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Effect of Morphine Derivatives on Lipid Metabolism in *Staphylococcus aureus*

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SUMMARY

Heroin at a concentration which inhibits the transport of lysine into *Staphylococcus aureus* stimulates the incorporation of glycerol into the phospholipid fraction of the cells. The stimulation is accompanied by an increased turnover of the major phospholipid, phosphatidylglycerol. Chromatographic examination of the lipid appears to show the presence of new phospholipids, judged from changes in the profile of elution from silicic acid columns and the appearance of new radioactive spots on silicic acid-impregnated paper chromatograms. Chromatographic analysis of the new components and their hydrolytic products shows that they contain phosphatidylglycerol and diphosphatidylglycerol. (*N*-Methyl-^{14}C)-heroin is rapidly taken up by staphylococcal cells, but examination of that part of the radioactivity appearing in phospholipids shows that this is contained in glycerol residues. (7,8-^{3}H)-Dihydroheroin is also taken up rapidly, and ^{3}H is found in association with phospholipids; over 50% of this ^{3}H dissociates from phospholipid on deacylation and chromatography in polar solvents. Free heroin reacts with phosphatidylglycerol *in vitro* to give complexes whose elution profiles are the same as those obtained from drug-treated cells; the "new components," however, are found only when phospholipid turnover takes place in the presence of the drug to give diphosphatidylglycerol.

Similar results have been obtained with levorphanol, dextrorphan (the optical isomer of levorphanol), levallorphan, and naloxone. The elution profiles of phospholipid extracts differ with the drug used but can again be reproduced by reaction of drug and phosphatidylglycerol *in vitro*. "New components" are produced in each case and yield, after deacylation and chromatography in 1-propanol–ammonia–water, mixtures of phosphatidylglycerol and diphosphatidylglycerol. Since the same effects are obtained with narcotics (heroin, levorphanol), morphine antagonists (levallorphan, naloxone), and dextrorphan, which is inactive in man, it would appear that the antibacterial effects are not related to the effects of morphine derivatives in man.

INTRODUCTION

Heroin, levorphanol, and levallorphan have a variety of effects on the transport of amino acids into *Staphylococcus aureus*, some systems being stimulated, some inhibited, and others unaffected (1). Simon, Cohen, and Raina (2) found that levorphanol brought about a decrease in the putrescine content of *Escherichia coli* and suggested that this could be due to leakage of the amine out of the cell. Greene and Magasanik (3) have shown that levorphanol and levallorphan cause *E. coli* to leak nucleotides and galactosides although the permeability changes do not affect all the small molecular weight substances in the "pool" of the cells. These re-

sults suggest that the drugs affect the membranes of the cells, and the present paper shows that a number of morphine derivatives produce changes in the composition and the rate of turnover of the phospholipid fraction of *S. aureus*.

MATERIALS AND METHODS

The organism used was *S. aureus* Duncan grown for 18 hr at 25° and prepared in washed suspension as previously described (4). Incubation conditions, extraction, fractionation, and determination of lipids were also performed as previously described (5). The majority of the experiments were carried out using a phosphate-buffered salts solution (4); for investigation of the incorporation of ^{32}P-phosphate, the solution was buffered with 0.1 M tris(hydroxymethyl)aminomethane adjusted to pH 7.6 with 0.1 N HCl and total phosphate adjusted to 1.0 mM.

Labeled compounds

(1-^{14}C)-Glycerol (specific activity, 1.57 mCi/mmole), sodium (1-^{14}C)-acetate (2.9 mCi/mmole), ^{32}P-phosphate, and (*N*-methyl-^{14}C)-morphine hydrochloride (16.5 mCi/mmole) were purchased from the Radiochemical Centre, Amersham, England. (7,8-^{3}H)-Dihydromorphine hydrochloride (specific activity, 2.5 Ci/mg) was kindly given to us by Dr. H. W. Kosterlitz, Physiology Department, University of Aberdeen. The labeled morphine and dihydromorphine were converted to heroin and diacetyldihydromorphine, respectively, by solution, with 4–6 mg of carrier, in 0.5 ml of pyridine and 0.5 ml of acetic anhydride at 37° for 3 days, followed by removal of the solvents, solution in the minimum amount of ethyl acetate, and addition of petroleum ether (40–60° fraction) until a slight turbidity appeared; on standing overnight in the ice chest, the acetylated products crystallized with 90–93% yield. ^{14}C-Labeled phosphatidylglycerol was prepared by incubating cells with (1-^{14}C)-glycerol (5), extracting the lipid, and fractionating the lipid on silicic acid columns as described below (CA1).

Estimation of Radioactivity

The incorporation of ^{14}C-labeled glycerol or ^{32}P-phosphate was routinely estimated by a Geiger-Müller end window counter with conventional scaler equipment; 1 mμmole of glycerol with a specific activity of 1.57 mCi/mmole = 284 cpm. Incorporation into specific phospholipids was estimated by monitoring chromatograms after location of components by radioautography. Estimations of the ^{32}P:^{14}C ratio in doubly labeled materials and the distribution of ^{14}C and ^{3}H on chromatograms were made either by cutting the chromatograms into 1-cm strips or by cutting out components after location by radioautography, placing the paper strips in vials, adding 0.25 ml of 2,5-bis[2(5-*tert*-butylbenzoxazolyl)]-thiophene (BBOT) (4 mg/ml), and estimating activity in a Packard Tri-Carb scintillation spectrometer, model 3375. When chromatograms were run on silicic acid-impregnated paper, quenching effects made it impossible to estimate with any accuracy ^{14}C and ^{3}H on the same sample; experiments were therefore run in parallel with one label in each incubation mixture. Under the conditions used, ^{14}C was estimated with 55% and ^{3}H with 2% efficiency.

