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Siderophores in Microbially Processed Cheese

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Siderophore activity, equivalent to about 5–10 mg per kilogram, was detected in mold ripened cheese. The siderophore activity in Oregon Blue cheese behaved in its physical and biological properties as ferrichrome. A crystalline specimen of the latter siderophore was obtained from commercial blue mold powder. In contrast, *Penicillium roqueforti*, when grown in low iron medium, excreted another well-characterized fungal siderophore, namely, coprogen.

Siderophores, defined as low-molecular-weight, high-affinity ferric ion transport agents, are excreted by a variety of aerobic and facultative anaerobic microorganisms growing in *low iron* environments (Neilands, 1974). The siderophores generally can be relegated into two groups: the catechols and the hydroxamic acids. The former are typically produced by bacteria, especially the enteric species such as *Escherichia coli*, *Aerobacter*, and *Salmonella*, while the latter are commonly synthesized by the yeasts and fungi. The large stability constants for ferric ion ($K_s = 10^{30}$–10^{50} M) enables siderophores to compete effectively for iron complexed as an oxyhydroxide polymer, the form of ferric ion predominating at physiological pH.

Aerobic and facultative anaerobic microorganisms are utilized in the production of various foodstuffs. The fungi *Penicillium roqueforti* and *Penicillium camemberti* are used to ripen the blue and camembert-type cheeses, respectively, and *Aspergillus oryzae* and *Rhizopus* are used to ferment many of the oriental beverages, condiments, and sauces prepared from beans and rice (Gray, 1970). Cheese has an iron content ranging from 2 to 10 ppm (Aitzetmüller and Wirotama, 1974). In addition, milk and milk products contain the iron-binding protein lactoferrin which can deny iron to microorganisms (Oram and Reiter, 1968).

Because cheese contains only small amounts of available iron, we reasoned that the ripening of this product could induce the synthesis of siderophores in the food substance. Several members of the ferrichrome family of siderophores have been detected in sake (Tadenuma and Sato, 1967). Moreover, *P. camemberti* is known to produce the siderophore coprogen when grown in a synthetic, iron-deficient medium (Zähner et al., 1963).

We report here: (1) the detection of siderophore activity in fungally ripened cheese; (2) the identification of the siderophore coprogen (I) from the culture medium of a strain of *P. roqueforti* isolated from blue cheese; (3) the crystallization of ferrichrome from commercial blue mold powder; and (4) the partial purification and characterization of a substance from Oregon Blue cheese which resembles ferrichrome (II) in its physical properties and biological activities.

A preliminary account of this work has been presented elsewhere (Ong and Neilands, 1976).

EXPERIMENTAL SECTION

Materials. Cheeses were obtained from local food stores. "Midwest" *P. roqueforti* Blue Mold Powder was purchased from Dairyland Food Laboratories Inc., Waukesha, WI. Coprogen was provided by the Lederle Laboratories, while coprogen B and dimerum acid were gifts of H. Diekmann. Ferrichrome was isolated from *Ustilago sphaerogena* (Neilands, 1952) and albomycin was

donated by J. Turková. All chemicals were reagent grade and deionized water was employed.

Cultures. The bacterial strains *Salmonella typhimurium* LT-2 TA2443 (*enb-7*) (Pollack et al., 1970) and TA2701 (Luckey et al., 1972) and *E. coli* K-12 were employed. Strain TA2701 is a *sidA* derivative of *enb-7*. Phage ϕ80 vir. and *E. coli* CSCG856 were obtained from laboratory stocks (Wayne and Neilands, 1975). A culture of *P. roqueforti* was furnished by Robert Burman, Microgarden, University of California, Berkeley; a second strain was isolated from Oregon Blue cheese.

Methods. *Siderophore Assay.* The ability of extracts prepared from the cheeses to support growth of the mutants on medium E was examined by placing 6-mm diameter filter paper disks on pour plates seeded with 2 drops of an overnight nutrient broth culture of the mutant (Roth, 1970). Each disk contained 20 μL or 40 μL of the extract prepared as described below. Disks containing methanolic extracts were allowed to dry prior to their assay. The radius of visible growth was measured after 24 h of incubation at 37 °C, using ferrichrome as a standard.

