Microbial Growth on C₁ Compounds

THE ROLE OF FOLATE IN THE METABOLISM OF *PSEUDOMONAS* AM1

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(Received 28 September 1965)

1. The growth of *Pseudomonas* AM1 is much more sensitive to inhibition by sulphanilamide when methanol, rather than succinate, acts as the sole carbon and energy source; a sulphanilamide concentration of 1 mM, which causes almost complete inhibition of growth on methanol, has little effect in a succinate medium. 2. Similar results have been obtained with sulphadiazine and sulphathiazole. Sulphanilic acid has little effect. 3. A similar differential sensitivity to sulphanilamide is shown by *Protaminobacter ruber* and *Pseudomonas extorquens* when grown on methanol media as compared with succinate. 4. Sulphanilamide inhibited the growth of *Pseudomonas oxalaticus* on formate, oxalate and succinate media to about the same extent. 5. Inhibition of growth of *Pseudomonas* AM1 by sulphanilamide is accompanied by an accumulation of glycine in the cells. 6. Inhibition of growth by sulphanilamide can be reversed by *p*-aminobenzoic acid. 7. Microbiological assays of the folate content of *Pseudomonas* AM1 have been performed after growth on both methanol and succinate, and the results are discussed in terms of differences in metabolism.

Peel & Quayle (1961) have described the isolation of an aerobic organism, *Pseudomonas* AM1, which can utilize methanol as sole carbon source, the oxidation of this compound providing the energy supply (Quayle, 1963). The organism is also capable of growth on a wide variety of carbon compounds, including succinate, as sole source of carbon and energy.

Kinetic experiments have shown the first stable product of [¹⁴C]methanol fixation by whole cells to be serine (Large, Peel & Quayle, 1961); the pathway of carbon assimilation is thought to involve the condensation of glycine with a C₁ unit derived from methanol (Large, Peel & Quayle, 1962). Growth of a similar organism *Pseudomonas* PRL-W4 on methanol (Kaneda & Roxburgh, 1959) may also involve an analogous pathway. A comparison of activities of formate-tetrahydrofolate ligase (ADP) (tetrahydrofolate formylase EC 6.3.4.3), methylene-tetrahydrofolate dehydrogenase (EC 1.5.1.5) and serine hydroxymethylase (EC 2.1.2.1) in extracts of methanol-grown *Pseudomonas* AM1 with those in extracts of organisms grown on succinate has led to tetrahydrofolate being regarded as the C₁-unit carrier (Large & Quayle, 1963). This pathway is summarized in eqn. (1).

The present paper describes experiments which support this idea. A preliminary account of part of this work has been presented (Hollinshead, 1965).

MATERIALS AND METHODS

Maintenance and growth of organisms. Cultures of *Pseudomonas* AM1, *Protaminobacter ruber* and *Pseudomonas oxalaticus* were a gift from Professor J. R. Quayle (Department of Microbiology, University of Sheffield). The culture of *Pseudomonas extorquens* was a gift from Dr L. Janota-Bassalik (Institute of Plant Physiology, Warsaw).

Pseudomonas AM1 was maintained on methylamine hydrochloride-agar slopes and grown on methanol or succinate as described by Peel & Quayle (1961). The same media were used for the growth of *Pseudomonas extorquens* (Bassalik, 1913) and *Protaminobacter ruber* (Weaver, Samuels & Sherago, 1938) (N.C.I.B. no. 2879). *Pseudomonas oxalaticus* (OX1) (N.C.I.B. no. 8642) was grown as described by Quayle & Keech (1959, 1960).

Chemicals. Amethopterin (methotrexate, 4-amino-N¹⁰-methylpteroylglutamic acid) and leucovorin (citrrovorum factor) as the calcium salt were supplied by Lederle Laboratories Ltd. (Pearl River, N.Y., U.S.A.), aminopterin (4-aminopteroylglutamic acid) was supplied by Koch-Light Laboratories Ltd. (Colnbrook, Bucks.), and folic acid by Sigma Chemical Co. (St Louis, Mo., U.S.A.). Other

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chemicals were obtained from British Drug Houses Ltd. (Poole, Dorset), AnalaR grade being used when obtainable.

