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Sulphate Activation and its Control in *Escherichia coli* and *Bacillus subtilis*

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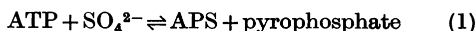
The first stage in the assimilation of sulphate by micro-organisms is its activation by ATP to form adenosine 5′-sulphatophosphate (APS) (Gregory & Robbins, 1960):



$$ATP + SO_4^{2-} \rightleftharpoons APS + pyrophosphate \quad (1)$$

In micro-organisms utilizing sulphate as sulphur source only, and not dependent on it as a terminal electron acceptor (Ishimoto & Fujimoto, 1959; Peck, 1959), this is followed by further reaction with ATP to yield adenosine 3′-phosphate 5′-sulphatophosphate (PAPS) (Gregory & Robbins, 1960):

$$APS + ATP \rightarrow PAPS + ADP \quad (2)$$

These enzymes (adenosine triphosphate–sulphate adenylyltransferase, EC 2.7.7.4, and adenosine triphosphate–adenylylsulphate 3′-phosphotransferase, EC 2.7.1.25, respectively), which have previously been demonstrated in extracts of *Neurospora* (Hilz & Lipmann, 1955; Ragland, 1959), yeast (Bandurski, Wilson & Squires, 1956; Robbins & Lipmann, 1956) and other fungi (Kaji & McElroy, 1958; Spencer & Harada, 1960), have now been shown to be present in two bacterial species, *Escherichia coli* and *Bacillus subtilis*. The control of sulphate activation by cyst(e)ine, the end product of sulphate reduction, has been investigated. When *E. coli* or *B. subtilis* is grown on cyst(e)ine instead of sulphate, the ability of extracts to synthesize PAPS is repressed (Pasternak, 1961). This effect explains the observations of Roberts, Abelson, Cowie, Bolton & Britten (1955) that incorporation of [^{35}S]sulphate into the proteins of *E. coli* is abolished by the presence of cystine in the growth medium.

MATERIALS AND METHODS

Growth of organisms. Stock cultures of *Escherichia coli* A.T.C.C. 9723 and *Bacillus subtilis* N.C.T.C. 1379 were maintained on slopes of Oxoid nutrient agar CM 4 at 4°. The medium for growth of *E. coli* was that described by Davis & Mingioli (1950) except that $MgSO_4,7H_2O$ was replaced by $MgCl_2,6H_2O$ (0·12 g./l.) and that $(NH_4)_2SO_4$ was replaced by NH_4Cl (2·5 g./l.). K_2SO_4 (British Drug Houses Ltd.) or

L-cystine (Roche Products Ltd., Welwyn Garden City) was added to the medium before autoclaving at 15 lb./in.^{2} for 20 min. Inhibition of growth of *E. coli* by cysteine (Roberts *et al.* 1955) was confirmed in the present experiments. L-Glutathione, oxidized or reduced (Sigma Chemical Co., St Louis 18, Mo., U.S.A.) was sterilized by filtration through Oxoid membrane filters and added to the medium after autoclaving. The medium for growth of *B. subtilis* was 1·3% (w/v) Oxoid nutrient broth CM1 to which sterile glucose (0·5%) had been added after autoclaving. L-Cysteine (British Drug Houses Ltd.) and glutathione were sterilized by filtration through Oxoid membrane filters and added to the medium after autoclaving. For enzyme preparations, media (400 ml. in 1 l. flasks) were inoculated with 1–2 ml. of a starter culture in logarithmic phase and shaken for 6–12 hr. on a reciprocal shaker at 37°. For testing the incorporation of ^{35}S or the inhibition by selenate, media (5 ml. in $\frac{5}{8}$ in. × 6 in. test tubes) were inoculated with 0·05–0·1 ml. of a starter culture and shaken at 10° to the horizontal on a reciprocal shaker at 37° for 5–6 hr. The [^{35}S]sulphate (SJS1) was obtained from The Radiochemical Centre, Amersham, and sterilized by heating at 100° for 10 min. Sodium selenate and sodium molybdate (British Drug Houses Ltd.) were sterilized by autoclaving. Growth was measured with an EEL Unigalvo type 20 nephelometer by using the blank filter B for cultures of *E. coli* and the red filter OR2 for cultures of *B. subtilis* containing broth; in each case the instrument was adjusted to give a reading of 25 with the Perspex standard.