Growth of Cultures. Nutrient broth (Difco) was employed for overnight culture and storage of bacteria. Medium E was prepared as described by Vogel and Bonner (1956). Liquid cultures were grown aerobically with shaking at 37 °C. Fungi were maintained on slants containing: 3.0 g of $NaNO_3$, 0.5 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 20.0 g of sucrose, 20.0 g of agar per liter (Koffler et al., 1945). Suspensions of conidia in sterile water were used as inocula. Fungi were cultured for 3 weeks with shaking at 24 °C in an iron deficient, synthetic medium (Garibaldi and Neilands, 1955).

Isolation of Penicillium roqueforti from Cheese. Isolation of the fungus was carried out in petri dishes containing: 1.0 g of yeast extract (Difco), 2.0 g of glucose, and 15.0 g of agar per liter. Small portions of Oregon Blue cheese were placed on the agar plates, and the tip of the mycelium or aerial conidia were sequentially transferred until a pure culture was obtained. The taxonomic identity of the fungus was verified by Wicklow (1977).

Extraction of Siderophore Activity from Cheese. A 50-g sample of cheese was homogenized with 100 mL of water for 5 min and then centrifuged for 10 min at 10 000 rpm. The supernatant was filtered through Whatman No. 1 paper. Exactly 5 mL of the filtrate was combined with 5 mL of methanol and then centrifuged for 15 min at top speed in a clinical centrifuge. The clarified supernatant was assayed for siderophore activity.

In larger scale work, 5 kg of Oregon Blue cheese was homogenized for 10 min with 2.5 L of water. The resulting homogenate was centrifuged for 15 min at 10 000 rpm. The supernatant was filtered through Whatman No. 1 paper, and the above process was repeated on the solid fraction. The filtered supernatants were combined and the pH was

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$$
\begin{array}{c}
CH_3 \\
| \\
C = O \\
| \\
NH
\end{array}
$$

HO(CH$_2$)$_2$C(CH$_3$)=CHCON(OH)(CH$_2$)$_3$CHCOO(CH$_2$)$_2$C(CH$_3$)=CHCON(OH)

HO(CH$_2$)$_2$C(CH$_3$)=CHOC(HO)N(CH$_2$)$_3$

I, deferricoprogen

—NHCH$_2$CONHCH$_2$CONHCH$_2$COCNHCHCO—

(CH$_2$)$_3$

N—O$^-$

C=O

CH$_3$

Fe^{+3}

II, ferrichrome

adjusted to 7 with 2 N NaOH. The temperature of the extract was raised to 80 °C and the extract stirred for 15 min, and the volume of the extract was concentrated to ca. 1 L under reduced pressure at 40 °C. The resulting extract was dialyzed vs. water for 36 h with three changes of the dialysate. The volume of the pooled dialysate was reduced to 2 L under reduced pressure and 40 g of (NH$_4$)$_2$SO$_4$/100 mL was added. The siderophores were extracted into benzyl alcohol in the usual way (Neilands, 1952). The water extract was taken to dryness at 40 °C, redissolved in 200 mL of water, and chromatographed on a column (o.d. 4.6 cm) packed with 450 g of Amberlite XAD-2 in H$_2$O, as described by Horowitz et al. (1976). The 40% methanol eluant containing the siderophore activity was reduced to dryness, redissolved in 4 mL of H$_2$O, and then chromatographed on a column (105 × 2.5 cm) of Bio-Rad P2 (100–200 mesh) in H$_2$O. The fractions containing siderophore activity were pooled, and the volume was reduced to 5 mL under reduced pressure at 40 °C. After addition of 25 mg of FeSO$_4$·7H$_2$O and 0.5 g of (NH$_4$)$_2$SO$_4$, the siderophores were extracted into benzyl alcohol and then returned to water as before. The extract was then subjected to preparative paper electrophoresis at pH 6.5 in 0.1 M phosphate buffer. Neutral and negatively charged orange-colored bands appeared and were eluted with methanol and chromatographed on EM silica gel plates (60F-254, glass support) in 90% methanol in water. The colored zones designated siderophores F and G (R_f 0.30 and 0.65, respectively), containing growth activity were eluted. The yield was 10 mL of solution with absorbancy of 0.52 and 1.07 at 430 nm for the neutral (F) and negatively charged (G) components, respectively.

