The Assimilation of Amino-acids by Bacteria

15. ACTIONS OF ANTIBIOTICS ON NUCLEIC ACID AND PROTEIN SYNTHESIS IN *STAPHYLOCOCCUS AUREUS*

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(*Received* 22 *May* 1952)

In previous papers of this series, experimental conditions have been described which will enable the following processes to be studied in washed suspensions of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*): internal accumulation of free glutamic acid (Gale, 1947*a*, *b*), extracellular accumulation of peptides containing glutamic acid (Gale & Van Halteren, 1951), accumulation of combined glutamic acid within the cells (Gale, 1951*b*) and synthesis of protein and nucleic acid (Gale & Folkes, 1953). Aureomycin and chloramphenicol inhibit the increase of cellular combined glutamate at lower concentrations than those necessary to prevent the accumulation of free glutamic acid within the cells, whereas sodium azide, 2:4-dinitrophenol and 8-hydroxyquinoline affect these processes to approximately the same extent (Gale & Paine, 1951). Penicillin and bacitracin have no effect upon the accumulation of free or combined glutamic acid in washed suspensions of cells, but if either of these antibiotics is added to the growth medium an hour before harvesting, the resulting cells are unable to accumulate either free or combined glutamic acid (Gale & Taylor, 1947; Gale & Paine, 1951; Paine, 1951). Hotchkiss (1950) has described a disorganization of protein synthesis by penicillin which results, under the conditions of his experiments, in extracellular accumulation of peptides when *Staph. aureus* is incubated with amino-acid mixtures.

Mitchell & Moyle (1951) followed the nucleic acid content of *Staph. aureus* during normal growth and in the presence of penicillin. During the normal phase of accelerated growth they found that nucleic acid reproduced more rapidly than cell dry weight, suggesting that nucleic acid controlled the rate of cell synthesis. A disturbance of nucleic acid metabolism occurred in the presence of penicillin and was accompanied by an intracellular accumulation of extractable nucleotides. Krampitz & Werkman (1947) and Gros & Macheboeuf (1948*a*, *b*) have previously described inhibitions of nucleic acid metabolism in bacterial cells by high concentrations of penicillin, while Gros, Beljanski & Macheboeuf (1951) have found that penicillin inhibits the breakdown of guanosine by sensitive strains of *Staph. aureus*. Park & Johnson (1949) described the accumulation of labile phosphate compounds in *Staph. aureus* growing in the presence of penicillin, and Park (1952) identified three substances accumulating under these conditions; one of these is a derivative of uridine-5′-pyrophosphate and an *N*-acetylaminosugar, and the other two possess the same basic structure in combination with either L-alanine or a peptide containing L-lysine, D-glutamic acid and DL-alanine.

METHODS

The organism, growth medium, amino-acid and purine-pyrimidine mixtures (*A*, *P* and *P*′), methods of estimation of free or combined glutamic acid, protein, nucleic acid, purine and pyrimidine bases and rates of fermentation are the same as those described in the preceding paper (Gale & Folkes, 1953).

Growth inhibition. Concentrations of antibiotics inhibitory to growth were determined by adding serial dilutions of the drugs to a fully nutrient medium containing 3% tryptic digest of casein, 0·1% Marmite and 1% glucose. Tubes were inoculated with approx. 10^6 cells/10 ml. medium and growth inspected after 48 hr. incubation at 37°.

RESULTS

The action of a number of antibiotics and inhibitors has been tested on the following processes in washed suspensions of *Staph. aureus* Duncan: (1) fermentation in the presence of glucose; (2) accumulation of free glutamic acid within the cells when incubation takes place in the presence of glucose and glutamic acid, the latter being the only amino-acid present; (3) protein synthesis when glucose and the complete amino-acid mixture *A* are present; (4) protein and nucleic acid synthesis when the incubation mixture contains glucose and mixtures *A* and *P* or *P*′. In general, it has been found that, except in the case of penicillin, the degree of inhibition of protein synthesis is the same whether mixture *P* is present or not.

