Since the method involves a difference of extinctions it is important to select the weight of sample so that the extinctions of neither sterol at 440 $m\mu$ is too low. The method is not considered suitable for the determination of less than 0.025 mg. of cholesterol and 0.075 mg. of coprosterol. Furthermore, it was found that optimum values for E_{440} and E_{560} are between 0.450 and 1.100. The sample quoted represents a typical determination, where 0.8765 mg. of lipid, containing 6.39 % of cholesterol and 49.40 % of coprosterol, had values of E_{440} 0.750 and E_{560} 0.707.

The difference in the spectra also reveals the presence of an unknown impurity with a peak at approx. 460 $m\mu$, resulting in errors of 0.01 and 0.02 mg. for cholesterol and coprosterol respectively. Tests carried out on other faecal sterols (Table 1) show errors of 3.1 and 1.7 % for E_{440} and E_{560} respectively. With the faecal fat just described (fat-free diet with corn-oil supplement) the corrected extinctions should therefore be E_{440} 0.727 and E_{560} 0.695, and the recalculated proportions are therefore cholesterol 6.41 % and coprosterol 47.01 %, thus giving estimated errors of 0.31 % for cholesterol and 4.8 % for coprosterol. These seem to be almost solely attributable to cholestanol, which would appear in the determination chiefly as coprosterol.

Table 1 shows that the molecular extinction of β -sitosterol is relatively high. The effect of this on the accuracy of the method in cases where this compound is part of the diet is difficult to assess. Assuming the ingestion of 2 g. of corn oil (i.e. approx. 10 %) daily, the β -sitosterol intake would amount to 10 mg. From the work of Gould (1955), who estimated β -sitosterol absorption in the intestine to be one-tenth of that of cholesterol, and from the work of Cook & Thomson (1951) on intestinal absorption of cholesterol, it is estimated that rats could absorb 12 mg. of β -sitosterol/day. Furthermore Coleman & Baumann (1957) produced evidence that approx. 70 % of the β -sito-

sterol is hydrogenated in the intestine. These considerations make it appear likely that β -sitosterol will not interfere appreciably with the method unless the amounts administered are greatly in excess of those quoted above. Nevertheless, the possibility of error, particularly when faecal components other than those mentioned above are present, cannot be ruled out and the value of the method must then be reassessed.

SUMMARY

1. A method based on that of Zak *et al.* (1957) is described for the simultaneous quantitative determination of cholesterol and coprosterol, both free and total, in faecal lipids.

2. The method was tested on mixtures of known composition and it was found that the standard deviations, based on percentage recovery, were ± 2.80 and ± 3.41 % for cholesterol and coprosterol respectively, and the mean percentage recoveries were 100.4 and 99.8 %.

3. Tests carried out on faecal lipids indicate that other sterols normally present in faeces do not materially affect the results.

4. The method is suitable for determinations on batches of six to eight samples.

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5-Fluorouracil and Mucopeptide Biosynthesis by *Staphylococcus aureus*

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The accumulation of uridine mucopeptides has been shown to occur when some strains of *Staphylococcus aureus* are treated with penicillin (Park, 1952a-c; Park & Strominger, 1957; Strominger, 1957). In the strain examined, the most compli-

cated of these compounds contained uridine diphosphate linked to one molecule each of 3-carboxy-ethylglucosamine (muramic acid), D-glutamic acid and L-lysine together with three molecules of alanine; two of the three alanine molecules were in

the D-configuration (Strominger & Threnn, 1959*a*). It has been suggested (Park & Strominger, 1957) that these compounds are precursors of the cell-wall mucopeptides. Other evidence (Park, 1958; Mandelstam & Rogers, 1959) shows that penicillin and some other antibiotics inhibit the synthesis of cell-wall mucopeptides and may therefore be expected to lead to the accumulation of precursors. Uridine mucopeptides also accumulate when cell-wall formation is prevented by the absence of an essential amino acid. For example, when a strain of staphylococcus is incubated in the absence of lysine a mucopeptide containing no lysine accumulates (Strominger & Threnn, 1959*b*). The present work has been designed to examine further, by the use of uridine analogues, the hypothesis that uridine compounds are precursors of the cell-wall mucopeptides. The results of this work are also interesting in relation to the possible mode of action of penicillin.

Duschinsky, Plevin & Heidelberger (1957) first synthesized 5-fluorouracil as a tumour-inhibiting compound. Low concentrations of it were also found (Scheiner, Kostelak & Duschinsky, 1957; Heidelberger *et al.* 1957) to inhibit the growth of a variety of bacteria. It has since been shown that 5-fluorouracil is incorporated into the ribonucleic acids of tobacco mosaic virus (Gordon & Stachlin, 1958), of mouse liver, spleen and tumour (Chaudhuri, Montage & Heidelberger, 1958) and of *Escherichia coli* (Horowitz & Chargaff, 1959). It is also converted into 5-fluorouridine mono-, di- and tri-phosphate together, probably, with 5-fluorouridine diphosphoglucose, although the last-named compound has not been positively identified (Chaudhuri *et al.* 1958). These reactions of the fluorinated compound have been summarized by Harbers, Chaudhuri & Heidelberger (1959) and by Mandel (1959). Ribosidation and ribotidation of fluorouracil have been achieved in a cell-free system (Sköld, 1958). The incorporation of 5-fluorouridylic acid blocks the incorporation of uracil into ribonucleic acid (Melnick, Cantarow & Paschkis, 1958) but allows a net increase of this nucleic acid to occur (Cohen, Flaks, Barner, Loeb & Lichtenstein, 1958). The incorporation of orotic acid into the thymine of deoxyribonucleic acid, on the other hand, is almost completely stopped (Edinoff, Knoll & Klein, 1957), and in *E. coli* 5-fluorouracil and its ribotide completely prevent deoxyribonucleic acid synthesis. These effects on deoxyribonucleic acid formation can be reversed by thymine and thymidine, which also allow growth to occur in the presence of 5-fluorouracil.

