The present investigation indicates the content of this C_{19} fatty acid in butterfat to be approx. 0·01 % of the total weight of fatty acids.

Although 2,6,10,14-tetramethylpentadecanoic acid has not formerly been isolated from natural fat, Smith & Boyack (1948) converted phytol into phytene-1 and then into a fatty acid with formula $C_{19}H_{38}O_2$ which they named 'apophytoic' acid and which may correspond with the butterfat constituent reported in this paper.

SUMMARY

1. 2,6,10,14-Tetramethylpentadecanoic acid has been isolated from butterfat and identified by mass and infrared spectrometry and gas–liquid chromatography.

2. It was present to the extent of approx. 0·01 % of the total weight of fatty acids.

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Effect of Trace Elements on the Production of Pigments by a Pseudomonad

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Several workers (Georgia & Poe, 1931; Burton, Campbell & Eagles, 1948; Grossowicz, Hayat & Halpern, 1957) have demonstrated the essentiality of Mg^{2+} ions for the production of various pigments by different species of *Pseudomonas*, though earlier reports (Jordan, 1899; Sullivan, 1905; Tanner, 1918) were contradictory. The effect of other trace-element ions such as Fe^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} etc. (Burton *et al.* 1948; Grossowicz *et al.* 1957) or the replacement of Mg^{2+} by other metal ions (Thumm, 1895) on the production of pigments in a variety of pseudomonads has also been studied, but not without some contradictory results. However, data on the effects of trace-element ions on the production of the water-soluble fluorescent pigments (fluorescein) by species of the *Pseudomonas*

fluorescens group are lacking. The present study was therefore carried out to obtain more conclusive evidence on the role of Mg^{2+} and other metal ions on the production of the water-soluble yellow–green-fluorescent pigments and on the growth of a member of this group. During the work one of the fluorescent pigments was crystallized and found to be a pteridine derivative that has been tentatively named 'compound P' (Chakrabarty & Roy, 1964*b*).

EXPERIMENTAL

Organism and media. The present organism is a strain of *Pseudomonas fluorescens–putida* intermediate that produces a number of water-soluble yellow–green-fluorescent pigments. Details about the maintenance of the organism have been given by Chakrabarty & Roy (1964*a*). For the propagation of the organism and to see the effects of various added metal ions, a synthetic medium was used (Chakrabarty & Roy, 1964*a*) which contained (per l.): $(NH_4)_2HPO_4$, 2·0 g.; KH_2PO_4, 1·0 g.; NaCl, 1·0 g.; $MgSO_4,7H_2O$, 0·5 g.; L-asparagine, 5·0 g.; adjusted to pH 7·0. Sterile glucose (final concn. 0·5%, w/v) was added after autoclaving.

Culture conditions. The inoculum was grown in 30 ml. of synthetic medium, free of any other trace-metal ions, dispensed in 250 ml. Erlenmeyer flasks for 24 hr. on a rotary shaker (120 rev./min.) at room temperature (25–30°). After the required growth period the cells were centrifuged at 1200*g* at 0° and washed eight to ten times with cold sterile deionized and double-distilled water, and then 3 drops of a suspension of the cells in the same water containing 1 mg. of cells/ml. were used to inoculate 100 ml. Erlenmeyer flasks containing 15 ml. of the synthetic medium. Incubation was for 5 days under stationary conditions in the dark at 30°.

Cell growth. The cells from 15 ml. of medium were centrifuged, washed twice with distilled water, and finally dried at 105° for 16 hr. and weighed, cell weight being the measure of growth.

Determination of compound P. After the removal of the cells, a portion of the medium was chromatographed on Whatman no. 1 paper in butanol-1-ol–ethanol–water (10:3:7, by vol.) and the green-fluorescent band of compound P was located under an ultraviolet lamp. This was then cut out, eluted with water in the dark and estimated from its extinction at 405 mμ in a Beckman model DU spectrophotometer (extinction coefficient, $E^{1\%}_{1\,cm.}$ 96·6).

Removal of trace-element ions from the medium. The water used in all experiments was deionized and distilled twice from an all-glass still. The conductivity of such water varied from 2·8 to 3·0 μmhos at 30° when measured with a Leeds and Northrup conductivity bridge. Only Pyrex glassware was used in these experiments. It was first rinsed with a detergent, then washed repeatedly with tap water, soaked in conc. HNO_3 for 24 hr. and then washed at least 30 times in tap water. Final washings were made with distilled water at least ten times and finally three or four times with deionized double-distilled water. The pipettes and other materials were cleansed by first soaking in a dilute solution of EDTA (disodium salt) and then in conc. HNO_3, after which exhaustive washings were made as described above.

