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Effects of Diacetylmorphine and Related Morphinans on Some Biochemical Activities of *Staphylococcus aureus*

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SUMMARY

Heroin (*O,O'*-diacetylmorphine) at concentrations of 30–60 mM prevents the growth of *Staphylococcus aureus*, and inhibits protein and nucleic acid synthesis by 40–50% and glucose oxidation by 25–35%; the same concentrations have negligible effect on amino acid incorporation in a system *in vitro* containing ribosomes and sap from the same cells. In this concentration range heroin increases the rate of accumulation in the free amino acid "pool" of aspartate, glutamate, and alanine, inhibits the accumulation of lysine and proline, and has no effect on the accumulation of five other amino acids tested. Levorphanol (*l*-3-hydroxy-*N*-methylmorphinan) and levallorphan (*l*-*N*-allyl-3-hydroxymorphinan) show a pattern of effects similar to that of heroin and are approximately 10 times more effective on a molar basis.

INTRODUCTION

Simon (1) noted in 1963 that levorphanol (*l*-3-hydroxy-*N*-methylmorphinan) inhibited the growth of *Escherichia coli* and, in a series of papers (2–4), observed that the drug inhibited the synthesis of protein and nucleic acid in this organism. Fractionation of the nucleic acid indicated that the synthesis of ribosomal RNA was more sensitive than that of the other macromolecules, but the drug was found to have no significant effect on RNA synthesis in cell-free systems. Greene and Magasanik (5) confirmed that levorphanol, as well as levallorphan (*l*-*N*-allyl-3-hydroxymorphinan), inhibited macromolecular synthesis in *E. coli*, but suggested that the primary effect resided in some reaction preceding macromolecule formation. They found that the addition of 5 mM levallorphan was followed by a rapid decrease in the ATP content of cells and leakage of adenosine nucleotides into the medium; they suggested that the drug increased the rate of breakdown of ATP but were unable to obtain direct proof that this was so. The present paper deals with the actions of heroin (diacetylmorphine), levorphanol, and levallorphan on *Staphylococcus aureus*, with particular reference to the transport of amino acids into the cells. Morphine itself was too insoluble to use in these experiments.

MATERIALS AND METHODS

Organism and conditions of culture. The organism used was *S. aureus* Duncan grown and prepared in washed cell suspension at 2.0 mg dry weight of cells per milliliter, as previously described (6). Unless otherwise stated, growth took place for 18 hr at 25° so that the cells were harvested in the middle of the exponential phase. Concentrations of drugs inhibitory to growth were determined by serial dilution into tubes containing 5 ml of nutrient medium; each tube was then inoculated with 1 drop (10^5–10^6 cells) of a fully developed culture and incubated at

37°; the lowest drug concentration inhibiting visible growth was recorded after 24, 48, and 72 hr.

Suspension density. The density of cultures and suspensions was determined turbidimetrically on a Hilger Absorptiometer calibrated against the dry weight of the organism concerned.

Oxidation of glucose. Warburg manometers were set up containing 1.5 ml of buffered salts solution [NaCl, 3.0 g; $MgSO_4 \cdot 7H_2O$, 2.1 g; KH_2PO_4, 3.0 g; Na_2HPO_4, 10.0 g; water, 1 liter; pH 7.0 (6)], 0.3 ml of washed suspension of cells at 2.0 mg (dry weight) per milliliter, 0.9 ml of water or drug solution, with 0.3 ml of 10% (w/v) glucose in the side arm and 0.2 ml of N KOH in the center well. Manometers were equilibrated at 20° or 37°, the glucose was tipped into the main vessel, and oxygen consumption was measured in the conventional manner. When drug solutions contained alcohol, equivalent amounts of alcohol were added to the control manometers.

Uptake of labeled amino acids. The transport of ^{14}C-labeled amino acids and their concentration within the cells were determined as previously described (7). The concentration gradient is expressed empirically as P/S = ratio of counts in the hot water extract to counts in the supernatant medium (7). In all cases estimates were made after 3, 9, and 27 min at 20°.

Incorporation of amino acids and nucleic acid precursors. Protein synthesis was measured by incorporation of ^{14}C-labeled aspartate into the hot trichloracetic acid precipitate, and nucleic acid synthesis by incorporation of either ^{14}C-labeled guanine or uracil into the cold trichloracetic acid precipitate (8, 9). In both cases cells were incubated in the presence of glucose and the complete mixture of amino acids required for protein synthesis (8). In the cell-free system, 0.5 ml of the buffered extract was incubated in a total volume of 1.0 ml with 0.02 ml of 60 mM ATP, 0.1 ml of 100 mM creatine phosphate, 0.1 ml of creatine phosphokinase (1 mg/ml), and 10 μCi of algal protein hydrolysate (2 mCi/mg) and incubated at 37°. Reaction was stopped at 5 or 10 min by the addition of 1.0 ml of 10% trichloracetic acid.