Drugs

Heroin (diacetylmorphine), levorphanol (*l*-3-hydroxy-*N*-methylmorphinan), dextrorphan (*d*-3-hydroxy-*N*-methylmorphinan), and levallorphan (*l*-*N*-allyl-3-hydroxymorphinan) were made into solution as described in the previous paper (1). Naloxone (*l*-*N*-allyl - 7,8 - dihydro - 14 - hydroxynormorphinone), a morphine antagonist, was a product of Endo Laboratories Pharmaceuticals Inc., Garden City, N. Y., and given to us by Dr. Kosterlitz; a stock 0.1 M solution was prepared in water. The drugs were used, in each case, over that range of concentration which produces approximately 50% inhibition of the rate of transport of lysine into *S. aureus* (1).

Chromatographic Analysis of Lipids

For convenience, the stages used for investigation of the lipid fraction are set out below and will be referred to in the text by the abbreviations CA1, etc.

CA1. Lipid extract in chloroform chromatographed on silicic acid columns eluted with chloroform containing 0% (fraction A), 6% (B), 15% (C), and 40% (D) methanol (5).

CA2. Eluates from CA1 taken down to a small volume and chromatographed on silicic acid-impregnated paper [Whatman SG81 or prepared according to Marinetti, Erbland, and Kochen (6)] and developed in diisobutyl ketone–acetic acid–water (40:20:3) (7).

CA3. Components on CA2 paper located by radioautography, eluted with approximately 5 ml of chloroform–methanol (50:50 by volume) in stoppered tubes, and the solutions saturated with gaseous ammonia; left for 5 days at room temperature and the ammonia removed under vacuum. The solvent residue was shaken with 1.0 ml of water and 10 ml of ether; the aqueous phase was separated and freeze-dried.

CA4. Water-soluble hydrolysis products from CA3 chromatographed on Whatman No. 1 paper developed in (a) 1-propanol–ammonia (sp. gr. 0.88)–water (60:30:10 by volume), (b) 2-propanol–ammonia–water (70:6:30 by volume), or (c) 2-propanol–concentrated HCl–water (17:44:36 by volume).

CA5. Components on CA4 paper located by radioautography and eluted with water; hydrolyzed for 2 hr in 2 N HCl at 105°; HCl removed *in vacuo*.

CA6. Hydrolysis products from CA5 chromatographed on Whatman No. 1 paper in 1-propanol–ammonia–water as for CA4 (a).

CA7. Water-soluble products from CA3 chromatographed in two dimensions using *sec*-butyl alcohol–formic acid–water (70:10:20 by volume) in the first direction, followed by phenol–water–ammonia (80:20:0.3 by volume) in the second.

Chromatography of Morphine Derivatives

This was carried out as described by Munier, Macheboeuf, and Cherrier (8) on 0.5 M KCl-impregnated paper developed in 1-butanol–concentrated HCl (98:2 by volume) saturated with water.

Cell-Free Extract

Approximately 500 mg, dry weight, of washed staphylococci were suspended in 2 ml of ice-cold medium containing 0.005 M KCl, 0.01 M magnesium acetate, 0.01 M 2-mercaptoethanol, and 0.1 M Tris-KH_2PO_4 buffer, pH 7.9. An approximately equal volume of glass Ballotini beads was added, and the mixture was blended at 0° in a high-speed micro-blender for three periods of 6 min, allowing 2-min intervals in the ice bath between treatments. The beads were filtered off on a sintered glass filter, and the broken material was centrifuged for 20 min at 2000 × *g*. The clear supernatant fluid was taken as the cell-free extract. In the experiments described below, 0.3 ml of this extract was mixed with 10^4 cpm of ^{14}C-labeled phosphatidylglycerol in 0.02 ml of ethanol solution and 3–30 mM heroin or 2–10 mM levorphanol, etc. Reaction was stopped by plunging the reaction tubes into a CO_2-acetone freezing mixture; the contents of the tubes were freeze-dried and lipid was extracted as described for cell pellets.

RESULTS

Effects of Drugs on Incorporation of Glycerol into Lipid

Figure 1a shows the effects of heroin, levorphanol, and levallorphan, at a concentration in each case which gives 40–50% inhibition of lysine accumulation (1), on the incorporation of glycerol into the lipid fraction. In each case a marked increase in incorporation occurred and, as demonstrated previously (5), the radioactivity was associated solely with glycerol residues in the lipid. The extent of the stimulation varied from experiment to experiment. Of the drugs tested, heroin gave the greatest increase and at concentrations of 30–60 mM sometimes doubled the incorporation of glycerol. Figure 1b shows the corresponding labeling of the fatty acid fraction by ^{14}C-acetate; under the same conditions the drugs showed inhibitory effects, heroin again having the most marked effect.