In the present work it is the first of the above functions of the 5-fluoro compound, as an analogue of uracil, and not the second, as an inhibitor of thymine synthesis, which is studied. Hence the

present interest is in its incorporation into various compounds in place of uridine, rather than in the simultaneous inhibition of thymine and deoxyribonucleic acid formation which also occurs. A preliminary report of some of the earlier stages of this work has already appeared (Rogers & Perkins, 1960) and a report of preliminary investigations with *E. coli* has been published (Tomasz & Borek, 1960).

MATERIALS AND METHODS

Organisms. *Staphylococcus aureus* strain 524/SC (Rogers, 1953) was used for most of the work but some experiments were also done with strain Oxford. Both strains were maintained as dried cultures.

Growth of bacteria and preparation of cell suspensions. These were as described by Mandelstam & Rogers (1959).

Chemicals. Amino acids labelled at C-1 with ^{14}C were supplied by The Radiochemical Centre, Amersham, and diluted with unlabelled material so that 1 mg. contained 1 μC . These were then dissolved to give stock solutions of 1 mg./ml. 5-Fluorouracil and 5-fluoro[2- ^{14}C]uracil were supplied through the courtesy of Dr Morrison of Roche Products Ltd., Welwyn Garden City; 6-azauracil was from California Research Inc.; 6-azathymine was from L. Light and Co. Ltd. and 8-azaguanine from Sigma Chemical Co.

Cell-wall biosynthesis. This was measured by the incorporation of labelled amino acids according to the techniques described by Mandelstam & Rogers (1959). All four amino acids present in the cell-wall mucopeptide (i.e. glutamic acid, glycine, lysine and alanine) were always present; when necessary one was supplemented with the labelled compound. Chloramphenicol (50 $\mu\text{g./ml.}$) was always present. Likewise the preparations of hot-trichloroacetic acid precipitates and cell walls from the organisms were as described by Mandelstam & Rogers (1959).

Soluble N-acetylhexosamine-containing compounds in the cells. These were extracted with trichloroacetic acid and their content of bound N-acetylhexosamine was measured according to Strominger (1957) including the brief preliminary period of hydrolysis with HCl.

Amino acids, glucosamine and muramic acid. These were measured as before (Perkins & Rogers, 1959).

Phosphorus. This was determined by the method of Allen (1940).

Paper chromatography

Samples to be examined by paper chromatography for purine and pyrimidine bases were first hydrolysed in 88% (v/v) formic acid at 165° for 1 hr. The formic acid was then removed *in vacuo*.

Amino acids and amino sugars. The following solvent systems were used: (a) butan-1-ol-acetic acid-water (63:10:27), upper phase; (b) butan-1-ol-pyridine-water (6:4:3) (Jeanes, Wise & Dimler, 1951).

Nucleotides and purine and pyrimidine bases. The following solvents were used: (a) butan-1-ol-ammonia (MacNutt, 1952); (b) isopropanol-HCl (Wyatt, 1951); (c) isobutyric acid-ammonia (Zetterström & Ljunggren, 1951); (d) ammonium acetate-ethanol, pH 7 and pH 3.8 (Paladini & Leloir, 1952).

Column chromatography

Dowex-1 ($\times 2$, chloride form, 50–100 mesh). This material was first treated with 2*N*-NaOH until no further chloride was present in the effluent, washed with water, treated with 2*N*-HCl until no sodium (by flame test) was eluted and then washed with water until the effluent was chloride free. The samples were applied in about 20 ml. of solution to a column of the resin (15–20 cm. long and 1.5 cm. in diameter) and allowed to flow at a rate of 0.5 ml./min. The column was then washed at the same rate with about 20 ml. of water. After this the first solvent was applied (0.002*N*-HCl). This and the succeeding solvents were those used by Strominger (1957) to fractionate the compounds, containing hexosamine, accumulated by staphylococci when they are acted upon by penicillin. The rate of flow was maintained at 0.5 ml./min. and 5.0 ml. fractions were collected. The whole operation was conducted at 0–4°. All samples thought to contain material of interest were neutralized to pH 7.0 with *N*-NaOH and not allowed to stand at an acid pH for longer than 3 hr.

NN-Diethylaminoethylcellulose. This was obtained from Kodak Ltd., Kirkby, Lancs. It was first suspended in water and the finer material carefully removed. This is most important if reasonable flow rates in columns are to be achieved. The larger material was then suspended in 0.01 *M*-ammonium acetate, pH 6.5, and *N*-acetic acid added drop by drop until the pH was restored to 6.5. A glass column about 10 cm. $\times$ 1.5 cm. with a no. 3 sintered disk at the bottom and a little glass wool above it was then packed to give a column about 7 cm. high. This was washed with 0.01 *M*-ammonium acetate at pH 6.5 until the inflowing and outflowing liquid had the same pH. The sample, dissolved in about 10 ml. of cold 0.01 *M*-ammonium acetate, pH 6.5, was then applied and allowed to flow through the column at about 0.5 ml./min. After the column was washed, a gradient of ammonium acetate was applied by running a 0.5 *M*-solution of the salt (for the expt. in Fig. 6 a *M*-solution was used), adjusted to pH 6.5, into 1 l. of 0.01 *M*-solution at the same pH contained in a flask fitted with a magnetic stirrer. Fractions of 5 ml. were collected at a flow rate of 0.5 ml./min. and the whole operation was conducted at 0–4°.

The extinction of all the samples coming from both the Dowex-1 and diethylaminoethylcellulose (DEAE) columns was measured in 1 cm. cells at a wavelength of 260 μ in a Unicam SP. 500 spectrophotometer. A sample (usually 0.4 ml.) of each alternate tube was briefly hydrolysed (Strominger, 1957) and the *N*-acetylhexosamine content measured. In acidifying these samples for hydrolysis, it was necessary to increase the amount of acid added to allow for the increasing concentration of ammonium acetate buffer in the samples. In peaks containing amino sugar compounds every tube was estimated. In the experiments in which 5-fluoro[2-¹⁴C]uracil was used 0.1 ml. of each alternate tube was plated and its radioactivity measured; again a sample from each tube was measured in the regions of activity.

