CONSEQUENCES OF *BACILLUS SUBTILIS* IN IRON DEFICIENCY

by

WALTER JOSEPH PETERS

B.S.A. (Microbiology),
University of British Columbia, 1965

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN THE DEPARTMENT

OF

MICROBIOLOGY

We accept this thesis as conforming to the

required standard

THE UNIVERSITY OF BRITISH COLUMBIA

May, 1968

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and Study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Microbiology

The University of British Columbia
Vancouver 8, Canada

Date April 29, 1968.

ABSTRACT

Cultures of Bacillus subtilis growing in an iron-deficient medium produced coproporphyrin III (coproporphyrin) and phenolic acids. (2,3-dihydroxybenzoylglycine (DHBG), 2,3-dihydroxybenzoic acid (DHB), or both). (DHB(G) refers to DHB or DHBG, or both compounds). Phenolic acid production was proportional to the amount of iron present, and occurred logarithmically, parallelling growth. In the presence of DHB, lower levels of iron inhibited phenolic acid production, so that the actual inhibition of synthesis may involve the Fe^{+3}:$(DHB(G))_3$ complex. Accumulation of DHB(G) was influenced by the levels of aromatic amino acids, anthranilic acid, and histidine in the medium. In vitro experiments demonstrated that DHB was formed from chorismic acid. In vivo and in vitro experiments with strain B-1471 showed that DHB was coupled to added glycine to form DHBG. Disappearance of DHB(G) was observed in all strains studied, but oxidation did not occur.

Phenolic acid production always preceded coproporphyrin production. Phenolic acids have very strong affinities for ferric iron. Their production may therefore allow the scavenging of the last traces of iron from the medium for hemin synthesis. The relationship between phenolic acid and coproporphyrin production was borne out by the following observations: (i) a higher level of iron was required to prevent coproporphyrin production than phenolic acid production (ii) the $Fe^{3+}(DHB(G))_3$ complex was a more potent inhibitor of coproporphyrin production than iron alone (iii) a mutant blocked at δ-aminolevulinic acid synthetase did not produce phenolic acids during iron-deficient

growth (iv) serine auxotrophs produced much lower levels of coproporphyrin and phenolic acids than the wild-type strain (v) some mutants defective in phenolic acid production produced low levels of coproporphyrin, whereas one strain of this type produced elevated levels of coproporphyrin.

Compounds known to inhibit normal functioning of the tricarboxylic acid cycle decreased coproporphyrin production in all strains studied. These inhibitors reduced DHBG excretion, but had no effect on DHB production. A number of analogs of DHB inhibited DHB(G) accumulation to varying degrees, depending upon their structure. The most potent inhibitors were m-substituted derivatives of benzoic acid. Two sideramines, ferrichrome and ferrioxamine, inhibited DHBG production in strain B-1471. The inhibitory action of ferrichrome was shown to be due to its ability to mediate cellular uptake of low levels of iron.

The capacity of B. subtilis for iron uptake was increased about 20-fold by growing the cells in an iron-deficient medium. Under these conditions, the addition of low levels of phenolic acids increased both the rate and extent of iron uptake. Mutants unable to synthesize normal levels of phenolic acid were shown to have a reduced capacity for iron uptake after growth in an iron-deficient medium. Mutants resistant to 8-hydroxyquinoline had an increased capacity for iron-uptake under these conditions.

TABLE OF CONTENTS

Table of Contents (Continued)

Table of Contents (Continued)

Table of Contents (Continued)

Table of Contents (Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES - continued

ACKNOWLEDGEMENTS

To Professor R. A. J. Warren for his unique and enthusiastic approach to scientific discovery, and for his demonstration of the framework on which science must be built. To Professor E. W. Nester for allowing me to draw upon his experience and knowledge of Bacillus subtilis. To Professor F. Gibson for providing many of his experimental observations prior to their publication. To W. W. Kay for valuable assistance with the planning and interpretation of transport experiments. To A. M. B. Kropinski whose general knowledge of microbial metabolism was continuingly available. To the other co-workers of Room 14 for constant discussion and criticism which, in many cases, led to experimental elucidation. To Mrs. Rosemary Morgan for generously typing this thesis. To Mrs. Rita Rosbergen for overcoming the administrative difficulties encountered during the past two weeks.

INTRODUCTION

An alteration of metabolism as a consequence of iron deprivation has been reported for a number of microbial species. In many instances, this alteration is manifested by the production of high levels of ferric iron complexing agents (40). Thus, Ustilago sphaerogena (17) produced the complex trihydroxamates, ferrichrome and ferrichroma A, while Bacillus megaterium produced a secondary monohydroxamic acid ("schizokinen") (10) under iron deficiency.

Other organisms have been shown to excrete phenolic acids when grown in a medium containing limiting iron. Mycobacterium smegmatis produced salicylic acid; Aerobacter aerogenes and a strain of Escherichia coli produced 2,3-dihydroxybenzoic acid (DHB) (50); another strain of E. coli produced 2,3-dihydroxybenzoylserine (DHBS) (17). Extracts of this second strain of E. coli formed DHBS from DHB and serine, and the level of this enzyme (DHBS synthetase) was dependent upon the level of iron in the growth medium (17). Brot and Goodwin (6) have suggested that iron may act as a corepressor in the system controlling the synthesis of DHBS synthetase.

Itoic acid, 2,3-dihydroxybenzoylglycine (DHBG) was found to be excreted by Bacillus subtilis (18). Accumulation of this compound depends upon the level of iron in the medium: cultures devoid of iron did not grow; high levels of iron prevented its production (27); low levels of iron lead to the excretion of more than 200 mg of DHBG per litre of culture fluid (40).

