# METABOLISM OF POLYAMINES BY *STAPHYLOCOCCUS*

SANFORD M. ROSENTHAL AND DONALD T. DUBIN[1]

*National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland, and Laboratory of Investigation, Hospital del Nino, Lima, Peru*

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ABSTRACT

ROSENTHAL, SANFORD M. (National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.) AND DONALD T. DUBIN. Metabolism of polyamines by *Staphylococcus*. J. Bacteriol. **84**:859–863. 1962—Although spermine and spermidine are not detectable in staphylococci grown in purified medium, considerable amounts (0.7 to 1.4 μmole/g wet wt) were demonstrated in cells grown in the presence of these amines. Conjugation of spermine, and to a lesser extent of spermidine, was demonstrated. Putrescine and cadaverine added to purified medium are not appreciably taken up by staphylococcal cells. However, high percentages were recovered in the medium in conjugated form. These conjugates were shown to be monoacetylputrescine and probably monoacetylcadaverine. Meat infusion broth, neopeptone, spermine, and spermidine were highly inhibitory to this acetylation. No evidence of utilization of these polyamines was obtained.

*Escherichia coli*, which normally contains putrescine and spermidine (Tabor, Rosenthal, and Tabor, 1958; Weaver and Herbst, 1958), also contains acetyl derivatives of these amines (Dubin and Rosenthal, 1960). When grown in the presence of spermine, which is not a normal constituent of *E. coli*, this amine and its acetyl derivatives are concentrated in the bacterial cells, largely replacing the amines normally present; acetyl derivatives of spermine were also demonstrated in the culture medium. A review of the biochemistry of spermine and spermidine has recently appeared (Tabor, Tabor, and Rosenthal, 1961).

Most gram-positive organisms, when grown on purified media, contain very low concentrations of polyamines (Herbst, Weaver, and Keister, 1958). In our experiments with *Staphylococcus aureus*, only putrescine, in trace amounts, could be demonstrated by chromatographic analysis. It was therefore of interest to study the behavior of polyamines when added to the culture media of staphylococci.

## MATERIALS AND METHODS

*Chemicals.* The nonradioactive putrescine was purchased from Eastman Kodak Co. (Rochester, N.Y.), and the cadaverine, spermidine, and spermine from Hoffmann-La Roche, Inc. (Nutley, N.J.). Putrescine-1,4-$C^{14}$ (specific activity: 75,000 counts per min per μmole) was synthesized by H. Tabor et al. (1958), and also obtained from New England Nuclear Corp. (Boston, Mass.; specific activity: 2,000,000 counts per min per μmole). Spermidine-$C^{14}$[$NH_2(CH_2)_4NHCH_2CH_2C^{14}H_2NH_2$, specific activity: 175,000 counts per min per μmole] and spermine-$C^{14}$[$NH_2C^{14}H_2CH_2CH_2NH(CH_2)_4NHCH_2CH_2C^{14}H_2NH_2$, specific activity: 350,000 counts per min per μmole] were prepared by E. L. Jackson (Jackson and Rosenthal, 1960). Cadaverine-$C^{14}$ (specific activity: 50,000 counts per min per μmole) was supplied by R. W. Schayer of the Merck Institute. Mono- and diacetylspermine were prepared by H. Bauer (Dubin and Rosenthal, 1960) and the acetyl derivatives of putrescine and spermidine by E. L. Jackson (1956). Acetokinase was supplied by W. Pricer of the National Institutes of Health (Bethesda, Md.).

*Bacteria and culture media. S. aureus* ATCC 152 (coagulase-negative) was used in most of these experiments. Studies were also carried out on the pathogenic Georgio strain and upon two strains of *Staphylococcus albus* (*S. aureus*, nonpigmented).

The culture medium was modified from that of Fildes et al. (1936). The 14 amino acids used by these authors were supplemented with tryptophan (70 mg/liter) and cystine (100 mg/liter). The amino acids were dissolved in distilled water

[1] Present address: Department of Bacteriology, Harvard Medical School, Boston, Mass.

with heating, neutralized with NaOH, and potassium phosphate buffer (pH 7.3) was added to make a 0.05 M solution. The metals Fe, Zn, Mn, Cu, and Mg, in the amounts recommended, were sterilized separately, as were the glucose (final concentration, 0.2%) and 1 ml/liter of a vitamin mixture (sterilized by filtration) containing, in $\mu$g: thiamine, 1,000; pyridoxine, 2,000; calcium pantothenate, 1,000; nicotinamide, 1,000; inositol, 20,000; choline chloride, 5,000; riboflavine, 200; folic acid, 10; biotin, 1; and *p*-aminobenzoic acid, 1.