Incorporation of ^{35}S. A preliminary experiment was carried out to determine the distribution of ^{35}S in *E. coli* after growth on [^{35}S]sulphate (260 mg. of S/l.) for 6½ hr. The fractionation procedure of Roberts *et al.* (1955) was employed, the residual protein being dissolved in 1 ml. of biuret reagent and estimated colorimetrically (Gornall, Bardawill & David, 1957) with crystallized bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne) as standard. This protein fraction contained 51% of the radioactivity; 23% was present in the ethanol-soluble protein fraction and 17% in the hot-trichloroacetic acid-soluble fraction. Thereafter, ^{35}S-labelled cells were merely washed five times with ice-cold 0·85% NaCl and dissolved in 1 ml. of biuret reagent. The content of protein and ^{35}S of this fraction was then determined. The same method was used to measure the ^{35}S content of *B. subtilis* after growth on [^{35}S]sulphate.

Assay of radioactivity. Samples (0·05–0·5 ml.) were plated on aluminium planchets (4·5 cm.^{2}), dried at 105° for 45 min. to yield infinitely thin specimens (< 0·1–5·0 mg.) and the radioactivity was measured with a G.E.C. Geiger–Müller end-window probe fitted to a type D657 scaling unit (Panax Equipment Ltd., Redhill, Surrey) or with a Nuclear–Chicago gas-flow automatic sample-changing counter. The efficiency of assaying ^{35}S by gas flow was approximately twice that obtained with the end-window probe.

Preparation of enzyme extracts. Cultures (0·2–1·0 mg. dry wt./ml.) were harvested towards the end of the logarithmic phase by centrifuging, washed twice with ice-cold 10 mM-cysteine–10 mM-EDTA buffer brought to pH 7·5 with NaOH and suspended in a minimal quantity (2–5 ml.) of buffer. Ultrasonic disintegration was carried out with a 600 w Mullard magnetostrictor oscillator operating at 25 kcyc./sec. for 2–3 min. at 0°. The resulting suspension was centrifuged in a Spinco model L refrigerated centrifuge at 105 000g for 60 min. and the supernatant fraction used.

Assay of enzyme extracts. The formation of PAPS was assayed by incubating 3 μmoles of ATP, 3·7 μmoles of $MgCl_2$, 0·3 μmole of K_2SO_4, 5–10 μc of $Na_2{}^{35}SO_4$, tris–HCl buffer (25 μmoles of tris, pH 8·8) and the supernatant fraction (0·05–0·1 ml., 0·7–1·7 mg. of protein) in a final volume of 0·5 ml. for 45 min. The reaction was terminated by heating in a boiling-water bath for 2 min., protein removed by centrifuging and the [^{35}S]PAPS content measured after separation by paper electrophoresis as described by Pasternak (1960). Confirmation of its structure was obtained by analysis of the adenosine (based on u.v. absorption measured with a Unicam SP. 500 spectrophotometer), [^{35}S]sulphate and phosphate (Dryer, Tammes & Routh, 1957) content after chromatography on Dowex 1 (with a LKB 3400 Radi-Rac automatic fraction collector, Gallenkamp and Co. Ltd.) as described in the Results section. Adenosine 3′,5′-diphosphate was determined by the spectrophotometric method of Gregory & Lipmann (1957) by using the high-speed supernatant fraction of rat liver purified by several passages through Dowex 1; ADP lot no. 603 of Pabst Laboratories (Pasternak, 1960) was used as standard. The same enzyme was employed to measure the transfer of [^{35}S]sulphate from [^{35}S]PAPS to p-nitrophenol (Pasternak, 1960). Protein in the reaction mixtures was determined by the biuret method (Gornall *et al.* 1957).

Table 1. *Incorporation of* [^{35}S]*sulphate by growing cultures of* Escherichia coli

Cultures were grown for 3 hr. in the presence of unlabelled K_2SO_4, cystine or glutathione as indicated. $Na_2{}^{35}SO_4$ (19 μc) was added (cell density 0·12 mg. dry wt./ml.), the cultures were grown for a further 2½ hr. and harvested, and the ^{35}S content of washed cells was measured.