During the past few years, a relationship has been demonstrated between phenol excretion and aromatic metabolism. Pittard et al

(47, 48) showed that washed cell suspensions of a number of aromatic amino acid auxotrophs of A. aerogenes excreted o-dihydroxy phenols, amongst them DHB, when incubated in minimal medium. In one such strain, NC3, blocked at anthranilate synthetase, formation of o-dihydroxy phenols was inhibited completely by exogenous tryptophan. Furthermore, it has been shown that while a wild type strain of A. aerogenes produced DHB only under iron deficiency, a mutant of this strain blocked after anthranilate synthetase produced DHB regardless of the level of iron in the medium (51).

The precise alteration of aromatic metabolism brought about by iron deficiency and causing the formation of phenolic acids is unclear. Ratledge and Winder (52) have suggested that M. smegmatis may synthesize salicylic acid from shikimic acid by steps analogous to those involved in anthranilic acid formation (60), but with the introduction of a hydroxyl group instead of an amino group. Under iron deficiency, the hydroxyl-insertion reaction could be accentuated, possibly by a requirement for iron by a competing pathway. This suggestion was supported by the observation that either magnesium or iron was required for the conversion of shikimic acid-5-phosphate to anthranilic acid by an extract of E. coli (59). Work with A. aerogenes, however, has indicated that Fe^{+2} ions inhibit anthranilic acid synthesis (53). In addition, an iron requirement has not been shown for the anthranilate synthetase prepared from E. coli (5, 58).

Cox and Gibson (12) showed that DHB was a growth factor for certain multiple auxotrophs of E. coli, but only in iron-deficient medium. Young et al (67) found that extracts of E. coli were

capable of forming DHB from chorismate, and that the activity of these preparations was dependent upon the level of iron.

In many microorganisms, growth in iron-deficient media leads to a disturbance of porphyrin metabolism (31). When U. sphaerogena was grown in iron-limiting medium, no porphyrin compounds were excreted. Extracts prepared from these cells contained less δ-aminolevulinate dehydratase activity than extracts from iron sufficient cells (29). In other organisms, there was excretion of high levels of a porphyrin. B. subtilis (18), Corynebacterium diphtheriae (23) and Micrococcus lysodeikticus (62) produced coproporphyrin III. Furthermore, it has been reported (25) that the production of coproporphyrin III by B. subtilis was always accompanied by the excretion of large amounts of DHBG. As DHBG has a very strong affinity for ferric iron (26) it has been suggested that this phenolic acid may be excreted into the medium to make iron available to the cell (40). In this regard, it is interesting that o-dihydroxy phenols are required for the growth of M. lysodeikticus (56). Similarly, low levels of these compounds have been shown to replace the tyrosine requirement of a species of Sarcina (22).

The work described in this thesis was undertaken in an attempt to define an overall approach to the study of iron-deficiency in B. subtilis.

It was necessary, first of all, to determine whether DHB(G) was produced during active growth of the organism or if it accumulated only during the stationary phase. If it were produced only during the stationary phase it would be much harder to design and interpret

experiments on the control of its production. Aromatic compounds which, like DHB, are synthesized by the cell from chorismic acid, were examined for their effects on DHB production. Such studies would indicate whether or not DHB production was regulated by the control mechanisms known to operate in the aromatic pathway in B. subtilis.

The relationship of DHB(G) production to coproporphyrin production was then examined. Iron is required for the formation of hemin and of non-heme iron proteins. It was possible, therefore, that the production of coproporphyrin and DHB(G) might be related in some other way besides a lack of iron.

Analogs of DHB were tested for their effects on DHB production. Those analogs which were markedly inhibitory might then be useful in studying the enzymology of DHB(G) synthesis and its control.

DHB and DHBG are known to bind iron strongly (26) so that a possible function of these compounds might be to serve as iron-transport factors. A study was made, therefore, of iron uptake by B. subtilis and of the effects of DHB(G) on this process. In addition, the effects of sideramines on DHB(G) production and on iron uptake were studied. Sideramines are thought to function as iron transport factors in other microorganisms (39, 41).

MATERIALS AND METHODS

I. Bacterial strains

1. Wild type strains

The source of each wild-type strain employed is shown in Table I. Strain WB-746 was selected (45) as a spontaneous prototrophic revertant of a tryptophan auxotroph strain (68).

2. Mutant strains

Mutant strains were selected as spontaneous derivatives or as N-methyl-N^1-nitro-N-nitrosoquanidine (NTG)-induced mutants (Table II).

(a) Spontaneous mutants

Strains resistant to 8-hydroxyquinoline (HQ^r), 5-methyltryptophan (MT^r), and to various antibiotics were selected by spreading log phase cells on minimal medium supplemented with a level of inhibitor which prevented the growth of the wild type strain. The supplementation level required for each class of mutants is presented in the text.

(b) NTG-induced mutants

Washed, log phase cells were suspended in tris-maleate buffer (1) and treated with NTG according to the procedure of Lorence and Nester (33).

Auxotrophs were selected as minute colonies on minimal medium containing a limiting level of the appropriate supplement: serine-glycine auxotrophs, 0.3 μg serine per ml; δ-aminolevulinate (ALA)

Table 1: B. subtilis wild type strains

Strain	Source
B-1471	J. B. Neilands, University of California, Berkeley.
WB-746 WB-443	E. W. Nester, University of Washington, Seattle.
W-23	J. Spizizen, Scripps Clinic and Research Foundation, La Jolla, California.
6051	American Type Culture Collection (ATCC)
6633	ATCC
6455	ATCC
12696	ATCC
14807	ATCC

