3. Taking into account the specificities of the two methods and the reactions they involve, it is difficult to explain such results except as being due to the presence of dehydroascorbic acid. These procedures do not suggest the presence of diketogulonic acid in human plasma.

4. The administration of adrenocorticotrophic hormone to human subjects resulted in a rise in the concentration of plasma total ascorbic acid and of plasma ascorbic acid with a diminution in the concentration of plasma dehydroascorbic acid 2-4 hr. after intramuscular injection.

5. The oral administration of cortisone to human subjects resulted in no rise in the concentration of plasma total ascorbic acid, but in a rise in ascorbic acid of such a magnitude that dehydroascorbic acid temporarily disappeared from the plasma 0.5-2 hr. after administration.

6. The significance of these alterations is discussed both with respect to the origin of the increment of total ascorbic acid appearing in the plasma and to the mechanisms involved in the reduction of dehydroascorbic acid to ascorbic acid.

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Note added in proof. In a few experiments it has now been found that, following the administration of cortisone or ACTH and coincident with the resulting diminution of the plasma dehydroascorbic acid concentration, there is a decrease in the reduced glutathione concentration of the whole blood. When, later, the original dehydroascorbic acid concentration is restored, the reduced glutathione of the blood also increases. The sum of the reduced and oxidized glutathione remains constant throughout.

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Long-chain Unsaturated Fatty Acids as Essential Bacterial Growth Factors: Further Studies with *Corynebacterium* 'Q'

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Previous work (Pollock, Howard & Boughton, 1949) on an oleic acid-requiring diphtheroid bacterium (*Corynebacterium* 'Q') showed that a number of related unsaturated fatty acids from natural sources (elaidic, linoleic, linolenic, petroselinic and palmitoleic acids) would support growth as well as oleic acid, although all saturated fatty acids tested were quite inactive. During the last few years there have been a number of reports along similar lines on species of *Lactobacillus* many of which require unsaturated fatty acids related to oleic acid for growth in the absence of biotin (Williams, Broquist & Snell, 1947; Axelrod, Hofmann & Daubert, 1947; Hassinen, Durbin & Bernhart, 1950; Cheng,

Greenberg, Deuel & Melnick, 1951), although there is some variation in the extent of replaceability of oleate by analogues. Thoma & Peterson (1950) have shown that unsaturated fatty acids are similarly necessary for the growth of *Clostridium sporogenes* without biotin. Long-chain saturated fatty acids have occasionally been known to produce some stimulation of growth in the presence of unsaturated acids, but this is more likely to be simply a protective effect against toxic properties of the unsaturated acids. In the presence of an adequate source of carbon, long-chain saturated fatty acids have not so far been found essential for the growth of and known strains of micro-organisms.

Apart from a few strains of some species of *Lactobacillus*, reported by Kitay & Snell (1950) to have an absolute requirement for oleic acid, and *Corynebacterium* 'Q' itself, all fatty acid-dependent bacteria so far examined need unsaturated fatty acids only in the absence of biotin. It seems likely that biotin plays a part in the biosynthesis of unsaturated fatty acids by lactobacilli and clostridia, but its exact role has not yet been elucidated. *Corynebacterium* 'Q' would therefore seem to be a better organism than most others to use for study of the specificity of unsaturated fatty acids as growth factors. Although biotin is not essential for growth of this organism it was included in the medium in order to minimize the risk of non-specific factors affecting oleic acid-requirements.

The apparently universal occurrence of unsaturated fatty acids in living tissues shows that organisms which do not need them for growth must synthesize them from simpler compounds. Thus, the fact that some cells require them 'ready-made' in the environment is an expression of a metabolic deficiency rather than an indication of the existence of some unusual metabolic process.

It was therefore decided to extend our previous study by the investigation of a wide range of possible substitutes for oleic acid in the growth of *Corynebacterium* 'Q' in the hope that the pattern of active and inactive compounds might, if more complete, throw further light both on the pathway by which oleic acid or its equivalent could be synthesized in non-exacting species and on the subsequent fate and function of unsaturated fatty acids in the organism. The new compounds tested were nearly all synthetic, partly in order to be more certain of their purity and partly because, in many cases, they were not available from natural sources. The results here reported, though of some intrinsic interest as a study in biological specificity, have not justified our earlier hopes that they might seriously contribute to the solution of the problem of biosynthesis of unsaturated fatty acids in micro-organisms.