Isolation and Characterization of Siderophores from Penicillium roqueforti Culture Medium. A 10-L, 3-week culture of the fungus obtained from Oregon Blue cheese was centrifuged to remove cellular debris, and 5.0 g of FeSO$_4$·7H$_2$O was added to the cell-free supernatant. The volume of the spent medium was evaporated to 2 L under reduced pressure at 40 °C. The resulting solution was saturated with (NH$_4$)$_2$SO$_4$, and the siderophores were

purified by extraction into benzyl alcohol as described above. The water extract was dried under reduced pressure at 40 °C and then chromatographed (Pidacks et al., 1953). Three orange-colored zones were resolved: a large, diffuse band was followed, in order, by a small, sharp band and a minor, diffuse band. Each band was reextracted into water and then neutralized with dilute NH$_4$OH. The first band was further chromatographed on a 3-cm o.d. column containing 50 g of silica gel (Kieselgel-Camag) in 90% aqueous methanol, pressurized at 14 psi (Leong and Raymond, 1974). The purity of the first band was ascertained by thin-layer chromatography in several solvent systems. Chromatograms were examined under ultraviolet light and developed with iodine or the Folin-Ciocalteau reagent (Subramanian et al., 1965). Yield: 73 mg band 1; 5.8 mg band 2; 2.7 mg band 3, designated siderophores A, B, and C, respectively. A siderophore (D) from a 1 L culture of *P. roqueforti* obtained from the Microgarden, University of California, was also isolated by benzyl alcohol extraction.

The purified major siderophore (A) from the Oregon Blue *P. roqueforti* culture was characterized by visible, infrared, and NMR spectroscopy. Visible spectra were measured in methanolic solutions on a Beckman Model 25 spectrophotometer. Infrared spectra were obtained in a KBr pellet in a Perkin-Elmer Model 257 infrared spectrophotometer. NMR spectroscopy was carried out on samples in D$_2$O with a Varian EM 360 NMR spectrometer. Iron was removed prior to the NMR analyses as previously described (Llinas et al., 1970).

The siderophore was further characterized by paper electrophoresis at several pH values and by thin-layer and paper chromatography in several solvent systems.

For mild alkaline hydrolysis of the *P. roqueforti* siderophore, approximately 10 mg of the deferri form in 0.5 mL of water was mixed with 0.5 mL of 1 N NaOH and allowed to stand at room temperature for 1 h. The solution was then neutralized to pH 6.5 with 1 N HCl and extracted with a few milliliters of benzyl alcohol. The two solvent phases were separated and saved. The benzyl alcohol

phase was diluted with ether and extracted with water in the usual way and concentrated on the rotary evaporator to ca. 0.1 mL (sample 1). The neutralized aqueous phase was acidified to pH 2 and the benzyl alcohol extraction repeated to yield sample 2. The samples were analyzed by thin-layer chromatography and paper electrophoresis, with 1% $FeCl_3$ as detection spray.

Isolation and Characterization of a Siderophore from Commercial Blue Mold Powder. The contents of two 1-lb cans of Midwest Blue Mold Powder (10^8–10^9 viable *P. roqueforti* spores/gram; G. E. Staehler, personal communication) were suspended in 8 L of water. The bread was allowed to settle and the supernatant containing the conidia was decanted through four layers of cheesecloth. The resulting suspension was centrifuged, the supernatant discarded, and the pellet resuspended in 2.75 L of chloroform-saturated water. After shaking for 5 h at 30 °C, the suspension was centrifuged, the supernatant reduced to 100 mL in vacuo at 45 °C, and 20 mg of $FeSO_4 \cdot 7H_2O$ and 40 g of $(NH_4)_2SO_4$ were added, and the siderophore activity was extracted into three 50-mL portions of benzyl alcohol. The color was returned to water by the addition of five volumes of ether. The aqueous solution, concentrated to 2 mL, was applied to a 88 × 2.5 cm column of Bio-Gel P2 equilibrated in water. The orange-colored band was collected, concentrated to dryness, redissolved in 0.6 mL of 70% methanol–water, and applied to a 2.5-cm o.d. column of Kieselgel (30 g) equilibrated with the same solvent and pressurized at 14 psi as described above. The main portion of the orange-colored band was evaporated from methanol to yield 19.4 mg of crude crystals. After recrystallization from methanol, the product (siderophore E) was examined microscopically, analyzed spectrally, chromatographed in several solvent systems, and checked for biological activity with *S. typhimurium* TA2443 and TA2701 and for antagonism vs. albomycin.