Preparation of cell-free extract. Approximately 300 mg dry weight of cells were taken up in 4 ml of buffer containing 0.05 M tris-(hydroxymethyl)aminomethane adjusted to pH 7.6 with 0.1 N HCl, 0.05 M KCl, 0.01 M magnesium acetate, and 0.01 M 2-mercaptoethanol. An approximately equal volume of glass Ballotini beads was added, and the mixture was blended at 0° in a micro-blender for two periods of 7 min, the mixture being held at 0° between the two periods to ensure cooling. The broken cell mix was filtered through a sintered glass filter and centrifuged at 2000 × *g* for 20 min. The supernatant liquid was used as extract.

Labeled compounds. All labeled compounds were purchased from the Radiochemical Centre, Amersham, England, and used at concentrations set out below. For the amino acid transport experiments, aspartate, lysine, or proline uniformly labeled with ^{14}C was used at 20 μM concentration with the specific activity adjusted to 2–4 mCi/mmole. Radioactivity was determined with an end window Geiger-Müller tube with conventional scaler equipment: 1 mμmole of aspartate with a specific activity of 3.12 mCi/mmole = 563 cpm.

Drugs. Heroin was obtained from May and Baker Ltd., Dagenham, England, and stock solutions (3 M) were prepared in 50% (v/v) ethanol; levorphanol and levallorphan tartrate were kindly given to us by Roche Products Ltd., Welwyn Garden City, England, and stock solutions (0.1 M) were prepared in 30% ethanol (levorphanol) or water. In all cases the pH was adjusted to approximately 6.

RESULTS

Action on growth. No visible growth of *S. aureus* from an inoculum of 10^5–10^6 cells in 5 ml occurred in 24 hr at 37° in the presence of 50 mM heroin or 5 mM levorphanol or levallorphan. In the case of heroin, turbidimetric measurements showed that the growth rate was approximately halved by 10 mM drug while a slight granular growth was visible after 72 hr at 100 mM. The growth occurring at 20 mM heroin was serially subcultured into media containing higher concentrations, and after 10 passages a culture was obtained which would grow in 24 hr in 200 mM heroin. This organism would also grow in 30 mM

levorphanol. It was not possible to show any drug dependence at these levels.

Action on protein synthesis and respiration. Figure 1a shows the effect of heroin on the oxidation of glucose and the incorporation of aspartate into the protein fraction of intact staphylococci. At concentrations less than 100 mM the oxidation of glucose is less sensitive than aspartate incorporation, although not markedly so. The figure shows that amino acid incorporation in the cell-free system is not significantly inhibited by heroin at concentrations less than 100 mM; at higher concentrations addition of the narcotic produced precipitation in the cell extract. Figure 2 shows that a similar situation holds for inhibition of glucose oxidation and aspartate incorporation in intact cells in the presence of levorphanol or levallorphan. Greene and Masasanik (5) reported that 5 mM levallorphan had no inhibitory effect on glucose oxidation in *E. coli* while protein synthesis was completely stopped; their experimental conditions were, however, different, in that respiration was followed in a growth medium and chloramphenicol was added to the control but not to the cells in the presence of levallorphan. In the washed staphylococcal cells, concentrations of the drugs which prevent growth in 24 hr gave rise in each case to 40–50% inhibition of protein synthesis and 25–35% inhibition of the rate of glucose oxidation. The inhibition of incorporation of ^{14}C-uracil or ^{14}C-guanine into the nucleic acid fraction of intact cells was the same as that of aspartate into the protein fraction.

Action on amino acid transport. The accumulation against a concentration gradient of glutamate and aspartate in the "pool" of *S. aureus* is an energy-requiring process while the accumulation of lysine involves two processes, one of which is energy-dependent (7, 10). Figure 3 shows the effect of heroin on the concentration of free aspartate, lysine, and