Measurement of growth of organisms. A starter inoculum was prepared by incubating 10 ml. of methanol or succinate liquid medium for 24 hr. at 30° after sterile transfer of bacteria from the methylamine slopes. Growth rates were determined in cultures containing 40 ml. of liquid medium and 1 ml. of starter inoculum. The culture vessels consisted of 250 ml. conical flasks (fitted with optically matched side arms) which were shaken on a New Brunswick Gyrotory Shaker (speed 200 rev./min.) at 30°. Growth was followed by measurement of the extinction at 540 m μ with a Unicam SP. 600 spectrophotometer. The ratio of the mean generation time of the control flask to the mean generation time of the experimental flask was used as a measure of inhibition of growth of the organisms. The mean generation times of control cultures grown on methanol or succinate were 3–4 hr. and 4–5 hr. respectively.

Serine and glycine assays. Extracts of methanol- and succinate-grown *Pseudomonas* AM1 were prepared by 5 min. ultrasonic disintegration (Measuring and Scientific Equipment Ltd., Ultrasonic Disintegrator Cabinet model 60—power output 60 w, 20 kcy/sec.) of a suspension of about 4 g. wet wt. of cells in 5 ml. of water and centrifugation at 25 000g for 10 min. at 2°. To the supernatant was added absolute ethanol (4 vol.) with shaking. After standing at 0° for 30 min. the suspension was centrifuged at 25 000g for 15 min. and the pale-pink supernatant evaporated until about 1 ml. of a cloudy pink suspension remained. This was clarified by centrifugation at 37 000g for 30 min. and the colourless supernatant evaporated further and streaked on to a paper chromatogram of Whatman no. 52 paper. The chromatogram was developed in butan-2-ol-formic acid-water (15:3:2, by vol.) (Hausmann, 1952) and the area corresponding to unresolved glycine and serine was eluted. Glycine and serine were separated by electrophoresis on 60 cm. strips of Whatman no. 3MM paper at 30 v/cm. in 7.5% (v/v) formic acid (Hartley & Jevons, 1962) for 1 hr.

Amino acids were located by dipping the electrophoretograms in 0.25% (v/v) ninhydrin in acetone, drying and heating at 100° for 5 min. The identities of the spots corresponding to glycine and serine were confirmed by chromatography in butan-1-ol-acetone-water-diethylamine (10:10:5:2, by vol.) (Hardy, Holland & Nayler, 1955).

The ninhydrin colour was made permanent by formation of the copper complex (Kawerau & Weiland, 1951). Stained areas corresponding to serine and glycine were eluted with 4 ml. of methanol and the extinction of the eluate was measured at 510 m μ (Gräser, 1964). To correct for any variation in ninhydrin colour intensity, calibration curves were prepared for each electrophoretogram relating amino acid concentration to extinction at 510 m μ , with standard solutions containing 0.0–0.5 μ mole of serine and 0.1–0.4 μ mole of glycine.

Methanol assay. The procedure was that of Powell & Ellam (1964), with Cavett flasks and the micro-diffusion technique of Neish (1957). Sodium starch glycolate (Peat, Bourne & Thrower, 1947) was used as indicator and the extinction of solutions read at 590 m μ . Culture medium was assayed for methanol after harvesting the cells. The range of sensitivity of the assay is 0.1–0.4 mg. of methanol/ml.

Microbiological assay of folate derivatives. *Streptococcus faecalis* (A.T.C.C. no. 8043; N.C.I.B. no. 8123) and *Pedococcus pentosaceus* (*Leuconostoc citrovorum*) (A.T.C.C. no.

8081; N.C.I.B. no. 7837) were employed for folic acid assay. The organisms were maintained on Bacto Micro Assay-Culture Agar (Difco), *S. faecalis* on a slope and *P. pentosaceus* on a stab culture. Transfers were made at weekly intervals, incubated at 37° for 24 hr. and stored at 2°.