Table II: Description of B. subtilis mutant strains

Strain	Genotype	Enzyme defect	Source
trp-1	trp^-	anthranilate synthetase	B-1471[a]
trp-1-MT^r-1	trp^-MT^r	anthranilate synthetase	trp-1[b]
trp-1-MT^r-2	trp^-MT^r	anthranilate synthetase	trp-1[b]
trp-2	trp^-	tryptophan synthetase	B-1471[a]
trp-3	trp^-	InGP synthetase	B-1471[a]
trp-4	trp^-	anthranilate synthetase	B-1471[a]
trp-5	trp^-	tryptophan synthetase	B-1471[a]
trp-6	trp^-	tryptophan synthetase	B-1471[a]
trp-7	trp^-	PRA isomerase	B-1471[a]
hem 1	ALA^-	ALA synthetase	B-1471[a]
phe-1	phe^-		B-1471[a]
phe-2	phe^-		B-1471[a]
MT^r-1	MT^r		B-1471[b]
MT^r-2	MT^r		B-1471[b]
HQ^r 1-6	prototroph		B-1471[b]
ser 1-4	ser^-		B-1471[a]
dhb-1-5			prototrophic revertants[b] of DHB auxotroph[a] of B-1471
aro-1	shk^-		B-1471[a]
SB-168	trp^-	InGP synthetase	E.W. Nester, WB-746
SB-194	trp^-	anthranilate synthetase	E.W. Nester, WB-746
SB-194-MT^t-1	trp^-	anthranilate synthetase	SB-194[b]
SB-194-MT^r-2	trp^-	anthranilate synthetase	SB-194[b]
SB-30	tyr^- trp^-		E.W. Nester, WB-746
WB 2102	shk^-	DAHP synthetase	E.W. Nester, WB-746
SB 167	shk^-	DHQ synthetase	E.W. Nester, WB-746
MT^r-1	MT^r		WB-746[b]
MT^r-2	MT^r		WB-746[b]

Table II (continued)

Strain	Genotype	Enzyme defect	Source
HQ^r 1-6	prototrophs		WB-746[b]
MT^r-1	MT^r		W-23[b]
MT^r-2	MT^r		W-23[b]

a: spontaneous derivative; b: NTG - induced derivative.

Abbreviations used: InGP, indoleglycerol phosphate; PRA, phosphoribosylanthranilic acid; Shk, shikimic acid; DAHP, 3-deoxy-D-arabino-heptulosonic acid 7-phosphate; DHQ, dehydroquinic acid; ALA, -aminolevulinic acid.

auxotrophs, 0.1 µg ALA per ml, with 10 g citric acid per litre replacing glucose as the carbon source; DHB auxotrophs, 0.05 µg DHB per ml, with citrate being omitted from the medium in this case because it has been shown that citrate can replace DHB as a growth requirement for a multiple aromatic auxotroph of Escherichia coli (67). Before plating for mutant selection, the mutagenized cells were diluted and plated on the appropriate selection medium to score for survivors. To avoid lysis of the treated cells during the incubation of plates for scoring survivors, it was necessary to store the cells at 4 C in minimal medium containing 5% glycerol (33). Under these conditions about 90% of the cells remained viable three days.

All strains were stored on Difco TAM sporulation agar slants in screw-cap vials.

II. Media

Difco trypticase soy broth supplemented with 1% Difco yeast extract (TSY medium) was used for the growth of inocula. The minimal medium used for the production of porphyrin and phenolic acids was a modification of the medium of Neilands and Garibaldi (42). It contained, in g per litre: K_2HPO_4, 1.0; ammonium acetate, 3.0; $MgSO_4.7H_2O$, 0.08; glucose, 10.0. It was supplemented with 5 ml per litre of a 10% solution of Difco yeast extract in distilled water, autoclaved with alumina prior to use (10), and with 0.1 ml per litre of the Neurospora trace elements solution of Vogel (65), from which the iron was omitted. The medium was made up with glass-distilled water, and was adjusted to pH 7.4 before autoclaving.

The glucose was sterilized separately as a 40% solution in glass-distilled water.

Extraction of the medium with 8-hydroxyquinoline (HQ) (65) yielded erratic results, presumably due to microquantities of HQ remaining in the medium after chloroform extraction. This procedure was therefore not employed.

III. Cultural conditions

All glassware was autoclaved twice with glass-distilled water prior to use. Erlenmeyer flasks (250 ml), fitted with side arms and containing 25-ml quantities of the medium, were incubated at 37 C in a New Brunswick Metabolyte water bath (New Brunswick Scientific Co., New Brunswick, N.J.), rotating at 250 rev/min. All experiments were performed by use of a 0.5% inoculum from a 14-hr culture, which was one transfer away from the stock slant. The time of onset of DHB(G)* production was constant under these conditions. Additions to flasks were made at the times mentioned in the text.

IV. Determinations

1. Growth

Growth was followed turbidimetrically, by use of a Klett-Summerson colorimeter with a540 filter. Readings were converted to cell numbers by use of a standard curve prepared with the organism.

* DHB(G) refers to DHB, or DHBG, or both compounds.

For higher densities it was necessary to dilute the culture with complete medium. A cell density of 10^8 per ml gave a reading of 42 Klett units. Growth curves were followed during all experiments.

2. Phenolic acids

DHB(G) was assayed by adding 0.5 ml of a ferric iron solution (0.5 mg per ml) to 2.0 ml of cell-free medium adjusted to pH 7.6, centrifuging, and measuring the optical density (O.D) of the supernatant at 510 mμ, 1.0 cm light path, with a Beckman Model B spectrophotometer. Blank corrections were always made, using 2.0 ml of cell free medium and 0.5 ml distilled water. Under these conditions, 1.0 O.D. unit represented a concentration of 364 μg DHB(G) per ml of culture supernatant. Production of DHB and DHBG was confirmed spectrophotometrically and chromatographically (27) by comparison with authentic specimens. Descending chromatographic analyses were performed using two solvent systems; (1) n-butanol, acetic acid, water 4/1/5; (2) t-butyl alcohol, methyl ethyl ketone, water, diethyl amine 10/10/5/1. The RF values are given in Table V.