Chloramphenicol. Fig. 1 shows that protein synthesis is very sensitive to chloramphenicol, 90% inhibition being produced by that concentration necessary to prevent growth of the organism in a complete medium. The processes of fermentation, respiration, free glutamic acid accumulation and nucleic acid synthesis are not inhibited by chloramphenicol except in very high concentrations. Chloramphenicol (30–100 μg./ml.) produces complete inhibition of protein synthesis and a significant increase in the rate of increase of nucleic acid (see also Table 1). Fig. 2, which shows progress curves for the increase in protein and nucleic acid of washed cells of *Staph. aureus* Duncan, also shows the effect of 30 μg. chloramphenicol on these processes. Gale & Folkes (1953) found little or no increase in the nucleic acid content of such cells when incubated with glucose and the purine-pyrimidine mixture P' in the absence of amino-acids, but the addition of chloramphenicol gives rise to a significant increase in nucleic acid under these conditions; the stimulation produced by the antibiotic is approximately the same whether the control synthesis is promoted by amino-acids or not. Table 1 shows that the increase in nucleic acid, which occurs in the presence of chloramphenicol and amino-acids, resides in the ribonucleic acid fraction since there is no increase in the thymine content.

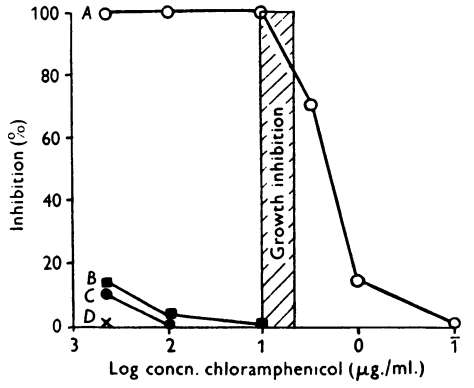

Fig. 1. Actions of chloramphenicol on amino-acid assimilation in *Staph. aureus* Duncan. Cells grown for 16 hr. at 30°; made into washed suspension and tested at final suspension density of 2·0 mg./ml. in the following systems. Curve A; rate of increase in protein-glutamate in cells (analyses on TCA precipitates) studied over 1 hr. incubation at 37° in buffered salt solution containing 1% glucose, amino-acid mixture A (each amino-acid at concentration of 0·2 mg./ml.) and purine-pyrimidine mixture P' (each component at concentration of 0·02 mg./ml.). Rate of increase in absence of any inhibitor = 12·5 μmoles protein-glutamate/hr./100 mg. dry wt. of cells. Curve B; rate of increase of nucleic acid content of cells treated as for curve A. Rate of increase in absence of any inhibitor = 6·5 mg. nucleic acid/hr./100 mg. dry wt. of cells. Curve C; rate of accumulation of free glutamic acid within the cells studied over 30 min. incubation in buffered salt solution containing 1% glucose and 2·7 μmoles sodium glutamate/ml. Rate of accumulation in absence of any inhibitor = 42 μmoles glutamic acid/hr./100 mg. dry wt. of cells. Curve D, rate of fermentation of glucose in bicarbonate buffer at pH 7·2. Rate in absence of any inhibitor = Q_{CO_2}, 95. Growth inhibition shows range of drug concentration preventing growth of inoculum of approx. 10^6 cells/10 ml. complete medium. Temperature, 37° in all cases.

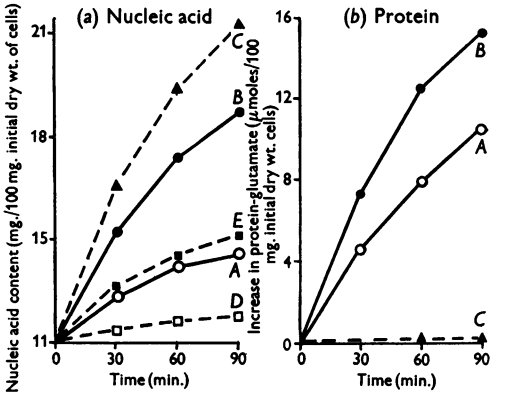

Fig. 2. Effect of chloramphenicol on (*a*) nucleic acid synthesis and (*b*) protein synthesis in *Staph. aureus* Duncan incubated at 37° in buffered salt solution containing 1% glucose and additions as follows. Curve A, amino-acid mixture A; Curve B, amino-acid mixture A + purine-pyrimidine mixture P'; Curve C, as (B) + 30 μg. chloramphenicol/ml.; Curve D, mixture P'; Curve E, as (D) + 30 μg. chloramphenicol/ml. Cells grown for 16 hr. at 30° in 'deficient' medium; made into washed suspension and incubated at final suspension density of 2·0 mg. dry wt. of cells/ml. in above mixtures. Final concentration of each amino-acid = 0·2 mg./ml., of each purine or pyrimidine = 0·02 mg./ml. After incubation, cells precipitated in the cold with 5% TCA. Protein estimated by glutamic acid content of precipitate after acid hydrolysis. Nucleic acid extracted from precipitates with hot 5% TCA and estimated spectrophotometrically.

