3. In nerves degenerating after section and incubated under the same conditions, there was a large increase in the specific activity of the lipid P. During the early stages of the degeneration (8 and 16 days after nerve section) there was a decrease in the specific activity of the PNA and ROP. At 8, 16 and 32 days after nerve section the activity of the lipid P and PNA/100 mg. nerve was greater than the corresponding figure for control nerves.

4. In regenerating nerves 32 days after nerve crush, the specific activity of each of the four P-containing fractions did not differ significantly from that of the corresponding fraction 32 days after nerve section. By 96 days after the operation the specific activity of the lipid P was significantly less than that of the lipid P of nerves at the same time interval after section. The activity/100 mg. nerve was considerably greater.

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REFERENCES

- Bodian, D. & Dziewiatkowski, D. (1950). *J. cell. comp. Physiol.* **35**, 155.
- Burt, N. S., McNabb, A. R. & Rossiter, R. J. (1950). *Biochem. J.* **47**, 318.
- Causey, G. & Palmer, E. (1953). *J. Anat., Lond.*, **87**, 30.
- Davidson, J. N. & Smellie, R. M. S. (1952). *Biochem. J.* **52**, 599.
- Dawson, R. M. C. (1953). *Biochem. J.* **55**, xii.
- DeLuca, H. A., Rossiter, R. J. & Strickland, K. P. (1953). *Biochem. J.* **55**, 193.
- Feng, T. P. & Gerard, R. W. (1930). *Proc. Soc. exp. Biol., N.Y.*, **27**, 1073.
- Findlay, M., Rossiter, R. J. & Strickland, K. P. (1953). *Biochem. J.* **55**, 200.
- Findlay, M., Strickland, K. P. & Rossiter, R. J. (1954). *Canad. J. Biochem. Physiol.* (in the Press).
- Folch, J. & LeBaron, F. N. (1951). *Fed. Proc.* **10**, 183.
- Fries, B. A., Schachner, H. & Chaikoff, I. L. (1942). *J. biol. Chem.* **144**, 59.
- Hammarssten, E. (1947). *Acta med. Scand.* **128**, Suppl. 196, 634.
- Hollinger, D. M. & Rossiter, R. J. (1952). *Biochem. J.* **52**, 659.
- Hollinger, D. M., Rossiter, R. J. & Upmalis, H. (1952). *Biochem. J.* **52**, 652.
- Johnson, A. C., McNabb, A. R. & Rossiter, R. J. (1949). *Biochem. J.* **45**, 500.
- Kühne, W. & Chittenden, R. H. (1889). *Z. Biol.* **26**, 291.
- Logan, J. E., Mannell, W. A. & Rossiter, R. J. (1952a). *Biochem. J.* **51**, 470.
- Logan, J. E., Mannell, W. A. & Rossiter, R. J. (1952b). *Biochem. J.* **51**, 482.
- Magee, W. L. & Rossiter, R. J. (1954). *Fed. Proc.* **13**, 257.
- Mullins, L. J. & Grenell, R. G. (1952). *J. cell. comp. Physiol.* **39**, 164.
- Samuels, A. J., Boyarsky, L. L., Gerard, R. W., Libet, B. & Brust, M. (1951). *Amer. J. Physiol.* **164**, 1.
- Schachner, H., Fries, B. A. & Chaikoff, I. L. (1942). *J. biol. Chem.* **146**, 95.
- Streicher, E. (1953). *Fed. Proc.* **12**, 139.
- Strickland, K. P. (1952). *Canad. J. med. Sci.* **30**, 484.
- Strickland, K. P. (1954). *Canad. J. Biochem. Physiol.* **32**, 50.

Actinomycete Metabolism: Origin of the Guanidine Groups in Streptomycin

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Despite the great interest that streptomycin has evoked in recent years, very little is known about the biochemical pathways leading to its production by *Streptomyces griseus*. Karow, Peck, Rosenblum & Woodbury (1952) have demonstrated the incorporation of the labelled carbon of [¹⁴C]glucose into streptomycin. They did not, however, submit their product to chemical degradation, so that the distribution of the radioactivity between the various parts of the molecule is unknown. Nor is it clear from their results whether glucose functions as a direct precursor of the trisaccharide molecule or is first degraded into smaller molecular fragments.