Antibiotic Cross Test. Solutions (10^{-4} M) of albomycin, ferrichrome, the siderophore from blue mold powder, and the siderophore fractions from cheese (concentration based on biological activity) were used to impregnate 0.8 × 5 cm strips of Whatman No. 1 filter paper. The strips were applied in a crosswise manner on nutrient broth pour plates seeded with 2 drops of an overnight nutrient broth culture of *E. coli* K-12. Incubation was for 12 h at 37 °C.

Phage Adsorption Assay. Adsorption of *φ80* vir to *E. coli* CGSC856 was determined as previously described (Wayne and Neilands, 1975).

Characterization of Siderophores from Cheese. The partially purified compounds, F and G, with siderophore activity were characterized by thin-layer and paper chromatography in various solvent systems. Spots were detected visually or with the Folin spray for hydroxamates (Subramanian et al., 1965). Growth factor activity for *S. typhimurium* TA2443 was also monitored by dividing each lane into 1-cm squares and eluting the activity with 0.1 mL of water.

Visible adsorption spectra were also obtained for the partially purified siderophores.

RESULTS

Siderophore Assay. The square of the radius of diameter of growth was linearly proportional to the log of the initial concentration of ferrichrome over the range 0.1–10 μM. The ability of the extracts prepared from cheese to support the growth of the *S. typhimurium* LT-2 TA2443 and TA2701 mutants on medium E agar plates was examined (Table I). TA2443 is known to utilize at least 15 hydroxamate-type siderophores while TA2701, its *sidA* derivative, responds to none of them. Both mutants

Table I. Growth Stimulation of *Salmonella typhimurium* LT-2 TA2443 and TA2701 by Extracts Prepared from Cheese[a]

| | growth response[b,c] | |
extract source	TA2443	TA2701
Brie (Marco)	+ +	−
Camembert (Rouge et Noir)	+ +	−
Camembert (Ile de France)	+ + +	−
Camembert (Bordens)	+ + +	−
Blue (Treasure Cave)	+ + + +	−
Roquefort (Montco)	+ + +	−
Oregon Blue (Rogue River Valley Creamery)	+ + + +	−
Gorgonzola (Co-op)	+ + +	−
Jarlesberg (Norwegian Dairies Sales Association)	−	−
Longhorn (Co-op)	−	−
Mild Cheddar (Co-op)	−	−
Kraft American (Kraft Inc.)	−	−
Monterey Jack (Co-op)	−	−
Swiss (Co-op)	−	−

[a] Filter paper disks (6 mm diameter) containing 40 μL of the extract were applied to Medium E plates seeded with 2 drops of an overnight culture of the appropriate mutant. Incubation was for 24 h at 37 °C. [b] Measured as radius of growth (mm). [c] Scoring method [growth response, (radius of growth (mm)), ppm siderophore (relative to a ferrichrome standard): ±, (<6); +, (6-10); + +, (11-15), <1; + + +, (16-20), 1-8; + + + +, (>20), >8.]

Table II. Proton Nuclear Magnetic Resonance Analysis of the Major Siderophore from the Culture Medium of *Penicillium roqueforti* Isolated from Cheese[a]

| | proton integration | |
δ	predicted[b]	observed[c]
6.2	10	12
4.3	16	15
3.8	30	34
2.4	18	19
1.9	68	65

[a] Exactly 28 mg of the deferrated siderophore was dissolved in 0.2 mL of D_2O and 0.01 mL of Me_4Si. The spectrum was obtained on a Varian Anespect EM360 NMR Spectrometer. [b] Zähner et al. (1963). [c] These are not actual proton counts, but are arbitrary numbers taken from an enlargement of the spectrum published by Zahner et al. (1963) and normalized to sum to approximately the same number of ^1H resonances found in the product from *P. roqueforti.*

will grow on their native catechol siderophore, enterobactin, as well as on iron salts, 2,3-dihydroxybenzoylserine, ascorbate, and ethylenediaminetetraacetic acid (Luckey et al., 1972). Of the cheese extracts tested, only those which were prepared from mold-ripened cheese showed positive activity with TA2443. None of the cheese extracts supported the growth of TA2701.

