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The Formation of Choline *O*-Sulphate by *Pseudomonas* $C_{12}B$ and other *Pseudomonas* Species

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Pseudomonas $C_{12}B$ and other *Pseudomonas* species released larger amounts of a ^{35}S-labelled metabolite into the medium when cultured on growth-limiting concentrations of Na_2SO_4 as opposed to growth in SO_4^{2-}-sufficient media. The metabolite was found at all stages of the culture cycle of *Pseudomonas* $C_{12}B$ and maximum quantities occurred in stationary-phase culture supernatants. The metabolite was not detected when the bacterium was cultured on growth-limiting concentrations of potassium phosphate. The amount of the metabolite present in the medium greatly exceeded that which could be extracted from intact cells and, except for choline chloride, it was independent of the carbon source used for growth. If choline chloride was present in high concentration, then larger amounts of the metabolite were found in the culture medium. The metabolite was not detected extracellularly or intracellularly when the bacterium was grown in SO_4^{2-}-deficient media containing 5mM-L-cysteine. The same metabolite was also synthesized *in vitro* only when *Pseudomonas* $C_{12}B$ extracts were incubated with choline chloride, ATP, $MgCl_2$ and $Na_2{}^{35}SO_4$. The metabolite-forming system was not subject to repression by Na_2SO_4 and was completely inhibited by 0.5mM-L-cysteine and activated by Na_2SO_4 (up to 1.0mM). The metabolite was identified as choline *O*-sulphate by electrophoresis, chromatography and isotope-dilution analysis. Another ^{35}S-labelled metabolite was also detected in culture supernatants, but was not identified.

Choline *O*-sulphate has a wide distribution in nature and has been detected in high concentration in plants (Nissen & Benson, 1961), lichens (Lindberg, 1955*a*; Harper & Letcher, 1966), red algae (Lindberg, 1955*b*), and in spores and mycelia of the higher fungi (Woolley & Peterson, 1937; De Flines, 1955; Stevens & Vohra, 1955; Kaji & McElroy, 1958; Harada & Spencer, 1960; Takebe, 1960; Ballio *et al.*, 1960). The ester was not detected in fungi representative of the Mucorales and Endomycetales or in various bacteria of diverse genera (including *Pseudomonas*) when these microorganisms were grown in chemically undefined culture media (Harada & Spencer, 1960). Similar negative results were also observed in fungi by Ballio *et al.* (1960).

The purpose of the present investigation was two-fold: (*a*) to identify choline *O*-sulphate as a metabolite which was synthesized by a number of *Pseudomonas* strains and (*b*) through studies on the regulation of choline *O*-sulphate formation in *Pseudomonas* $C_{12}B$, to provide a possible explanation as to why the ester was not detected previously in this genus.

Materials and Methods

Bacteria

Unless otherwise indicated *Pseudomonas* $C_{12}B$ (Payne, 1963), an isolate from soil enriched with sodium dodecyl sulphate (Payne & Feisal, 1963), was used throughout. *Pseudomonas aeruginosa* strains O.S.U. 64 and Holloway no. 1 were obtained through the courtesy of Professor R. G. Eagon of this department. Other bacteria were obtained from the National Collection of Industrial Bacteria (N.C.I.B.), Torry Research Station, Aberdeen, U.K., or the American Type Culture Collection (A.T.C.C.), Rockville, Md., U.S.A.

Culture conditions

Bacteria were maintained on nutrient agar slants (Difco Laboratories, Detroit, Mich., U.S.A.) at room temperature with monthly transfers. Inocula were prepared from cells cultured in nutrient broth (Difco) for 16h at 30°C and washed twice with sterile deionized water. The size of each inoculum was adjusted so that after inoculation the standard medium had an initial extinction of approx. 0.02. Bacteria were grown at 30°C with shaking in the standard medium which contained carbon and sulphur in the form and concentration described for each experiment. The other components of this medium were as follows: KH_2PO_4 (1.5g), K_2HPO_4 (3.5g), NH_4Cl (0.5g), $MgCl_2,6H_2O$ (0.15g), NaCl (0.5g) and deionized water (1 litre). Phosphates, sulphates and chlorides were autoclaved separately; carbon sources, other sulphur sources and $Na_2{}^{35}SO_4$ were sterilized by Millipore filtration. The pH of the medium was 7.0.