The major component of the lipid which was labeled under these conditions was phosphatidylglycerol, which, as shown previously (5), is in a state of turnover. Figure 2 shows the effect of suspending labeled cells in excess unlabeled glycerol with and without heroin after incorporation had proceeded for 20 min. The release of radioactivity from the lipid fraction was markedly accelerated by the presence of heroin whether the first incorporation took place in the presence or absence of drug. Similar results were ob-

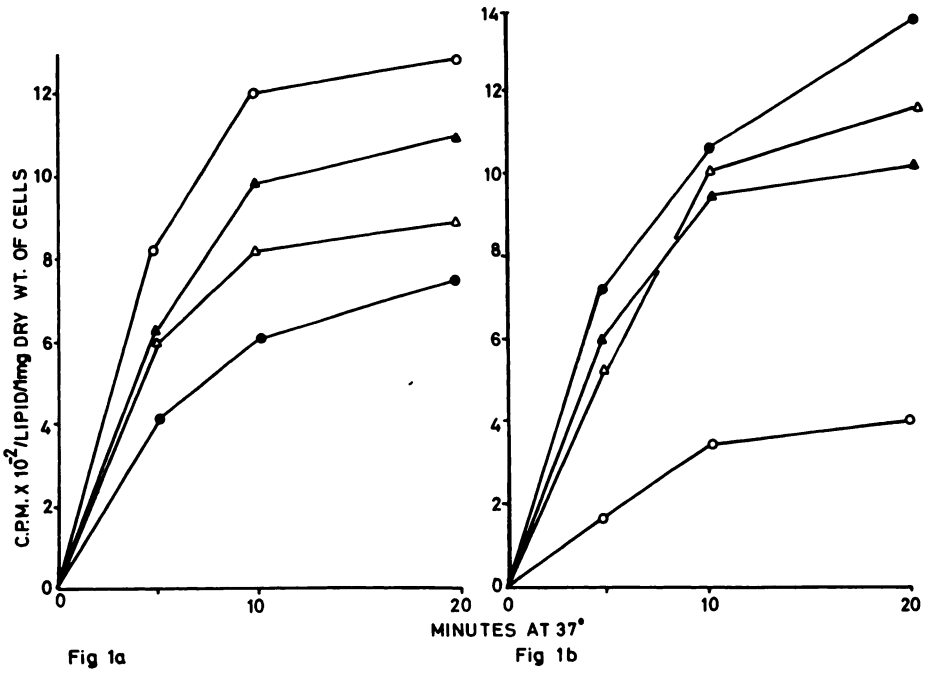

FIG. 1. *Effect of drugs on the incorporation of (1-^{14}C)-glycerol (a) and (1-^{14}C)-acetate (b) into the lipid fraction of S. aureus*

Cells were incubated at 0.2 mg dry weight per milliliter in buffered salts solution containing 0.3 mM glucose, 0.1 mM glycerol-(1-^{14}C) (specific activity, 1.57 mCi/mmole) in part a (unlabeled glycerol in part b), and 0.05 mM sodium acetate-(1-^{14}C) (specific activity, 2.9 mCi/mmole) in part b (unlabeled acetate in part a) with the drugs shown. Reaction stopped by rapid cooling, and lipid was extracted and counted. ●, no drug; ○, 30 mM heroin; ▲, 2 mM levorphanol; △, 2 mM levallorphan.

tained with levorphanol, dextrorphan, naloxone, or levallorphan at 2 mM concentration. No significant turnover, in the presence or absence of heroin, could be demonstrated for the labeling of the lipid fraction taking place as a result of the incorporation of ^{14}C-acetate.

Examination of the Lipid Fraction

Figure 3a (upper graph) shows the elution profile obtained on eluting the glycerol-labeled lipid fraction from silicic acid columns with methanol–chloroform mixtures (CA1). Fraction A, eluted with chloroform alone, contains neutral lipid; B (6% methanol–94% chloroform), further neutral lipid and phosphatidic acid; C (15% methanol–85% chloroform), phosphatidylglycerol and diphosphatidylglycerol; D (40% methanol–60% chloroform), further phosphatidylglycerol and its lysyl ester. Diphosphatidylglycerol and the lysyl ester of phosphatidylglycerol form only minor components under the experimental conditions used here, with a short incubation period in the absence of added amino acids. It can be seen from Fig. 3a that 30 mM heroin resulted in little change in the radioactivity eluted in fractions A and B but gave a marked increase in C and D, the elution profile in C being different from the control in that a new component was eluted within the first three samples collected after change of solvent. The eluates from each fraction were further examined by chromatography on silicic acid-impregnated papers developed with diisobutyl ketone–acetic acid–water (CA2). Radioautography showed the expected spots of phosphatidylglycerol (R_f = 0.45) in fractions C and D, but the lipids from heroin-treated cells showed further components with R_f = 0.6 in both C and D. These new substances in C and D found after heroin treatment will be referred to as the "H components" below.