Identification of Coprogen as a Siderophore from Penicillium roqueforti. The major siderophore (A) isolated from the spent medium of *P. roqueforti* had a visible spectrum with a broad peak centered at 435 nm, characteristic of α–β unsaturated siderophores such as coprogen. The infrared spectrum was essentially similar to that of authentic coprogen. Finally, both the chemical shifts and proton integration of the NMR spectrum (Figure 1, Table II) correspond well with those derived from a published spectrum for coprogen (Zähner et al., 1963).

Paper electrophoresis (pH 6.5, 0.1 M phosphate buffer) of the mild alkaline hydrolysate of the *P. roqueforti* deferrisiderophore indicated that sample 1 contained, as a major component, a neutral substance which gave a red-brown color with the iron spray and which appeared
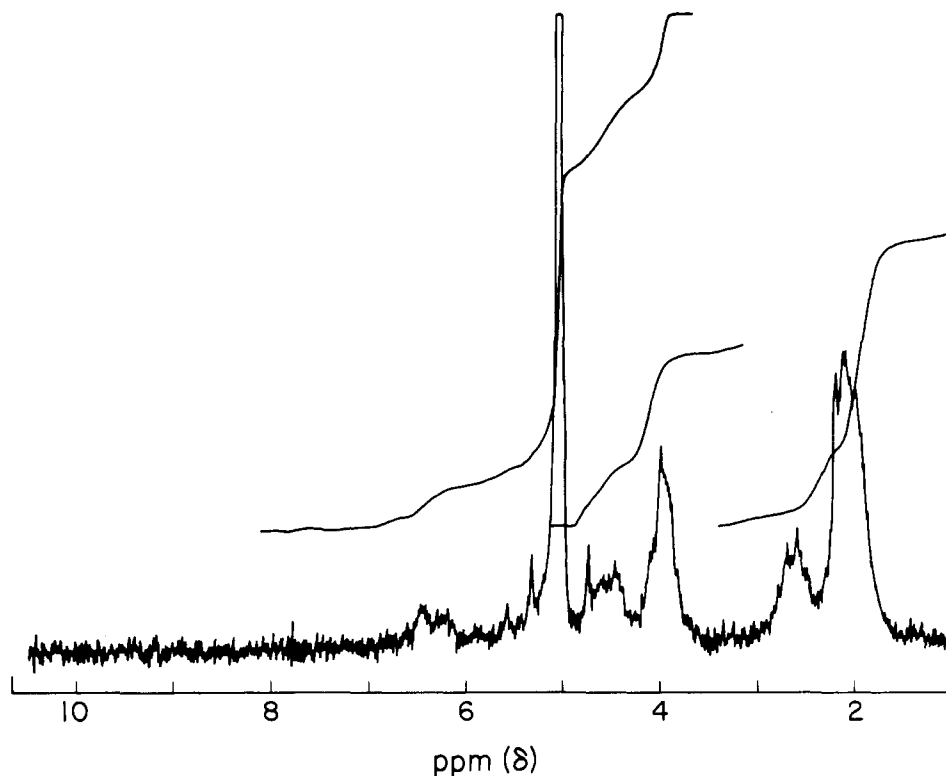
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Figure 1. Proton nuclear magnetic resonance spectrum of a siderophore obtained from the spent medium of *Penicillium roqueforti*. The conditions were as reported in Table II.