METHODS

Bacteriological. About 50000 viable washed cells were inoculated into each 50 ml. flask containing 10 ml. of a medium consisting of 'vitamin-free' casein hydrolysate, salts and growth factors; and growth was measured turbidimetrically after 4 days' aerobic incubation at 35°.

The general technique of cleaning glassware, medium preparation, inoculation, incubation and measurement of bacterial growth were exactly as previously described (Pollock *et al.* 1949) with the following minor alterations:

(1) The 'B' group of growth factors, which included purines, pyrimidines and folic acid, was omitted from the medium since it had little effect beyond a very slight stimulation of growth rate during the early period of incubation.

(2) Flasks were shaken at a rate of 100/min. on a mechanical agitator instead of being left stationary on the racks. This increased the rate of growth and the final yield to some extent, and gave more reproducible results, but did not alter the general picture appreciably.

(3) Aluminium caps were used instead of glass-wool plugs. The caps were easier to prepare and clean and were just as efficient as plugs in preventing contamination, even during shaking. Evaporation was slightly less than with plugs.

Fatty acids. Apart from the *cis*- and *trans*-octadec-11-enoic (vaccenic) acids supplied by Dr F. M. Strong (Ahmad, Bumpus & Strong, 1948) all the synthetic fatty acids were provided by Dr R. E. Bowman. The *cis*- and *trans*-octadec-9-enoic (oleic and elaidic) acids and the *cis*- and *trans*-heptadec-9-enoic acids were synthesized by Ames & Bowman (1951*a*); the *cis*-undec-9-enoic acid by Ames & Bowman (1952); the *cis*- and *trans*-tetradec-9-enoic (myristoleic) acids, *cis*- and *trans*-hexadec-9-enoic (palmi-toleic) acids, *cis*- and *trans*-eicos-9-enoic (gadoleic) acids, and *cis*- and *trans*-octadec-4-enoic acids by Boughton, Bowman & Ames (1952); and the saturated pentadecanoic and heptadecanoic acids were synthesized by Dr R. E. Bowman. All these samples were of a high degree of purity as were also 12-methyloleic and 5:7:13:17-tetramethyloleic acids prepared by Ames & Bowman (1951*b*). The samples of 9-methyloleic acid and 7:11-dimethyloleic acid (Ames & Bowman, 1951*b*) contained about 10 and 25% impurity respectively. The undec-10-enoic (undecylenic) acid was a commercial sample purified by Dr Bowman; the nonanoic (pelargonic) and nonane-1:9-dioic (azelaic) acids were also commercial samples purified by redistillation and recrystallization respectively. *cis*-Octadec-6-enoic (petroselinic) acid from natural sources prepared from the methyl ester as before (Pollock *et al.* 1949) was retested with the modified incubation technique since, previously, it had supported appreciably less growth than oleic acid. The 'natural' oleic and elaidic acids used as a reference were prepared and purified as previously (Pollock *et al.* 1949).

RESULTS

Unsaturated fatty acids. The growth-promoting effects, in terms of yield of organism, of those acids tested and found to be active, are summarized in Fig. 1. Other acids tested and found to be completely unable to support growth in concentrations up to 25 µg./ml. were: *cis*-undec-9-enoic, *cis*-undec-10-enoic, *cis*- and *trans*-gadoleic, *cis*-myristoleic and *trans*-octadec-4-enoic acids. *cis*-Octadec-4-enoic and *trans*-myristoleic acids both gave faint traces of opacity at concentrations of 5 and 25 µg./ml., in both cases equivalent only to about 40 µg. dry bacterial wt./ml. This amounted to between one-sixth and one-tenth of the growth occurring with the same amount of oleic acid, and was more than would be expected on the maximum amount of impurity thought likely. In the case of *cis*-octadec-4-enoic acid the uninoculated medium developed a slight cloudiness on incubation, and it is thus very doubtful if appreciable growth occurred with this compound. With *trans*-myristoleic acid, however, no cloudiness appeared in the uninoculated medium

and the extent of growth was not altered either by reducing or by increasing the size of the inoculum tenfold. The effect with *trans*-myristoleic acid thus appears to be genuine though very feeble.