Radioactive-counting technique. Sufficient of the dry solid materials (i.e. precipitates, insoluble in hot trichloroacetic acid, and cell walls) was packed in 0.3 cm.² planchets to allow the assumption of an infinitely thick layer. A thousand counts or more above background count were counted, giving an error of $\pm 5\%$. For samples from chromatograms of which 0.1 ml. was plated on detergent-treated metal

planchets to give an infinitely thin layer, counting was not continued until 1000 counts above background had been registered unless the sample was likely to be of subsequent importance (e.g. in the region of the peaks). The count registered in 15 min. was usually recorded. This latter procedure does not allow accurate assessment of any weakly active samples but this sacrifice was necessary where several hundred samples were to be counted. The efficiency of the ordinary end-window counter and scaling apparatus used for this work was such that a standard 1 cm.² polythene disk containing 1 μ C/g. gave 960 counts/min.

RESULTS

Inhibition of mucopeptide synthesis

Fig. 1 shows the effect of various concentrations of 5-fluorouracil upon the incorporation of [1-¹⁴C]-glutamic acid into the material, insoluble in hot trichloroacetic acid, prepared from cells which had been incubated for 100 min. in the usual system. Incorporation by both strains (524/SC and Oxford) is inhibited, although the effect on 524/SC is greater than that on strain Oxford. A considerable number of experiments of this type have been done, particularly with 524/SC, and the effect of 0.1 μ mole of 5-fluorouracil/ml. has been found to vary from about 25 to 60%. Attempts to prevent this variation by first starving the cells of intracellular uridine compounds by preincubating suspensions in salt solutions alone, salts and glucose, or salts and glucose and fluorouracil, have not been successful. The results of the experiments with strain Oxford seemed less variable although the inhibition was not as great.

Previous results (Mandelstam & Rogers, 1959) with strain 524/SC treated with penicillin showed that cell-wall synthesis was at first partially inhibited and only completely stopped after incuba-

Fig. 1. Effect of various concentrations of 5-fluorouracil upon the incorporation of [1-¹⁴C]glutamic acid into the material insoluble in hot trichloroacetic acid. The radioactivity of the materials was measured at infinite thickness. ○, Strain 524/SC; ●, strain Oxford.

Fig. 2. Rate of incorporation of [1-¹⁴C]glutamic acid by strain 524/SC into the material insoluble in hot trichloroacetic acid in the presence (○) and absence (●) of 0.06 μmole of 5-fluorouracil/ml.

tion for about 45 min. Fig. 2 shows the effect of 0.06 μmole of 5-fluorouracil/ml. upon the course of incorporation of [1-¹⁴C]glutamic acid into the material from cells of 524/SC, which is insoluble in hot trichloroacetic acid. Although there may be some tendency for synthesis in the presence of fluorouracil to decrease more rapidly with time than it does in the control cells, there is no very dramatic difference between the two curves in this respect. 5-Fluorouracil appears to be almost fully effective in the inhibition of synthesis from the start of the experiment. Examination of the effect of fluorouracil upon the incorporation of the four cell-wall amino acids shows (Table 1) that they are all affected, although to slightly different extents. Examination of the composition of the separated cell walls before and after the incubation in the presence of 0.4 μmole of 5-fluorouracil/ml. showed no difference in the molar proportions of the amino acids and amino sugars. Previously, different molar ratios had been found in the mucopeptides from penicillin-treated cells of strain 524/SC (Mandelstam & Rogers, 1959).

Work described above has been done with material insoluble in the hot trichloroacetic acid rather than with isolated cell walls since it was shown earlier (Mandelstam & Rogers, 1958, 1959) that when *Staphylococcus aureus* strain 524/SC is incubated in the presence of chloramphenicol and labelled

Table 1. Effect of 5-fluorouracil upon the incorporation of the four mucopeptide amino acids into the material insoluble in hot trichloroacetic acid from *Staphylococcus aureus* strain 524/SC

The washed cells (1 mg./ml.) were incubated with 200 μg./ml. each of alanine, lysine, glycine and glutamic acid, 1% (w/v) glucose, 50 μg. of chloramphenicol/ml. and 0.05M-potassium phosphate buffer, pH 7.0. The amount of labelled amino acid added was equal to 0.02 μc/ml. Incubation was for 90 min. at 35° with shaking to aerate, and 25 ml. of the suspensions were contained in 100 ml. conical flasks. At the end of the incubation period the flasks were cooled, 2.5 ml. of 50% (w/v) trichloroacetic acid was added to each flask. The resulting precipitates were washed with the appropriate unlabelled amino acid and treated with 5% (w/v) trichloroacetic acid at 90° as described by Mandelstam & Rogers (1959).

Amino acid labelled	Concn. of 5-fluorouracil (μmole/ml.)	Counts/min. at infinite thickness	Inhibition (%)
Glutamic acid	0	144.7	—
	0.05	113.8	22.0
	0.50	94.7	34.5
Glycine	0	1029	—
	0.05	861	17.0
	0.50	749	26.2
Lysine	0	60.2	—
	0.05	53.2	11.9
	0.50	48.3	19.9
Alanine	0	481	—
	0.05	419	13.0
	0.50	344	28.8

Table 2. Effect of 5-fluorouracil upon the incorporation of [1-¹⁴C]glutamic acid into material, insoluble in hot trichloroacetic acid, and into cell walls of *Staphylococcus aureus* strain 524/SC

The conditions of incubation and preparation of the material insoluble in hot trichloroacetic acid were as in the legend to Table 1 except that incubation was continued for 2 hr. The concentration of 5-fluorouracil was 0.4 μmole/ml. The cell walls were prepared from a separate sample of the incubated suspension by the method of Cummins & Harris (1956).