Table III. Thin-Layer and Paper Chromatography of Authentic Siderophores and Siderophores Isolated from *Penicillium roqueforti* (A, B, C, D), Blue Mold Powder (E), and Oregon Blue Cheese (F, G)

siderophore	source	color	R_f value solvent system[a]					
			1	2	3	4	5	6
ferrichrome	authentic	yellow-orange	0.29	0.29	0.14	0.26	0.60	0.36
coprogen	authentic	red-orange	0.50	0.45	0.23	0.21	0.66	0.48
coprogen B	authentic	orange-red	streak	0.19	0.15	0.00	streak	0.27
A	fungus isolated from cheese	red-orange	0.49	0.44	0.23	0.19	0.68	0.50
B	fungus isolated from cheese	red-orange	0.71	0.28	0.21	0.06	0.73	0.18
C	fungus isolated from cheese	orange-red	streak	0.18	0.15	0.00	streak	0.33
D	fungus from UC Berkeley Microgarden	red-orange	0.50	0.44	0.23	0.22	0.66	0.49
E	blue mold powder	yellow-orange	0.29	0.28	0.15	0.27	0.60	0.38
F	Oregon Blue cheese	yellow-orange	0.29	0.30	0.14	0.26	0.60	0.36
G	Oregon Blue cheese	red-orange	0.71	0.28	0.21	0.06	0.73	0.18

[a] 1, 9:1 (v:v) methanol/water on silica gel; 2, 9:6:5 (v:v:v) 1-butanol/1-propanol/water on cellulose; 3, 4:1:1 (v:v:v) 1-propanol/acetic acid/water on silica gel; 4, 2:1:1 (v:v:v) chloroform/methanol/benzyl alcohol on silica gel; 5, 2:1 (v:v) *p*-dioxane/0.33 N acetic acid on silica gel; 6, 4:1:5 (v:v:v) 1-butanol/acetic acid/water on Whatman No. 1, ascending.

to be identical with that of authentic dimerum acid (Diekmann, 1970). Sample 2 contained, as a major component, a negatively charged substance which probably corresponds to fusarinine. Thin-layer chromatography on Brinkmann silica sheets in 1-butanol/acetic acid/water (4:1:1, by vol) indicated that sample 1 contained dimerum acid and a substance with an R_f of 0.6, whereas sample 2 contained a compound with an R_f of 0.43, probably corresponding to fusarinine (Emery, 1965), and a substance with an R_f of 0.6, probably representing unhydrolyzed deferricoprogen.

Thin-layer and paper chromatography of the intact ferric siderophore in several solvent systems (Table III) showed the *P. roqueforti* compound, A, to migrate with an R_f equivalent to that of authentic coprogen.

Finally, paper electrophoresis at several values of pH (pH 4.9, in pyridine acetate buffer; pH 6.5 in 0.1 M phosphate buffer; and pH 2 in formic acetate buffer) indicated that the compound is, like coprogen, neutral.

The culture medium of the strain of *P. roqueforti* obtained from the University of California Microgarden also contained a compound (D) which comigrated with coprogen in several solvent systems (Table III).

Identification of the Crystalline Siderophore from Commercial Blue Mold Powder as Ferrichrome. The product crystallized from methanol in long, thin, orange-red needles characteristic of ferrichrome. The electronic absorption spectral curve was identical with that of ferrichrome and the ϵ_{mM} at 425 nm was 2.85 (literature 2.89, Neilands, 1952). The R_f and color of the visually observed spot on thin-layer and paper chromatography in several solvent systems (E, Table III) was identical with that of authentic ferrichrome from *Ustilago sphaerogena*. Similarly the compound was active in the antibiotic cross test and fed iron to *S. typhimurium* LT-2 TA2443 but not TA2701.

Characterization of the Siderophore from Oregon Blue Cheese. Chromatography of the cheese extract on P-2

Table IV.   Competition with $\phi80$ vir. for the Ferrichrome Receptor of E. coli Strain CGSC856[a]

addition	concn,[b] M	inhibition, %
ferrichrome	2×10^{-7}	100
	2×10^{-8}	85
	2×10^{-9}	50
cheese dialysate	5×10^{-7}	100
	5×10^{-8}	100
	5×10^{-9}	35

[a] The assay was performed as described by Wayne and Neilands (1975).   [b] The relative concentration of siderophore in the dialysate was measured as siderophore growth factor activity for S. typhimurium LT-2 TA2443.

enabled sizing of the active substance(s). The activity was found to lie well within the bounds of the molecular weights of most known siderophores (500–1000 daltons).

Paper and thin-layer chromatography of the cheese extract purified through P-2 proved difficult as the preparation was quite viscous. The viscosity was significantly reduced when a gummy substance precipitated out of a methanolic solution of the extract which was allowed to stand for three weeks at 0 °C.