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Our first points of attack have been the guanidine side chains of the streptomycin molecule and the results set out here show that the carbon atoms concerned are derived largely, if not entirely, from carbon dioxide. The mode of biosynthesis of the guanidine groups as a whole does not present as clear a picture, but there is an indication that arginine takes part in the process.

EXPERIMENTAL

Fermentation. The organism, *Streptomyces griseus*, *albus* mutant (Dulaney, Z38) is described in the patent covering its production (Dulaney, 1951). The development of a vegetative inoculum in a special meat medium and its use to inoculate a soybean medium are also described in example 1

of the same patent, which includes details of the broth media and the conditions of growth.

A clear synthetic medium ('Lumb's medium', Chesters & Robinson, 1951) was also used, particularly when mycelium was required free from medium constituents. In such instances, mycelium from 48 hr. cultures (1 vol. medium) was washed 3 times with 0.01M potassium phosphate pH 7.0 (1 vol.), and resuspended for further experiments in a suitable buffer (1 vol. 0.01M potassium phosphate, pH 7.0).

A special shake flask, the details of which are shown in Fig. 1, was constructed for the growth of cultures in the presence of $^{14}\text{CO}_2$. The gas was continuously circulated during each experiment by means of a small pump; other components of the circuit were a large gas reservoir and a simple apparatus for generating $^{14}\text{CO}_2$ from $\text{Ba } ^{14}\text{CO}_3$. The culture flask was enclosed in a wire cage, screwed at its base to a rotary shaker and held in position by tightly packing the rest of the cage with cotton wool. At harvest, CO_2 was removed from the system by altering the circulation so as to absorb the gases in saturated aqueous $\text{Ba}(\text{OH})_2$.

Fig. 1. Shake flask used for growing cultures in the presence of a circulated stream of gas. (A, ground glass cones and sockets; B, cotton-wool plugs; C, 250 ml. conical flask.)

Streptomycin was assayed biologically by a large cup-plate method similar to that used by Brownlee *et al.* (1948). *Staphylococcus aureus* was used as the test organism. Under these conditions, 95–100% of the detected microbiological activity is accounted for by streptomycin.

Isolation of radioactive streptomycin. After removing a small portion for bioassay, the contents of the fermentation flask (40 ml.) were acidified to pH 2 with phosphoric acid. The flask was allowed to stand for 30 min. at room temperature, kieselguhr (2 g.) was added, and the mycelium was removed by filtration and washed well with water. About 1 g. of the pure calcium chloride complex of streptomycin

was added to the combined filtrate and washings, which were then brought to neutrality by the addition of 40% (w/v) KOH. After further filtration if necessary, the volume of the solution was adjusted to 500 ml. with distilled water. The solution was percolated slowly (1 ml./min.) through a column containing 5 g. Amberlite IRC-50 cation-exchange resin (The Rohm and Haas Co., Philadelphia, Pa.) in the sodium cycle (Karow *et al.* 1952). All the streptomycin was retained when the solution was added at this rate. The resin was finally washed with water (50 ml.).

Elution was carried out very slowly with N-HCl (0.2 ml./min.); the initial neutral fraction (approx. 20 ml.) contained nearly all the ^{14}C streptomycin. This fraction was immediately added to 2% aqueous ammonium reineckate (150 ml.) preheated to 65°. After cooling slowly to 30°, calcium reineckate was removed by filtration and the filtrate and aqueous washings (5 ml.) were allowed to stand at 0° for 24 hr. The resultant precipitate of ^{14}C streptomycin reineckate was filtered off and washed with a small volume of ice-cold water. After recrystallization by dis-

Fig. 2. Degradation of streptomycin.