5-Fluorouracil	Counts/min. at infinite thickness	
	Material insoluble in hot trichloroacetic acid	Cell walls
Absent	374	880
Present	180	564
Inhibition by 5-fluorouracil (%)	52	36

cell-wall amino acids, 95% of the incorporated activity appears as the corresponding amino acid in the cell wall, the remaining 5% of activity being present in soluble material from the cell, presumably protein. Therefore, any inhibitory effect of fluorouracil on the 5% of the amino acid which is incorporated into protein is unlikely to influence the results. Table 2 shows that the major part of the

effect of fluorouracil in the system is upon cell-wall synthesis. In this experiment cell walls were separated by the technique of Cummins & Harris (1956). The degree of inhibition of incorporation by 5-fluorouracil is less when measured in isolated cell walls than in material insoluble in hot trichloroacetic acid. The reason for this is not known, but the results differ by more than the maximum total experimental error of the test, which is of the order of 10%. However, it is clear that cell-wall synthesis is inhibited.

Prevention of inhibition. Inhibition of cell-wall synthesis by 5-fluorouracil could be prevented by the simultaneous presence of either uracil or uridine. Both of these compounds were about equally effective on a molar basis. For example, inhibition by 0.06 μ mole of 5-fluorouracil/ml. was 50% reversed by an equimolar amount of either uracil or uridine. In a single test 0.06 μ mole of thymine/ml. was completely without effect.

Other analogues. The effect of other purine and pyrimidine analogues on strain 524/SC was then tested in the usual systems and again the incorporation of [14 C]glutamic acid into the material, insoluble in hot trichloroacetic acid, was followed. 8-Azathymine, 8-azaguanine, 6-azauracil and 6-azauracil ribotide were examined, each at an arbitrary concentration of 20 μ g./ml. and compared with 5-fluorouracil at concentrations of 15.5 and 2.6 μ g./ml. Whereas the latter compound inhibited cell-wall synthesis to the extent of 38 and 15% at these two concentrations, none of the other compounds showed any inhibition of amino acid incorporation. To the extent of this examination the effect of 5-fluorouracil can be said to be specific.

Accumulation of compounds containing amino sugar in inhibited cells

Cells of *S. aureus*, strain 524/SC, were incubated in the usual system with and without 0.4 μ mole of 5-fluorouracil/ml. Samples of the suspension were withdrawn at the times indicated in Fig. 3 and cooled. The cells were centrifuged, washed with an equal volume of cold water and then extracted at 0° with cold 10% trichloroacetic acid (1 ml. of acid/20 mg. of cells, for 1 hr.). After this, the insoluble material was deposited by centrifuging and washed. The extract and washings were freed from trichloroacetic acid by ether extraction and the ether was removed in a stream of air.

Samples of the extract were made up to a known volume and estimated for *N*-acetylhexosamine (cf. Methods section). Fig. 3 shows that a very large increase in the soluble compounds containing amino sugar occurred in the presence of 5-fluorouracil. The effect of increasing concentration of the fluoro compound upon the accumulation process is shown in Table 3. These results were obtained after

Fig. 3. Rate of formation of material, soluble in trichloroacetic acid and containing bound *N*-acetylhexosamine, by strain 524/SC incubated in the presence (●) and absence (○) of 0.4 μ mole of 5-fluorouracil/ml.

Table 3. Effect of increasing concentration of 5-fluorouracil upon the accumulation of trichloroacetic acid-soluble compounds containing bound *N*-acetylhexosamine in *Staphylococcus aureus* strain 524/SC

The suspensions were incubated under the conditions shown in the legend to Table 1 for 1 hr. At the end of this period the suspensions were cooled to 0°, and centrifuged. The cells from 25 ml. were then washed once with 10 ml. of ice-cold water, mixed with 1.0 ml. of ice-cold 10% (w/v) trichloroacetic acid and stood at 0–4° for 1 hr. At the end of this period the tubes were centrifuged, the precipitates resuspended in 0.5 ml. of cold 10% (w/v) trichloroacetic acid and centrifuged again. The supernatant solutions and washings were combined, extracted three times with 3 ml. of ethyl ether and the remaining ether was removed in a stream of warm air. The samples were then adjusted to 2.0 ml. and *N*-acetylhexosamine was estimated by the technique of Reissig, Strominger & Leloir (1955) after hydrolysis as suggested by Strominger (1957). The acid used for hydrolysis was neutralized with about 0.05 ml. of *N*-NaOH with phenolphthalein as internal indicator before the estimation.

Concn. of 5-fluorouracil (μ mole/ml.)	Concn. of <i>N</i> -acetylhexosamine* (μ g./100 mg. dry wt. of cells)
0	26.4
4×10^{-3}	60.0
4×10^{-2}	142.0
2×10^{-1}	432.0
4×10^{-1}	506.4

* The results are expressed in terms of *N*-acetylglucosamine.

incubation for 1 hr. which is after the accumulation process has ceased to occur at a maximum rate with the higher concentrations of inhibitor (see Fig. 3). It was necessary, however, to use this longer time, rather than the ideal of about 20–30 min. in order to obtain sufficiently large amounts of *N*-acetylhexosamine to make estimations accurate at the lower concentrations of fluorouracil.

Prevention of 5-fluorouracil effect. If uracil or uridine were present together with 5-fluorouracil during the incubation, accumulation of compounds containing *N*-acetylhexosamine was prevented. As is shown in Fig. 4 the riboside is slightly more effective than the free base on a molar basis. The molar ratio of 5-fluorouracil to uracil for 80% prevention of inhibition is about 1:1, whereas for the uridine it is about 0.7. The addition of an equimolar amount of thymidine simultaneously with the 5-fluorouracil caused very much less suppression (35–40%) of the accumulation of soluble compounds containing *N*-acetyl amino sugar.