The cultures were grown at 37 C with mechanical shaking or with a magnetic stirrer.

*Estimation of amines.* The bacterial cells were obtained by centrifugation. After draining, the cells were weighed and washed two or three times with a solution of 0.4% NaCl plus 0.5% KCl. Extraction was made with 4 to 9 volumes of 10% trichloroacetic acid; the acid was removed by extraction with several volumes of ether. The medium was extracted with *t*-butanol after the addition of a $Na_2SO_4$-$K_3PO_4$ salt mixture (McIntire, Roth, and Shaw, 1947; Rosenthal and Tabor, 1956). The butanol extract was acidified with HCl and evaporated to dryness in a vacuum desiccator.

The amines were characterized by a combination of ion-exchange and paper chromatography as previously described (Tabor et al., 1958; Dubin and Rosenthal, 1960). Gradient elution was carried out on Dowex 50-$H^+$ columns with 0.5 to 2.5 N HCl (method B of Tabor et al., 1958). Paper chromatography was done with several systems, as previously reported (Dubin and Rosenthal, 1960); the most useful were *n*-butanol–acetic acid–pyridine–water (4:1:1:2) on Schleicher and Schuell paper no. 598, and, for acetylputrescine, methanol–pyridine–water (20:1:5) on Schleicher and Schuell paper no. 507.

Colorimetric assay was accomplished on the eluates with 2,4-dinitrofluorobenzene (Rosenthal and Tabor, 1956), and color development on paper with a ninhydrin dip (Dubin and Rosenthal, 1960). Radioactivity in the eluates or in the paper strips was determined with a Nuclear-Chicago Corp. (Des Plaines, Ill.) gas-flow counter.

Acid hydrolysis was carried out with 6 N HCl in sealed tubes at 110 C overnight.

## RESULTS

*Spermine.* Experiment A (Table 1) demonstrated the high uptake of $C^{14}$-spermine added to the culture medium of *S. aureus*. At pH 8, 89% of the radioactivity, amounting to 0.7 $\mu$mole per g (wet wt) of cells, was recovered in the bacteria. At pH 6 (experiment B), the yield of organisms was one-half, but the concentration of amine per g of cells was similar. This experiment was performed before acetylation had been demonstrated, but chromatography on Dowex 50 showed all of the counts in the spermine-monoacetylspermine area.

In experiment C, in addition to the labeled spermine, 7 $\mu$moles of nonradioactive spermine were added to the medium. After 42 hr of incubation, 8% (1.4 $\mu$moles/g of cells) were taken up. Chromatography of the trichloroacetic acid extract on Dowex 50 revealed 40% of the counts in the monoacetylspermine area, and this was converted to spermine after acid hydrolysis. Insufficient counts were available for paper chromatography of this compound. The remaining counts in the cells, as well as all of the counts in the medium, behaved as spermine in both types of chromatography.

*Spermidine.* In experiment D (Table 1), 1.25 $\mu$moles of spermidine-$C^{14}$ plus 7 $\mu$moles of nonradioactive compound were added to 200 ml of culture medium. Half of the culture was analyzed after 24 hr of incubation and the remainder after 42 hr. At 24 hr, 3% of the added amine (0.86 $\mu$mole/g of cells) was recovered in the trichloroacetic acid extract. Dowex 50 chromatography of the extract revealed all of the counts in the spermidine-monoacetylspermidine area; on paper chromatography, only spermidine was detected, but insufficient counts were available for accurate assay. All of the remaining counts were recovered in the medium, where 5% of the radioactivity migrated with monoacetylspermidine on paper chromatography, and was converted into spermidine by acid hydrolysis. The other 95% of the counts in the medium behaved as spermidine.