Aureomycin and terramycin. These antibiotics have inhibitory actions similar to those of chloramphenicol except that, at high concentrations, they inhibit the processes of fermentation, accumulation of free glutamic acid and synthesis of nucleic acid (Figs. 3 and 4, Table 2). Fig. 3 shows that protein synthesis is markedly more sensitive to aureomycin than is the accumulation of free glutamic acid within the cells, and that growth is prevented by a concentration of the antibiotic which produces approx. 90% inhibition of the rate of protein synthesis. Concentrations of either antibiotic which inhibit protein synthesis, but do not inhibit nucleic acid synthesis, produce a significant stimulation of the rate of nucleic acid synthesis. The

Table 1. *Composition of nucleic acid fraction of* Staphylococcus aureus *Duncan treated with various antibiotics*

(*A. Staph. aureus* Duncan grown for 6 hr. at 37° with and without the addition of penicillin (10 international units/ml.) to the medium after 4 hr. growth. Cells harvested, washed, precipitated with 5% (w/v) trichloroacetic acid (TCA) in the cold; precipitates washed with cold 5% TCA, then extracted three successive times with 5% TCA at 90° for 10 min. Extracts combined for analysis of bases, etc. *B. Staph. aureus* Duncan grown for 16 hr. at 30° in deficient medium; harvested and made into washed suspension. Cells incubated for 1 hr. in buffered salt solution containing glucose, amino-acid mixture, purine-pyrimidine mixture *P'* and additions as shown below. After incubation, cells washed and nucleic acid extracts prepared as in *A*.)

	Culture *A*		Culture *B*				
				Additions to incubation medium			
	Control (% dry wt.)	Penicillin treated (% control)	Initial (% dry wt.)	None	Chloramphenicol (30 μg./ml.)	Penicillin (3000 units/ml.)	Bacitracin (2·5 mg./ml.)
				(Results expressed as % initial values)			
Total nucleic acid	14·1	89	10·30	126	148	114	110
Adenine	1·34	90	0·86	125	143	117	107
Guanine	1·68	91	1·02	126	144	116	115
Cytosine	0·90	92	0·66	125	139	116	108
Uracil	0·62	90	0·535	126	151	115	108
Thymine	0·22	90	0·25	108	95	110	92

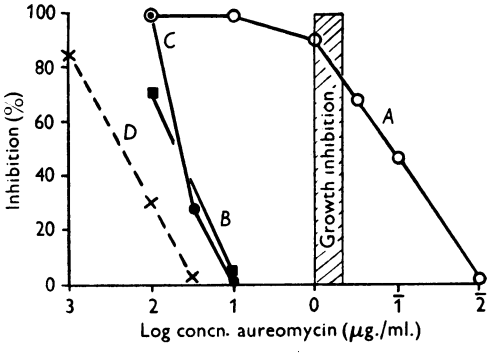

Fig. 3. Actions of aureomycin on amino-acid assimilation in *Staph. aureus* Duncan. Conditions as for Fig. 1.

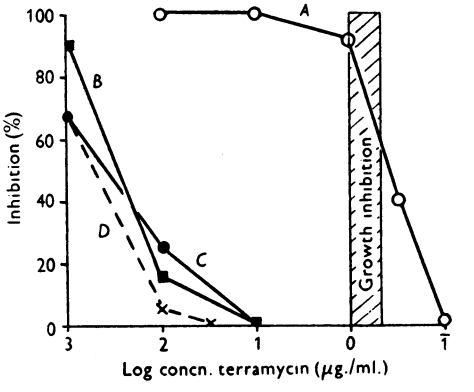

Fig. 4. Actions of terramycin on amino-acid assimilation in *Staph. aureus* Duncan. Conditions as for Fig. 1.

Table 2. *Effect of antibiotics and inhibitors on protein and nucleic acid synthesis, accumulation of free glutamic acid and glucose fermentation in* Staphylococcus aureus

(Conditions as for Fig. 1 using washed suspensions for all tests other than growth.)