Reversal of 5-fluorouracil effect. The above results show that either uracil or uridine can prevent the accumulation of compounds containing amino sugars which are soluble in trichloroacetic acid when they are present simultaneously with the fluorinated analogue. It remained to be shown whether these compounds, accumulated in the presence of fluorouracil, could be used by the cell for wall-mucopeptide synthesis when the fluoro compound was removed. To test this the cells were first treated with 5-fluorouracil and the compounds containing amino sugar allowed to accumulate; the cells were then removed by centrifuging, washed and incubated with uridine. Only a slight fall

occurred in the concentration of the preformed compounds. Similar results were obtained when the pretreated cells were incubated with different combinations of amino acids and glucose (cf. Table 4). Thus it appears that once formed these compounds are not readily used for synthesis. If labelled glycine were included in the second incubation mixture it could be shown that mucopeptide synthesis was proceeding in the cells which had first been treated with 5-fluorouracil, washed and subsequently reincubated in the normal system. Thus the compounds containing amino sugar, which accumulated, did not disappear rapidly even though cell-wall synthesis was going on.

Nature of the compounds containing amino sugar

These compounds were allowed to accumulate in larger volumes of suspensions of the strain 524/SC in the presence of 5-fluorouracil and the cells were then extracted with cold trichloroacetic acid in the usual way. The volumes of the extracts obtained were sometimes reduced by evaporation from the frozen state at low pressure after the trichloroacetic acid had been removed by ether extraction and the

Table 4. *Influence of various conditions of re-incubation on cells which have already accumulated compounds soluble in trichloroacetic acid and containing amino sugar*

The compounds containing amino sugar were first allowed to accumulate in the cells by incubating a suspension under the usual conditions for 1 hr. at 35° with 0.4 μ -mole of 5-fluorouracil/ml. These cells were then washed once with ice-cold 0.1M-potassium phosphate buffer at pH 7.0 and resuspended in the presence of the substances shown below. The four cell-wall amino acids and glucose were at the concentrations shown in the legend to Table 1. Chloramphenicol (50 μ g./ml.) and phosphate (0.05M, pH 7.0) were always present. Uridine was present at a concn. of 0.4 μ mole/ml. The experiment was done in duplicate and the suspensions were reincubated at 35° for 1 hr. with shaking.

Conditions of incubation	Bound <i>N</i> -acetylhexosamine* (μ g./100 mg. dry wt.)	Change from control† (μ g./100 mg. dry wt.)
Control (not incubated)	375, 358	—
1. Potassium phosphate buffer (pH 7.0) alone	320‡	–46
2. As (1) + glucose	277‡	–89
3. As (2) + four cell-wall amino acids	338, 318	–36
4. As (3) + uridine	263, 283	–93

* Expressed in terms of *N*-acetylglucosamine.

† Mean values.

‡ Duplicates lost.

Fig. 4. Prevention of the accumulation of materials, soluble in trichloroacetic acid and containing bound *N*-acetylhexosamine, by uracil (□) and uridine (●). The point at Δ represents the test with a single concentration of thymidine. The concentration of 5-fluorouracil was 0.2 μ mole/ml. in each test.

pH adjusted to 7.0. The details of these experiments were as follows:

Cell suspensions (1600 ml., 1 mg./ml.) containing the usual four amino acids, glucose, phosphate, 50 μ g./ml. of chloramphenicol and 0.1 μ mole of 5-fluorouracil/ml. were incubated for 60 min. at 35° in eight 1 l. conical flasks (200 ml./flask) with constant shaking to ensure aeration. At the end of this time the flasks were cooled and the contents centrifuged. The cells were washed and the trichloroacetic acid extract was made in the usual way. In a typical experiment the final volume of the extract, after removal of trichloroacetic acid and ether, was 38 ml. which contained a total of 3.46 mg. of bound amino sugar, estimated as *N*-acetylglucosamine.

This extract was then applied to a Dowex-1 column which was washed and developed as described in the Methods section.

Examination of the samples from the column at a wavelength of 260 μ m showed the presence of several peaks likely to contain nucleotides, but when the bound *N*-acetylhexosamine was measured only two of these peaks contained both types of substance (see Fig. 5). In the particular experiment under consideration peak I contained 49% of the bound *N*-acetyl amino sugar and peak II 35%, giving a total recovery of 84% of the bound amino sugar applied to the column. In addition, a further 5–10% of the compounds containing amino sugars ran through the column with the washings and was presumed to be free *N*-acetylhexosamine. The two

peaks containing hexosamine, obtained from the examination of extracts from cells treated with 5-fluorouracil, correspond exactly, in elution position, with the two peaks that Strominger (1957) found in similar extracts from cells treated with penicillin.

The samples from each of the peaks were combined to give two pools, and passed through small carbon–Celite columns (the carbon was Ultra-sorb ZF, British Carbo-Norit Union Ltd., London Road, West Thurrock, Grays, Essex). These columns were then washed with about 100 ml. of water and eluted with 40% (v/v) aqueous ethanol containing 1% (v/v) ammonia solution (sp.gr. 0.880) (Cabib, Leloir & Cardini, 1955). The yield of bound amino sugar from this process was never better than about 70% and often as low as 60%. However, no better method for concentrating the substances was found. Alcohol and ammonia were removed from these eluates under the vacuum given by a water pump

Fig. 5. Elution pattern obtained from a column of Dowex-1 (Cl-form) after application of the trichloroacetic acid extracts from cells of strain 524/SC which had been incubated for 1 hr. with 0.1 μ mole of 5-fluorouracil/ml. added to the usual system. —, Extinction measured at 260 μ m; - - - - - , *N*-acetylhexosamine measured after brief acid hydrolysis of the samples.

Fig. 6. Elution pattern obtained from DEAE columns when material in peak I (see Fig. 5) from Dowex-1 had been applied. Elution was with a gradient of ammonium acetate at pH 6.5, starting with a concentration of 0.01 M. —, Extinction at 260 μ m; - - - - - , *N*-acetylhexosamine after brief acid hydrolysis.

Fig. 7. Elution pattern obtained from DEAE columns when material in peak II (see Fig. 5) had been applied (see legend to Fig. 6 for remaining details).

at a bath temperature of less than 40°. Finally they were dried *in vacuo* over calcium chloride.