The neutral siderophore component (F) obtained from cheese, unlike the negatively charged fraction (G), was capable of protecting E. coli K-12 in the cross test. Coprogen had no protective capacity.

As shown in Table IV, the dialysate prevented adsorption of $\phi80$ vir. and the protective potency correlated reasonably well with the growth factor activity.

The sum of these data indicate that siderophores A and D are coprogen, E is ferrichrome, as is also probably F, while B, C, and G remain uncharacterized.

DISCUSSION

The presence of the siderophore ferrichrome in Oregon Blue cheese was strongly indicated by both biological and physical data. The behavior on gel permeation chromatography, the relative mobility in several solvent systems, the visible absorption spectrum, and the ability to protect E. coli K-12 from the antibiotic analogue of ferrichrome, albomycin, and the phage $\phi80$ vir. support this conclusion.

Siderophore activity, as measured by growth of TA2443, was detected in all of the cheeses ripened by either P. roqueforti or P. camemberti. This material can probably be attributed to true siderophore activity since strain TA2701, which did not respond to the extracts, has been shown unable to utilize most known hydroxamate-type siderophores (Luckey et al., 1972). A more efficient extraction process applied to cheese might reveal the presence of somewhat higher levels than the estimated 5–10 mg/kilogram siderophore (as ferrichrome) detected in the present study.

The isolation of crystalline ferrichrome from commercial blue mold powder indicates that this product may be a convenient source of small amounts of this interesting metabolite.

Coprogen was found in the spent culture medium of P. roqueforti, whereas ferrichrome was discovered in cheese and in mold powder. This is not a surprising result since most fungi synthesize more than one siderophore and since the relative yields may be influenced by the environment. Coprogen and ferrichrome are both typically produced by the Penicillia. All the Penicillium strains which Winkelmann has examined produce ferrichrome, and many synthesize coprogen (G. Winkelmann, personal communication). Finally, the origin of the siderophore could also account for these differences. Horowitz et al. (1976) found coprogen in the culture medium of Neurospora crassa, whereas ferrichrome C and ferricrocin were isolated from

the conidia and the fungal mycelium. Thus the bulk of the siderophore isolated from cheese may actually have arisen from the fungal conidia and/or mycelium.

The P. roqueforti siderophore, coprogen, was accompanied by two other components: a negatively and a positively charged substance, both having growth factor activity for TA2443. Chromatographic analysis showed that the positively charged compound might be related to coprogen B, whereas the negative component resembled the acidic substance isolated from the cheese. These latter compounds remained negative even at pH 2 as measured by paper electrophoresis in formic acid–acetate buffer. Their behavior does not correspond to that of any known siderophore.

What is the relevance of the presence of siderophores in food? There are several points of interest. Positively speaking, siderophores may play a role in solubilizing iron in the gut and thus make it more available (transportable). Brown et al. (1978) have demonstrated ferric acid–acetohydroxamate to be a potent iron source for the anemic rat.

Negatively speaking, siderophores in foods might complex iron and render this important nutrient unavailable to the body. Neither deferrioxamine nor its iron complex, the siderophore of Streptomyces pilosus, are absorbed from the gut (Anderson and Hiller, 1976).

Finally, hydroxamate siderophores such as ferrichrome could be modified in vivo by hydrolysis or oxidation to yield toxic products. Hydrolysis will afford an alkyl hydroxylamine, the latter moderately mutagenic and hence possibly carcinogenic. Chemical oxidation of benzohydroxamic acid leads to a significant yield of N,O-dibenzoylhydroxylamine (Emery and Neilands, 1962), thus illustrating the potential for conversion of the hydroxamic acid to an acylating and mutagenizing agent.

Siderophores have now been identified in both sake (Tadenuma and Sato, 1967) and cheese. Obviously, the significance of the presence of these substances in the human diet are questions which await clarification.