Since the chief source of trace-element impurities lies in sugars, amino acids and salts, a separate purification of glucose was followed as described by Walker (1953) by passing glucose solution (7·5%, w/v) twice through a column of Dowex 50 (H^+ form), which had previously been washed exhaustively with deionized double-distilled water. The effluent glucose solution had pH 4·8. Other chemicals used were 'guaranteed reagents' from E. Merck and Co. To minimize heavy-metal contamination, $(NH_4)_2HPO_4$, KH_2PO_4, $MgSO_4,7H_2O$, NaCl and asparagine were weighed, dissolved in a suitable amount of deionized double-distilled water and the medium was further purified by the dithizone procedure as described by Donald, Passey & Swaby (1952). Exhaustive washing with thrice-distilled chloroform ensured that the chelating agent was completely removed from the medium. In general, the metal ion whose effect was to be tested was added in graded doses before autoclaving the medium. For testing the effect of Mg^{2+} ions, $MgSO_4,7H_2O$ was omitted from the medium, the sulphur deficiency being made good by the addition of equivalent amount of $(NH_4)_2SO_4$.

RESULTS

Effect of bivalent cations. Tables 1 and 2 show the effects of Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+} and Co^{2+} ions. In general a wide range of concentrations of the

Table 1. *Effect of* Fe^{2+}, Zn^{2+} *and* Cu^{2+} *ions on the production of compound P and growth of the pseudomonad*

A 15 ml. portion of the synthetic medium containing graded doses of the metal ions was inoculated with cells from a 24 hr. culture as described in the Experimental section. After a fermentation period for 5 days at 30°, the cells were centrifuged and dried for the measure of growth, and the compound P in the broth was estimated as described in the Experimental section. —, Not detected.

Concn. of metal ion (μg./ml.)	$FeSO_4,7H_2O$		$ZnSO_4,7H_2O$		$CuSO_4,5H_2O$	
	Cell weight (mg./15 ml.)	Compound P (μg./ml.)	Cell weight (mg./15 ml.)	Compound P (μg./ml.)	Cell weight (mg./15 ml.)	Compound P (μg./ml.)
0·00	3·0	10·4	3·2	10·7	3·6	9·1
0·01	3·6	12·1	4·19	16·8	3·5	15·6
0·10	4·1	9·3	3·8	17·2	2·72	16·8
1·0	5·8	3·5	3·4	15·3	2·68	12·7
5·0	6·3	0·75	2·82	19·8	2·81	2·85
50·0	5·2	—	2·25	10·1	1·65	—
100·0	5·4	—	2·10	8·4	1·60	—

Table 2. *Effect of* Mn^{2+}, Co^{2+} *and* Ni^{2+} *ions on the production of compound P and growth of the pseudomonad*

Growth measurement and the determination of compound P were done as described in Table 1. —, Not detected.

Concn. of metal ion (μg./ml.)	$MnSO_4,H_2O$		$CoSO_4,7H_2O$		$NiSO_4,7H_2O$	
	Cell weight (mg./15 ml.)	Compound P (μg./ml.)	Cell weight (mg./15 ml.)	Compound P (μg./ml.)	Cell weight (mg./15 ml.)	Compound P (μg./ml.)
0·0	3·1	11·10	3·95	9·8	3·48	10·32
0·01	4·4	14·62	4·38	9·8	3·28	10·7
0·10	4·76	17·8	4·05	9·6	3·20	9·72
1·0	5·00	18·64	4·10	7·2	3·50	9·6
5·0	4·58	16·75	3·26	4·6	3·38	7·8
50·0	4·46	12·4	2·90	—	2·95	3·95
100·0	4·62	10·6	2·76	—	3·10	4·2

Table 3. *Effect of dichromate, molybdate and borate on the production of compound P and growth of the pseudomonad*

Growth measurement and the determination of compound P were done as described in Table 1. —, Not detected; n.d., not determined.

Concn. of added ion (μg./ml.)	$K_2Cr_2O_7$		$(NH_4)_2Mo_7O_{12},12H_2O$		H_3BO_3	
	Cell weight (mg./15 ml.)	Compound P (μg./ml.)	Cell weight (mg./15 ml.)	Compound P (μg./ml.)	Cell weight (mg./15 ml.)	Compound P (μg./ml.)
0·0	2·75	10·7	2·75	10·7	2·75	10·7
0·5	2·59	7·6	2·25	6·2	2·96	11·4
1·0	2·28	5·3	2·50	4·8	2·61	9·6
5·0	2·83	—	3·20	—	2·40	10·9
50·0	—	n.d.	3·09	—	n.d.	n.d.

Table 4. *Replaceability of* Mg^{2+} *ions by other metal ions in the production of compound P and growth of the pseudomonad*

Growth measurement and the determination of compound P were done as described in Table 1. —, Not detected.