Material from each of the dried concentrates was then dissolved and passed down a separate column of DEAE. Elution was with a gradient of ammonium acetate at pH 6.5 (cf. Methods section). Figs. 6 and 7 show that both the peaks from the Dowex-1 separation were complex with respect to nucleotide material but the second peak was more complex with respect to bound amino sugar substances than the first. DEAE columns appeared to be satisfactory for the separation of the substances when developed as described in the Methods section. No experiments, however, were done to ascertain whether or not this substituted cellulose was satisfactory for the separation of nucleotides in general. Korn (1959) has described the use of another cellulose product for nucleotide analysis.

Dowex-1 peak I material. About 80 % of all the bound hexosamine was contained in a single sharp peak obtained from DEAE (Fig. 6, peak IA). The samples comprising this peak were pooled and dried *in vacuo* during which process the ammonium acetate was removed. Examination of the resulting material by paper chromatography showed a single spot. Samples were then hydrolysed and examined by paper chromatography with separate solvent systems for amino acids, amino sugars and pyrimidine bases. The amino acids present were lysine, glutamic acid and alanine, the amino sugar was muramic acid and the base 5-fluorouracil.

Also present in the DEAE fractions was the very small peak, containing bound hexosamine, at B in Fig. 6. The amount of material here was very small but when hydrolysed by the same methods as those used for the material from peak A, the amino acids recognized were glycine, glutamic acid and alanine, and the amino sugar was muramic acid. The base could not be identified owing to the small amounts of material available. These same two mucopeptides in the 'Dowex-peak 1' material could also be separated by direct paper chromatography in butanol-acetic acid. Consideration of the quantitative data on peak IA from DEAE will be delayed

until the experiments with labelled 5-fluorouracil are described.

Dowex-1 peak II material. When material from the second peak from Dowex-1 was run on DEAE three peaks, containing both nucleotide material and bound amino sugar, were found. The samples in these peaks were combined to give three pools and the materials they contained were examined as before. Table 5 summarizes the qualitative results obtained. Both uracil and 5-fluorouracil were to be found associated with *N*-acetylglucosamine in the absence of amino acids. Also 5-fluorouracil and uracil were associated with muramic acid and alanine. Estimations showed that this peak IIC, which was clearly still heterogeneous, contained more muramic acid than alanine on a molar basis (see Table 6).

Experiments with 5-fluoro[2-¹⁴C]uracil

The labelled 5-fluorouracil as received had a specific activity of 10.6 $\mu\text{C}/\mu\text{mole}$. This material (1.88 μmoles) was diluted with unlabelled material (400 μmoles) and incubated at a final concentration of 0.4 $\mu\text{mole/ml}$ in the usual system for studying the biosynthesis of cell wall; strain 524/SC was used. The fluorouracil concentration was four times as high as that in the previous experiments. This change was originally made in order to increase the amount of the compounds, containing bound amino sugar, available for analysis (cf. Table 3). About 20 % of the fluorouracil supplied to the organisms was in the trichloroacetic acid extracts from the cells and 50–60 % remained in the supernatant solutions prepared from the incubated suspensions. The remaining 20–30 % was presumably mostly incorporated into the ribonucleic acid of the cells although this was not examined.

The trichloroacetic acid extracts were treated and analysed exactly as before. The distribution of radioactivity due to 5-fluorouracil, bound amino sugar and material absorbing light of 260 $m\mu$ wavelength in the samples from the Dowex-1 column was examined. Three peaks containing radioactivity appeared. The first accounted for about

Table 5. *Components recognized as present in the compounds in the peaks from the diethylaminoethylcellulose columns*

The columns were run and the samples hydrolysed under the conditions given in the Methods section. The symbol (–) means not present.

Dowex peak	DEAE peak	Amino acid				Amino sugar	Base
		Glutamic acid	Glycine	Lysine	Alanine		
I	A	+	–	+	+	Muramic acid	Fluorouracil
	B	+	+	–	+	Muramic acid	Not identified
II	A	–	–	–	–	Glucosamine	Uracil
	B	–	–	–	–	Glucosamine	Fluorouracil
	C	–	–	–	+	Muramic acid	Fluorouracil, uracil

4-5 % of the applied material but contained no bound amino sugar and was ignored. The second (referred to previously and subsequently as peak I) contained amino sugar and accounted for 53 % of that which had been applied to the column and 31 % of the radioactivity. The third peak containing radioactivity (i.e. the second containing both bound amino sugar and radioactivity, peak II) accounted for 50 % of the applied amino sugar and 46 % of the radioactivity. Hence the total recovery of radioactivity (presumably fluorouracil) was 77 % and of bound amino sugar 103 %. No further solvents were run through the column, in view of these high yields.

The solutions comprising these two peaks were then concentrated by adsorption and elution from carbon as before and applied to columns of DEAE. Figs. 8 and 9 show the results obtained. Both the pictures are more simple than those shown in Figs. 6 and 7. Qualitative examination of IA (Fig. 8) showed that it exactly corresponded to the previous peak IA. Recovery in this peak was 73 % of the radioactivity applied and 98 % of the bound amino sugar. No peak corresponding to IB (Fig. 6) appeared. Peak II (Dowex) material split into two instead of three peaks containing bound amino sugar and nucleotide. The first, called IIB, contained 35 % of the bound amino sugar and IIC, still obviously heterogeneous, contained 48 % giving a total yield of 83 % of the applied material. The corresponding figures for radioactivity were 44 and 37 %.

Thus the use of the higher concentration of fluorouracil had eliminated the material (IIA in Fig. 7) which contained uracil and *N*-acetylglucosamine, leaving that containing the fluorouracil and *N*-acetylglucosamine.