The microbiological assay procedure was that described in the Difco Manual (1953), with Bacto Folic Acid Assay Medium (Difco) and Bacto CF Assay Medium (Difco) for *S. faecalis* and *P. pentosaceus* respectively. *S. faecalis* responds to pterate, pteroylglutamate and their derivatives; *P. pentosaceus*, however, requires a reduced form of folic acid showing no response with any unreduced compound (Jones, Guest & Woods, 1961). Folic acid was used for the construction of a response curve with the former and citrovorum with the latter.

Extracts of methanol- or succinate-grown *Pseudomonas* AM1 were prepared by crushing the bacterial paste from a Sharples centrifuge in a Hughes (1951) press which had been cooled to –25°. The material was then stirred in ice-cold water (4 vol.) for 30 min. and the viscous solution formed centrifuged at 37 000g for 30 min. The supernatant was stored in a deep-freeze until assayed.

All glassware used in the microbiological assay techniques was cleaned in Calgon according to Barton-Wright (1963).

RESULTS

Inhibition of growth of *Pseudomonas* AM1 by sulphonamides. A comparison was made of the effect of sulphanilamide on the growth of *Pseudomonas* AM1 on either methanol or succinate. The culture conditions are as described in the Materials and Methods section, the sulphonamides being included in the medium. The ratio of the mean generation time of a control culture to which no sulphonamide had been added to that of the culture to which sulphonamide had been added was used as a measure of growth inhibition.

The effect of sulphanilamide over the concentration range 0–30 mm is shown in Fig. 1(a). The organism was markedly more sensitive to sulphanilamide when growing on methanol than when growing on succinate. A concentration of 1 mm-sulphanilamide caused almost complete inhibition of growth on methanol but had little effect on growth on succinate.

Fig. 1(b) shows that a similar effect is observed in the presence of sulphathiazole. The differential sensitivity in the presence of sulphadiazine in the concentration range used was smaller (Fig. 1c). Experiments at higher concentrations were limited by the poor solubility of this sulphonamide.

Inclusion of sulphanilic acid in the medium (the pH of which was corrected to 7.0 with n-sodium hydroxide) up to a concentration of 20 mm caused no inhibition in growth of the organisms on succinate, but about 50% inhibition when grown on methanol (Fig. 1d).

Inhibition of growth of other organisms in the presence of sulphonamides. The experiment de-

Fig. 1. Inhibition of growth of *Pseudomonas* AM1 on methanol or succinate by sulphonamides. Cultures contained 40 ml. of media and were grown at 30° as described in the text. (a) Sulphanilamide, (b) sulphathiazole, (c) sulphadiazine and (d) sulphanilic acid. O, Methanol medium; ●, succinate medium.

scribed above was repeated with the organisms *Pseudomonas extorquens* and *Protaminobacter ruber* (Fig. 2). Both organisms showed a differential sensitivity to sulphanilamide between growth on succinate and methanol, similar to that observed with *Pseudomonas* AM1. *Pseudomonas oxalaticus*, however, showed no such differential sensitivity, 1 mM-sulphanilamide causing almost complete inhibition of growth on formate, oxalate or succinate.

Growth of Pseudomonas AM1 in the presence of folate antagonists. The presence of Amethopterin (methotrexate) or aminopterin up to a concentration of 0.5 mg./ml. in the culture media caused no increase in mean generation time when grown on methanol. The only observed effect was a tendency for a slight increase in the duration of the lag phase before commencement of growth at the higher concentrations. Microscopic examination revealed no morphological changes such as filamentous growth in the organism when grown in the presence of folate antagonists.

Fig. 2. Inhibition of *Protaminobacter ruber* by sulphanilamide when grown on methanol or succinate media. Cultures contained 40 ml. of media and were grown at 30° as described in the text. O, Methanol medium; ●, succinate medium. A similar sensitivity was observed with *Pseudomonas extorquens*.

Addition of sulphanilamide to Pseudomonas AM 1 during exponential growth. Addition of sulphanilamide to a final concentration of 1, 2 or 5 mM to an exponentially growing culture of *Pseudomonas AM 1* on methanol caused a sharp cessation of growth after 5 hr. (Fig. 3). Addition of sulphanilamide to give a final concentration 0.5 mM caused a less sharp cessation of growth after about 7 hr. The presence of 5 mM-serine in the medium, although causing a slight stimulation of growth in control cultures, had no effect on the sharp cessation of growth caused by addition of sulphanilamide to exponentially growing cultures of the organism.