Formation of metabolites A and B in vivo

Unless otherwise indicated, 0.5μl of an aqueous solution of $Na_2{}^{35}SO_4$ (sp. radioactivity approx. 50mCi/mmol) was added per ml to each culture medium to give a final concentration of 0.01mM.

For studies designed solely to demonstrate the presence of metabolites A and B in culture media, bacteria were cultured in 20ml of the standard medium in 125ml Erlenmeyer flasks fitted with side arm cuvettes. Growth was measured at 650nm with a Bausch and Lomb Spectronic 20 spectrophotometer. At various time-intervals after inoculation, samples (0.3ml) of the culture medium were taken and centrifuged. Depending upon the amount of radioactivity present, between 5 and 30μl of the clear supernatant was subjected to electrophoresis.

For studies designed to assess the occurrence of metabolite A both in the medium as well as in cell extracts, bacteria were grown in 500ml Erlenmeyer flasks each containing 250ml of standard medium. Growth was measured at 650nm with an Hitachi Perkin–Elmer u.v.-vis spectrophotometer model 139. At the termination of growth cells were collected by centrifugation and washed once with 5ml of uninoculated medium lacking $Na_2{}^{35}SO_4$. The wash fluid was shown by electrophoresis to contain the same relative proportion of ^{35}S-labelled components as that present in the culture medium. The cell pellet was then resuspended in 2ml of cold (5–10°C) water by rapid agitation for 10min with a Vortex mixer. The smooth cell suspension was centrifuged and the cell pellet was treated with water as before except that the cells were left in contact with water overnight (approx. 16h) at 4°C. Supernatants obtained by centrifuging each cell suspension were combined and concentrated to 0.5ml by freeze-drying. Concentrates were rendered non-mucoid by the addition of 0.1ml of trichloroacetic acid (2%, w/v) followed by centrifugation at 4°C. The resulting concentrate is termed the cold water extract.

Hot water extracts were prepared as follows: the cold water extracted cell pellet was resuspended in 3ml of hot (70–80°C) water and maintained at that temperature for 5min with occasional stirring. Cell debris was removed by centrifugation at 4°C and the cloudy supernatant was treated with 0.5ml of cold trichloroacetic acid (4%, w/v) and re-centrifuged at 4°C. The cell debris pellet was treated with hot water as before and the trichloroacetic acid supernatants were combined and concentrated to 0.5ml by freeze-drying to yield a hot-water-extract concentrate. Cell debris which remained after hot water extraction was further treated directly with 0.2ml of trichloroacetic acid (4%, w/v) and centrifuged at 4°C. Electrophoresis of 30μl of the supernatant did not reveal the presence of metabolite A.

Concentrates (cold and hot water extracts) were stored at 4°C and metabolite A was stable under this condition for at least 3 weeks. Extraction of pooled cold- and hot-water-extract concentrates with diethyl ether to remove trichloroacetic acid (see Fitzgerald & Dodgson, 1971*b*), did not increase the quantity of metabolite A present in the extract. Moreover, an aqueous solution of authentic choline *O*-sulphate (1%, w/v) containing the same concentration of trichloroacetic acid was found to be stable to acid hydrolysis even after incubation at 50°C for 2h. The $BaCl_2$–gelatin method (Dodgson, 1961) was employed to detect inorganic $SO_4{}^{2-}$ ion release under these conditions.

Formation of metabolite A in vitro

Pseudomonas $C_{12}B$ was grown in 1-litre Erlenmeyer flasks each containing 400ml of standard medium. Bacteria were collected by centrifugation (4°C) at the termination of growth (turbidimetric measurement at 650nm). Cells were immediately suspended in 1ml of appropriate buffer (2–5°C) without washing and ruptured by passage through a chilled (2–5°C) French pressure cell operating at 138MPa (20000lb/in^2). Treatment was continued until broken-cell suspensions became translucent and non-mucoid (usually three passes were required). Cell debris was removed by centrifugation in a Sorvall RC2-B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn., U.S.A.) employing the SS-34 rotor. The rotor was operated for 1h at 2°C and 23628*g* ($r_{av.}$ 3.25in). Clear supernatants were then assayed directly or dialysed at 4°C against 1 litre of the same buffer changed six times during a period of 6h.