Sulphur source (mg. of S/l.)				Growth (mg. dry wt./ml. at harvesting)	Percentage of added [^{35}S]sulphate incorporated	Total [^{35}S]sulphate incorporated (mg. of S/l.)
K_2SO_4	$Na_2{}^{35}SO_4$	Cystine	Glutathione (reduced)			
26·7	0·013	—	—	0·92	13·4	3·58
26·2	0·013	0·52	—	0·93	13·2	3·46
21·5	0·013	5·2	—	0·69	4·7	1·02
—	0·013	26·7	—	0·88	<1·5	<0·0002
26·7	0·013	26·7	—	0·71	<0·5	<0·0001
—	0·013	—	6·4	0·83	84·0	0·011
—	0·013	—	26·7	0·94	84·0	0·011
—	0·013	—	64·0	0·79	87·0	0·011

RESULTS

Incorporation of [^{35}S]*sulphate by* Escherichia coli *and* Bacillus subtilis. Cystine in the growth medium at a concentration of 26·7 mg. of S/l., with or without sulphate, completely abolished the incorporation of [^{35}S]sulphate by cells of *E. coli*; but glutathione, at a concentration of 6·4–64·0 mg. of S/l., allowed 85 % of added tracer [^{35}S]sulphate to be incorporated (Table 1). This experiment confirms the conclusion of Roberts *et al.* (1955) that glutathione is not an intermediate in the incorporation of [^{35}S]sulphate into cell constituents and suggests that *E. coli* prefers cystine to sulphate and sulphate to glutathione as a source of cellular sulphur. With *B. subtilis* both cysteine (26·7 mg. of S/l.) and glutathione (26·7 mg. of S/l.) decreased the incorporation of [^{35}S]sulphate (Table 2). The mechanism by which these effects are exerted was investigated by examining the ability of extracts of *E. coli* and *B. subtilis* to form PAPS, an intermediate in the assimilation of sulphate.

Synthesis of adenosine 3′-phosphate 5′-sulphatophosphate by ultrasonic extracts of Escherichia coli *and* Bacillus subtilis. Ultrasonic extracts of *E. coli* catalysed the formation of [^{35}S]PAPS from [^{35}S]-sulphate, ATP and Mg^{2+} ions (Table 3). Fluoride was inhibitory. All the activity was in the supernatant fraction obtained after centrifuging at 105 000*g* for 60 min. The optimum pH was between 8 and 9 (Fig. 1). [^{35}S]PAPS was the only labelled product of the reaction. The identity of [^{35}S]PAPS was established by isolating the radioactive product of a reaction mixture containing 45 μmoles of ATP, 56 μmoles of magnesium chloride, 4·5 μmoles of potassium [^{35}S]sulphate (150 μc) and the supernatant enzyme fraction (48 mg. of protein) prepared from *E. coli* grown in the presence of glutathione, after incubation for 45 min. Protein was removed by centrifuging after heating at 100° for 1 min. The supernatant fraction was purified by chromatography on Dowex 1 as described by Pasternak (1960). Two radioactive fractions were obtained (Fig. 2). The first (tubes 64–66) was shown to be inorganic sulphate. The second (tubes 84–90) was purified further by adsorption on charcoal (Norit A), washing with water to remove inorganic ions and eluting with ethanol–water–aq. ammonia (sp.gr. 0·88) (20:20:1, by vol.). The eluate was identified as [^{35}S]PAPS on the basis of its spectral properties ($\lambda_{max.}$ at 257 mμ, E_{250}/E_{260} of 0·87 and E_{280}/E_{260} of 0·24), its constituents (adenosine:[^{35}S]sulphate: phosphate:adenosine 3′,5′-diphosphate molar proportions of 1·00:1·09:2·12 [< 0·1 mol.prop. liberated by 1 N-sulphuric acid at 100° in 10 min.]:1–2) and its ability to transfer more than 52 % of its [^{35}S]sulphate to *p*-nitrophenol enzymically. The reduction of PAPS to sulphite by *E. coli* has been demonstrated by Fujimoto & Ishimoto (1961) and Peck (1961).

When the synthesis of [^{35}S]PAPS by extracts of *E. coli* which had been grown on cystine as the sole

Table 2. *Incorporation of* [^{35}S]*sulphate by growing cultures of* Bacillus subtilis

Cultures were grown for 2½ hr. on broth with added cysteine or glutathione as indicated. $Na_2{}^{35}SO_4$ (20 μc) was added (cell density 0·09 mg. dry wt./ml.), the cultures grown for a further 2½ hr. and harvested, and the ^{35}S content of cells was measured.