	Concentration which inhibits growth (μg./ml.)	Concentration producing 50% inhibition (μg./ml.)				
		Protein synthesis	Purine-stimulated protein synthesis	Nucleic acid synthesis	Free glutamic acid accumulation	Glucose fermentation
Aureomycin	0·5–1·0	0·2	0·2	50	50	200
Terramycin	0·5–1·0	0·4	—	300	500	600
Chloramphenicol	5–10	2·0	2·0	*	*	*
Penicillin	0·05	*	200	2000	*	*
Bacitracin	50–100	1000	1000	1000	*	*
Streptomycin	1–2	3000	—	*	*	*
Polymyxin	*	*	*	*	*	*
2:4-Dinitrophenol	Approx. 120	60	—	60	60	6000
Sodium azide	Approx. 600	320	—	320	260	3200

* Little or no inhibition at 1000 μg./ml.

synthesis of nucleic acid has the same sensitivity as the accumulation of free glutamic acid. In general the actions of terramycin are very similar to those of aureomycin except that the former does not differentiate clearly between glucose fermentation and the processes of nucleic acid synthesis or glutamic acid accumulation.

Uncoupling agents. In a previous paper of this series (Gale, 1951*a*) it was shown that the drugs, sodium azide and 2:4-dinitrophenol, known to act as uncoupling agents in mitochondrial preparations, inhibit free glutamic acid accumulation at concentrations significantly smaller than those required to inhibit respiration or fermentation. This was confirmed in the present studies, and it was found (Table 2) that protein and nucleic acid syntheses had significantly the same sensitivity to both these drugs as glutamic acid accumulation.

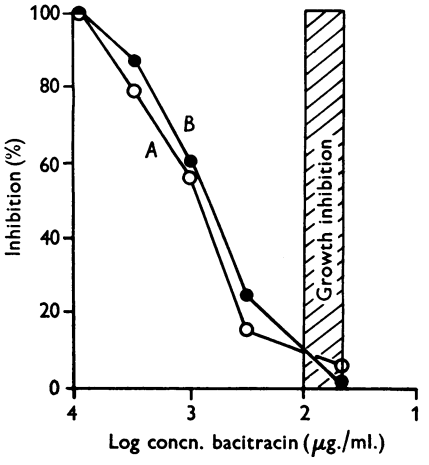

Fig. 5. Inhibition of protein (*A*) and nucleic acid (*B*) syntheses by bacitracin in *Staph. aureus* Duncan. Conditions as for Fig. 1.

Streptomycin. Table 2 shows that none of the processes studied in these experiments is inhibited by concentrations of streptomycin of the order of those preventing growth. The synthesis of protein is more sensitive than either free glutamic acid accumulation or nucleic acid synthesis, but 1 mg. streptomycin/ml. produces less than 50% inhibition of the rate of protein synthesis.

Polymyxin. Polymyxin in concentrations of 100 μg./ml. had no effect on any of the processes studied here in *Staph. aureus*.

Bacitracin. Staph. aureus Duncan is unable to grow if 50–100 μg. bacitracin/ml. are added to the growth medium; concentrations of this order have little effect upon either protein or nucleic acid synthesis by washed cells, whereas concentrations of 2–10 mg. bacitracin/ml. have marked inhibitory actions on both protein and nucleic acid synthesis (Fig. 5), the two processes having significantly the same sensitivity to the antibiotic.

Penicillin. It has not been possible to demonstrate, in previous experiments of this series, any action of penicillin even in high concentrations (300 international units/ml.) on the accumulation of free or combined glutamic acid by washed suspensions of *Staph. aureus* Duncan. In view of the reports by Krampitz & Werkman (1947), Gros & Macheboeuf (1948*a*, *b*), Gros *et al.* (1951) and Mitchell & Moyle (1951), it seemed desirable to re-investigate the effects on protein synthesis, especially when such synthesis took place in the presence of added purines and pyrimidines. The results are summarized in Fig. 6: penicillin has little or no effect on the synthesis of protein by washed cells when the incubation mixture is devoid of purines and pyrimidines but, in high concentrations, abolishes the additional synthesis promoted by the addition of mixture *P*. The additional nucleic acid synthesis taking place on the addition of mixture *P* is also decreased by the presence of penicillin, but it can be seen that a concentration of penicillin which completely abolishes the additional protein formation,