The activities of the definitely 'positive' compounds were of the same order though the vaccenic, palmitoleic and petroselinic acids were appreciably less active than oleic and elaidic acids. There was no significant difference between the synthetic and natural oleic acid. As observed previously, *cis*-palmitoleic acid was inhibitory to growth at concentrations above 15 $\mu\text{g./ml.}$, in marked contrast to its

completely without action. It was thought just possible that oleic acid might be biosynthesized by condensation of two molecules of a saturated fatty acid with exactly half the number of C atoms as oleic acid. For this reason we tested pelargonic and azelaic acids together and separately, but they were inactive.

All compounds which were without effect up to 40 $\mu\text{g./ml.}$ were tested, as previously, for possible toxicity, in the presence of 25 $\mu\text{g./ml.}$ of oleic acid, but in no case was any significant inhibition of growth observed.

Fig. 1. Extent of growth of *Corynebacterium* 'Q' on different unsaturated fatty acids. 4 days' incubation at 35° in a basal medium of casein hydrolysate + growth factors. Key: ○—○, 'natural' oleic acid (A); ⊙—⊙, synthetic oleic acid (B); □—□, 'natural' elaidic acid (C); ⊠—⊠, synthetic elaidic acid (D); ●—●, *trans*-heptadec-9-enoic acid (E); ⊗—⊗, *cis*-heptadec-9-enoic acid (F); ●—●, petroselinic acid (G); △—△, *cis*-vaccenic acid (H); △—△, *trans*-vaccenic acid (I); ×—×, *trans*-palmitoleic acid (J); ⊗—⊗, *cis*-palmitoleic acid (K).

trans isomer which was not inhibitory at all at 40 $\mu\text{g./ml.}$

Substituted oleic acids. A number of synthetic methyl-substituted oleic acids happened to be available and were also tested, although not in detail. Two monomethyloleic acids, 9- and 12-methyloleic acid, both gave approximately the same yield of growth as oleic acid itself; but a di- (7:11) and a tetra- (5:7:13:17) substituted methyloleic acid were quite inactive.

Saturated fatty acids. Synthetic pentadecanoic and heptadecanoic acids, which also happened to be available, were tested; but, as with their homologues having an even number of C atoms, they were

DISCUSSION

With the possible exception of *trans*-myristoleic acid, the difference between fatty acids that can support the growth of *Corynebacterium* 'Q' and those that cannot, is fairly clear-cut; since the growth-promoting ability in terms of final yield per unit weight of fatty acid is not greatly different for all active compounds. In fact, the 'all-or-none' principle applies here fairly well. In addition, the following points are worth noting:

(1) The *cis*- and *trans*-heptadec-9-enoic acids with seventeen C atoms are as active as oleic acid. It had been thought possible that fatty acids with

an odd number of C atoms, in view of their extreme rarity in natural sources of fat, might be unable to replace oleic acid.

(2) Fatty acids with the *trans* configuration around the double bond are in general as active as their *cis* isomers, although *trans*-vaccenic is appreciably less effective than the *cis* compound.

oleic acids was not large enough to allow any general conclusions to be drawn, it is of interest to note that while a methyl group substituted on C atom 12 did not cause any loss in activity, an OH group in the same position (ricinoleic acid) completely abolished activity (Pollock *et al.* 1949). It is not, however, surprising to find that the polar OH

Fig. 2. Unsubstituted mono-unsaturated fatty acids able to act as growth factors for *Corynebacterium* 'Q'. Fatty acids with symbols shown inside the circle are those found to be mutually replaceable as growth factors for 'Q', while those outside are inactive. The position of the symbol on the chart is a guide to the chain length and position of the double bond. Key: The two 'arms' of the symbol are proportional in lengths to those of the C chains on either side of the double bond (represented by the round spot). The horizontal tag represents the carboxyl group and the *cis* and *trans* isomers are indicated by the different configurations around the double bond.