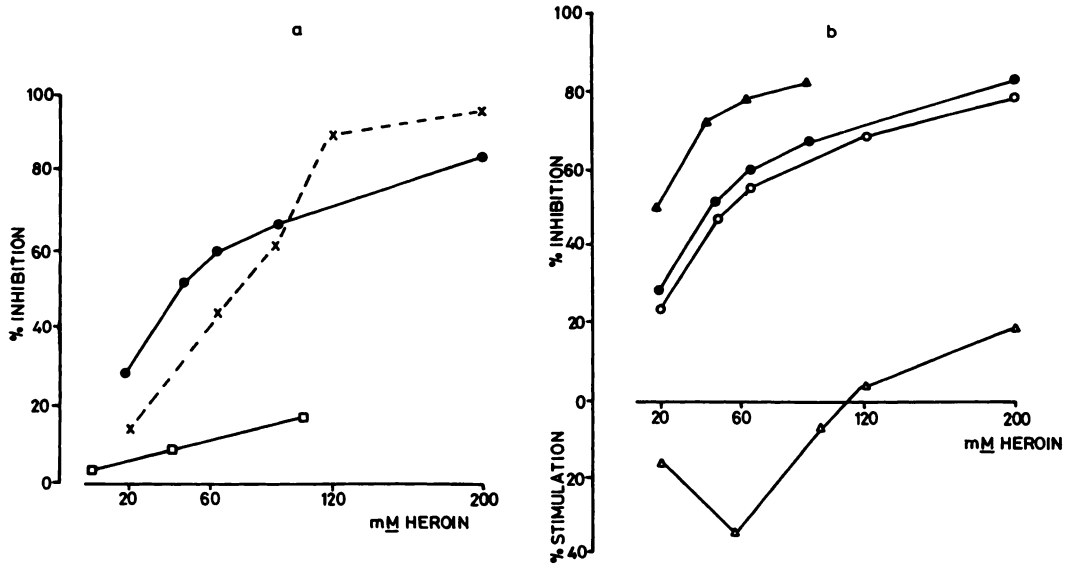

FIG. 1. *Effects of heroin on S. aureus*

a. Inhibition by heroin of glucose oxidation, Q_{O_2} measured over 60 min (×- - - ×), and incorporation of aspartate into the protein fraction (●——●) of intact cells at a density of 0.4 mg dry weight per milliliter and 37°. The effect on incorporation of ^{14}C-labeled amino acids into the cell-free protein-synthesizing system (□——□) at 37° is also shown. For preparation and quantities, see MATERIALS AND METHODS.

b. Effect of heroin on rates of aspartate incorporation into the protein fraction (●——●) and accumulation in the amino acid "pool" of aspartate (△), lysine (○), and proline (▲). Intact cells at 0.4 mg dry weight per milliliter were incubated at 20°. Rates of accumulation of amino acids were measured over the initial 10 min at 20°.

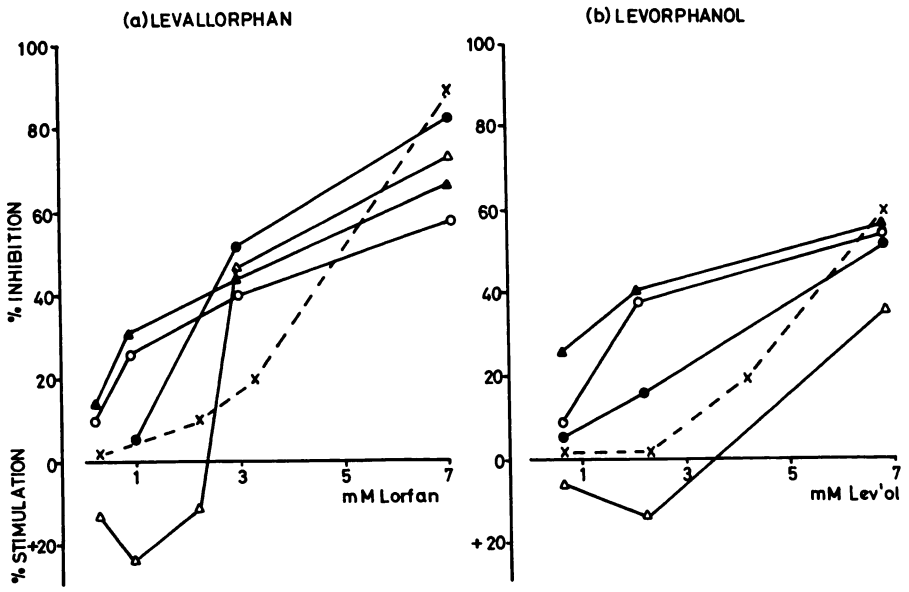

FIG. 2. *Effects of levallorphan (a) and levorphanol (b) on S. aureus*
Intact cells at 0.4 mg dry weight per milliliter were incubated at 20°. ×, glucose oxidation; ●, incorporation of aspartate into the protein fraction; accumulation in the "pool" of aspartate (△), lysine (○), and proline (▲).