Growth of Pseudomonas AM 1 in the presence of sulphanilamide and p-aminobenzoic acid. Growth of *Pseudomonas AM 1* was followed in media containing sulphanilamide and p-aminobenzoic acid.

Fig. 3. Effect of addition of sulphanilamide to an exponentially growing culture of *Pseudomonas AM 1* on methanol. Cultures (40 ml.) were grown at 30° as described in the text. ●, Flask to which sulphanilamide was added at the position marked by the arrow to give a final concn. 2 mM; ○, control flask to which no sulphanilamide was added.

The experiment was performed at sulphanilamide concentrations of 0.5, 1, 2 and 5 mM, under the conditions described previously. The presence of p-aminobenzoic acid in the medium reversed inhibition of growth on methanol by sulphanilamide. The criterion for complete reversal of inhibition was a mean generation time equal to that of a control culture containing neither sulphanilamide nor p-aminobenzoic acid. The minimum p-aminobenzoic concentrations required to reverse completely inhibition at the sulphanilamide concentrations studied are shown in Table 1. Within the sulphanilamide concentration range studied, a constant ratio of sulphanilamide concentration:minimum p-aminobenzoic concentration to cause complete reversal of inhibition was observed. Reduction in the concentration of p-aminobenzoic acid below this value caused an increase in mean generation time, further reduction also causing an increase in lag phase; at very low concentrations, no growth was observed even after 5 days. Above a sulphanilamide concentration of 5 mM it was not possible to reverse completely inhibition of growth with p-aminobenzoate.

Serine/glycine ratios in extracts of Pseudomonas AM 1. Cultures were in 600 ml. vessels each inoculated with a 10 ml. starter culture, and about 4 g. of wet cells was used for each assay. Assays were made at three different stages in growth: (a) stationary phase (after 48 hr. growth); (b) exponential phase (after 36 hr. growth); (c) cultures in which exponential growth had been stopped by addition of sulphanilamide (sulphanilamide was added to give a final concentration of 2 mM after 30 hr. incubation and the cells were harvested 6 hr. later). Three different cultures were assayed in each of the three stages of growth and the results expressed as the serine/glycine molar ratio (Table 2).

Cells in the stationary phase contained very little ninhydrin-positive material compared with exponentially growing cells. In all cases, the most abundant amino acids were glutamine and glutamate. When in the stationary phase very little

Table 1. *Reversal of sulphanilamide inhibition of growth of Pseudomonas AM 1 by p-aminobenzoic acid*

Cultures of *Pseudomonas AM 1* were grown on methanol in the presence of sulphanilamide and p-aminobenzoic acid as described in the text. The concentrations of p-aminobenzoic acid were the lowest that restored the mean generation time to the normal value.

Sulphanilamide concn. (mM)	p-Aminobenzoic acid concn. required to reverse inhibition completely (mM)	Ratio sulphanilamide concn.: p-amino- benzoic acid concn.
0.5	0.1	5
1	0.2	5
2	0.4	5
5	1.0	5

Table 2. *Relative concentrations of intracellular serine and glycine in Pseudomonas AM 1*

The serine and glycine concentrations of extracts of the cells were determined as described in the text. Cells in the stationary phase were harvested after 48 hr. growth and cells in the exponential phase after 36 hr. growth. Sulphanilamide-inhibited cultures were those in which growth had ceased after addition of sulphanilamide to a final concn. of 2 mM, as described in the Results section. Each value represents a separate culture. Methanol assays were performed, as described in the text, on the culture supernatant after harvesting the cells. (The methanol concentration of the medium when inoculated was 5 mg./ml.)