(3) While the hydrocarbon 'tail' of oleic acid can be shortened by at least two C atoms (palmitoleic acid) without affecting its growth-promoting properties, addition of two C atoms (gadoleic acid) completely abolishes activity.

(4) The double bond can be shifted away from the 9:10 position of oleic acid at least to the 11:12 position (vaccenic acid) and to 6:7 (petroselinic acid), but not as far away as 4:5 (octadec-4-enoic acid) without loss of activity.

(5) Although the series of methyl-substituted

group has altered the properties of a compound much more drastically than a CH_3 group.

These findings have confirmed and extended those made originally with this organism, and are in general agreement with those reported by the American workers for certain species of two very different genera of bacteria, *Clostridium* and *Lactobacillus*, when grown in the absence of biotin. In a most interesting and extensive investigation on lactobacilli, Cheng *et al.* (1951) studied a range of C_{18} unsaturated fatty acids which were able to

replace oleic acid for *L. arabinosus* and their results were very similar to ours though they did not find quite so definite an 'all-or-none' effect. They reported that all the *cis*-octadecenoic acids tested (with the double bond ranging from the 6 to the 12 position) were more or less equivalent; but, while elaidic was equivalent to oleic acid, other *trans* isomers yielded less growth than oleic acid, the divergence being the more marked the further the double bond was separated from the 9:10 position of elaidic acid.

A summary of all our findings with mono-unsaturated fatty acids and 'Q' is shown in Fig. 2.

It was hoped that the pattern of active and inactive compounds would provide a more obvious clue to the interpretation of its basis than is shown by this scheme. It is, unfortunately, not possible to conclude more than that the greater the difference in chain length, or in position of the double bond from that of oleic acid, the less likely is the compound in question to be capable of supporting growth of this species of bacterium. It must also always be borne in mind that a 'negative' compound might possibly owe its inactivity to the inability to penetrate into the cell, and thus its lack of effect could be due to quite different properties from those of substances that cannot actually be metabolized. It is conceivable that such factors contribute to the apparently arbitrary selection shown in Fig. 2. The divisions between the groups of active and inactive fatty acids do not correspond to any obviously sudden change in physical or chemical properties. Yet the specificity is far from being absolute; and if *cis*-palmitoleic acid is able to replace oleic acid, why should *cis*-myristoleic acid fail? It is possible that further light would be thrown on the problem by a more detailed study of the 'borderline' compounds such as myristoleic acid, and of a systematic series of substituted oleic acids. More valuable still, however, would be information on the fate and

function of those fatty acids that are active in supporting growth. Preliminary work by one of us (Boughton, 1952) using [^{14}C] oleic acid has indicated that the acid is taken up by the cell during growth and incorporated in the lipid material of the cell, as might be expected. Unfortunately, work with isotopically labelled oleate could not be continued, although clearly this type of approach is at the moment likely to yield more information than most others on the fate and function of unsaturated fatty acids in micro-organisms.

SUMMARY

1. A number of synthetic long-chain unsaturated fatty acids have been tested for their ability to replace oleic acid for the growth of *Corynebacterium* 'Q'.

2. *cis*- and *trans*-Palmitoleic, *cis*- and *trans*-heptadec-9-enoic, and *cis*- and *trans*-vaccenic acids were all approximately equivalent in growth-promoting ability to oleic acid; *trans*-myristoleic acid gave very slight growth in the higher concentrations; while *cis*- and *trans*-gadoleic, *cis*-myristoleic, and *cis*- and *trans*-octadec-4-enoic acids were inactive.

3. These results show that unsaturated fatty acids with chain lengths two carbon atoms longer, or four carbon atoms shorter than oleic acid or with the double bond one or two atoms nearer the carboxyl than the 6:7 position are incapable of replacing oleic acid for the growth of this organism. Within these limits oleic acid can be replaced by several fatty acids with either the *cis* or the *trans* configuration.

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