solving in 1% aqueous ammonium reineckate (15 ml.) at 60° and cooling to 0°, the ^{14}C streptomycin reineckate was collected, washed with ice-cold water (5 ml.) and air-dried. The radioactivity of this material was unchanged after further recrystallization. Recoveries were rather variable (60–90%, based on the bioassay of the original broth and of the added crystalline calcium chloride complex). A second crop of material of lower radioactivity was obtained from the combined mother liquors by co-crystallization with added unlabelled streptomycin reineckate. Conversion of the reineckate into sulphate was carried out, where necessary, by acidification of an aqueous solution with 2N- H_2SO_4 , and

removal of Reinecke's acid by extraction into amyl acetate. Streptomycin sulphate was obtained from the aqueous phase by freeze-drying and crystallized from aqueous methanol.

Chemical degradation of streptomycin. The chemical structure of streptomycin and relevant degradation products are shown in the accompanying formulae (Fig. 2).

Streptomycin sulphate was dissolved in the minimum quantity of $N-H_2SO_4$ and allowed to stand for 1 week at room temperature. An equal volume of methanol was added and the solution was allowed to stand a further day when addition of 1 vol. of acetone completed the precipitation. After a few hours at 0° , the colourless rod-shaped crystals of streptidine sulphate were filtered off and recrystallized from a small volume of water.

Streptobiosamine dimethyl acetal was isolated from the mother liquors after removal of the sulphuric acid with Amberlite IR-4B (The Rohm and Haas Co., Philadelphia, Pa.) by evaporation *in vacuo* to remove the organic solvents and final freeze-drying. Although it proved to be unnecessary in the present work, this substance may be further degraded to *L-N*-methylglucosamine by acid hydrolysis (cf. Brink, Kuehl, Flynn & Folkers, 1946).

The streptidine sulphate was further degraded by boiling for 2 days with an excess of saturated aqueous $Ba(OH)_2$ in the absence of atmospheric CO_2 . The mixture of barium salts (carbonate, sulphate and silicate from the glass of the apparatus) was then separated by centrifuging. The CO_2 was liberated from this by warming with 20% aqueous lactic acid and carried over in a CO_2 -free air stream into saturated $Ba(OH)_2$. The resultant $Ba^{14}CO_3$ was again isolated by centrifuging, washed with water and acetone and dried in a vacuum oven at 50° . After separation of the insoluble barium salts, the excess of barium was removed from the original hydrolysate as $BaCO_3$ by addition of solid CO_2 . Streptamine sulphate was then precipitated after acidification with sulphuric acid by addition of an equal volume of acetone. It was purified by crystallization from aqueous acetone.

Paper-chromatographic techniques. For the separation of compounds containing guanidine groups, the system used by Winsten & Eigen (1948) was modified. The solvent used for development contained *p*-toluenesulphonic acid (2%, w/v) dissolved in a mixture of water-saturated *n*-butanol (99 vol.) and piperidine (1 vol.). The same system was also used for the separation of α -amino acids. Other conventional systems that were found useful for this purpose included phenol-water (4:1, v/v) and lutidine-collidine-water (1:1:2, v/v). For the detection on paper of compounds containing guanidine groups, the diacetyl spray (Halliday, 1952) as modified by Foster & Ashton (1953) was used, and a freshly prepared 0.1% ninhydrin solution in ethanol for amino acids. All chromatograms were run on Whatman no. 4 paper.

Assay of ^{14}C . All radioactivity measurements were done on 2 cm.² Polythene disks with solid samples of 'infinite thickness' (Popják, 1950).

RESULTS AND DISCUSSION

Incorporation of $^{14}CO_2$ into streptomycin

After 2 days' growth of *Streptomyces griseus* on the soybean medium (40 ml.), $^{14}CO_2$ was liberated from barium [^{14}C]carbonate (16 mg., containing 85 μC)

and circulated continuously over the fermenting culture for 5 more days. Unreacted CO_2 was then trapped as barium carbonate (8.70 g., specific activity $1.75 \mu C/m\text{-mole}$). Bioassay of a small portion revealed that the amount of streptomycin present in the broth was 75.0 ± 5.2 mg. Extraction as previously described after addition of unlabelled streptomycin calcium chloride complex containing 795 ± 20 mg. of streptomycin (bioassay) as carrier gave 1.70 g. of recrystallized streptomycin reineckate as the main crop. Assay for radioactivity showed, after making allowance for the dilution with unlabelled material, that the original undiluted streptomycin had a specific activity of $2.91 \mu C/m\text{-mole}$. This represents an incorporation of between 0.4 and 0.5% of the $^{14}CO_2$ into streptomycin.