Figure 3b (lower graph) shows the cor-

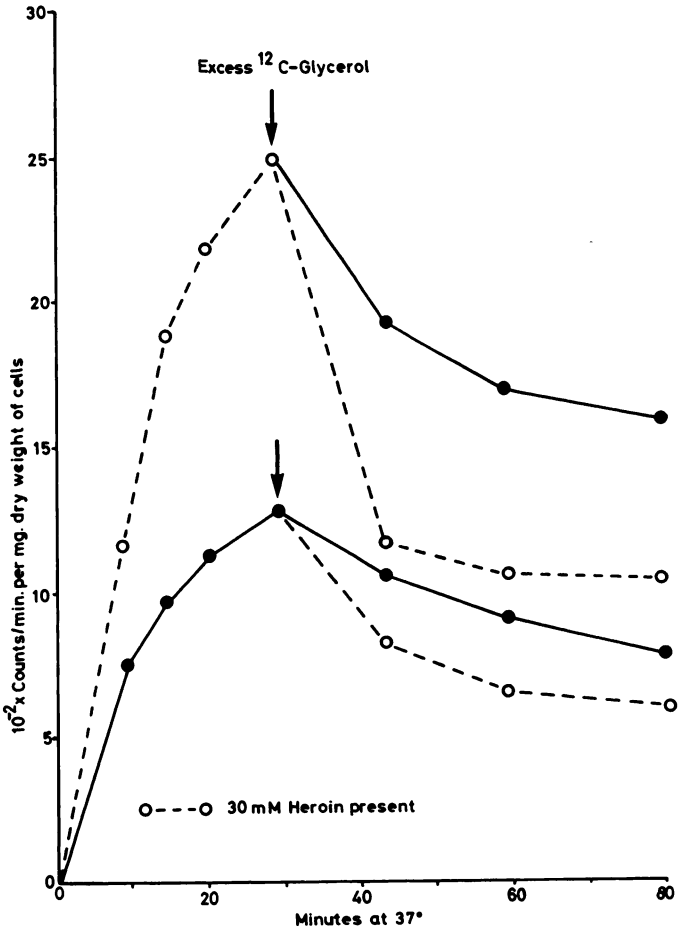

FIG. 2. *Turnover of ^{14}C-glycerol in lipid of S. aureus in the presence and absence of heroin*

Cells were incubated at 0.2 mg dry weight per milliliter in buffered salts solution containing 0.3 mM glucose and 0.1 mM ^{14}C-glycerol (1.57 mCi/mmole) alone (●) or with 30 mM heroin (○). After 30 min at 37°, cells were centrifuged down and resuspended in 0.3 mM glucose and 10 mM glycerol with (○) or without (●) 30 mM heroin.

responding profiles for extracts from cells labeled with glycerol in the presence of either levallorphan or levorphanol. In the case of levallorphan, three major changes from the control can be seen: a marked increase in radioactivity eluting in fraction B; a decrease in the total activity in fraction C, with evidence of a new component eluting in a position similar to that obtained with heroin; and a marked decrease in the radioactivity of fraction D. Radioautograms (CA2) again showed new material with R_F = 0.6 (sometimes streaking to 0.8) in fractions B and C. In the extracts obtained from levorphanol-treated cells, the elution profile (CA1) sometimes resembled that of heroin-treated cells and sometimes appeared as shown in Fig. 3b, with enhanced activity in fraction C eluting in sample tubes 3–5; radioautograms (CA2) again showed the presence of material with R_F = 0.6 in fractions C and D. Table 1 demonstrates the distribution of radioactivity in the various fractions obtained by column and paper chromatography; the proportion of phospholipid radioactivity found in new components running at R_F = 0.6 varies from 12 (6 mM naloxone) to 61% (2 mM levallorphan).

Table 1 shows that the radioactivity appearing in the front of the 15% methanol

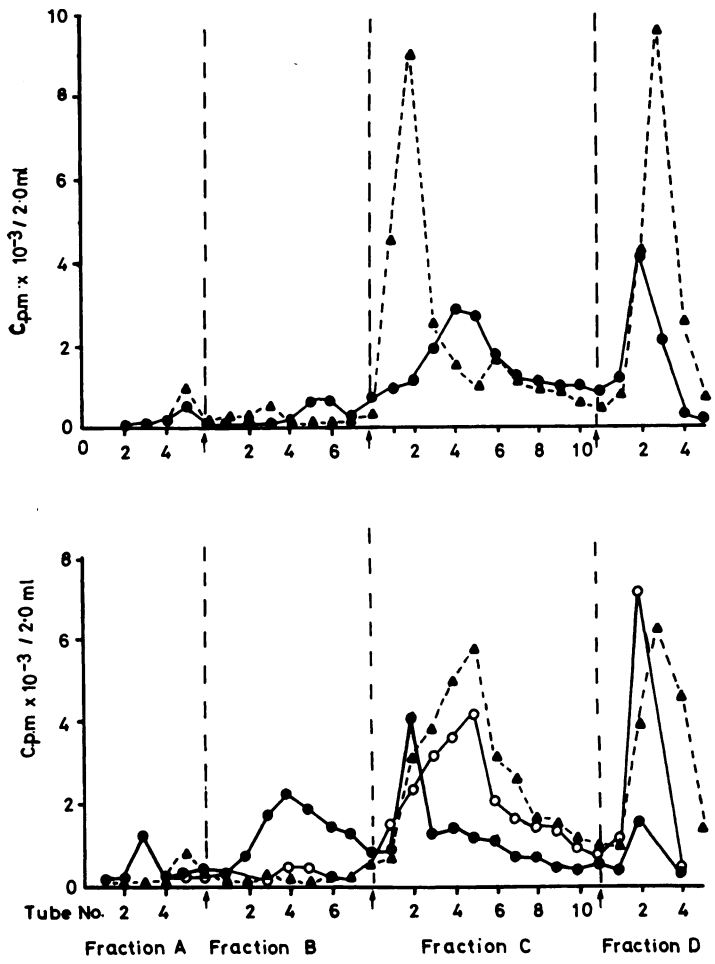

FIG. 3. *Elution profile of lipid extract from S. aureus after treatment with (a, upper) 30 mM heroin (▲- - -▲) or (b, lower) 2 mM levallorphan (●——●) or 2 mM levorphanol (▲- - -▲), or with no drug (a, ●——●); (b, ○——○)*