After dialysis, extracts were immediately assayed for the ability to form metabolite A by incubating 200μl of extract with 200μl of complete assay medium at 34°C for 6h. The reaction was terminated by the addition of 50μl of trichloroacetic acid (4%, w/v) and the reaction mixture was clarified by centrifugation. Samples (5μl) of the clear supernatant were subjected to electrophoresis. The composition of the complete assay medium was as follows: ATP (disodium salt, crystalline, 0.03g); choline chloride (crystalline, 0.12g); $MgCl_2,6H_2O$ (0.15%, w/v, 2ml); $Na_2{}^{35}SO_4$ (sp. radioactivity approx. 916mCi/mmol, 10μl) and 0.1M-K_2HPO_4–KH_2PO_4 buffer, pH7.0 (2ml).

Electrophoresis and chromatography

The identification and quantitation of metabolite A was assessed by electrophoresis of samples on Whatman no. 1 paper for 45min at 400V in 0.1M-sodium acetate–acetic acid buffer, pH4.5, or 0.1M-ammonium acetate, pH8.6. Samples with low salt content were further assessed for the presence of metabolite A by ascending chromatography on Whatman no. 1 paper at 23–25°C. The following

solvents and running times were employed: solvent 1, phenol–water (8:2, v/v), 24h; solvent 2, acetone–water (9:1, v/v), 3h; solvent 3, butan-1-ol–acetic acid–water (4:1:2, by vol.), 24h. Localization and quantitative determination of radioactive areas on papers were as described by Dodgson *et al.* (1961).

Sulphate esters

Unlabelled choline *O*-sulphate was prepared by the method of Schmidt & Wagner (1904). The i.r. spectrum of the product was identical with that of choline *O*-sulphate published by Takebe (1960). Choline *O*-[^{35}S]sulphate was prepared by using 3mCi of $Na_2{}^{35}SO_4$ (sp. radioactivity approx. 50mCi/mmol) by the method of Segel & Johnson (1963) as modified by Spencer *et al.* (1968). A sample of an aqueous solution of the product was mixed with unlabelled choline *O*-sulphate and recrystallized four times from ethanol–water (3:1, v/v). Constant specific radioactivity was maintained throughout.

Protein determination

The method of Lowry *et al.* (1951) was employed to determine soluble protein in cell extracts. Dry bovine serum albumin, fraction V powder (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), was used as the standard.

Experimental and Results

Formation of metabolites A and B in vivo by Pseudomonas $C_{12}B$

Electrophoresis of a cell-free culture fluid sample, taken at the termination of growth in the presence of 0.1mM-$Na_2{}^{35}SO_4$ (mixture of 0.01mM labelled and 0.09mM unlabelled compound) revealed the presence of three ^{35}S-labelled components. One component (metabolite A) remained at the origin and evidence for its identity as choline *O*-sulphate will be presented below. The component with the greatest anodic mobility was identified by coincidence of migration as inorganic $^{35}SO_4{}^{2-}$. This result was confirmed by electrophoresis of the culture fluid sample in a 0.1M-barium acetate–acetic acid buffer, pH4.5. Under this circumstance, inorganic $SO_4{}^{2-}$ remain at the origin (see Fitzgerald & Dodgson, 1971*a*). A third ^{35}S-labelled component (metabolite B, electrophoretic mobility relative to inorganic $SO_4{}^{2-}$ 0.48) has not as yet been identified.

Metabolites A and B were present in the culture medium in quantities as high as 91.2% and 18.6% of the total radioactivity respectively, when *Pseudomonas* $C_{12}B$ was cultured on growth-limiting concentrations of $Na_2{}^{35}SO_4$ (Table 1). Under these cultural conditions, inorganic $^{35}SO_4{}^{2-}$ could not be detected. The percentage of metabolite A in culture media declined sharply when the bacterium was grown in the presence of Na_2SO_4 concentrations (>0.01mM) which

Table 1. *Influence of Na_2SO_4 concentration of the culture medium on the growth and the release of metabolites A and B into the culture medium by Pseudomonas $C_{12}B$*

Bacteria were grown to the stationary phase in the standard medium containing sodium citrate (1%, w/v) and $Na_2{}^{35}SO_4$. For concentrations of $Na_2{}^{35}SO_4$ >0.01mM, unlabelled Na_2SO_4 was mixed with 0.01mM-$Na_2{}^{35}SO_4$ to give the final stated concentration. All other concentrations were prepared from appropriate dilutions of $Na_2{}^{35}SO_4$ alone.