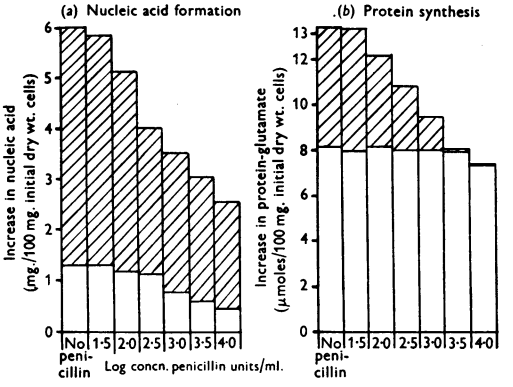

Fig. 6. Effect of penicillin on (*a*) nucleic acid and (*b*) protein synthesis by *Staph. aureus* Duncan in the absence (unshaded portion) and presence (unshaded + shaded) of purine-pyrimidine mixture *P*. Cells grown for 16 hr. at 30° in deficient medium; harvested and made into washed suspension. Cells, at suspension density of 2·0 mg. dry wt./ml. incubated for 1 hr. at 37° in buffered salt solution containing 1% glucose, amino-acid mixture *A* (each amino-acid at final concentration of 0·2 mg./ml.) with and without mixture *P* (each component at 0·02 mg./ml.). After incubation, cells precipitated in the cold with 5% TCA (final concn.). Protein-glutamate determined on portion of precipitate after acid hydrolysis; nucleic acid extracted with hot 5% TCA from remainder of precipitate and estimated spectrophotometrically. Initial values: nucleic acid = 11·2 mg./100 mg. dry wt. of cells; protein-glutamate = 33·5 μmoles/100 mg. dry weight of cells.

does no more than halve the rate of additional nucleic acid synthesis. If there is a metabolic connexion between the additional nucleic acid formation and the additional protein synthesis on the inclusion of mixture *P* in the incubation mixture, it might be that the material formed in the presence of *P* and penicillin (and which is measured simply as the 260 mμ.-absorbing material in the nucleic acid fraction) is not normal nucleic acid. However, it is clear from Table 1 that there has been no gross alteration in the relative proportions of the purine and pyrimidine bases, within experimental error, in the nucleic acid fraction.

DISCUSSION

The effects of amino-acid and purine-pyrimidine mixtures on increases in the protein and nucleic acid contents of cells clearly suggest a metabolic connexion between the synthesis of protein and nucleic acid (Gale & Folkes, 1953). There is little or no increase in the nucleic acid of the cells unless amino-acids are present in the incubation mixture; if an amino-acid mixture which promotes net protein synthesis is present, then a rapid increase in nucleic acid occurs; single amino-acids or simple mixtures thereof have smaller effects which might be correlated with their ability to promote protein turnover within the cell or otherwise 'spare' protein catabolism. Conversely, the presence of nucleic acid precursors accelerates the synthesis not only of nucleic acid but also of protein, while the addition of penicillin decreases the synthesis of nucleic acid and abolishes the purine-stimulated protein formation. These findings accord well with the various theories that have been put forward during recent years concerning a role of nucleic acid in protein synthesis (reviewed by Chantrenne, 1952). If nucleic acid acts as an organizer or template for protein synthesis, then increased nucleic acid will mean increased protein synthesis, and the reverse relationship may also hold (Caldwell & Hinshelwood, 1950). There are many indications in the literature that penicillin plays some interfering part in nucleic acid metabolism (Krampitz & Werkman, 1947; Gros & Macheboeuf, 1948*a*, *b*; Hotchkiss, 1949; Mitchell & Moyle, 1951; Gros *et al.* 1951; Park, 1952; Hotchkiss, 1952); this might result in an inhibition of nucleic acid synthesis as a whole or of some anabolic process which would lead to formation of an altered nucleic acid. The transformation of sensitive organisms to resistant ones (Hotchkiss, 1952) and the continued formation of adaptive penicillinase after removal of the substrate (Pollock, 1950) would suggest the latter mode of action. No gross changes in the base composition of the nucleic acid formed in the presence of penicillin could be shown in these studies (Table 1), but many alterations may take place in a complex structure such as nucleic acid without resulting in alterations in this type of analysis. If we accept a catalytic role of nucleic acid in protein synthesis, bacitracin would appear to inactivate the nucleic acid already formed in addition to preventing further synthesis of nucleic acid or protein; in this it differs from penicillin.