Added sulphur source (mg. of S/l.)		Growth (mg. dry wt./ml. at harvesting)	Percentage of added [^{35}S]sulphate incorporated
Cysteine	Glutathione (reduced)		
—	—	0·55	0·35
26·7	—	0·39	0·03
—	26·7	0·41	0·05

Table 3. *Synthesis of adenosine 3′-phosphate 5′-sulphatophosphate by ultrasonic extracts of* Escherichia coli

The supernatant fraction (0·7 mg. of protein/0·05 ml.) of an extract of *E. coli* grown on K_2SO_4 was incubated with ATP (3 μmoles), $MgCl_2$ (3·7 μmoles), K_2SO_4 (0·3 μmole), $Na_2{}^{35}SO_4$ (5·6 μc) and tris–HCl buffer (25 μmoles of tris, pH 8·4) in 0·5 ml. and analysed for [^{35}S]PAPS formation.

Incubation medium		Incubation time (min.)	Enzyme added (ml.)	[^{35}S]PAPS formed (μm-moles/mg. of protein)
Additions	Omissions			
—	—	0	0·05	1·4
—	—	15	0·05	14·5
—	—	30	0·05	25
—	—	45	0·05	34
—	—	45	0·025	7·5
—	—	45	0·1	43
—	—	45	0·15	63
—	ATP	45	0·05	1·0
—	$MgCl_2$	45	0·05	26
8·6 mM-NaF	—	45	0·05	22
28·6 mM-NaF	—	45	0·05	4·9
86 mM-NaF	—	45	0·05	3·6

sulphur source was assayed, no activity could be detected (Table 4). This result is not due to inhibition either by cystine (cysteine itself is stimulatory) or by a metabolite of it present in the enzyme extract, as the addition of inactive (cystine-grown) extract to active (sulphate-grown) extract did not diminish [^{35}S]PAPS synthesis significantly (Table 4). Growth of *E. coli* on glutathione (oxidized or reduced) did not repress the PAPS-synthesizing system, but yielded rather more active extracts (Table 4). With *B. subtilis*, addition of either cyst(e)ine or glutathione (oxidized or reduced) to the growth medium resulted in repressed synthesis of the sulphate-activating enzymes (Table 5). The results summarized in Tables 4 and 5 are thus in complete agreement with the experimental findings on the incorporation of [^{35}S]sulphate by growing cultures (Tables 1 and 2), namely that cystine abolishes [^{35}S]sulphate uptake by *E. coli* and that cysteine and glutathione decrease that of [^{35}S]-sulphate by *B. subtilis*.

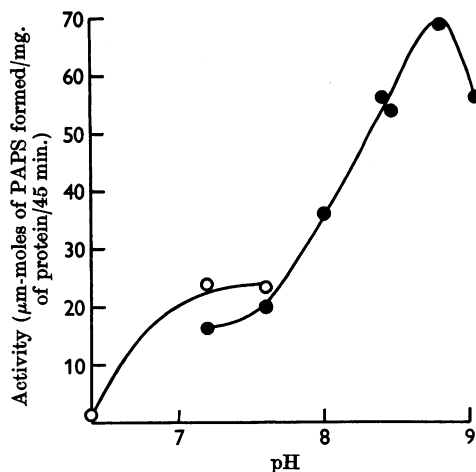


Fig. 1. Effect of pH on the formation of adenosine 3′-phosphate 5′-sulphatophosphate by ultrasonic extracts of *Escherichia coli*. The supernatant fraction of an ultrasonic extract of *E. coli* grown on K_2SO_4 was incubated with ATP, $MgCl_2$, K_2SO_4 and [^{35}S]sulphate with either sodium phosphate (25 μmoles of phosphate) (○), or tris–HCl (25 μmoles of tris) buffer (●), and analysed for ^{35}[S]PAPS formation.