After 42 hr of incubation, the yield of bacteria as well as the uptake of spermidine had approximately doubled (0.94 $\mu$mole/g of cells); on chromatographic analysis of the cell extract, only spermidine was found, but a small percentage of conjugate may have escaped detection. Nearly all of the remaining counts were recovered in the medium, which was accidentally lost for further analysis.

*Putrescine.* Little or no uptake of putrescine by staphylococci was found. Practically all of the radioactivity was recovered in the medium,

TABLE 1. *Metabolism of polyamines by Staphylococcus aureus 152 in purified medium**

| Expt | pH | Vol | Incubation time | Wet wt of cells | Amine added | | $C^{14}$ recovered | | Amines recovered† | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $C^{14}$ | Carrier | Cells | Medium | Cells | Medium |
| | | *ml* | *hr* | *g* | *μmoles* | *μmoles* | % | % | | |
| *Spermine* | | | | | | | | | | |
| A | 8.0 | 200 | 20 | 0.50 | 0.4 | — | 89 | | | |
| B | 6.0 | 200 | 20 | 0.26 | 0.4 | — | 41 | | | |
| C | 7.3 | 200 | 42 | 0.43 | 0.34 | 7.0 | 8.4 | 76 | 60% Spm.<br>40% Acetyl | 100% Spm. |
| *Spermidine* | | | | | | | | | | |
| D | 7.3 | 100 | 24 | 0.14 | 0.63 | | 3.5 | 97 | Spd.<br>? Acetyl | 95% Spd.<br>5% Acetyl |
| | 6.2 | 100 | 42 | 0.24 | 0.63 | 3.5 | 5.5 | 84 | Spd.<br>? Acetyl | |
| *Putrescine* | | | | | | | | | | |
| E | 7.3 | 250 | 20 | 0.49 | 10.7 | — | 0.025 | 94 | — | 15% Put.<br>85% Acetyl |
| F | 7.3 | 20 | 24 | — | 2.0 | — | — | 96 | — | 45% Put.<br>55% Acetyl |
| | 6.2 | 20 | 42 | — | 2.0 | — | — | 100 | — | 4% Put.<br>96% Acetyl |
| G | 7.3 | 650 | 20 | 0.95 | 4.0 | 455.0 | 0 | 100 | — | 25% Put.<br>75% Acetyl |
| *Cadaverine* | | | | | | | | | | |
| H | 7.3 | 5 | 24 | — | 1.4 | — | — | 100 | — | 7% Cadav.<br>93% Conjugate |

* The specific activities of the isotopic compounds are given in the text. (The putrescine-$C^{14}$ used in these experiments had a specific activity of 75,000 count per min per μmole.) The pH represents the value at the beginning of incubation, except in experiments D and F, where half of the culture was analyzed at 24 hr and the remainder at 42 hr; the value given at 42 hr was determined at the end of the first day.

† Abbreviations: Spm., spermine; Spd., spermidine; Put., putrescine; Cadav., cadaverine.

largely as a conjugate which has been identified as monoacetylputrescine.

Representative experiments with *S. aureus* 152 are shown in Table 1. The extent of acetylation is demonstrated in experiment G, where a large amount of carrier putrescine was added to the medium. After 20 hr of incubation, 325 μmoles (75%) were present in the medium as a conjugate, and the remainder as putrescine. The identity of the conjugate was established in two ways. (i) Chromatography on Dowex 50 and on paper, with several solvent systems, was employed. Techniques for paper chromatography, and $R_F$ values for the polyamines have been described for three solvent systems (Dubin and Rosenthal, 1960). In addition, the system methanol-pyridine-water (20:1:5) on Schleicher and Schuell paper no. 507 was used for acetylputrescine; the chromatogram was run a distance of 21 cm, and the $R_F$ values for putrescine, cadaverine, spermidine, spermine, monoacetylspermidine, and monoacetylspermine were 0.02 to 0.05, while monoacetylputrescine was 0.73. (ii) The medium from experiment E was reduced to 30 ml in a flash evaporator. This was made alkaline, and extracted four times with 10 ml of *t*-butanol, after the addition of salt mixture. The butanol extracts were pooled, acidified with HCl, and evaporated to dryness. The residue was dissolved in water and chromatographed on a Dowex 50 column (8 by 1.2 cm). After gradient elution (2.5 N HCl, 300 ml of water in the mixing vessel), the eluates in the acetylputrescine area (94 to 120 ml) were evaporated. This material was