3. Coproporphyrin III*

A 2.0 ml sample of culture supernatant was adjusted to pH 5.0 with acetic acid. The coproporphyrin was extracted with a known volume of ether. The absorption of the ether extract was measured at 408 mμ using a Beckman DB spectrophotometer (15). Care was taken to ensure that, at the time of measurement, the volume of

* Coproporphyrin III is referred to as coproporphyrin.

the ether was equal to that originally added to the acidified culture supernatant.

4. Identification of glycine

Electrophoresis was performed with a model D high-voltage electrophorator (Gilson Medical Electronics, Middleton, Wisc.), using 2.5% formic acid and 7.8% acetic acid buffer (pH 1.9), at 2000 v for 45 min. Glycine was identified by co-electrophoresis with an authentic specimen.

5. Protein concentration

Protein was assayed by the method of Lowry et al (35).

V. Preparation of extracts

After 12-14 hr growth, cultures were harvested by centrifugation at 20 C, washed once with complete medium, and the cells resuspended at a concentration of 1 g wet weight per 5 ml in 0.05 M potassium phosphate - 0.01 M mercaptoethanol, pH 7.5. Lysozyme (100 μg per ml), deoxyribonuclease (10 μg per ml) and $MgCl_2$ (5×10^{-3} M) were added to the suspension and the mixture incubated for 30 min at 37 C. The resulting extract was sonicated for 2 min at a probe intensity of 70 using a Biosonik probe oscillator, (Bromwill Scientific, New York, N.Y.), and then centrifuged at 25,000 x g for 15 min at 4 C. The supernatant fluid was used directly for enzyme assays.

VI. Enzyme assays

1. Synthesis of DHB

The formation of DHB from chorismic acid was measured by the method of Young et al (67). DHB production was estimated at a standard curve prepared using commercial DHB (67). Under these conditions, an O.D. at 318 mμ of 0.1 represented 0.12 μmoles DHB.

2. Synthesis of DHBG

The synthesis of DHBG from DHB and glycine was determined by the method of Brot et al (7), except that glycine was substituted for serine in the reaction mixture.

VII. Respiration studies

Cells were harvested at 20 C at the times indicated in the text, washed twice with 0.005 M Mg^{+2} - 0.1 M tris(hydroxymethyl)aminomethane (Tris)-chloride (pH 7.4), and resuspended in the same buffer at a concentration of approximately 5 mg dry weight of cells per ml. Respiration studies were performed at 37 C in Warburg vessels which contained a final concentration of 0.005 M Mg^{+2} - 0.05 M Tris pH 7.4, one ml of cell suspension, and 5 μmoles of acetate or citrate.

VIII. Iron transport studies

1. Preparation of cells

Cultures were grown to a density of 5.5×10^8 cells/ml (unless stated otherwise), then used immediately for transport studies. For iron-sufficient growth, the medium was supplemented with 1 μg of iron/ml.

2. Iron uptake

(a) Iron-deficient cells

Ten ml of culture were transferred to a 250 ml Erlenmeyer flask. The flask was incubated at 37 C (unless stated otherwise) in a New Brunswick Model G-77 Metabolyte water bath (New Brunswick Scientific Co., New Brunswick, N.J.), rotating at 100 rpm. Additions were made after 10 min.

(b) Iron-sufficient cells

Ten ml of culture were filtered through a 0.45 μ Millipore membrane. The cells were washed with 10 volumes of prewarmed, iron-deficient medium, and the membrane transferred to 10 ml prewarmed iron-deficient medium in a 250 ml Erlenmeyer flask. The cells were resuspended by gently blowing medium over the membrane with a pipette. The flask was incubated as for iron-deficient cells, with additions again being made after 10 min. In both cases, additions were made in a total volume of 0.5 ml glass-distilled water. When uptake was measured at 0 C, glycerol was added to a concentration of 5% to prevent lysis of the cells. Unless stated otherwise, iron was used at a concentration of 5.0 mμg/ml.

3. Assay of iron uptake

Samples of 1.0 ml were withdrawn with hypodermic syringes, filtered through 0.45 μ Millipore membranes and the cells washed with 2.0 ml prewarmed, iron-deficient medium. The membranes were

dried, placed in vials containing 10 ml scintillation fluid (Liquifluor, New England Nuclear Corp.) and assayed for radioactivity in a liquid scintillation counter (Nuclear Chicago Model 225). Unless stated otherwise, the rate of uptake was measured over the interval 3 min to 8 min after iron addition. Radioactivity in the cold TCA-insoluble fraction of cells was measured by the method of Roberts et al (53). A 1.0 ml sample was added to 1.0 ml of 10% TCA at 0 C. After 30 min at 0 C, the suspension was filtered through a 0.45 μ Millipore membrane, and the membrane washed with two volumes of iron-deficient medium. The membrane was dried and assayed for radioactivity.

IX. Chemicals

Chorismic acid was purified from the cultural supernatant of A. aerogenes 62-1 according to the method of Gibson (19) except that the acid was precipitated from the Dowex effluent as the barium salt (18). Free chorismic acid was obtained by sedimenting most of the barium as the phosphate salt, and by subsequent addition of Dowex-50 to the supernatant. DHBG was synthesized by a modification of the dicyclohexylcarbodiimide method of Sheehan and Hess (26). Phosphoribosylanthranilic acid (1-(O-carboxyphenylamino)-1-d-D-ribulose-5-phosphate) was synthesized according to the method of Doy (13).

Ferrichrome was obtained from J. B. Neilands of the University of California, Berkeley. The iron was removed from this compound by the method of Emery and Neilands (14). Ferrioxamine was obtained from W. Keller-Schierlein, of Eldg.

Technische Hochschule, Zurich, Switzerland.