Cells were incubated with ^{14}C-glycerol as in Fig. 2; a 10-mg sample was extracted and lipid was poured onto a silicic acid (1.5 g) column; eluted with chloroform containing 0% (fraction A), 6% (fraction B), 15% (fraction C), or 40% methanol (fraction D). Cuts were taken at 2.0 ml, and radioactivity was determined.

elution resolves into two components, of which the major one corresponds in R_F value with phosphatidylglycerol. Naloxone differed from the other drugs tested in that the amount of radioactivity incorporated into the lipid fraction as a whole was significantly decreased compared with the control without drug; the distribution obtained was similar to that obtained with heroin.

Figure 2 shows the course of the release of radioactivity from labeled lipid when excess unlabeled glycerol, with or without heroin, is added to the incubation mixture. The lipid extracts have been examined (a) after labeling for 20 min with ^{14}C-glycerol, (b) after a further 30 min in the presence of excess unlabeled glycerol, and (c) as in (b) with the addition of 30 mM heroin. Chromatographic analysis (CA2) of CA1 fractions C and D from (a) and (b) showed the expected pattern with phosphatidylglycerol as the major component, but the lipid from (c) showed the presence of H components as well as phosphatidylglycerol. Similar results were ob-

TABLE 1

Distribution of ^{14}C-labeled glycerol in phospholipid fractions of S. aureus after incubation in the presence of heroin or related drugs

Washed cells (10 mg, dry weight) were incubated at 37° and a density of 0.2 mg/ml in buffered salts solution containing 0.3 mM glucose, 0.1 mM ^{14}C-labeled glycerol (specific activity, 1.57 mCi/mmole), and drug at the concentrations given below. Reaction was stopped after 30 min by cooling; lipid was extracted and chromatographed first (CA1) on a silicic acid column (approximately 1.5 g), eluted with chloroform–methanol mixtures (as in Fig. 3), and collected in 2-ml samples. Eluates were combined as shown and chromatographed (CA2) on silicic acid-impregnated paper developed in diisobutyl ketone–acetic acid–water; components were detected by radioautography, eluted, and counted. Values are normalized to initial activities.

Drug	CA1 fraction B (6% methanol), tubes 1–9		CA1 fraction C (15% methanol)				CA1 fraction D (40% methanol), tubes 1–5		Total phospholipid
			Tubes 1–3		Tubes 4–11				
	R_F 0.45[a]	R_F 0.6	R_F 0.45	R_F 0.6	R_F 0.45	R_F 0.6	R_F 0.45	R_F 0.6	
	cpm	cpm	cpm	cpm	cpm	cpm	cpm	cpm	cpm
Experiment 1									
None	750		2,150[b]		11,300[b]		6,740[b]		20,940
		120		170					290
30 mM heroin	170		14,510		1,970		9,980		26,630
		1,550		2,950		770		200	5,470
2 mM levorphanol	200		3,975		4,245		3,540		11,960
		3,900		2,045		1,055		1,260	8,260
2 mM levallorphan	780		4,250		2,280		440		7,750
		7,500		4,050		760		200	12,510
Experiment 2									
None	850		3,450		15,990		10,740		31,030
		190		30		120			340
30 mM heroin	220		16,100		4,830		11,070		32,220
		1,740		3,940		680		330	6,690
6 mM naloxone	300		2,250		6,090		5,730		14,370
		740		660		90		550	2,040

[a] Stage CA2 R_F values are shown throughout.

[b] Identified as phosphatidylglycerol.

tained if the "chase" experiment was performed in the presence of levallorphan or levorphanol: in the former case components with R_F 0.6 again were eluted in fraction B as well as C. The new components are thus obtained not only when glycerol is incorporated in the presence of the drugs but also when previously labeled lipid turns over in their presence. Gale and Folkes (5) have shown previously that there is a net loss of radioactivity from the lipid fraction during "chase" experiments of this nature; Table 2 shows that this net loss is considerably increased when the chase is carried out in the presence of heroin or levallorphan. The "lost" radioactivity can be recovered quantitatively from the medium supernatant in all cases. The flux of glycerol through the lipid fraction is therefore increased in the presence of these drugs.

Effect of Removal of Drug

Experiments 3 and 4 of Table 2 show the effect of incubating cells with labeled glycerol in the presence of either heroin or levallorphan, centrifuging the cells down, and resuspending them in the absence of drug for a further period of incubation. In the heroin-treated cells, the second incubation in glucose and unlabeled glycerol resulted in a redistribution of radioactivity (CA1 and CA2) so that most now appeared in the normal positions for phosphatidylglycerol; there was considerable loss of radioactivity from the lipid fraction as a whole, the loss occurring mainly in the components running on silicic acid paper at R_F 0.45. A similar redistribution occurred when the second incubation took place in buffer alone, but the loss was then smaller. Similar changes were found with

TABLE 2

Effect of heroin and levallorphan on the distribution of ^{14}C-labeled glycerol in phospholipid fractions of S. aureus during lipid turnover

Cells were incubated in 10-mg quantities at a density of 0.2 mg dry weight per milliliter in buffered salts solution containing 0.3 mM glucose and 0.1 mM ^{14}C-labeled glycerol (specific activity, 1.57 mCi/mmole) with or without drugs as shown below. After 20 min at 37°, cells were centrifuged down and one 10-mg sample was taken for lipid examination; other samples were resuspended at 0.2 mg/ml for 20 min in (a) buffered salts solution alone, (b) buffered salts solution containing 0.3 mM glucose and 10 mM glycerol, (c) as in (b) + 30 mM heroin, or (d) as in (b) + 2 mM levallorphan. Fractionation and estimations were performed as described in Table 1.