$10^{-1}\times Na_2SO_4$ concn. (mM)	Release of ^{35}S-labelled metabolites (% total ^{35}S in medium)			
	Metabolite			
	A	B	$^{35}SO_4{}^{2-}$	E_{650} of culture
—	—	—	—	0.21
0.02	82.5	17.5	0	0.56
0.04	84.5	15.5	0	0.56
0.06	81.4	18.5	0	0.78
0.10	91.2	8.8	0	1.22
0.50	22.9	35.2	41.9	1.38
1.0	11.4	26.9	61.7	1.35
10.0	1.5	3.6	94.9	1.35
20.0	0.6	1.3	98.1	1.38
30.0	0.0	0.0	100.0	1.35

allowed the attainment of maximum culture extinction values (Table 1). As expected, the percentage of inorganic $^{35}SO_4^{2-}$ ions found in the medium after stationary-phase growth increased with the increasing concentrations of Na_2SO_4 supplied initially. The effect of Na_2SO_4 on the occurrence of metabolite B was similar, except that the percentage of this component was highest in a culture medium in which the Na_2SO_4 concentration was sufficient for maximum growth (Table 1). Metabolites A and B were not detected in stationary-phase culture supernatants when *Pseudomonas* $C_{12}B$ was cultured on growth-limiting concentrations of inorganic PO_4^{3-} in SO_4^{2-}-sufficient culture media (3.0mM-Na_2SO_4).

Fig. 1 shows that the percentages of metabolites A and B in the culture medium increased sharply during mid-exponential-phase growth on 0.01 mM-$Na_2{}^{35}SO_4$. The release of metabolite A into the medium increased at a decreased rate thereafter and reached a maximum value at the termination of growth. In contrast, the percentage of metabolite B present in the medium decreased with increased growth. It should also be noted that metabolite A was detected in culture supernatants at the first indication of visible growth (Fig. 1).

Metabolite A could also be extracted from intact cells by suspending washed bacteria in cold or hot water (Table 2, see the Materials and Methods section for details). Owing to differences in total radioactivity, the amount of this metabolite present in culture supernatants was always considerably higher than the quantity that could be extracted from intact bacteria. Table 2 shows that the percentage of metabolite A present in cell extracts as well as culture supernatants decreased as bacteria were grown on increasing concentrations of Na_2SO_4. Except for choline chloride, the effect of Na_2SO_4 on metabolite A production was independent of the carbon source used for growth (Table 2). Moreover, the same relative proportions of the metabolite were found intracellularly and extracellularly when bacteria were grown in the presence of either sodium citrate, sodium acetate or asparagine. These observations suggest that it is unlikely that the high content of metabolite A in culture medium was due to altered cell permeability

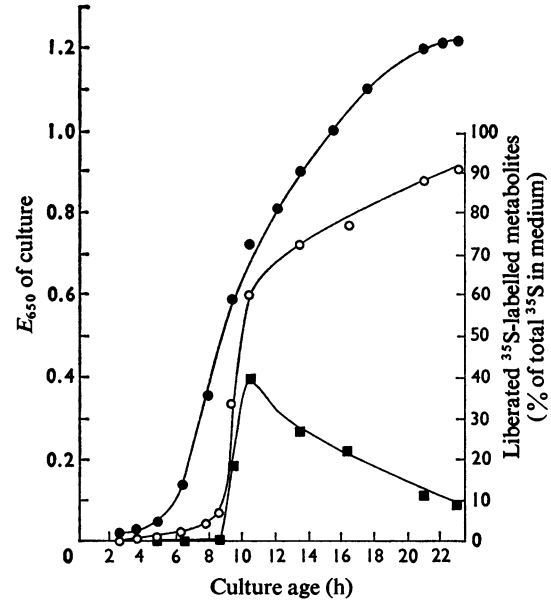

Fig. 1. *Relationship between culture extinction values and the liberation of some ^{35}S-labelled metabolites into the medium by Pseudomonas $C_{12}B$*

●, E_{650} of culture; ○, metabolite A; ■, metabolite B. Bacteria were grown in the standard medium containing sodium citrate (1%, w/v) and 0.01mM-$Na_2{}^{35}SO_4$ as the sole sources of carbon and sulphur.

Table 2. *Influence of some culture conditions on the formation of metabolite A in vivo by Pseudomonas $C_{12}B$*

Bacteria were grown to the stationary phase in the standard medium containing the stated compound as the sole carbon source (1%, w/v) and $Na_2{}^{35}SO_4$ as the sulphur source. $Na_2{}^{35}SO_4$ (0.1mM) was mixed with unlabelled Na_2SO_4 to give the final concentrations shown below.