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Action of Sodium Nitrite on Folic Acid and Tetrahydrofolic Acid

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Folic acid reacted with sodium nitrite in dilute aqueous solution at both pH 1.5 and 5.0 to yield exclusively N^{10}-nitrosofolic acid. At pH 1.5, the reaction was second order in nitrite, but at pH 5.0, the order in nitrite was one, and buffer anions participated in the nitrosation reaction. Tetrahydrofolic acid was rapidly oxidized by sodium nitrite at both pH 1.5 and 5.0 to produce p-aminobenzoylglutamate and several pterin products. Ascorbate had a protective effect on the oxidation of tetrahydrofolate by nitrite at pH 5.0, but even when the ascorbate was in 100-fold excess, tetrahydrofolate oxidation was not completely inhibited. 5-Methyltetrahydrofolate was oxidized by nitrite at pH 5.0 to produce several pterin products. 5-Formyltetrahydrofolate reacted with sodium nitrite at pH 5.0 to yield a single product which was probably the N^{10}-nitroso derivative.

The occurrence and formation of N-nitroso compounds, especially in foods, has received much attention recently (Scanlan, 1975; Gough et al., 1977). The widespread occurrence of both nitrite and folic acid and its derivatives in food material makes the interaction of these compounds of interest. We have shown that N^{10}-nitrosofolic acid (Figure 1) is a weak carcinogen for the rat (Wogan et al., 1975). N^{10}-Nitrosofolic acid is also a bacterial mutagen and is active in a mammalian cell transformation assay (Purchase et al., 1978).

In addition to being a nitrosating agent, nitrous acid is a potent oxidizing agent. For example, it readily oxidizes ascorbic acid (Bunton et al., 1959; Archer et al., 1975). The naturally occurring forms of folic acid are reduced derivatives (Stokstad et al., 1977) and interaction of these forms with nitrous acid may hence lead to a loss of vitamin activity.

Because of the complex chemistry of tetrahydrofolate oxidation reactions, the multiplicity of products and rapid reaction rates (Chippel and Scrimgeour, 1970; Blair and Pearson, 1974), kinetic analyses have been difficult to perform. To overcome these problems, we have developed methods for the rapid, efficient analysis of folate and pterin (2-amino-4-hydroxypteridine) derivatives using high-performance liquid chromatography (Reed and Archer, 1976; Archer and Reed, 1979). These separations include the reduced and the N^5- or N^{10}-substituted folates, the products of nitrosation of folic acid, and pterins that result from the oxidative breakdown of tetrahydrofolic acid (xanthopterin, pterin-6-carboxaldehyde, and pterin). Analysis of microgram samples of these derivatives which can be injected sequentially at 5–10-min intervals has thus

become possible. We present here our work on the nitrosation of folic acid and oxidation of 5,6,7,8-tetrahydrofolic acid by nitrous acid using these methods. We also present some preliminary work on the reaction of nitrous acid with 5-methyl- and 5-formyltetrahydrofolate.

MATERIALS AND METHODS

Important Safety Note. N-Nitroso compounds have been shown to be carcinogenic in test animals and all experimental work should be done in an efficient fume hood. Safety gloves should be worn whenever these compounds are being handled. The gloves should not be re-used.

Folic acid, dihydrofolic acid, tetrahydrofolic acid, 5-methyltetrahydrofolic acid, p-aminobenzoylglutamate, xanthopterin, pterin, and pterin-6-carboxylic acid were purchased from Sigma (St. Louis, MO). 5-Formyltetrahydrofolic acid was purchased from Grand Island Biochemical Co. (Grand Island, NY). Pterin-6-carboxaldehyde was prepared by the method of Waller et al. (1950); N^{10}-nitrosofolic acid was prepared by the method of Cosulich and Smith (1949); 2-hydroxy-N^{10}-nitrosofolic acid and 2-hydroxyfolic acid were prepared by the method of Angier et al. (1952).

High-performance liquid chromatography of the various folic acid derivatives and pterins has been described previously (Reed and Archer, 1976; Archer and Reed, 1979).

In the kinetic experiments, folic acid was dissolved in 0.01 M citrate/0.02 M phosphate buffer adjusted to either pH 5.0 with NaOH or pH 1.5 with perchloric acid. Some experiments were also performed in acetate buffer, pH 5.0, at concentrations in the range of 0.025 to 0.2 M. Sodium nitrite was added to start the reactions. Reaction mixtures were maintained in a constant temperature bath (25 or 30 °C) with stirring. In the reaction at pH 5, samples were removed at various time intervals and immediately analyzed by liquid chromatography. Dilution of reactants caused by the high eluant flow rate had the effect of

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