Expt.	First incubation	Second incubation	CA1 fraction B (6% methanol), tubes 1–9		CA1 fraction C (15% methanol)				CA1 fraction D (40% methanol), tubes 1–5		Phospholipid	
					Tubes 1–3		Tubes 4–11					
			R_F 0.45[a]	R_F 0.6	R_F 0.45	R_F 0.6	R_F 0.45	R_F 0.6	R_F 0.45	R_F 0.6	Total	Loss
			cpm	cpm	cpm	cpm	cpm	cpm	cpm	cpm	cpm	cpm
1	No drug		910		1,420		15,860		6,500		24,690	
				130		80					210	
	No drug	(b)	820		1,250		10,770		7,900		20,740	
				100		500					600	3,560
	No drug	(c)	640		3,020		1,060		4,140		8,860	
				200		2,280		700		300	3,480	12,560
2	No drug		1,935		1,440		10,450		5,550		19,375	
				50		30					80	
	No drug	(b)	1,880		1,610		6,590		5,990		16,690	
				80		450		90			620	2,665
	No drug	(d)	1,770		3,780		1,230		370		7,150	
				2,360		4,450		350		50	7,210	5,095
3	30 mM heroin		2,850		14,980		4,190		4,350		26,370	
				1,050		4,320		290		130	5,790	
	30 mM heroin	(b)	290		730		9,070		8,890		18,980	
				2,910		2,410		430		250	6,000	7,180
	30 mM heroin	(a)	310		1,100		17,070		5,800		24,280	
				2,730		1,840		550		1,240	6,360	1,620
4	2 mM levallorphan		10,820		1,720		3,010		2,460		18,010	
				8,000		1,360		360		160	9,880	
	2 mM levallorphan	(b)	570		3,985		5,760		2,960		13,275	
				4,290		375		360		1,760	6,785	7,830
	2 mM levallorphan	(a)	1,260		600		1,750		4,620		8,230	
				5,900		1,660		530		160	8,250	11,410
	2 mM levallorphan	(d)	1,160		490		750		450		2,850	
				13,760		530		790		180	15,360	9,680

[a] Stage CA2 R_F values are shown throughout.

levallorphan-treated cells although, in the experiment quoted, the net loss was less when glucose and glycerol were present during the second incubation than when this took place in buffer alone. A further buildup of material running with R_F 0.6 took place when drug was present in the second incubation as well as the first.

Incorporation of ^{32}P-Phosphate

Experiments were carried out, as above, with ^{14}C-labeled glycerol and ^{32}P-phosphate in the presence and absence of heroin and levallorphan; the lipids were extracted and chromatographed (CA1 and CA2), and the ^{32}P:^{14}C ratio was determined on materials running with R_F 0.45 and 0.6 on CA2. In

controls without drug, the main phosphatidylglycerol (R_F 0.45) component gave a ratio of 3.7 (mean of six values; range 3.5–4.1); in the presence of either heroin or levallorphan, the R_F 0.45 components gave essentially the same ratio while the R_F 0.6 components gave 5.9 (n = 6; range, 4.1–6.7). Tables 1 and 2 show that small amounts of activity occur at R_F 0.6 in the controls; this gave a ^{32}P:^{14}C ratio of 6.5.

Nature of the New Components

Chromatographic examination of deacylated products. Preparations of phospholipid were made from cells incubated, as above, with ^{14}C-labeled glycerol in the presence and absence of heroin or related drugs. Fractions were examined by chromatography (CA1–CA6). Phosphatidylglycerol from control incubations without drug gave, at stage CA4 in 1-propanol–ammonia solvent, a single spot with R_F 0.52, corresponding to glycerophosphorylglycerol. Extracts from drug-treated cells gave two components at stage CA2, varying in the CA1 elution according to the drug as described above, and running with R_F values 0.45 and 0.6. At stage CA4, the CA2 components with R_F 0.45 all gave a single spot, R_F 0.52, corresponding to glycerophosphorylglycerol. The CA2 components with R_F 0.6 ran at stage CA4 with R_F 0.4, with sometimes a faint spot visible at R_F 0.25–0.3. Thus the H components gave rise to H1 (glycerophosphorylglycerol) and a second material, H2, with R_F 0.4 in CA4. No differences at stage CA4 were observed between the "new components" obtained after heroin treatment and those obtained after treatment with other drugs, despite the different CA1 elution profiles. The CA4, R_F 0.4 components (H2 and equivalent components obtained with the other drugs) ran in the three solvents listed in MATERIALS AND METHODS as CA4 (a–c) with the same R_F values as those for the minor phospholipid occurring in extracts from control cells and running with R_F 0.6 at stage CA2.