Carbon source	$Na_2{}^{35}SO_4$ concn. (mM)	Metabolite A (% total ^{35}S) Culture medium	Cellular cold water extract	Cellular hot water extract
Citrate	1.5	0.9	0.5	1.5
Choline	1.5	14.9	3.2	2.7
Citrate	0.15	9.4	1.2	4.6
Citrate	0.05	24.3	6.1	4.3
Acetate	0.05	21.9	5.4	3.7
Citrate	0.02	49.2	11.4	10.2
Asparagine	0.02	57.4	8.3	5.4

as a consequence of growth in the presence of sodium citrate. This chelating agent tends to induce 'leakiness' in other pseudomonads especially at a concentration as high as that used in the present study (R. G. Eagon, personal communication). Finally, the presence of 5mM-L-methionine in culture media containing sodium citrate (1%, w/v) and 0.02mM-$Na_2{}^{35}SO_4$ caused only a small decrease in the formation of metabolite A when compared with determinations made on culture media and extracts after growth in the absence of this amino acid (4.3% and 1.1% decrease in culture medium content and hot-water-extract content of metabolite A, respectively). In contrast, metabolite A could not be detected in culture supernatants or cell extracts in a parallel experiment in which 5mM-L-cysteine hydrochloride (pH7.0) replaced L-methionine.

A 15-fold increase in percentage of metabolite A in the culture medium was noted after growth of *Pseudomonas* $C_{12}B$ with choline chloride as the sole carbon source in a culture medium containing a relatively high concentration of Na_2SO_4 (1.5mM, Table 2). The quantity of metabolite A that could be extracted from intact cells was also higher than that extracted from bacteria grown on sodium citrate as the only carbon source. Results of a further examination of the influence of choline chloride (Table 3) revealed that *Pseudomonas* $C_{12}B$ formed approximately twice as much of this metabolite when grown in sulphur-sufficient media containing sodium citrate and choline chloride (1%, w/v) than bacteria grown in the same medium lacking choline chloride. It should be noted that the percentage of metabolite B in culture supernatants remained relatively constant regardless of the presence or absence of choline chloride (Table 3).

Formation of metabolite A in vivo by other Pseudomonas species

The capacity for metabolite A production is not confined to *Pseudomonas* $C_{12}B$. Of a number of arbitrarily selected *Pseudomonas* strains, all were found to liberate a ^{35}S-labelled compound into the culture medium which remained at the origin after electrophoresis (metabolite A, Table 4). The concentration of Na_2SO_4 selected to act as the sole sulphur source was growth-limiting only for *P. reptilivora* and *P. stutzeri* and these two species formed the highest amounts of metabolite A. Although the percentage of $^{35}SO_4{}^{2-}$ remaining in the medium at the termination of growth varied, the quantity of metabolite A in culture supernatants was approximately the same after growth of the other strains tested. The remainder of the ^{35}S content of each culture medium consisted of two other components (electrophoretic mobilities relative to inorganic $^{35}SO_4{}^{2-}$ 0.44 and 0.65 respectively).

Formation of metabolite A in vitro by Pseudomonas $C_{12}B$

In good agreement with results on the formation of choline *O*-sulphate by *Aspergillus oryzae* (Spencer & Harada, 1960), extracts of *Pseudomonas* $C_{12}B$ formed metabolite A only in the presence of choline chloride, ATP, $MgCl_2$ and $Na_2{}^{35}SO_4$ (Table 5). A deletion of any one component from the assay medium resulted in a significant decrease in the quantity of metabolite A detected even when undialysed extracts were employed (i.e. 69.6% and 89.6% decreases observed when choline chloride was omitted with undialysed and dialysed extracts, respectively). Dialysis of extracts in the presence or absence of 1mM-2-mercaptoethanol resulted in a 76% increase in metabolite A-forming activity. However, extracts were very unstable losing most of their activity after 48h even when prepared in and dialysed against buffer containing 1mM-2-mercaptoethanol. Metabolite A was not formed after incubation of extracts in the presence of 0.05mM-L-cysteine hydrochloride (pH7.0) or after incubation of extracts dialysed against buffer containing this amino acid. In contrast,

Table 3. *Influence of choline chloride concentration of the culture medium on the formation of metabolite A in vivo by Pseudomonas $C_{12}B$*

Bacteria were grown to the stationary phase in the standard medium containing 0.05mM-$Na_2{}^{35}SO_4$ (mixture of 0.04mM unlabelled and 0.01mM labelled compound), sodium citrate (1%, w/v) and the stated concentrations of choline chloride. Cells were extracted with hot water only.