Quantitative examination of the fractions containing muramic acid. Table 6 shows the analysis obtained for the materials isolated from peaks IA and IIC. It will be seen that IA contains fluorouracil, phosphorus, muramic acid, glutamic acid, lysine and alanine in the approximate ratios 1:2:1:1:1:3. Thus the analysis of this compound corresponds to that of 'compound 3' isolated by Park (1952*b*) from staphylococci treated with penicillin with the exception, of course, that uracil is replaced by fluorouracil. This compound is presumably the fluorouridine analogue of the Park (1952*b*) mucopeptide 3. The material from peak IIC contains muramic acid and phosphorus in the molar ratio of approximately 1:2 together with a small proportion of alanine. This was re-examined by the use of a longer (20 cm.) column of DEAE adjusted to pH 5.0 and eluted with a gradient of ammonium acetate buffer (0.01-0.5*M*) also at pH 5.0. Two partially separated peaks were obtained containing both fluorouracil and *N*-acetylhexosamine. Ana-

Fig. 8. Elution pattern obtained from DEAE column after trichloroacetic acid extracts from cells of 524/SC treated with 0.4 μ mole of labelled 5-fluorouracil/ml. had been passed through Dowex-1 (Cl-form) and the material in peak I (see Fig. 5) isolated. For the method of elution of the DEAE column see the legend to Fig. 6. - - - -, Radioactivity expressed as counts/min./ml.; —, extinction at 260 $m\mu$; - · - · -, amounts of *N*-acetylhexosamine/ml., measured after brief acid hydrolysis.

Fig. 9. Elution pattern obtained from DEAE columns after the application of material from peak II from Dowex-1 (cf. Fig. 5). For the remainder of the details of this experiment see the legend to Fig. 8.

Table 6. Quantitative analysis of fractions, containing muramic acid, isolated by the use of diethylaminoethylcellulose columns from trichloroacetic acid extracts of cells treated with 5-fluoro[2-¹⁴C]uracil

Preparation of the samples and the analytical methods are described in the text. The value for fluorouracil is taken as 1.00. The concentration of fluorouracil was estimated from the radioactive count.

Sample	Molar proportion						
	Fluorouracil	P	Glutamic acid	Lysine	Alanine	Muramic acid	Glucosamine
1A	1.00	2.21	0.99	1.05	2.83	1.12	None
2C	1.00	2.24	None	None	0.31	1.04	None

lysis showed that the first of these contained fluorouracil, muramic acid and alanine in the ratio 1.0:1.3:1.0, and the second fluorouracil and muramic acid in the ratio 1.0:1.0, but no alanine. Hence the material in peak IIC is a mixture of fluorouridine diphospho-*N*-acetylmuramic acid and this compound linked to alanine.

The compound containing N-acetylglucosamine. When the material in the peak II (Fig. 5) from the Dowex-1 column was examined by chromatography on the DEAE columns the sharp well-separated peak IIB emerged (cf. Fig. 9). When this material was hydrolysed it was found to contain fluorouracil and glucosamine but no amino acids or muramic acid. These were thought likely to arise from hydrolysis of fluorouridine diphospho-*N*-acetylglucosamine. Examination of the ratios of base:phosphorus:amino sugar, in the concentrated material from peak IIB, however, did not agree with this possibility. Further examination of the individual samples from the column showed that although the ratio of radioactivity to extinction at 260 m μ remained constant, that of radioactivity to *N*-acetylhexosamine rose steeply towards the end of the peak. This suggested that the high ratio of base and phosphorus to amino sugar which had been found in the pooled material might be due to a mixture of a fluorouridine phosphate with fluorouridine diphospho-*N*-acetylglucosamine. In consequence the material from peak IIB from the DEAE column was concentrated and the ammonium acetate was removed by evaporation from the frozen state in the usual way; the material was then dissolved in water and examined by paper chromatography. After being run for 3 days in the isobutyric acid-ammonia soln. solvent system, two ultraviolet-absorbing spots appeared, both of which contained radioactivity. Only one of these spots contained *N*-acetylglucosamine and it moved almost exactly the same distance as a marker of uridine diphospho-*N*-acetylglucosamine. This material was eluted with water and its radioactivity and *N*-acetylglucosamine content were measured. The usual brief period of hydrolysis was used to liberate the *N*-acetylglucosamine from the nucleotide. The ratio of base to *N*-acetyl amino sugar was 1.0:0.97

and it seems probable, therefore, that the amino sugar was bound as fluorouridine diphospho-*N*-acetylglucosamine.

DISCUSSION

As with other types of cell, 5-fluorouracil enters staphylococci easily and the internal concentration of further metabolites of the compound becomes considerable. When the cells are supplied with an external concentration of 0.4 μ mole of 5-fluorouracil/ml. the total internal concentration of the free and combined compound rises to a calculated value of about 1 μ mole/ml. of cell water. Of this material about 3–4 % is not retained by a Dowex-1 column and may be free fluorouracil or its riboside. Of the remainder, 70–80 % is present in combination with amino sugars and amino acids. About half this combined material is as a fluorinated nucleotide-mucopeptide compound which appears to be identical, except for the replacement of uridine by fluorouridine, with the largest of the three compounds isolated by Park (1952*a, b, c*). Of the remainder, some is present as fluorinated derivatives of fragments of this compound (i.e. fluorouridine diphospho-*N*-acetylmuramic acid and fluorouridine diphospho-*N*-acetylmuramic acid-alanine), and some as fluorouridine diphospho-*N*-acetylglucosamine. In the presence of 0.4 μ mole of fluorouracil these appear to be the only compounds, containing both nucleotide and *N*-acetyl amino sugars, which accumulate. It seems reasonable to suppose that these are uridine-activated precursors of cell-wall mucopeptide. The normal analogues of these compounds also accumulate in other strains of staphylococci treated with penicillin (Park, 1952*a, b, c*; Park & Strominger, 1957) or with a unique sample of gentian violet (Strominger, 1959). It was originally proposed that they were the precursors of the cell-wall mucopeptides because the molecular proportions of amino sugars and amino acids in them were the same as those in the cell walls of the organisms examined (Park & Strominger, 1957; Strominger, Park & Thompson, 1959). In the present instance it also seems reasonable to implicate the substances, accumulating in the presence of