Glycerophosphorylglycerol, H1, H2, and the CA4 R_F 0.52 and 0.4 components were hydrolyzed in acid and rechromatographed in stages CA5 and CA6 using glycerol and glycerophosphate as markers. All gave glycerol and glycerophosphate after hydrolysis; in one series of experiments, the glycerophosphate to glycerol ratio of radioactivity for glycerophosphorylglycerol, H1, and CA4 R_F 0.52 was 1.15 (0.9–1.4, n = 7), and for H2 and CA4 R_F 0.4 it was 2.4 (1.7–3.0, n = 6) whether obtained from cells treated with heroin, levorphanol, or levallorphan.

The results so far indicate that lipid from heroin-treated cells contains phosphatidylglycerol and an H component that gives, after deacylation, glycerophosphorylglycerol and another substance hydrolyzing in acid to yield a mixture of glycerol and glycerophosphate but containing a higher content of phosphate than phosphatidylglycerol itself. The chromatographic properties of this second substance correspond to a minor component of the phospholipid from control cells; this component runs in the same position as diphosphatidylglycerol. A sample of synthetic triglycerol diphosphate was obtained from Professor Baddiley; deacylated H2 corresponded in R_F value with triglycerol diphosphate in 2-propanol–HCl, 2-propanol–ammonia, and 1-propanol–ammonia (CA4). The two substances co-chromatographed in the two-dimensional system CA7. The same results were obtained with the corresponding components from cells treated with levallorphan, levorphanol, naloxone, or dextrorphan.

Results with labeled heroin. The results described in the previous paragraphs suggest that heroin, and the related drugs used, complex with phosphatidylglycerol and that these complexes separate as new phospholipid components in some of the chromatographic procedures used. To test this possibility, cells were incubated with (a) glucose, unlabeled glycerol, and 20 mM (N-methyl-^{14}C)-heroin and, in parallel, (b) glucose, ^{14}C-labeled glycerol, and 20 mM unlabeled heroin. The lipids were extracted and chromatographed on silicic acid columns and papers as usual. Immediate adsorption of labeled heroin onto the cells occurred; at heroin concentrations less than 7 mM there was a linear relationship between the amount of drug adsorbed and its concentration in the medium, and no significant increase took place during incubation over 30 min at 37°. With 10 mM heroin, zero time adsorption amounted to about 750 mμmoles/mg dry

weight of cells, and this increased to 880 on incubation. On fractionation, the drug was extracted in the lipid fraction and radioactivity was eluted from silicic acid columns (CA1) in all fractions: 12% in A, 70% in B, 16% in C, and 2% in D. On chromatography in CA2, free heroin smeared from the origin to around R_F 0.2. Radioautograms of lipid fractions C and D from cells incubated with (*N*-methyl-^{14}C)-heroin showed, in addition to material near the origin, radioactivity corresponding in position to phosphatidylglycerol and the H component. On stage CA4 chromatography radioactivity was found in positions corresponding to H1 and H2. After elution and hydrolysis CA5 and CA6 showed radioactivity in positions corresponding to glycerophosphate and glycerol, none at the R_F of heroin or morphine. Prolonged acid hydrolysis liberated all the radioactivity as glycerol: it appeared that demethylation of the *N*-methyl-^{14}C group had taken place followed by incorporation of 1-^{14}C residues into glycerol.

A parallel experiment was carried out with (7,8-^{3}H)-dihydroheroin; in CA2 chromatograms heavy concentrations of ^{3}H were found near the origin and peaks of radioactivity were also found in areas corresponding to phosphatidylglycerol and H components; assuming that this radioactivity represented dihydroheroin or its nor-*N* derivative, the ratio of drug to glycerol incorporated varied from 0.2 to 3.5 in different experiments and with different components. The areas corresponding to the lipids were eluted, and the material was carried to stage CA4; between 50 and 80% of the ^{3}H now ran with an R_F equal to that of heroin markers, the remainder being located in the glycerophosphorylglycerol or H2 position. Acid hydrolysis showed that this latter activity was associated with glycerol or glycerophosphate so that a small amount of ^{3}H had been incorporated into glycerol residues. Comparing the molar ratios of (*N*-methyl-^{14}C)-heroin to (7,8-^{3}H)-dihydroheroin on parallel samples, material running near the origin (CA2) in fraction C gave a value of 1 against 0.13 for that in fraction D. It seems probable that some demethylation of heroin or dihydroheroin occurred in these experiments and that some of the nor derivative was associated with phosphatidylglycerol and diphosphatidylglycerol at stage CA2 but separated at stages CA3 and CA4.

It was of interest to know whether heroin was deacylated in the course of incubation with *S. aureus*, especially in view of the effect of heroin on acetate incorporation (Fig. 1b). After the experiment in which cells were incubated with (*N*-methyl-^{14}C)-heroin, radioactive material was eluted from the chromatogram origins (CA2) for lipid fractions A, B, and C and also was recovered from the incubation medium; in all cases the labeled material ran with heroin markers in the chromatographic system of Munier *et al.* (8).