Effect of selenate and molybdate on growth of Escherichia coli. Selenate and molybdate are specific inhibitors of reaction (1) because they serve as substrates for the enzyme, though adenosine 5′-selenatophosphate and adenosine 5′-molybdophosphate are at once decomposed by water to yield AMP and pyrophosphate as the sole products of the reaction (Wilson & Bandurski, 1958). When sodium selenate was added to *E. coli* utilizing sulphate, growth was inhibited (Table 6). Slight inhibition also occurred with *E. coli* growing on

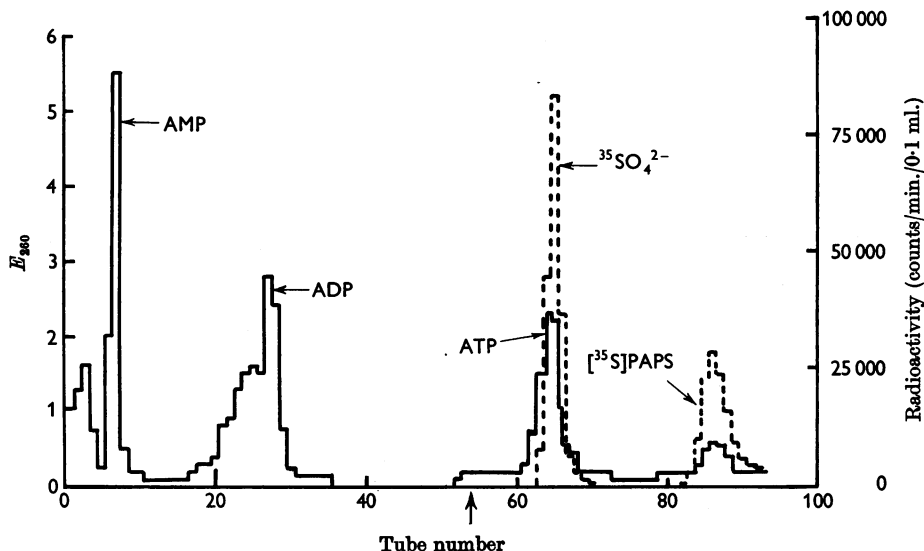


Fig. 2. Ion-exchange chromatography of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate. The deproteinized reaction mixture (see text) was diluted to 50 ml., adsorbed on a 1 cm. × 20 cm. column of Dowex 1 (X4; 200–400 mesh; formate form) and subjected to gradient elution with ammonium formate–formic acid at 4° as described by Pasternak (1960). The fraction volume was 10 ml. The start of the 1M-ammonium formate is indicated by the arrow. The ultraviolet absorption at 260 mμ (full line) and the radioactivity (broken line) of the effluent fractions were measured as described in the text. The separated compounds were identified on the basis of their adenosine:phosphate ratio.

Table 4. *Synthesis of adenosine 3′-phosphate 5′-sulphatophosphate by ultrasonic extracts of* Escherichia coli

Cultures were grown in the presence of each of the compounds indicated (each at a concentration of 26·7 mg. of S/ml.), harvested and assayed for ability to synthesize PAPS.

Sulphur source for growth	Enzymic activity (μm-moles of PAPS synthesized/mg. of protein/45 min.)
K_2SO_4	36·5
K_2SO_4	20·0*
Glutathione (red.)	54·3
Glutathione (ox.)	65·5
Cystine	<0·02
Cystine + K_2SO_4	0·02

* Extract (0·1 ml.) of cystine-grown cells added to extract (0·1 ml.) of K_2SO_4-grown cells.

Table 5. *Synthesis of adenosine 3′-phosphate 5′-sulphatophosphate by ultrasonic extracts of* Bacillus subtilis

Cultures were grown with the added sulphur compounds (each at a concentration of 26·7 mg. of S/l.) indicated, harvested and assayed for ability to synthesize PAPS.

Sulphur source for growth	Enzymic activity (μm-moles of PAPS synthesized/mg. of protein/45 min.)
Oxoid broth + K_2SO_4	30·6
Oxoid broth + K_2SO_4	17·9*
Oxoid broth + glutathione (red.)	0·9
Oxoid broth + glutathione (ox.)	4·4
Oxoid broth + cysteine	0·4
Oxoid broth + cystine	0·6
Oxoid broth + cysteine + K_2SO_4	<0·2

* Extract (0·1 ml.) of cysteine-grown cells added to extract (0·1 ml.) of K_2SO_4-grown cells.

Table 6. *Effect of sodium selenate on the growth of* Escherichia coli

Cultures were grown for 6 hr. in the presence of K_2SO_4, glutathione (reduced) or cystine (each at a concentration of 26·7 mg. of S/l.) and Na_2SeO_4 as indicated. The cell densities without inhibitor were 0·61, 0·23 and 0·62 mg. dry wt./ml. respectively.