The following chemicals were obtained from commercial sources: o- and m-tyrosine, δ-aminolevulinic acid (Sigma Chemical Co.); 2- and 3-fluoro, and 2,3-dimethoxybenzoic acid, DHB, NTG (Aldrich Chemical Co.); m-hydroxybenzoic acid, α-picolinic acid, and hippuric acid (J. T. Baker Chemical Co.); MT, 3-hydroxyanthranilic acid, hemin, serine-1-^{14}C (specific activity 10.3 μc per μmole) (Calbiochem Co.); salicylic acid (The British Drug Houses, Ltd.); ^{59}Fe as $FeCl_3$, specific activity 25.5 μc/μg (International Chemical and Nuclear Co.); glycine-1-^{14}C (specific activity 2 μc per μmole) (Merck & Co.).

RESULTS

Section I: - General properties of phenolic acid excretion

1. Excretion of phenolic acids by wild type strains

A number of wild type strains of B. subtilis were grown in iron-deficient medium to determine the compounds produced under these conditions. The strains fell into three groups according to their patterns of phenolic acid production: strains of group I produced only DHBG, those of group II only DHB, and those of group III both of these compounds (Table III). A strain representing each group was selected for further study. i.e. B-1471 (group I), WB-746 (group II) and W-23 (group III).

2. Influence of iron on DHBG production

Production of DHBG by strain B-1471, in the absence of added iron, started after about 8 hrs of growth and continued logarithmically, parallelling growth, until the early stationary phase was reached. Significant production of DHBG did not occur in the stationary phase. If the medium was supplemented with 1 mg of iron per litre, production of DHBG was inhibited completely (Fig. 1). At concentrations below 150 µg of iron per litre, DHBG production was inversely proportional to the level of iron in the medium. As the level of iron added was increased from 0 to 150 µg per litre of medium, the production of DHBG began proportionately later.in the growth period (Fig. 2). DHBG production was inhibited completely by the addition of 150 µg of iron per litre.

Table III. Excretion of phenolic acids by wild type strains

Group	Strain	DHB	DHBG	level produced (mg/l)
I	B-1471	-	+	300
	6633	-	+	50
	6455	-	+	60
	12696	-	+	150
II	WB-746	+	-	1000
	SB-443	+	-	100
III	W-23	+	+	250
	6051	+	+	200
	14807	+	+	100

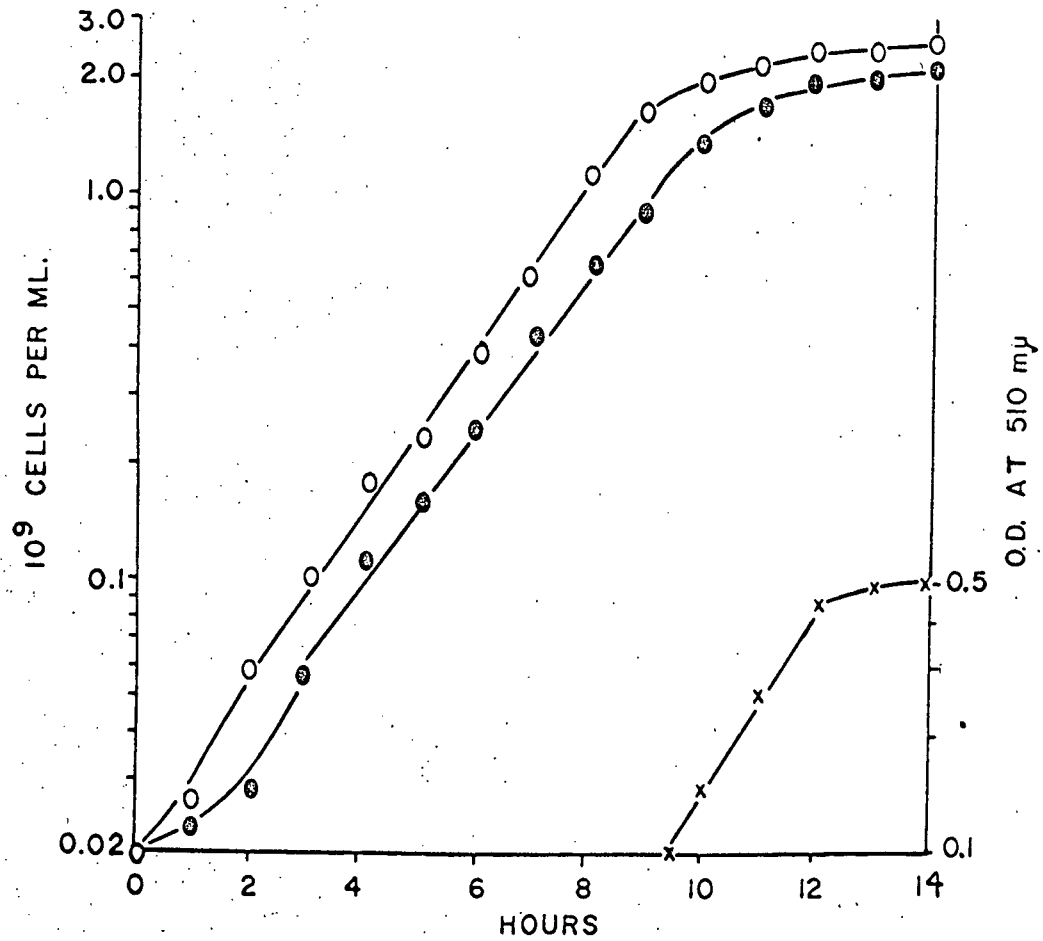

Figure 1. Growth and production of DHBG by strain B-1471. Cells were grown in the absence (●) and presence (O) of 1 mg of added iron per litre. DHBG was produced under conditions of iron deficiency (X) but was not produced in the presence of added iron.

3. Fe^{3+}: $(DHBG)_3$ complex and the control of DHBG production

When added to an iron-deficient culture at any time between 5 and 9 hrs after inoculation, 1 mg of iron per litre did not completely inhibit DHBG production. The addition of DHB or DHBG to an iron-deficient medium at zero time had no effect on subsequent DHBG production. (Fig. 3).