proline within *S. aureus*; the rate of accumulation of aspartate was initially increased in the presence of 60 mM heroin while the rates for lysine and proline were decreased. After 30 min the P/S value for aspartate accumulation in the presence of 60 mM heroin may, as in Fig. 3, be less than that obtained in the control without drug, but this was not invariably the case. Figure 1b shows the effect of heroin concentration; 20–100 mM heroin stimulated the transport of aspartate, measured over the first 10 min at 20°, while higher concentrations had some inhibitory action. The accumulation of proline within the cells was more sensitive than that of lysine at all heroin concentrations tested; both dinitrophenol-sensitive and -insensitive transport systems were affected (not illustrated). Figure 2 shows that the inhibitory actions of levorphanol and levallorphan display the same pattern as those obtained for heroin.

The accumulation of aspartate, glutamate, or alanine was accelerated, and that of lysine or proline inhibited, while that of valine, arginine, histidine, leucine, or glycine was not affected by 50 mM heroin. The drug did not cause leakage of previously accumulated amino acids under these conditions.

Figure 4 shows the effect on subsequent transport of prior treatment of cells with heroin. Cells were incubated at 37° for 30 min at a density of 0.4 mg dry weight per milliliter, centrifuged down, and resuspended under the normal conditions for aspartate or lysine accumulation. Cells treated in the absence of drug and then incubated with aspartate showed a rapid increase in the P/S value to a higher level than that obtained in freshly harvested cells; the presence of heroin during accumulation had little effect during the first 15 min. In cells treated in the presence of 30 mM heroin, the rate of aspartate accumulation in the absence of drug was markedly decreased for the first 15–20 min and then increased, until the impairment was reversed after a lag of about 30 min at 20°. The presence of heroin during accumulation by cells treated with the drug gave a marked stimulation during the first 10 min, after which aspartate accumulation effectively stopped.

Lysine accumulation in cells treated with

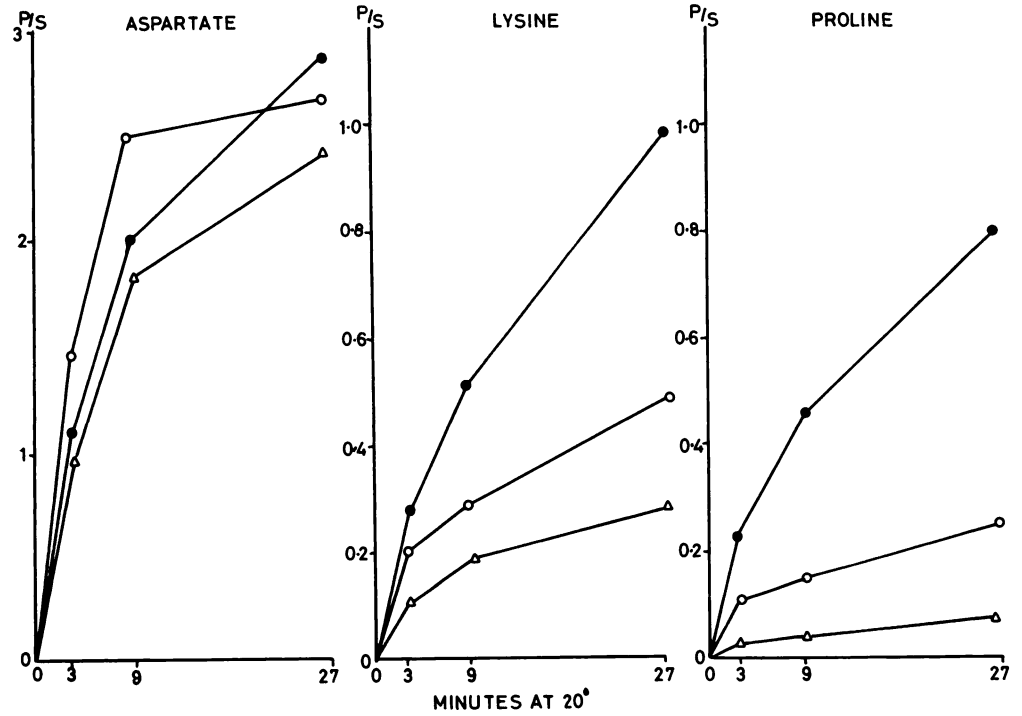

FIG. 3. *Effect of heroin on amino acid accumulation, measured by the empirical concentration gradient P/S, where P = total counts in hot water-extracted "pool" and S = total counts in supernatant medium* (7)

Cells were incubated at 20° and 0.4 mg dry weight per milliliter in buffered salts solution, 1% (w/v) glucose, 20 mM ^{14}C-labeled amino acid, and 50 μg of chloramphenicol per milliliter. ●, control; ○, 60 mM heroin; △, 120 mM heroin.

heroin ceased at a lower P/S value than in cells previously incubated in the absence of drug. The process was less sensitive to heroin present during accumulation when the cells had also been treated with the drug.