Concn. of choline chloride (%, w/v)	Metabolite A (% total ^{35}S)		Metabolite B (% total ^{35}S) in culture medium
	Culture medium	Cellular hot water extract	
—	21.8	8.9	38.1
0.1	20.6	8.2	37.2
0.5	27.8	10.3	38.6
1.0	54.8	21.6	36.3

Table 4. *Formation of metabolite A by some Pseudomonas species*

Bacteria were grown to the stationary phase in the standard medium containing sodium acetate (1%, w/v) and 0.07mM-$Na_2{}^{35}SO_4$ (mixture of 0.06mM unlabelled and 0.01mM labelled compound) as the sole sources of carbon and sulphur.

Species	Source and strain	Formation of ^{35}S-labelled metabolites (% total ^{35}S in culture medium)		E_{650} of culture
		Metabolite A	$^{35}SO_4{}^{2-}$	
P. reptilivora	A.T.C.C. 14836	81.1	0	0.48
P. stutzeri	A.T.C.C. 11607	57.1	0	0.35
P. fluorescens	N.C.I.B. 8248	14.6	43.5	1.21
P. aeruginosa	A.T.C.C. 10145	23.4	17.8	1.15
P. aeruginosa	N.C.I.B. 9685	14.8	19.4	1.30
P. aeruginosa	O.S.U. 64	20.9	27.2	1.05
P. aeruginosa	Holloway no. 1	12.3	34.4	0.65

Table 5. *Formation of metabolite A by extracts of Pseudomonas $C_{12}B$*

Bacteria were grown to the stationary phase in the standard medium containing sodium citrate (1%, w/v) and 0.01mM-Na_2SO_4 as the sole source of carbon and sulphur. The protein concentration of the extract was approx. 5mg/ml. See the text for assay procedure and composition of complete assay medium.

Assay conditions	Metabolite A (% total ^{35}S in incubation mixture)
Complete (extract dialysed against 25mM-K_2HPO_4–KH_2PO_4 buffer, pH7.0)	9.6
Minus $MgCl_2$	4.8
Minus $MgCl_2$, plus 1.0mM-EDTA	0.0
Minus ATP	0.3
Minus choline chloride	1.0
Complete (after storage of extract for 48h)	
at −20°C	1.1
at 2°C	3.1
Complete (extract dialysed against buffer containing 1mM-2-mercaptoethanol)	10.9
Complete (after storage of extract for 48h)	
at −20°C	2.3
at 2°C	3.4
Complete (extract dialysed against buffer containing 1mM-L-cysteine hydrochloride, pH7.0)	0.0
Complete (extract not dialysed)	2.3
Minus choline chloride	0.7

Orsi & Spencer (1964) found that the choline sulphokinase activity of *Aspergillus nidulans*, although highly unstable, was enhanced by 78% when extracts were prepared in the same buffer containing either 2-mercaptoethanol or cysteine.

In an attempt to correlate the formation of metabolite A *in vitro* with its production *in vivo* (see Table 1), *Pseudomonas* $C_{12}B$ was grown in the presence of increasing concentrations of Na_2SO_4 and extracts were assayed for metabolite A-forming activity. The results of this study (Table 6) show that metabolite A was formed by dialysed extracts derived from bacteria grown in the presence of Na_2SO_4 concentrations as high as 10mM. The variability in the quantity of metabolite A detected with each extract (Table 6) may reflect the instability of the metabolite A-forming system. Nevertheless, these results appear to exclude the possibility that inorganic $SO_4{}^{2-}$ (or its metabolic derivatives) repress the synthesis of the metabolite A-forming system. Moreover, this system was not particularly sensitive to inhibition by excess of inorganic $SO_4{}^{2-}$ (Table 7).

Table 6. *Influence of the Na_2SO_4 concentration of the culture medium on the formation of metabolite A in vitro by Pseudomonas $C_{12}B$*

Bacteria were grown to the stationary phase in the standard medium containing sodium citrate (1%, w/v) and the stated concentrations of Na_2SO_4 as the sole carbon and sulphur sources. Extracts were dialysed against 25mM-K_2HPO_4–KH_2PO_4 buffer, pH7.0, containing 1mM-2-mercaptoethanol. The protein concn. of each extract was adjusted to approx. 5mg/ml with the same buffer and extracts were assayed for metabolite A formation by using the complete assay medium as described in the text.