The simultaneous addition of 3.5×10^{-4}M DHB and 1 mg of iron per litre, 5 hrs after inoculation, resulted in the complete inhibition of DHBG production (Fig. 4). In fact, 80 µg of iron per litre was equally effective in the presence of 3.5×10^{-4}M DHB. When the iron-DHB mixture was added 7 or 9 hrs after inoculation, its inhibitory effect on DHBG production was not fully manifested for about 3 hrs (Fig. 4). It appeared therefore, that between 5 and 7 hrs in the growth cycle, an iron-deficient culture became committed to the production of some DHBG, regardless of subsequent addition of an iron-DHB mixture.

4. Effect of aromatic amino acids on DHBG production

The excretion of DHB by certain aromatic amino acid auxotrophs of A. aerogenes (43) prompted investigation of the effects of aromatic intermediates and end products on the production of DHBG by B. subtilis. None of the supplements, at the levels used, affected the growth rate of cultures.

Supplementation of media with p-hydroxybenzoic acid or p-aminobenzoic acid, at concentrations up to 10^{-3}M, had no effect on subsequent DHBG production. Addition of tryptophan at a concentration of 10^{-4}M caused earlier and significantly higher production of DHBG

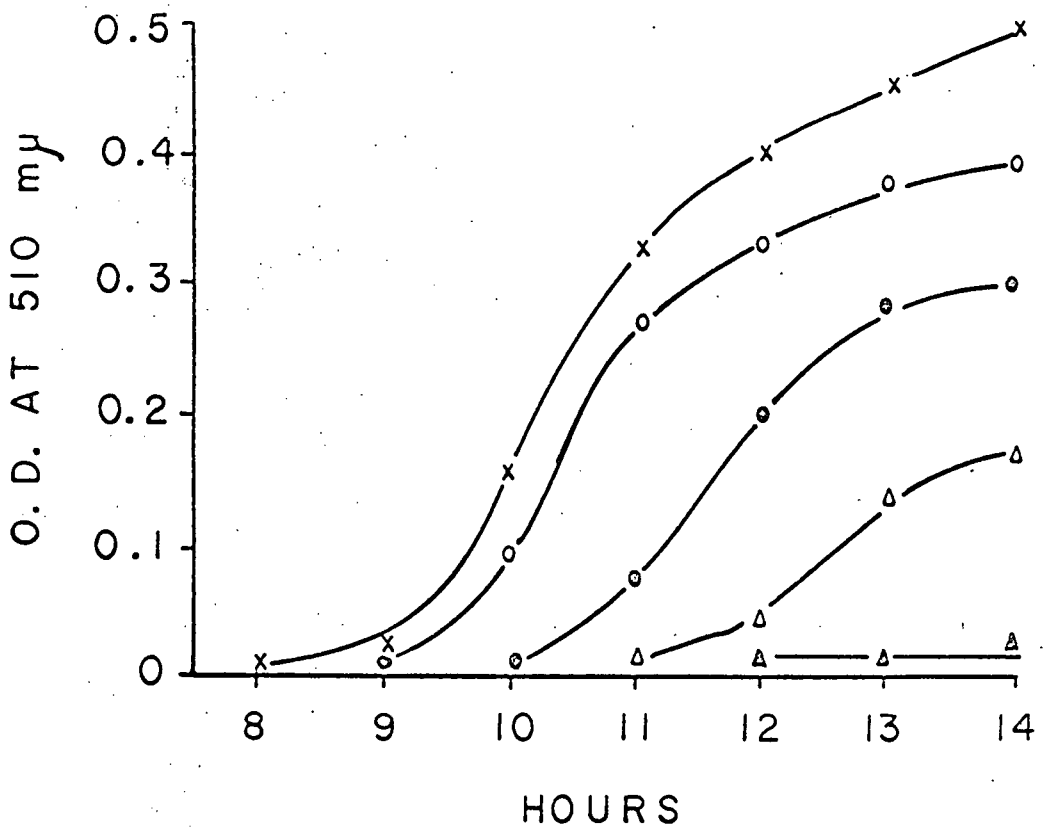

Figure 2. Effect of iron added at zero time on the production of DHBG by strain B-1471. The levels of iron added were, in μg per litre: 20 (0), 50 (●), 80 (▲), and 150 (▲). No iron was added to the control flask (X).