Further degradation, after conversion into streptomycin sulphate, showed that all the radioactivity was concentrated in the streptidine portion of the molecule. The streptidine sulphate was further diluted with unlabelled material before conversion into streptamine sulphate and barium carbonate. It was found that practically all the radioactivity of the streptidine was due to the carbon atoms of the guanidine side chains. The small amount found in the streptamine sulphate may have been due to contamination with a small quantity of unhydrolysed strepturea. The results are shown in Table 1.

It can be seen that the specific activity ($1.56 \mu C/m\text{-mole}$) of the barium carbonate obtained by degradation (and hence of the guanidine carbons) approaches very closely to the specific activity of the barium carbonate derived from the air circulating at the end of the original fermentation ($1.75 \mu C/m\text{-mole}$). The errors involved in the determination of these figures—bioassay determinations, measurement of broth volumes and radiochemical assays—are considerable, however, and the difference may not be significant. At first sight, it appears that the carbons of the guanidine side chains are entirely derived from CO_2 , but, if these carbon atoms are not in complete equilibrium with the carbon dioxide in the circulating gas, the rates of $^{14}CO_2$ dilution and streptomycin synthesis have to be considered. As the circulating CO_2 is largely derived

Table 1. *Radioactivity of streptomycin degradation products*

Details of the production of streptomycin in the presence of $^{14}CO_2$ and of the chemical degradation are given in the Experimental section.

Substance	Specific activity ($\mu C/m\text{-mole}$)
Streptomycin sulphate	2.91
Streptidine sulphate	3.06
Streptamine sulphate	0.028
Barium carbonate (derived from guanidine side chains)	1.56

from the reducing sugars in the medium, the figures given in Table 2 are of interest in this connexion. In this typical serial experiment, flasks were removed each day, and assayed for reducing sugars (Shaffer & Hartmann, 1920) and streptomycin content. It can be seen that much of the reducing sugar had already been utilized, and hence the amount of CO_2 would remain relatively constant while most of the streptomycin was being produced by the organism.

To gain further information on this point, a second experiment with $^{14}\text{CO}_2$ was carried out under exactly the same conditions as the first one, except that growth was allowed to proceed for only 5 days altogether. The $^{14}\text{CO}_2$ was of the same initial specific activity as before. The results of the two experiments are compared in Table 3. The weight of the recovered barium carbonate in the second experiment approaches that obtained in the first, confirming that the rate of dilution of the $^{14}\text{CO}_2$ is at its greatest much earlier than the time of maximum streptomycin accretion. This would seem to indicate that the carbons of the guanidine side chains of streptomycin are, in fact, very largely derived from CO_2 , but the lower degree of incorporation of radioactivity in the second (5-day) experiment poses a problem. Presumably, a proportion of the streptomycin, or, more probably, streptomycin precursors is elaborated by the organism during the first two days of growth. In the first experiment, where the culture was allowed to grow for seven days, the relatively large streptomycin production during the last two or three days of the fermentation would tend to obscure this effect.

Effect of delaying the passage of $^{14}\text{CO}_2$ on the extent of the incorporation of ^{14}C into streptomycin. In view

Table 2. *Content of reducing sugar and streptomycin in the soybean medium during growth of Streptomyces griseus*

Length of fermentation (days)	Reducing sugars (as glucose) (mg./ml.)	Streptomycin content ($\mu\text{g./ml.}$)
0	25.9	—
1	25.1	—
2	15.4	—
3	10.8	250
4	7.3	511
5	2.5	923
6	1.5	1306
7	1.25	1461
8	1.25	1257

of the above results, an attempt was made to determine whether further variation in the period during which $^{14}\text{CO}_2$ was supplied would result in any alteration in the extent of incorporation of ^{14}C into streptomycin. Three fermentations were carried out in which the passage of $^{14}\text{CO}_2$ was begun 2, 3 and 4 days after inoculation.