Na_2SO_4 concn. (mM)	Metabolite A (% total ^{35}S in incubation mixture)
0.01	11.6
0.1	8.4
0.4	12.3
1.0	3.1
2.5	4.6
5.0	6.3
10.0	8.4

Table 7. *Influence of the Na_2SO_4 concentration of the assay incubation medium on the formation of ^{35}S-labelled metabolite A by an extract of Pseudomonas $C_{12}B$*

Bacteria were grown to the stationary phase in the standard medium containing sodium citrate (1%, w/v) and 0.01mM-Na_2SO_4 as the sole carbon and sulphur sources. The extract was dialysed against 25mM-K_2HPO_4–KH_2PO_4 buffer, pH7.0, containing 1mM-2-mercaptoethanol. The protein concentration of the extract was adjusted to approx. 5mg/ml with the same buffer and the extract was assayed for metabolite A formation by using the complete assay medium as described in the text.

Na_2SO_4 concn. (mM)	Metabolite A (% total ^{35}S in incubation mixture)
—	6.1
0.15	8.8
0.30	14.4
1.0	11.1
3.0	8.3
4.0	5.6
8.0	0.8
10.0	0.0

Identification of metabolite A

The obligatory requirement of choline chloride for metabolite A formation *in vitro*, the ease with which the metabolite can be extracted from intact cells (cf. Harada & Spencer, 1960) and the finding that the inclusion of choline chloride in culture media enhanced the formation of metabolite A *in vivo* suggest its identity as choline *O*-sulphate. This possibility was confirmed in a number of instances in the following manner. Metabolite A, present in culture medium supernatants and incubation mixtures, was separated from other ^{35}S-labelled components by successive passage of samples through pads of Dowex-50 resin (H^+ form) formed on the surfaces of Büchner funnels lined with filter paper. Resins were washed twice with deionized water and filtrates were separated at the pump. To ensure that the cation concentration had been minimized, the final pooled acidic filtrate was neutralized with 0.1M-KOH and passed again through pads of the same resin until the pH of the pooled filtrate was approx. 5.6 (pH of deionized water used). The pooled filtrate was concentrated *in vacuo* at 65°C and its anion concentration was minimized by successive passage through pads of Dowex-1 resin (OH^- form). The pH of the pooled alkaline filtrate was adjusted to 7.0 with 0.1M-HCl and again passed through another pad of the same resin. The filtrate (pH7.0) was concentrated *in vacuo* at 65°C. A ^{35}S-labelled component was found to remain at the origin after electrophoresis of samples of the concentrate at pH4.5 and 8.6. Inorganic $^{35}SO_4^{2-}$ was detected after electrophoresis in sodium and barium acetate buffers of a sample of the concentrate that had been boiled in 3M-HCl to hydrolyse the sulphate ester bond. A single peak of radioactivity was detected after scanning of chromatograms of mixtures of the concentrate and authentic choline *O*-[^{35}S]sulphate developed in solvents 1, 2 and 3 (see the Materials and Methods section). Further confirmation of the identity of metabolite A was obtained by isotope-dilution analysis. A sample of the concentrate containing the ^{35}S-labelled metabolite was mixed with 1.0g of authentic choline *O*-sulphate and the whole recrystallized from ethanol–water (1:3, v/v) six times. Although roughly 30% of the existing solid was lost to the mother liquor at each crystallization, constant specific radioactivity was maintained throughout. This latter technique was also applied directly to confirm the identity of metabolite A present in culture medium supernatants in which this metabolite represented the major component (>80% of the total ^{35}S). The presence of choline *O*-sulphate (metabolite A) in cold-water-extract concentrates was detected as a routine by chromatography of the concentrate in solvent 1 [see Harada & Spencer (1960) for the resolving capacity of this solvent]. A ^{35}S-labelled component with the same mobility as authentic choline *O*-[^{35}S]sulphate (R_F 0.94–0.96) was detected and constituted approximately the same proportion of the total radioactivity as that determined by electrophoresis at pH4.5.

Discussion

Reports on the formation of sulphate esters by bacteria are rare. Apart from publications dealing with the identification of a sulphated glycolipid in *Halobacterium cutirubrum* (Kates *et al.*, 1967) and in some *Mycobacterium* species (Goren, 1970, 1971), results of the present study represent the only other instance of the occurrence of a sulphate ester in bacteria.