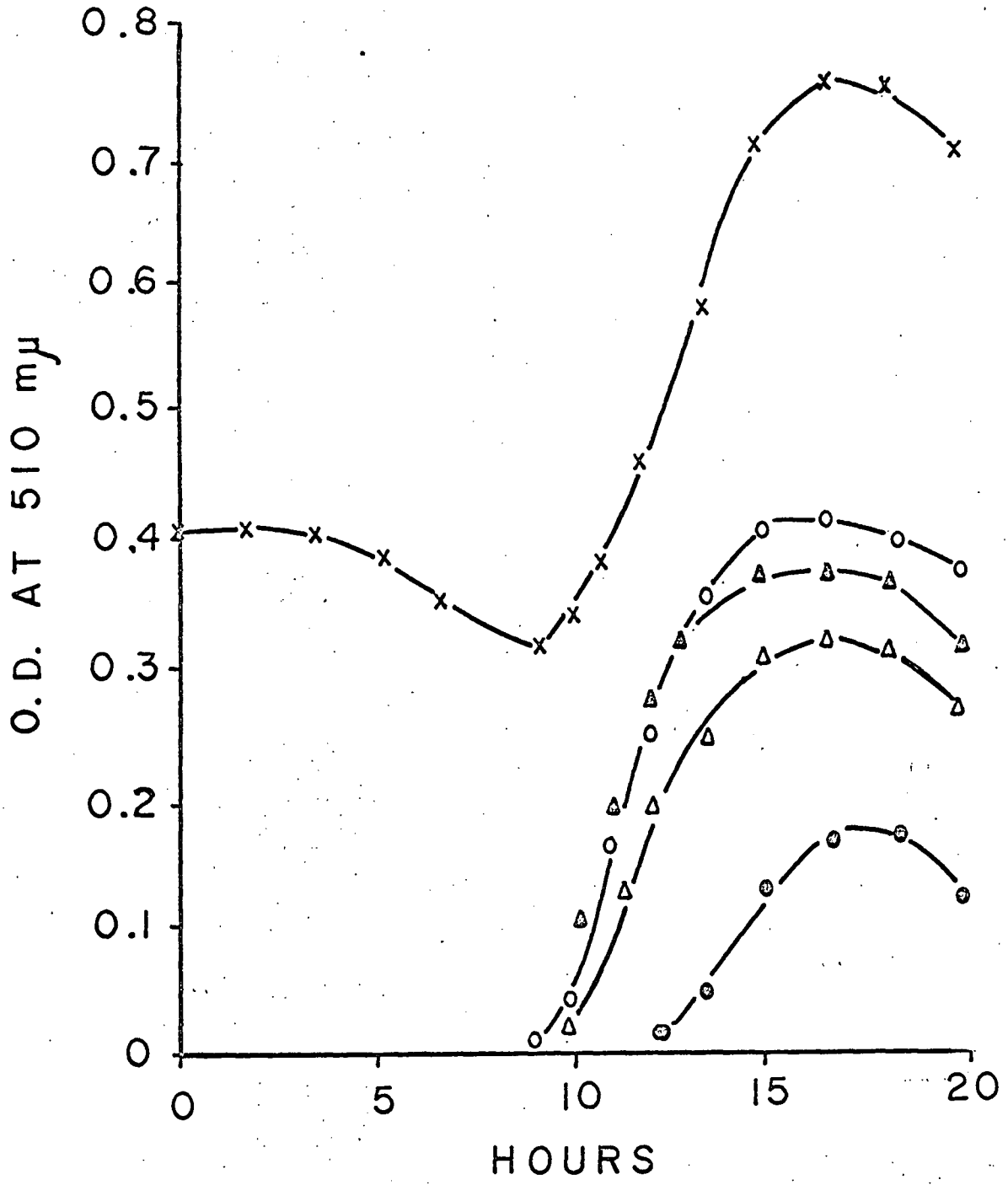

Figure 3. Effect on DHBG production of adding iron or DHB to strain B-1471. DHB (3.5×10^{-4} M) was added at zero time (X). Iron (1 mg per litre) was added at 5 hr (●), 7 hr (Δ), and 9 hr (▲). No iron or DHB was added to the control flask (O).

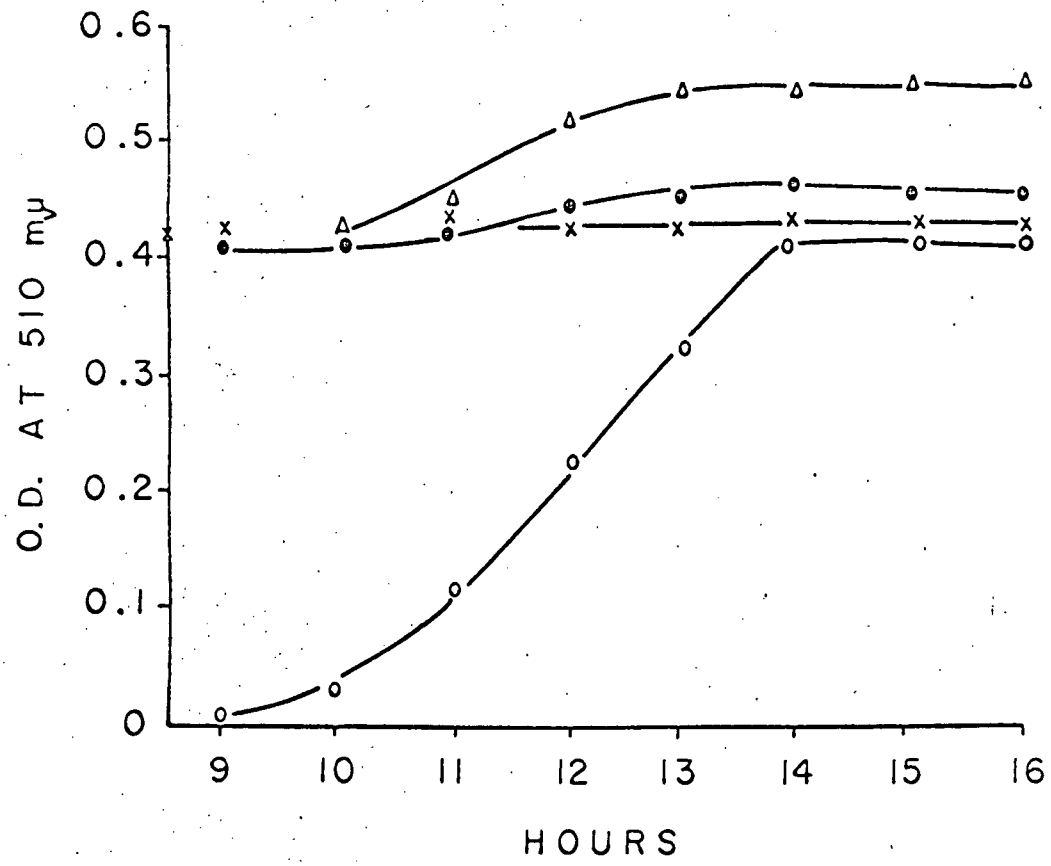

Figure 4. Effect of simultaneous addition of 3.5×10^{-4} M DHB or DHBG and 1 mg of iron per litre on DHBG production by strain B-1471. The additions were made at 5 hr (X), 7 hr (●), and 9 hr (Δ). No phenolic acids or iron was added to the control flask (○).

than in the control flask. As the level of tryptophan was increased beyond 10^{-4}M, DHBG accumulation was inhibited (Fig. 5). Phenylalanine and tyrosine reduced DHBG accumulation at 10^{-4}M, and to a greater extent at higher concentrations. Tyrosine was a more effective inhibitor than phenylalanine (Fig. 5). These inhibitory effects were not additive. The percentages of inhibition observed for each amino acid at 10^{-3}M, were tryptophan, 25; phenylalanine, 27; and tyrosine, 52. When all three amino acids were present together at 10^{-3}M, the inhibition was 50%.