Sulphur source	Concn. of Na_2SeO_4 (mM)	Growth (% of cell density without inhibitor)
K_2SO_4	—	100
	2	9
	10	9
Glutathione	—	100
	2	40
	10	32
Cystine	—	100
	2	120
	10	116

glutathione though all cellular sulphur was presumably derived from glutathione without participation of reaction (1). Selenate was without effect on the growth of *E. coli* on cystine. An explanation for this discrepancy may be that cystine, unlike glutathione, represses the enzyme catalysing reaction (1). Selenate cannot inhibit in absence of the enzyme, but in its presence it uncouples ATP metabolism and leads to a decrease in the energy supply available for maximal growth. That reaction (1) is inhibited when selenate is added to cultures growing on glutathione was shown by adding tracer [^{35}S]sulphate and measuring the incorporation into cell constituents: 0·9 mM-selenate was without effect on growth but decreased [^{35}S]sulphate incorporation by *E. coli* from 62 to 3·5%. The effects of molybdate on growth of *E. coli* were similar to those of selenate.

DISCUSSION

Repression of an enzyme early on in a metabolic sequence by the end product of that sequence is a common mechanism of control in bacteria (Pardee, 1959). It is not surprising therefore to find that cyst(e)ine represses sulphate activation in *E. coli* and *B. subtilis* (Tables 4 and 5). What is unexpected is that glutathione does not do so in *E. coli* even when it is the sole source of sulphur (Table 4). This implies either that glutathione metabolism to cysteine and methionine in protein does not go via free cyst(e)ine or that the rate of cyst(e)ine formation from glutathione is so slow that the normal pool size is not exceeded. The second alternative would seem to be more likely and receives support from the finding that unlabelled glutathione does not dilute the uptake of tracer [^{35}S]sulphate into proteins (Table 1). The repressive effect of glutathione in *B. subtilis* (Table 5) may be due to the action of glutathione itself or could result from repression by cyst(e)ine which may be formed more rapidly in *B. subtilis* than in *E. coli*. It has not yet been possible to determine which of the two enzymes concerned with sulphate activation are repressed by cyst(e)ine though the experiments with selenate (Table 6) discussed above indicate repression of adenosine triphosphate sulphurylase (reaction 1).

Sulphate activation is not the only step in sulphate assimilation which is repressed by cyst(e)ine. Mager (1960) has adduced evidence to show that a later stage, namely the reduction of sulphite, is also repressed. Since the concentrations of cysteine used by Mager were similar to those in the present study, it is possible that the mechanism of coordinate repression (Ames & Garry, 1959) is operative.

SUMMARY

1. The enzymes (adenosine triphosphate–sulphate adenylyltransferase, EC 2.7.7.4, and adenosine triphosphate–adenylylsulphate 3′-phosphotransferase, EC 2.7.1.25) catalysing the synthesis of adenosine 3′-phosphate 5′-sulphatophosphate have been shown to be present in ultrasonic extracts of *Escherichia coli* and *Bacillus subtilis*.

2. The incorporation of [^{35}S]sulphate by growing cultures of *E. coli* is prevented by cystine but not by glutathione. Incorporation by *B. subtilis* is reduced by cysteine and glutathione.

3. These results are explained by the finding that the enzyme system catalysing synthesis of adenosine 3′-phosphate 5′-sulphatophosphate is repressed by cystine in *E. coli* and by cyst(e)ine and glutathione in *B. subtilis*.

4. Growth of *E. coli* on sulphate as the sole source of sulphur is abolished by 2 mM-sodium selenate; growth on glutathione is inhibited 60% but growth on cystine is unaffected.

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Teichoic Acid from the Walls of *Staphylococcus aureus* H

2. LOCATION OF PHOSPHATE AND ALANINE RESIDUES*

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The teichoic acid that occurs in large amounts in the walls of *Staphylococcus aureus* H is a ribitol phosphate polymer to which are attached acylglucosamine and alanine residues (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958). The detailed structure of this and related teichoic acids is of particular interest in view of their serological properties (Sanderson, Juergens & Strominger, 1961) and the recognition that the group-specific antigens of at least two staphylococci are teichoic acids (Haukenes, Ellwood, Baddiley & Oeding, 1961).

Baddiley, Buchanan, RajBhandary & Sanderson (1962) showed that the alanine in this teichoic acid, like that in all other cases studied, has the D-configuration and is in labile ester linkage with

* Part 1: Baddiley *et al.* (1962).

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