The higher fungi can accumulate large quantities (0.2–1.5% dry wt.) of choline *O*-sulphate (De Flines, 1955; Stevens & Vohra, 1955; Takebe, 1960) and very little of it occurs extracellularly (Harada & Spencer, 1960). These data and other evidence (see also Takebe & Yanagita, 1959; Takebe, 1960) prompted Spencer & Harada (1960) to suggest that choline *O*-sulphate acts as a storage form of sulphate in these fungi. It is unlikely, however, that a similar physiological role can be ascribed to this ester in *Pseudomonas* since most of the choline *O*-sulphate that is synthesized by these bacteria is released into the culture medium. This finding was not unexpected. The storage of large quantities of a compound in an unpolymerized form imposes severe osmotic pressures on the cell surface especially of gram negative bacteria characteristic of the *Pseudomonas* genus. Species of this genus are prone to releasing a wide variety of utilizable metabolites into the culture medium throughout their entire culture cycle. Further experimentation often reveals that the excreted compounds can serve as carbon and/or sulphur sources for growth after subculture of the particular species (see, e.g. Gunsalus *et al.*, 1955; De Ley, 1960; Fitzgerald & Dodgson, 1970, 1971*a*,*b*).

It was surprising to find that *Pseudomonas* $C_{12}B$ formed large amounts of choline *O*-sulphate under conditions of SO_4^{2-} deprivation. Certainly, this unusual result was not restricted to this ester since another ^{35}S-labelled metabolite (metabolite B) was also formed and released into the culture medium when Na_2SO_4 was growth-limiting. Again this observation is not consistent with a sulphur storage function for choline *O*-sulphate in this bacterium. However, it may explain why the ester was not detected previously in *P. pyocyanus* and *P. fluorescens* N.C.T.C. 4725 (Harada & Spencer, 1960). These species were grown in chemically undefined media supplemented with 4.2mM-Na_2SO_4; a concentration at which choline *O*-sulphate formation was not detected with any of the eight *Pseudomonas* strains tested in the present study. Nevertheless, when the Na_2SO_4 concentration was lowered to 0.07mM, ester formation was detected with all of these latter strains (including *P. fluorescens* N.C.I.B. 8248, see Table 4).

The observation that *Pseudomonas* $C_{12}B$ formed maximum amounts of choline *O*-sulphate *in vivo* when grown in the presence of growth-limiting concentrations of SO_4^{2-} but not PO_4^{3-} was noteworthy in that it poses a problem which can best be stated in the form of a question. Why does this bacterium form the ester and release it into the medium under conditions in which it would be expected that the inorganic SO_4^{2-} ions in the medium would be used for growth rather than for choline *O*-sulphate formation? At first sight, it would appear that inorganic SO_4^{2-} might either repress the synthesis of (or inhibit the activity of) the choline *O*-sulphate-forming system. The formation of arylsulphatase (arylsulphate sulphohydrolase, EC 3.1.6.1) is known to be regulated in an analogous manner in this isolate (Fitzgerald & Payne, 1972). However the data of Tables 6 and 7 obtained with dialysed extracts appear to eliminate these possibilities, although it should be recognized that the formation *in vitro* of choline *O*-sulphate was not detected with undialysed extracts derived from bacteria grown on Na_2SO_4 concentrations exceeding 0.01mM (see also Table 5). This latter observation offers a clue to a partial explanation of the problem. Growth in the presence of Na_2SO_4 may generate a diffusible inhibitor(s) of the choline *O*-sulphate-forming system *in vivo* and *in vitro*. This inhibitor would be required for growth and would be depleted under cultural conditions in which SO_4^{2-} is growth limiting so allowing the formation of maximum amounts of the ester. When the concentration of SO_4^{2-} in the medium is in excess of growth requirements (SO_4^{2-}-sufficient media) the inhibitor would accumulate intracellularly and would inhibit choline *O*-sulphate formation *in vivo*. Of course the exact nature of the inhibitor(s) is unknown, but it might be predicted to be a metabolic derivative of SO_4^{2-} (i.e. cysteine). Although the components of the cysteine pathway have not been discerned in *Pseudomonas* $C_{12}B$, inorganic SO_4^{2-} is known to be converted into cysteine in *E. coli* (Roberts *et al.*, 1957). The observation that cysteine completely inhibits the formation of choline *O*-sulphate by *Pseudomonas* $C_{12}B$ *in vivo*, as well as *in vitro*, is consistent with its being one of the inhibitors in question.

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