DISCUSSION

The morphine analogues used in these experiments affect many biochemical activities in staphylococci. There is little difference in the sensitivities of respiration and macromolecular synthesis, so that the question could be asked whether inhibition of energy metabolism is sufficient to explain the other effects. It was for this reason that we first looked at amino acid transport and found the variety of effects described above rather than a general inhibition related to that of energy metabolism. Greene and Magasanik (5) found a variety of changes in permeability of *E. coli* in the presence of levorphanol and levallorphan: the cells did not become generally leaky but showed a loss of ability to retain specific substances. These changes suggest an alteration in the properties of the cell membrane, and this is investigated in the next paper (11). Greene and Magasanik (5) suggested that the common factor could be an accelerated breakdown of ATP; this might also be a factor in the results described in the present paper, although some of the changes in amino acid transport could not easily be explained in this way.

Greene and Magasanik (5) found rapid and complete recovery of *E. coli* after removal of levallorphan; Fig. 4 shows that the staphylococci exhibit a lag period before transport of aspartate is restored to that in untreated cells. Treatment of staphylococci with heroin results in an increased turnover of phospholipid, as shown in the following paper (11); under the conditions used in

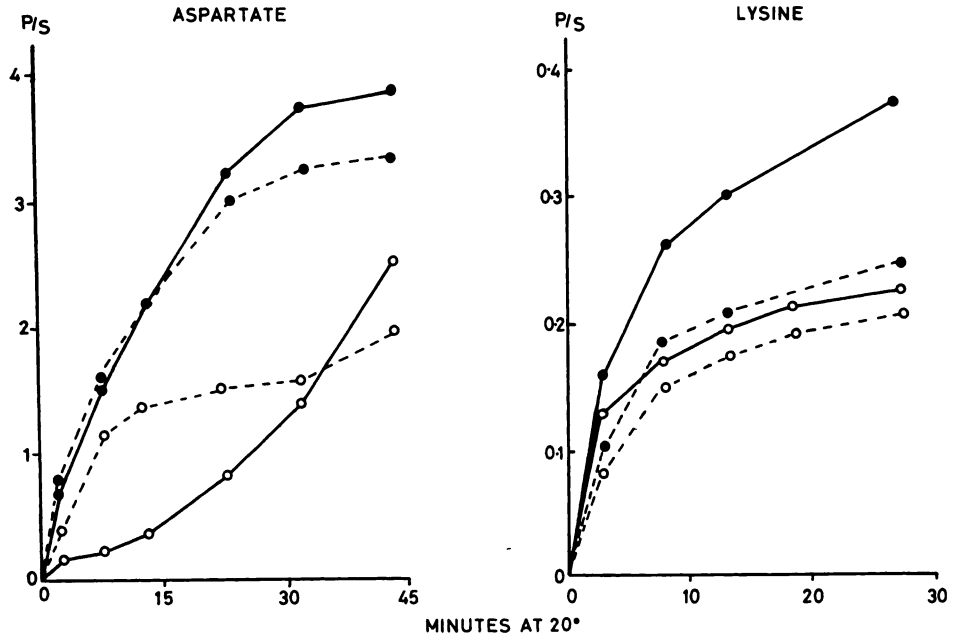

FIG. 4. *Effect of treatment of S. aureus with heroin on subsequent accumulation of free aspartate or lysine*
Cells were first treated by incubation for 30 min at 37° in buffered salts solution alone (●) or containing 30 mM heroin (○). Cells were centrifuged down and resuspended in the test medium at 20° (as for Fig. 3) with (- - -) or without (——) 30 mM heroin.

Fig. 4 there would be a marked breakdown of phospholipid during the prior treatment with heroin, followed by resynthesis under the conditions of aspartate uptake in the presence of glucose—the rate of this resynthesis being increased for a time by heroin. Thus there appear to be correlations between phospholipid metabolism and aspartate transport which are again of interest in view of the previous finding (7) that aspartate accumulation is increased by the addition of lipid or fatty acid fractions to staphylococcal suspensions.

Levallorphan is a morphine antagonist but no significant differences have been observed in the pattern of its actions and those of heroin or levorphanol in the systems investigated so far.

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