made alkaline and distilled upon a "cold finger" (Fieser, 1955) under high vacuum; the distillate was dissolved in 1 N HCl and evaporated to dryness over KOH. A sample of the material was mixed with authentic nonradioactive monoacetylputrescine, and recrystallized four times without change in activity (Table 2). Another portion, as well as some of the Dowex 50 eluate, was shown to liberate stoichiometric amounts of acetate upon acid hydrolysis (Table 3). Acetate was assayed by a micromodification (Dubin and Rosenthal, 1960) of the acetokinase method of Rose et al. (1954).

TABLE 2. *Effect of recrystallization of $C^{14}$ monoacetylputrescine on specific activity**

| Recrystallization | Specific activity (count per min per μmole) |
|---|---|
| Original | 1,137 |
| 1st | 1,203 |
| 2nd | 1,400 |
| 3rd | 1,300 |
| 4th | 1,180 |

* The isolated $C^{14}$ material plus 3 mg of monoacetylputrescine was dissolved in 0.1 to 0.2 ml of methanol acidified with methanolic HCl, and recrystallized by the addition of 2 to 4 ml of ethyl acetate and cooling.

TABLE 3. *Acetate and amino group assay of the putrescine conjugate**

| Source | Free amino groups | | Acetate released |
|---|---|---|---|
| | Before hydrolysis | After hydrolysis | |
| | *μmole* | *μmole* | *μmole* |
| Dowex 50 | 0.35 | 0.69 | 0.29 |
| Cold finger | 0.31 | 0.62 | 0.30 |

* Free amino groups were estimated with ninhydrin and acetate with acetokinase, as described in the text. Acid hydrolysis released stoichiometric amounts of acetate and amino groups.

An unusual feature of this acetylation was its abolition when staphylococci were grown in meat infusion broth (containing 2% neopeptone). Under these conditions, essentially all of the added putrescine could be recovered in the medium in unchanged form (Table 4). The following experiments indicate that this lack of acetylation results from inhibitors present in the broth and in the neopeptone. (i) It is not influenced by the addition to the broth of glucose, magnesium, and vitamins, in concentrations present in the purified medium. (ii) Addition of neopeptone broth, broth, or neopeptone to the purified medium depressed acetylation. The percentage inhibition, compared with a control in purified medium alone, was as follows: 20% neopeptone broth, 30 to 33% inhibition; 50% broth, 87% inhibition; 2% neopeptone, 88% inhibition.

The inhibitory substances have not been identified, but some evidence indicates that they are basic compounds, probably peptides and higher polyamines. A *t*-butanol extract of broth (with added salt mixture), representing 2.5 ml of broth, gave 30% inhibition in 5 ml of purified medium; since the salt mixture rendered the broth strongly alkaline, chiefly basic compounds would be extracted. Ether and acetone extracts of evaporated broth, as well as the ash of broth, were not inhibitory. Spermidine and spermine were strongly inhibitory, 0.04 μmole/ml in purified medium giving 85% inhibition.

*Cadaverine.* Experiments with cadaverine-$C^{14}$ in purified medium revealed a behavior similar to putrescine; nearly all of the added amine remained in the medium, mostly in conjugated form (Table 1). While no standard of monoacetylcadaverine was available for exact identification, evidence that the conjugate was the monoacetyl derivative was found in that it migrated with monoacetylputrescine on ion-exchange and paper chromatography, and was

TABLE 4. *Lack of acetylation of putrescine by Staphylococcus aureus 152 when grown in meat infusion broth plus 2% neopeptone*

| pH | Vol | Added $C^{14}$ | Wt of cells | $C^{14}$ in cells | $C^{14}$ in medium | Amines in medium | Incubation time |
|---|---|---|---|---|---|---|---|
| | *ml* | *count/min* | *g* | *count/min* | *count/min* | | *hr* |
| 7.2 | 150 | 350,000 | 0.37 | 60 | 310,000 | Put. only | 20 |
| 7.2* | 150 | 225,000 | 0.48 | — | 206,000 | Put. only | 20 |

* Glucose + Mg + vitamins added.

converted to cadaverine upon acid hydrolysis. Since putrescine and cadaverine are not distinguishable in these systems, it is probable that their acetyl derivatives would behave similarly.