	Experiment	Serine (μ mole/culture)	Glycine (μ mole/culture)	Ratio μ moles of serine/ μ mole of glycine	Methanol concn. of medium after harvesting cells (mg./ml.)
Cells in stationary phase	A	0.008	0.14	0.06	
	B	0.02	0.28	0.07	less than 0.01
	C	0.02	0.2	0.1	
Cells in exponential phase	A	0.07	0.03	2.3	
	B	0.15	0.08	1.9	2.7
	C	0.09	0.04	2.3	
Sulphanilamide-inhibited cells	A	0.05	0.065	0.77	
	B	0.05	0.06	0.82	3.2
	C	0.057	0.07	0.82	

Table 3. *Folate content of extracts of Pseudomonas AM 1*

Cell-free extracts of methanol- or succinate-grown organisms were assayed for pterate derivatives and reduced pterate derivatives by microbiological assay as described in the text. Because only *N*⁵-derivatives of tetrahydrofolate were stable in the assay conditions, the value for 'non-*N*⁵-reduced' folate has been derived by difference between total pterate content and the observed reduced pterate content.

Growth medium	μ g./g. dry wt. of bacteria		
	Total pterate (as folic acid)	Reduced pterate (as citrovorum factor)	'Non- <i>N</i> ⁵ -reduced' pterate (by difference)
Methanol	0.21	0.05	0.16
Succinate	0.36	0.4	(0) approx.

serine was present; consequently the serine/glycine ratio was very low. In the exponential phase however, the reverse was true, and the serine/glycine ratio was high. Cessation of exponential growth by addition of sulphanilamide caused a sharp fall in serine concentration after a total growth time of 36 hr. compared with the control exponential cultures. However, in the inhibited cultures, the serine concentration was not as low as in stationary-phase cultures.

Methanol content of culture media. Methanol remaining in the culture media after harvesting the cells was assayed as described in the Materials and Methods section. The results are shown in Table 2. No methanol (i.e. less than 0.1 mg./sample) was detected in 10 ml. samples of the media from cells in the stationary phase. The methanol concentration in 36 hr. cultures treated with sulphanilamide was a little higher than in control cultures.

Folate derivatives in extracts of Pseudomonas

AM 1. Microbiological assays of extracts of *Pseudomonas AM 1* were performed as described in the Materials and Methods section. Pterate, pteroylglutamate and their derivatives were assayed with *Streptococcus faecalis* and estimated in terms of folic acid. Reduced folate derivatives were assayed with *Pediococcus pentosaceus* and estimated in terms of citrovorum factor. Under the conditions of the assay, only reduced compounds substituted in the *N*⁵-position would be expected to be stable.

The assays were performed on both methanol- and succinate-grown organisms and the results are shown in Table 3. The total pterate content of succinate-grown organisms was higher than that of methanol-grown organisms.

With succinate-grown cells, all the pterate appeared to be in a form to which *Pediococcus pentosaceus* could respond and was therefore considered to be in the form of *N*⁵-reduced folate derivatives, or derivatives converted into such in the conditions of

the assay. However, in methanol-grown cells only about 25% of the pterate occurred in the N^5 -reduced forms or derivatives converted into them in the conditions of the assay, whereas about 75% occurred as either non-reduced pterate derivatives or reduced pterate derivatives which are unstable and were converted into non-reduced forms in the course of the experiments.

DISCUSSION

Growth of *Pseudomonas* AM1 on methanol has been shown to be considerably more sensitive to inhibition by sulphanilamide than growth on succinate; the incorporation of the latter substance is assumed to occur by a pathway which does not directly involve folate. The uptake of methanol, on the other hand, is thought to involve the condensation of a C_1 unit derived from methanol with glycine to form serine, folate being the primary C_1 carrier (Large *et al.* 1961, 1962). Sulphonamides competitively inhibit the utilization of *p*-aminobenzoate for the biosynthesis of folic acid (Brown, 1962). It is therefore to be expected that folate deficiency due to the presence of such compounds would result in more restricted growth on methanol, due to inability to utilize the carbon source, than on succinate.

Sulphathiazole was the most effective sulphonamide used, followed by sulphadiazine and then sulphanilamide; sulphanilic acid caused little inhibition even at a concentration of 20 mM. The effectiveness of these sulphonamides as growth inhibitors is similar to that described by Brown (1962) using *Escherichia coli*. However, the most striking differential sensitivity was obtained with sulphanilamide; hence this sulphonamide was selected for subsequent experiments.