The action of chloramphenicol, aureomycin and terramycin is to effect a clear-cut inhibition of protein synthesis which is accompanied at the same time by an increase in the rate of nucleic acid formation within the cell. If protein and nucleic acid syntheses pass through a common stage, then this must be followed by splitting away of protein, or its immediate precursor, from the nucleoprotein complex and it may be that this group of antibiotics acts at this point. On the hypothesis of Caldwell & Hinshelwood (1950) such action might well be accompanied by a stimulation of nucleic acid synthesis.

The uncoupling agents, sodium azide and 2:4-dinitrophenol, inhibit the accumulation of free glutamic acid and the syntheses of protein and nucleic acid at concentrations significantly less than those which inhibit fermentation; this differential action may be due to coupled phosphorylations being involved in the more sensitive reactions. Loomis (1950) found that aureomycin at concentrations of 100 μg./ml. will uncouple oxidative phosphorylation in mitochondrial preparations but that such concentrations are toxic to guinea pigs. Similar concentrations inhibit the accumulation of free glutamic acid by *Staph. aureus* while having little effect on glucose fermentation (see Fig. 3), but protein synthesis is very much more sensitive than either of these processes. Loomis (1950) found that chloramphenicol was not effective as an uncoupling agent in mitochondrial preparations, and it does not effect a differential inhibition of glucose fermentation and glutamic acid accumulation in these studies (Fig. 1).

The results obtained with penicillin in these experiments have been obtained with high concentrations, and it is difficult to interpret the significance of inhibitions produced by concentrations of drug several orders higher than those required to prevent growth of normal inocula. It is possible that inhibition of growth is caused by inhibition of the synthesis of a specific protein or nucleic acid whereas much higher concentrations of the antibiotic are required to inhibit the total protein and nucleic acid syntheses which have been studied here. Gros *et al.* (1951) find that the breakdown of guanylic acid by *Staph. aureus* is inhibited by penicillin but that, whereas 2000–3000 units/ml. are required to inhibit the reaction in washed suspensions, the addition of 3–10 units/ml. to the growth medium 1 hr. before harvesting gives rise to cells whose ability to attack guanylic acid is 10% of that of untreated control cells. They suggest that peni-

cillin penetrates the growing cell more readily than the 'washed resting' cell. It would, however, seem equally possible that the loss of ability to oxidize guanylic acid following growth in the presence of penicillin is a secondary effect following some primary disorganization, an explanation put forward to explain the somewhat similar results obtained for the effect of penicillin on glutamic acid accumulation (Gale & Rodwell, 1949).

Such difficulties of interpretation do not arise in the cases of chloramphenicol, aureomycin and terramycin where the limiting concentration affecting protein synthesis is the same as that affecting growth.

SUMMARY

1. Chloramphenicol, aureomycin and terramycin inhibit protein synthesis by washed suspensions of *Staphylococcus aureus*, at bactericidal concentrations. Such concentrations simultaneously stimulate nucleic acid synthesis.

2. Bacitracin inhibits protein and nucleic acid synthesis to the same extent. Complete inhibition requires a concentration of bacitracin 100 times that which prevents growth.

3. Penicillin in high concentrations prevents the acceleration of protein synthesis produced by the addition of purines and pyrimidines to the incubation mixture containing amino-acids and glucose.

The authors are indebted to Miss P. J. Samuels for assistance with chromatographic procedures and to Dr T. F. Paine for gifts of aureomycin, streptomycin and bacitracin.

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The Oxidation of Certain Dicarboxylic Acids by Peroxidase Systems in Presence of Manganese

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(*Received* 9 *August* 1952)

It has frequently been suggested that the physiological effects of manganese may be due to its capacity for valency change. Kenten & Mann (1950) have shown that Mn^{2+} is oxidized by peroxidase systems. Such oxidation takes place in plant extracts (Kenten & Mann, 1951) and preliminary results suggest that it also occurs *in vivo* in higher plants. Kenten & Mann (1949) suggested that the manganese oxidation product reacts with plant metabolites whereby the Mn^{2+} is involved in an oxidation-reduction cycle which could explain its effect on plant respiration. As yet, however, there is little evidence as to the nature of the metabolites oxidized.

A number of instances have been recorded where an activating effect of Mn^{2+} on oxidative reactions is