Streptomycin was isolated as usual after a total of 7 days' growth and the results are summarized in Table 4.

It can be seen that, when passage of $^{14}\text{CO}_2$ was begun 3 days after inoculation, the incorporation of ^{14}C into streptomycin was little different from that observed in the first experiment. However, if passage of $^{14}\text{CO}_2$ was delayed for 4 days, the labelling of the streptomycin was considerably lowered. The depression was greater than that to be expected if the effect were due solely to the streptomycin already synthesized, and we were again led to infer the presence of some intermediate acting as a depot for CO_2 and not in complete equilibrium with the gaseous phase. In view of the known role of arginine in transaminations in biological systems, we decided to investigate the effect of this amino acid on the incorporation of $^{14}\text{CO}_2$.

Effect of arginine on the incorporation of $^{14}\text{CO}_2$. In this experiment, passage of $^{14}\text{CO}_2$ through two flasks linked in series was begun 2 days after inoculation. To one of the flasks L-arginine hydrochloride (200 mg.) dissolved in water (1 ml.) was added at the same time and to the control flask 1 ml. of water only was added. At harvest, the streptomycin from each flask was isolated after reserving a small portion for bioassay. A very marked difference in the incorporation of ^{14}C was observed in the two cases. The specific activity of the streptomycin isolated from the flask to which the arginine had been added was $0.401 \mu\text{C/m-mole}$, whereas the product from the control culture had a specific activity of $0.627 \mu\text{C/m-mole}$. There was little difference in the amounts of streptomycin produced in each flask (28 and 33 mg.). If one assumes that the carbons of the guanidine side chains are almost entirely derived from CO_2 in a normal fermentation, it is difficult to see how the arginine could depress the incorporation of ^{14}C in this experiment except by furnishing carbon atoms to replace those normally supplied by the gas. In this experiment, the unlabelled carbon atoms

Table 3. *Incorporation of $^{14}\text{CO}_2$ into streptomycin*

Length of fermentation (days)	Initial wt. of $\text{Ba}^{14}\text{CO}_3$ (mg.)	Final wt. of $\text{Ba}^{14}\text{CO}_3$ (g.)	Final radioactivity of $\text{Ba}^{14}\text{CO}_3$ ($\mu\text{C/m-mole}$) A_1	Final radioactivity of streptomycin ($\mu\text{C/m-mole}$) A_2	A_2/A_1
7	16	8.70	1.75	2.91	1.7
5	30	7.30	3.19	3.16	0.99

present in the guanidine groups of the added arginine could have been used first for the synthesis of streptomycin, causing an apparent decrease in the extent of CO_2 fixation into streptomycin. Thus, these results suggest that arginine functions as an intermediate in the biosynthesis of streptomycin by a transfer of guanidine groups ('transamidation').

Experiments with washed mycelial suspensions

In these experiments, a study was made of the synthesis of substituted guanidines by the organism. Substrates were added at a level of 1 mg./ml. to the washed mycelial suspensions (40 ml., dry wt. of mycelium approx. 10 mg.) which were then incubated at 28° on a rotary shaker for 18 hr.

No conversion of streptamine (either hydrochloride or sulphate) into streptidine could be detected when washed suspensions were used under our conditions, whether arginine was present or not. However, by using the *p*-toluenesulphonic acid system and the diacetyl spray, a new unidentified compound containing at least one guanidine group was always detected by downward-flow paper

chromatography. Methanol extracts of freeze-dried filtrates were used for the application of the spots to the papers.