*Other strains of staphylococci.* Acetylation of putrescine was demonstrated with three other strains. The Georgio strain of *S. aureus* gave 41% acetylation in 28 hr with 0.1 μmole/ml of putrescine-$C^{14}$ in purified medium. One strain of *S. albus* gave 43%, and another 6% acetylation, under similar conditions. Less than 0.3% of the added radioactivity was recovered in the trichloroacetic acid extracts of the cells of these strains.

## DISCUSSION

While staphylococci do not form appreciable amounts of spermidine and spermine endogenously, it was shown with strain 152 that considerable amounts of these amines are concentrated in the cells when they are added to the culture medium. Although some conjugation occurs, there is no evidence of utilization, since, within the limits of assay methods, practically all of the added radioactivity can be recovered as the amine or its conjugate. The physiological function of these polyamines remains to be established, but they have been shown to have powerful effects on membrane and nucleic acid stability (Tabor et al., 1961).

Putrescine and cadaverine, on the other hand, are not concentrated in the cells but are largely converted to monoacetyl derivatives which accumulate in the medium. The site of this conjugation has not been established; although a cell-free filtrate of a 24-hr culture of strain 152 acetylated 2% of putrescine-$C^{14}$ (1.5 μmoles in 7 ml) in 20 hr at 37 C, this activity may have resulted from enzymes liberated from lysed cells.

There is also no evidence for the utilization of putrescine and cadaverine; essentially all of the initial radioactivity remains in the culture medium as the free or conjugated compounds. Few counts are found in the cell extracts or in the cell residues after trichloroacetic acid extraction. In one experiment (experiment E, Table 1), the $CO_2$ collected overnight from the culture showed no radioactivity. This behavior is different from *E. coli*, where putrescine added to the medium is actively taken up by the cells, where, along with endogenous putrescine, it is either stored, degraded, or incorporated into spermidine (Tabor et al., 1958).

## LITERATURE CITED

DUBIN, D. T., AND S. M. ROSENTHAL. 1960. The acetylation of polyamines in *Escherichia coli*. J. Biol. Chem. **235**:776–782.

FIESER, L. F. 1955. Experiments in organic chemistry, p. 89. D. C. Heath and Co., Boston.

FILDES, P., G. M. RICHARDSON, B. C. J. G. KNIGHT, AND E. P. GLADSTONE. 1936. A nutrient mixture suitable for the growth of *Staphylococcus aureus*. Brit. J. Exptl. Pathol. **17**:481–484.

HERBST, E. J., R. H. WEAVER, AND D. L. KEISTER. 1958. The gram reaction and cell composition: diamines and polyamines. Arch. Biochem. Biophys. **75**:171–177.

JACKSON, E. L. 1956. The preparation of spermidine and monoacetylspermidine. J. Org. Chem. **21**:1374–1375.

JACKSON, E. L., AND S. M. ROSENTHAL. 1960. The preparation of $C^{14}$-labeled spermine and $C^{14}$-labeled spermidine. J. Org. Chem. **25**:1055–1056.

MCINTIRE, F. C., L. W. ROTH, AND J. L. SHAW. 1947. Purification of histamine for bioassay J. Biol. Chem. **170**:537–544.

ROSE, I. A., M. GRUNBERG-MANAGO, S. R. KOREY, AND S. OCHOA. 1954. Enzymatic phosphorylation of acetate. J. Biol. Chem. **211**:737–756.

ROSENTHAL, S. M., AND C. W. TABOR. 1956. The pharmacology of spermine and spermidine. Distribution and excretion. J. Pharmacol. Exptl. Therap. **116**:131–138.

TABOR, H., S. M. ROSENTHAL, AND C. W. TABOR. 1958. The biosynthesis of spermidine and spermine from putrescine and methionine. J. Biol. Chem. **233**:907–914.

TABOR, H., C. W. TABOR, AND S. M. ROSENTHAL. 1961. Spermidine and spermine. Ann. Rev. Biochem. **30**:579–604.

WEAVER, R. H., AND E. J. HERBST. 1958. Metabolism of diamines and polyamines in microorganisms. J. Biol. Chem. **231**:637–646.