5-fluorouracil, as precursors of the cell-wall mucopeptide. In this instance, however, the molecular proportions of the components are quite different from those in the cell-wall mucopeptides of the organisms which accumulate them (Mandelstam & Rogers, 1959; Rogers & Perkins, 1959). In this connexion, the metabolic path of glycine in cells treated with fluorouracil must be considered. Rogers & Perkins (1960) reported that this amino acid is found in small concentrations in hydrolysates of materials from both the peaks obtained from Dowex-1. Nevertheless, it is not a component of any of the compounds isolated when the cells have been treated with the higher concentration of 5-fluorouracil of 0.4 μ mole/ml. A definite compound containing glycine, however, can be isolated from cells treated with the lower concentration of 5-fluorouracil of 0.1 μ mole/ml. (i.e. the small peak IIB). The nature of the base associated with this material could not be definitely identified owing to the small amount available. It cannot be concluded that the material in this small peak is not an important precursor of the cell-wall mucopeptide because large amounts of it do not accumulate in the presence of 5-fluorouracil. It might be that it is attached to a base other than uracil in which case it would not necessarily be expected to accumulate. This material contained glutamic acid, alanine and muramic acid as well as glycine but it contained no lysine and could be either another precursor of the cell-wall mucopeptide, or the precursor of a second mucopeptide present in the wall. If either of these possibilities is true the molecular proportions of the components of the wall mucopeptide or mucopeptides might be expected to differ from those of the major component accumulating with 5-fluorouracil.

The similarity between the substances accumulating in the presence of 5-fluorouracil and those accumulating in the presence of penicillin is remarkable. The only clear difference, apart from the expected abnormal bases, is the presence of the fluorinated derivative of uridine diphospho-*N*-acetylglucosamine when the cells have been treated with 5-fluorouracil. The normal compound has not been reported to accumulate in the presence of penicillin. One could suppose that penicillin either interfered with the metabolism of uridine diphospho-*N*-acetylmuramic acid compounds or suppressed the formation of uridine diphospho-*N*-acetylglucosamine. Either mechanism would interfere with the formation of the cell-wall mucopeptide. However, uridine diphospho-*N*-acetylglucosamine has been involved in the biosynthesis of mucopolysaccharides such as chitin (Glaser & Brown, 1957) and hyaluronic acid (Markovitz, Cifonelli & Dorfman, 1958, 1959) and these processes are not known to be penicillin sensitive. These facts do not neces-

sarily exclude the possibility that penicillin acts upon the *N*-acetylglucosamine pathway, but the emphasis could be thrown upon peculiarities of the enzymes involved in staphylococci and other penicillin-sensitive micro-organisms, rather than upon the substrates.

The present work, of course, does not suggest that 5-fluorouracil stops the growth of micro-organisms by interfering with cell-wall synthesis. It is much more likely that this happens by inhibition of deoxyribonucleic acid synthesis. It is probable, however, that fragility effects such as those observed (Tomasz & Borek, 1959) in *E. coli* as a result of exposure to the fluorouracil are due to inhibition of cell-wall synthesis, as suggested by Tomasz & Borek (1960). Fluorouracil is not unique among uracil analogues in its effects upon cell-wall synthesis by micro-organisms. For example, Otsuji & Takagi (1959) and Takagi & Otsuji (1958) have shown that when azauracil acts on *E. coli*, bound amino sugars, soluble in trichloroacetic acid, accumulate; these authors did not examine the effects upon mucopeptide formation nor did they fractionate the bound amino sugar compounds. Also Sells (1959) showed that 6-azauracil inhibited cell-wall formation in *Bacillus cereus*. In the present work 6-azauracil or its ribotide led to an accumulation of soluble bound amino sugar although the amounts were small; but at the concentrations used, and under our conditions, these compounds did not inhibit the formation of mucopeptide.

SUMMARY

1. 5-Fluorouracil partially inhibits cell-wall mucopeptide synthesis by *Staphylococcus aureus* strain 524. This inhibition can be prevented by the simultaneous presence of uracil or uridine.

2. During incubation in the presence of 5-fluorouracil, compounds accumulate which contain bound amino sugar and are soluble in trichloroacetic acid; this accumulation is prevented by the simultaneous presence of uracil or uridine.

3. The compounds containing bound amino sugars which have accumulated are not readily utilizable by the cell for mucopeptide synthesis when fluorouracil is removed.

4. Fractionation of the material, soluble in trichloroacetic acid and containing bound amino sugars, has revealed the presence of four compounds: (1) containing fluorouracil, phosphorus, muramic acid, alanine, glutamic acid and lysine with the molecular proportions of 1:2:1:3:1:1; (2) a fluorouridine-phosphorus-*N*-acetylglucosamine compound with molecular proportions of 1:2:1; (3) a mixture of fluorouridine-muramic acid-alanine compounds which has not been fractionated completely but is likely to be fluorouridine

diphospho-*N*-acetylmuramic acid and this compound linked to alanine.

5. The concentration of fluorouracil seems to decide whether or not non-fluorinated uridine compounds of similar composition also accumulate. At higher concentrations of fluorouracil these were not found.

6. The relationship of the accumulation of the fluorouridine compounds to the penicillin-induced formation of the corresponding non-fluorinated compounds is discussed.

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A Spectrophotometric Study of the Reaction Catalysed by Serine Transhydroxymethylase

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In previous papers (Blakley, 1958, 1959, 1960) evidence has been presented that formaldehyde reacts non-enzymically and reversibly with tetrahydropteroylglutamic acid. A comparison of the equilibrium constant of this reaction with those

obtained from the reaction of formaldehyde with other hydropteridines indicated that N^5N^{10} -methylenetetrahydropteroylglutamate is the product formed from tetrahydropteroylglutamate, although N^5 -hydroxymethyltetrahydropteroylglu-