Substituted guanidines all move slowly in this system, and it was only possible to obtain relative *R* values for them. These are shown in Table 5. Further investigations revealed that this same guanido-compound (GC) could also be obtained by incubating mycelial suspensions with streptidine hydrochloride or sulphate, so that streptamine is possibly converted first into streptidine by the mould and then rapidly into GC. We then found that GC could be obtained in the best yield from L-arginine (judging yield merely by the colour intensity of the diacetyl spot). It was also formed from creatine, creatinine, glycoyammine, guanidine, citrulline or ornithine. The function of this compound is not clear, but it may be involved in the biosynthesis of streptomycin.

GC was not detectable on spraying papers with the ninhydrin reagent under our conditions. Material containing GC and arginine, obtained from methanol extracts of freeze-dried filtrates, was subjected to alkaline hydrolysis by boiling with saturated aqueous barium hydroxide for 24 hr. On developing a methanol extract of the hydrolysate (after neutralization with sulphuric acid and freeze-drying), ninhydrin revealed the presence of ornithine and a further substance, presumably derived from GC. Using downward flow paper chromatography with phenol-water (4:1, v/v), this new compound had an *R* value of 1.39 relative to ornithine.

Table 4. *Effect of varying the time of $^{14}\text{CO}_2$ passage on the incorporation of ^{14}C into streptomycin*

For details of fermentations see text.

Time of $^{14}\text{CO}_2$ addition after inoculation (days)	Specific radioactivity of recovered BaCO_3 ($\mu\text{C}/\text{m-mole}$) A_1	Specific radioactivity of streptomycin ($\mu\text{C}/\text{m-mole}$) A_2	A_2/A_1
2	1.75	2.91	1.7
3	4.17	6.75	1.6
4	0.69	0.53	0.77

Table 5. *Comparative *R* values of substituted guanidines and α -amino acids in the *p*-toluenesulphonic acid system*

For details of the solvent system used see Experimental section.

Substance	R_A (relative to arginine)
L-Arginine	1.00
GC	1.43
Streptidine	3.45
Streptomycin	4.97
Mannosidostreptomycin	2.24
Creatine	0.87
Creatinine	0.96
Glycoyammine	0.81
Guanidine	3.50
L-Alanine	1.91
L-Lysine	0.66
L-Proline	1.16
L-Aspartic acid	0.35
L-Citrulline	0.45
DL-Ornithine	0.65, 0.69 (2 spots)

SUMMARY

1. Investigations carried out on *Streptomyces griseus* with the aid of $^{14}\text{CO}_2$ have shown that the carbons of the guanidine side chains in streptomycin are derived very largely, and possibly entirely, from carbon dioxide.

2. The maximum incorporation of $^{14}\text{CO}_2$ into streptomycin obtained was between 0.4 and 0.5%. A much lower degree of fixation of ^{14}C was obtained when no $^{14}\text{CO}_2$ was passed for the first 72 hr. of a fermentation.

3. L-Arginine possibly functions as an intermediate in the biosynthesis of the guanidine side chains of streptomycin.

4. A variety of compounds, either containing guanidine groups or readily convertible into such compounds, are converted by *Streptomyces griseus* into a further substance containing at least one guanidine group. This compound has not yet been identified, but may be involved in the biosynthesis of streptomycin by the organism.

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REFERENCES

- Brink, N. G., Kuehl, F. A., Flynn, E. H. & Folkers, K. (1946). *J. Amer. chem. Soc.* **68**, 2557.
- Brownlee, K. A., Delves, C. S., Dorman, N., Green, C. A., Grenfell, E., Johnson, J. D. A. & Smith, N. (1948). *J. gen. Microbiol.* **2**, 40.
- Chesters, C. G. C. & Robinson, G. N. (1951). *J. gen. Microbiol.* **5**, 559.
- Dulaney, E. L. (1951). U.S. Patent 2 571 693 (Merck and Co. Inc.).
- Foster, M. C. & Ashton, G. C. (1953). *Nature, Lond.*, **172**, 958.
- Halliday, W. T. (1952). *Nature, Lond.*, **169**, 335.
- Karow, E. O., Peck, R. L., Rosenblum, C. & Woodbury, D. T. (1952). *J. Amer. chem. Soc.* **74**, 3056.
- Popják, G. (1950). *Biochem. J.* **46**, 560.
- Shaffer, P. A. & Hartmann, A. F. (1920). *J. biol. Chem.* **45**, 349.
- Winsten, W. A. & Eigen, E. (1948). *J. Amer. chem. Soc.* **70**, 3333.

