. The linoleic acid added would therefore be sufficient to form about five monolayers on the area available. These calculations, therefore, do not exclude the possible formation of monolayers by the linoleic acid. The more pronounced effect of linolenic acid would be in keeping with this theory, because of the influence of the increased number of double bonds. On the other hand, methyl esters would differ in their adsorption properties and this might explain their ineffectiveness.

Apart from this indirect evidence, there is the fact that at least four of the compounds capable of reversing the inhibitory action of linoleic acid are surface-active. The effect of these compounds (lecithin, cholesterol, lumisterol and calciferol) could be explained by the fact that they could form complexes with linoleic acid, which would thus be removed from its original close contact with the bacterial surfaces. The ineffectiveness of ergosterol is also in keeping with the physico-chemical hypothesis, since this compound forms solid surface layers in which the molecular packing may be too close for complex formation with linoleic acid to occur. The ineffectiveness of saponin and sodium taurocholate may be due to lack of micelle formation

by these substances in the dilution employed. The activity of α -tocopherol and its acetate may reasonably be explained by a general similarity between the surface properties of cholesterol and the tocopherols (Askew, 1935; Moss, Cuthbertson, Danielli & Drummond, 1938).

The bacteriostatic action of linoleic and linolenic acids is of interest since linoleic acid is regarded as an unsaturated acid essential in the nutrition of higher animals.

The fact that the inhibitory action of linoleic acid is limited to Gram-positive organisms is interesting; most potent chemotherapeutic agents are similarly more active against Gram-positive bacteria.

With regard to the microbiological assay of riboflavin, our experiments allow of the following conclusions: (a) that removal of lipids by a fat-solvent, preferably chloroform, is essential for increasing the specificity of the assay; (b) that a standard inoculum of known concentration is an advantage, as it obviates the high blanks which result from the use of too heavy an inoculum; (c) that incubation for 72 hr. rather than for a shorter period tends to minimize differences due to the presence of unspecific interfering substances such as oleic acid.

SUMMARY

1. Oleic, linoleic and linolenic acids can cause complete inhibition of the growth and lactic acid production of *L. helveticus*. The extent of this inhibition depends on the concentration of bacteria, the time of incubation, the amount and nature of the fatty acid added, the presence of other lipids in the medium. Linoleic and linolenic acids exhibit this effect when added to an unextracted medium of the type used for the microbiological assay of riboflavin. Methyl esters of these acids do not show this inhibition.

2. Lecithin, cholesterol, calciferol (but not ergosterol), lumisterol, α -tocopherol and α -tocopherol acetate all reverse this inhibition. The fact that the reversal can be brought about by lecithin or cholesterol after inhibition for 48 hr. by linoleic acid indicates that the action is bacteriostatic. Calcium chloride reversed almost completely the inhibitory action of linoleic acid.

3. Possible mechanisms of the inhibitory action are discussed, and it is suggested that the evidence points to a physico-chemical interpretation.

4. The inhibitory action of linoleic acid was demonstrated in the case of certain other Gram-positive bacteria but not in the case of the Gram-negative *Proteus vulgaris* and *Esch. coli*.

5. For the microbiological assay of riboflavin it is recommended that a standard inoculum be employed, that incubation be of 72 hr. duration, and that the material under test be extracted with chloroform.

6. The inhibitory effect, upon Gram-positive bacteria, of diethyl stilboestrol and hexoestrol has been confirmed but was not reversed by lecithin or cholesterol. Dienoestrol is also inhibitory, but its dibenzoate and dipropionate esters are not.

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Antibacterial Substances Related to Pantothenic Acid

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It has already been shown that pantoyltaurine (Snell, 1941; McIlwain, 1939 *a, b*; McIlwain & Hawking, 1943) and other pantothenic acid derivatives (Barnett & Robinson, 1942; McIlwain, 1942) are *in vitro* antibacterial substances whose action can be antagonized by increasing the amount of pantothenic acid present in the medium. Further, it has been possible to demonstrate a therapeutic activity in rats infected with streptococci (McIlwain & Hawking, 1943) by administering large and frequent doses of pantoyltaurine. The rat was chosen because the level of circulating pantothenic acid was low enough for an effect of pantoyltaurine to be demonstrable with this animal; in mice the level was too high. The massive dosing was necessary to offset the rapid elimination of the drug from the blood stream and thus provide a concentration sufficient to antagonize the high level of pantothenic acid normally found in blood plasma. It is possible that the presence of the sulphonic acid group in pantoyltaurine was the cause of the rapid clearance rate and that a more favourable therapeutic effect might be obtainable if this group were replaced by a neutral or basic residue, retaining where possible

the β -aminoethyl configuration of the parent pantothenic acid. Recently Barnett (1944) has described certain N-pantoyl- β -aminoethylthiol, sulfoxide and sulphone compounds in which the sulphonic acid group of pantoyltaurine is replaced by other sulphur-containing groupings such as —S— , —SO— and $\text{—SO}_2\text{—}$, but although the substances inhibited the growth of *Lactobacillus arabinosus*, *in vitro*, and this effect was reversed by pantothenic acid, it was not possible to obtain any action in rats infected with *Streptococcus pyogenes*.

The present investigation was undertaken with the object of preparing and examining further examples of pantamides in the expectation that substances of some therapeutic value might result. The substances I–XI in Table 1, collectively referred to as pantamides, were made and tested.

The reference inhibitor used was pantoyltaurine: $\text{P—NH—CH}_2\text{—CH}_2\text{SO}_3\text{Na}$ (see Table 1). Other substances, such as hydrazine, phenylhydrazine, salicylhydrazide, salicylic acid, quinine and mepacrine, were included in the biological tests in order to determine by comparison whether the antibacterial activity of some of the substances enumerated