Reaction of heroin with phosphatidylglycerol in vitro. There is strong absorption of heroin by staphylococcal cells and the drug is extracted along with the lipids in the methods used above. Consequently lipid extracted from heroin-treated cells comes into contact with the drug during the extraction procedures and subsequent fractionation. The question therefore arises whether the complexes observed between heroin (or related drugs) and lipid in the presence of staphylococci are produced as a result of metabolism or direct interaction. ^{14}C-Labeled phosphatidylglycerol was prepared by incubating cells with ^{14}C-labeled glycerol, as above, extracting the lipid, and fractionating the extract on silicic acid columns (CA1). Phosphatidylglycerol was then incubated in Tris buffer, pH 7.5, with heroin or levallorphan with and without a cell-free extract of staphylococci prepared as described under MATERIALS AND METHODS. It was possible to produce all the changes in CA1 elution profiles described in Fig. 3 by mixing ^{14}C-phosphatidylglycerol, cell extract, and heroin or levallorphan and extracting at zero time. With levallorphan the elution profile shown in Fig. 3b was obtained in the absence of cell extract; with heroin the zero time changes were enhanced by the presence of cell extract. The heroin profile in Fig. 3a was obtained in the presence of 3 mM heroin; if the concentration was raised to 10–30 mM, the CA1 profile resembled that obtained with 2 mM levallorphan with a highly active peak in in fraction B. When (*N*-methyl-^{14}C)-heroin was added with unlabeled phosphatidyl-

glycerol, the radioactivity profile corresponded to that obtained in parallel samples containing labeled phospholipid and unlabeled heroin. In the tests with heroin, incubation for 30 min at 37° in the presence of cell extract enhanced the change in profile from the normal phosphatidylglycerol distribution toward the "heroin picture"; with levallorphan incubation generally had the opposite effect, in that a change toward the control distribution took place. Eluates corresponding to profile peaks were combined and chromatographed as CA2; with two exceptions described below, only one radioactive spot appeared on radioautograms, and that corresponded to phosphatidylglycerol. The associations of drug and phosphatidylglycerol that give rise to the CA1 profiles thus dissociate at stage CA2. In two experiments some degree of turnover of phosphatidylglycerol occurred as judged from a loss of radioactivity from the lipid extract and the appearance of a trace of activity corresponding to diphosphatidylglycerol at stage CA2 radioautograms; in these experiments small amounts of the H components could be seen on CA2 radioautograms. The formation of H components—and similar materials with other drugs—follows upon turnover of phosphatidylglycerol with the production of diphosphatidylglycerol (5), whose CA1 elution profile is again altered in the presence of the morphine derivatives.

Action of dextrorphan. At a late stage in these investigations dextrorphan, the isomer of the levorphanon structure, became available and was tested in the various systems described above. At equimolar concentrations, no significant differences could be observed in the effects of the two substances on the uptake of aspartate, lysine, or proline, the incorporation of glycerol, the turnover of phospholipid, or reaction with phosphatidylglycerol *in vivo* and *in vitro*.

DISCUSSION

Previous results (1–3) have suggested that drugs such as heroin, levorphanol, and levallorphan may affect the bacterial membrane. The work described in this paper shows that these drugs, together with naloxone and dextrorphan, have three major effects on the lipid of *S. aureus*: (a) an initial increase in the rate of synthesis of phospholipid, (b) an increased rate of turnover of phosphatidylglycerol, and (c) an increased flux of glycerol through the lipid fraction. Chromatographic analysis of lipid extracted from drug-treated cells apparently showed the presence of new phospholipid components. The CA1 elution profiles of these extracts differed with the drug used for treatment, but it has been found that the profiles can be reproduced by mixing phosphatidylglycerol and drug *in vitro* and so presumably reflect the elution properties of phospholipid-drug complexes. The fact that cell extract enhanced the formation of these complexes in some cases may be due to changes in the partition of the drug between lipid and aqueous phases on addition of the extract. On CA2 chromatography, phospholipid extracts from drug-treated cells showed the presence of components other than phosphatidylglycerol, but deacylation and chromatography in more polar solvents resolved these into mixtures of glycerophosphorylglycerol and triglycerol diphosphate. The new component obtained after treatment of cells with heroin gave rise at stage CA4 to both glycerophosphorylglycerol (H1) and triglycerol diphosphate (H2), with the release of ^{3}H-labeled material when ^{3}H-dihydroheroin was initially present. Experiments with labeled heroin showed that strong, nonspecific absorption of drug by cells takes place and that the drug is removed in the lipid extracts, so giving rise to the possibility of complexes forming *in vitro*, while results with (7,8-^{3}H)-dihydroheroin suggest that part, at least, of the morphine nucleus becomes associated with phospholipid at stages CA1 and CA2. Diphosphatidylglycerol is formed during the turnover of phosphatidyglycerol in *S. aureus* (5), and turnover is increased by the presence of the drugs used. The CA1 elution profiles of both phospholipids are altered by the presence of these drugs and there is no reason to suppose that the "new components" are anything more than phospholipids separating in new positions in CA1 and CA2 as a result of associations with the drugs or their nor derivatives. Whether the formation of such complexes gives rise to the increased turnover in the presence of the drugs remains to

be investigated. It is clear that the morphine drugs can form lipid-soluble complexes with phospholipid, and it seems probable that changes in membrane properties would follow the formation of these substances *in vivo*.

No differences have been observed between the effects of the various morphine derivatives tested; of these, levallorphan and naloxone are morphine antagonists while dextrorphan is without analgesic or toxic action in man. It therefore appears that the effects described for growth-limiting concentrations of *S. aureus* are not related to the effects obtained, at considerably lower concentrations, on the nervous system in animals. It is perhaps not surprising that *S. aureus* lacks the refined specificity seen in the central nervous system, and it may be that alteration of phospholipid metabolism plays a part at a more specific level in the mammalian system. It is of interest that Mulé (9) observed increased turnover of phospholipids in guinea pig central cortex slices in the presence of 1–10 mM morphine or nalorphine but found the drugs to be without effect at 0.01–0.1 mM.

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