Protaminobacter ruber and *Pseudomonas extorquens*, organisms similar to *Pseudomonas* AM1 (Peel & Quayle, 1961), showed a similar differential sensitivity to sulphanilamide with respect to growth on methanol and succinate. The metabolism of methanol by these organisms is also thought to involve the formation of a C_1 unit which is incorporated into serine by a reaction involving a folate carrier (J. R. Quayle, unpublished work).

In contrast, the organism *Pseudomonas oxalaticus*, which utilizes succinate, oxalate or formate as sole carbon source (Quayle, Keech & Taylor, 1961) by routes not considered to involve folate, showed very little differential sensitivity to sulphanilamide.

Amethopterin (methotrexate) and aminopterin, which are strong inhibitors of dihydrofolate reductase, had no bacteriostatic action on *Pseudomonas* AM1 when either methanol- or succinate-grown. This is probably due to impermeability of the cell wall to such folate antagonist molecules, but the possibility of a resistant enzyme system or a

detoxication mechanism must not be overlooked. Study of the incorporation of [^{14}C]methanol into serine by cell-free extracts in the presence of folate antagonists might clarify this problem; such work is at present limited by the requirement of a hydrogen acceptor for the first stage of the reaction, whereas reducing conditions are necessary for the stability of the reduced folate derivatives participating in the second stage (eqn. 1). *Pseudomonas* AM1 therefore does not belong to the class of bacteria in which growth in the presence of Amethopterin or aminopterin results in morphological changes or inhibition of growth (Webb, 1954).

The presence of a high serine/glycine ratio in exponentially growing cells when there is an adequate carbon source is in accordance with the proposed pathway of methanol uptake since glycine would be rapidly converted into serine. Similarly, when in the stationary phase, the medium of which has been shown to be depleted of methanol, an accumulation of the precursor of serine has been demonstrated.

Inhibition of exponential growth by addition of sulphanilamide also caused a reduction in the serine/glycine ratio even though the medium still contained an adequate methanol supply (Table 2). However, the loss of serine was significantly greater than the accumulation of glycine. The extent of the glycine accumulation was not as marked as in the stationary phase, but sulphanilamide-inhibited cells have methanol available which may be oxidized as an energy supply (Johnson & Quayle, 1964; Anthony & Zatman, 1964). This observation is consistent with the fact that less methanol remains in the medium after 37 hr. growth in uninhibited cells than those to which sulphanilamide has been added.

Sulphanilamide inhibition of the growth of *Pseudomonas* AM1 on methanol therefore appears to be caused by prevention of the primary utilization of carbon, probably owing to folate deficiency. This conclusion is supported by the experiments involving *p*-aminobenzoate.

Competitive reversal of sulphanilamide inhibition by *p*-aminobenzoic acid has long been known (Woods, 1940). This has now been demonstrated in *Pseudomonas* AM1, but the *p*-aminobenzoate concentration necessary to reverse completely the inhibition is about a thousand times that required by *Streptococcus haemolyticus* (Woods, 1940).

The higher folate content of cells grown on succinate than on methanol was unexpected, but this may be the result of succinate being a more favourable substrate for growth than methanol. However, the difference in the proportion of pterate which forms the N^5 -reduced form in the conditions of the assay is probably the important factor. The path of incorporation of the C_1 unit into serine is thought to involve N^{10} -formyltetrahydro-

folate, *N*^{5,10}-methenyltetrahydrofolate and *N*^{5,10}-methylenetetrahydrofolate (Large & Quayle, 1963); the first of these compounds at least would be converted into non-reduced folate in the conditions of the assay.

When cells are grown on methanol, about 75% of the total folate appears in a non-reduced form, whereas in succinate-grown organisms all the pterate derivatives appear to occur as the *N*⁵-reduced form. These results therefore could be explained in terms of differences in the metabolic roles of folate in the two media.

The results presented in this paper support the view that the growth of *Pseudomonas* AM1 on methanol involves the formation of serine, from glycine and a C₁ unit derived from methanol, by a pathway which has a requirement for reduced folate derivatives.

The author thanks Professor J. R. Quayle for his interest and many helpful discussions, and Mr. R. Bacon for technical assistance with part of this work.

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