Reduction of Dehydroascorbic Acid by Bacteria

3. ENZYMIC ACTIVATION OF DEHYDROASCORBIC ACID

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In the previous communications in this series (Eddy, 1952; Eddy, Ingram & Mapson, 1952) it has been shown that the mechanism by which *Escherichia coli* reduces dehydroascorbic acid (DHA) to ascorbic acid (AA) includes any of various dehydrogenases possessed by the organism and cytochrome b_1 , and it has been suggested that there may be a further enzyme which acts on DHA. Because cell-free extracts of *Esch. coli*, however prepared, would not reduce DHA, this hypothetical activating enzyme could not be studied directly, but there remained several lines of attack which could strengthen the evidence for its existence. These were the use of leuco-Nile blue as the immediate hydrogen donor (Tarr, 1940; Jebb, 1949); the determination of the specific chemical configurations which are capable of combining with the enzyme; the investigation of the chemical specificity and kinetic behaviour of inhibitory substances, and experiments to find typical Michaelis-Menten relationships between the concentrations and rates of reduction of DHA and related reducible substances.

METHODS AND MATERIALS

Methods

The organisms used for this part of the work were two strains of *Esch. coli*: a reducing strain isolated in this laboratory and a strain (NCTC 7277) which we shall denote as the non-reducing strain, although in fact it does reduce DHA very slowly. The cells were grown and harvested as described previously (Eddy, 1952).

Thunberg tube experiments with Nile blue were carried out as follows. Into the body of the Thunberg tubes were introduced 3.2 ml. of 0.2M phosphate buffer (pH 6.2) made from Na_2HPO_4 and KH_2PO_4 , 0.1 ml. of M lithium lactate

(to give the optimum concentration—Eddy, 1952, Fig. 2), 0.2 ml. of 0.2% (w/v) aqueous Nile blue sulphate (British Drug Houses Ltd.) and 1 ml. of bacterial suspension. A solution of 2 mg. DHA in 0.5 ml. of water was placed in the hollow stopper. The tubes were evacuated and placed in a water bath at 35°. When the dye had become reduced the DHA was added from the stopper and the oxidation of the dye measured by visual comparison with a series of colour standards. In a parallel series of tubes, serial AA estimations were carried out by opening the tube, adding 5 ml. 20% (w/v) metaphosphoric acid, diluting to a suitable volume and filtering. Samples were then titrated with 2.6-dichlorophenol indophenol.

Measurements of electrode potential were made in a 50 ml. beaker which was fitted with a rubber bung carrying a platinum electrode, a mercury-calomel half-cell and gas inlet and outlet tubes. It was immersed in a water bath at 35°. Into the beaker were put 17.5 ml. 0.2M phosphate buffer (pH 6.2), 5 ml. of bacterial suspension and 0.5 ml. of M glucose. Air was removed by bubbling N_2 through the suspension. When the potential had reached a steady state after about 20–25 min., 2 ml. of DHA solution (4 mg./ml.) were added, and the ensuing potential changes measured. From a duplicate system samples were removed at intervals for AA determinations.

Experiments on the effect of inhibitors and H-acceptor concentration on the rate of reduction were carried out as described by Eddy (1952), the method involving serial titrations of AA on samples withdrawn from a reaction mixture containing H-donor, bacterial cells, DHA and buffer at pH 6.2, held at 35° under anaerobic conditions.

Materials

Of the compounds cited in Fig. 4, L-ascorbic acid, dihydroxymaleic acid, glyoxal, diacetyl, pyruvic acid and dihydroxytartaric acid (the hydrate of dioxosuccinic acid) were available commercially, as was also D-araboascorbic acid. The hydroxytetronic and reductic acids and the 3-hydroxy-1:2-dioxo-3-phenylbutyric acid lactone were given