Additions (μg. of metal ion/ml.)	Cell weight (mg./15 ml.)	Compound P (μg./ml.)
None	0·92	0·2
$MgSO_4,7H_2O$ (25·0) + $MnSO_4,H_2O$ (25·0)	3·30	6·1
$MgSO_4,7H_2O$ (50·0)	3·95	11·1
$MnSO_4,H_2O$ (50·0)	1·08	Trace
$ZnSO_4,7H_2O$ (10·0)	0·75	0·95
$FeSO_4,7H_2O$ (1·0)	1·18	—
$NiSO_4,7H_2O$ (5·0)	1·64	—
$FeSO_4,7H_2O$ (1·0) + $ZnSO_4,7H_2O$ (5·0)	1·50	—
$FeSO_4,7H_2O$ (1·0) + $CoSO_4,7H_2O$ (5·0)	1·22	—
$CaCl_2,2H_2O$ (50·0)	0·90	—
$ZnSO_4,7H_2O$ (50·0)	0·65	2·34

metal ions was used to find out both the stimulatory as well as the inhibitory effects, if any. Although the greenish-fluorescent compound P was mainly taken as the measure of pigment formation, in some cases, as with Cu^{2+} or Ni^{2+} ions (above 5 μg./ml.), a yellow pigment appeared even when compound P ceased to be formed.

Effect of dichromate, molybdate and borate. These were tested over a narrow range of concentrations because of their likely presence in very low concentrations in other salts. The results are shown in Table 3. Borate was without effect but dichromate and molybdate were detrimental to the production of compound P. Molybdate, however, improved growth even when it inhibited the formation of compound P totally.

Replaceability of Mg^{2+} *ions by other metal ions.* Magnesium and other metal ions at different concentrations were added to the Mg^{2+} ion-free medium, singly or in combination, to determine whether Mg^{2+} ions were required for growth and compound P formation by the pseudomonad. To avoid contamination by molybdate etc. Lenhoff, Nicholas & Kaplan (1956) used beakers as covers, whereas in the present study cotton plugs were used throughout. This might have caused slight contamination by Mg^{2+} ions. Results in Table 4 demonstrate the need for Mg^{2+} ions both for growth as well as for pigmentation by the pseudomonad.

It is evident that the concentration of Mg^{2+} ions present in the original medium was the most suitable and that Mg^{2+} ions could not be replaced by Mn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, Cu^{2+} ions etc. However, Zn^{2+} ions, at higher concentrations, restored pigment formation to some extent.

DISCUSSION

The present study lends additional support to the previous observations (Georgia & Poe, 1931; Burton *et al.* 1948; Grossowicz *et al.* 1957) that Mg^{2+} ions are essential for pigment production by the *Pseudomonas* species. The apparent replaceability of Mg^{2+} ions by Ca^{2+} or Mn^{2+} ions (Thumm, 1895) might result from contamination of those salts by Mg^{2+} ions. In conformity with the findings of Grossowicz *et al.* (1957), Fe^{2+} and Co^{2+} ions were both inhibitory, although Fe^{2+} ions (0·01 μg./ml.) considerably stimulated the formation of compound P. Goodwin & McEvoy (1959) showed that at concentrations of 0·005–0·01 μg./ml. Fe^{2+} ions are stimulatory for flavinogenesis in *Candida* sp., whereas Schopfer & Knusel (1956) and also Goodwin & McEvoy (1959) have shown that Zn^{2+} ions have a stimulatory role in flavinogenesis at lower concentrations. This stimulatory effect of Fe^{2+} and Zn^{2+} ions has also been shown for vitamin B_{12} synthesis by Maitra & Roy (1960). That the production of fluorescein is related inversely to the logarithm of the concentration of Fe^{2+} ions has also been shown by Totter & Moseley (1953) in a strain of *Pseudomonas aeruginosa*.

Inorganic substances necessary for the production of pyocyanine by *Pseudomonas aeruginosa* have been previously studied, but only in a very few cases has the effect of trace-metal ions on the production of the characteristic water-soluble fluorescent pigments of the pseudomonads been studied. This is due to the lack of a standardized method for the estimation of these pigments (Lysenko, 1961; Elliott, 1958). The crystallization and quantitative recovery of the present pigment by chromatography (Chakrabarty & Roy, 1964*b*) has been utilized to follow the effects of trace-metal ions on the production of this pigment, and to provide additional information about the trace-element nutrition of the pseudomonad in relation to its ability to form fluorescent pigments.

SUMMARY

1. The effect of different trace-element ions on the production of pigments, particularly a pteridine derivative, by a pseudomonad was studied in media freed from contaminating metal ions with chelating agents.

2. Magnesium ions were essential both for pigment formation and growth of the organism and were irreplaceable by any of the other metal ions tested.

3. Ferrous ions were stimulatory to the pigment formation at low concentrations, but at higher concentrations they were inhibitory.

4. Manganese ions were stimulatory at all concentrations tested, but Zn^{2+}, Cu^{2+} and Ni^{2+} ions, though stimulatory at lower concentration, inhibited pigment formation at higher concentrations.

5. Cobalt ions were inhibitory at all concentrations. Borate was without any effect, whereas dichromate and molybdate were inhibitory at higher concentrations.

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