Inhibition of DHBG production, comparable to that seen with tyrosine, was produced by anthranilic acid. The production of DHBG was inversely proportional to the level of anthranilic acid in the medium (Fig. 6). This inhibition occurred in the presence of tryptophan, so that the effect of anthranilic acid was not produced as a consequence of its depriving the cell of tryptophan (Fig. 6).

5. Source of glycine in DHBG

A volume of 200 ml of medium was inoculated and incubated as described in Materials and Methods. Immediately after the initiation of DHBG synthesis, 50 µmoles of glycine-1-^{14}C (specific activity, 2 µc per µmole) were added. Four hours later, the cells were removed by centrifugation and the DHBG was purified according to the method of Ito and Neilands (29). A 30-mg sample (wet weight) of cells was fractionated according to the procedure of Roberts et al (14). Radioactivity of the various fractions was measured by use of a Nuclear Chicago model 181 A planchet counter (Table IV). The purified DHBG gave a single spot when chromato-

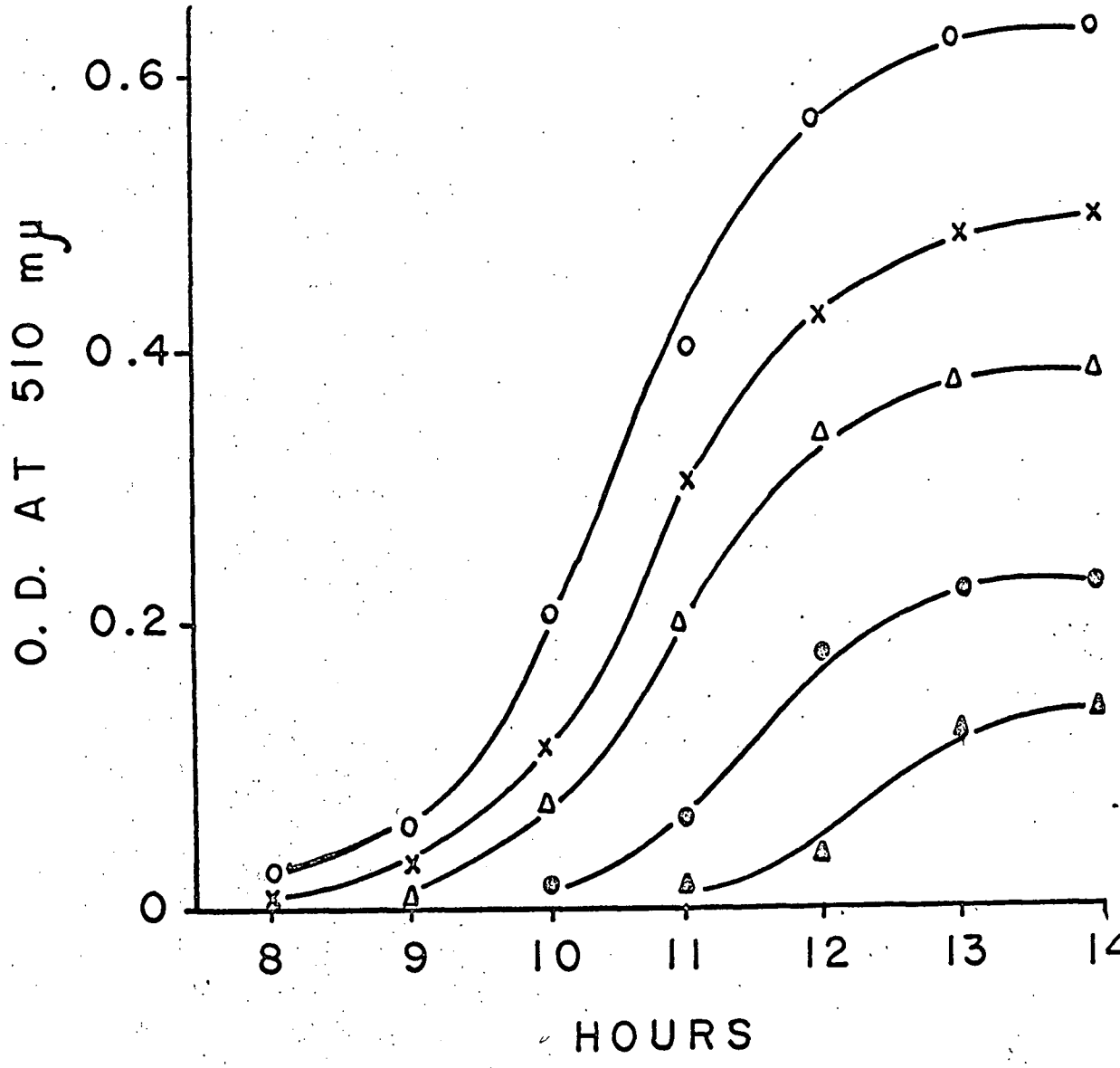

Figure 5. Effect of adding end product amino acids on DHBG production by strain B-1471. Flasks were supplemented at zero time with: 10^{-4} M tryptophan (0); 10^{-4} M, 10^{-3} M phenylalanine, or 10^{-3} M tryptophan (Δ); 10^{-3} M tyrosine (●); 2 x 10^{-3} M tyrosine, or 2 x 10^{-3} M of each of tyrosine, tryptophan and phenylalanine (▲). No additions were made to the control flask (X).

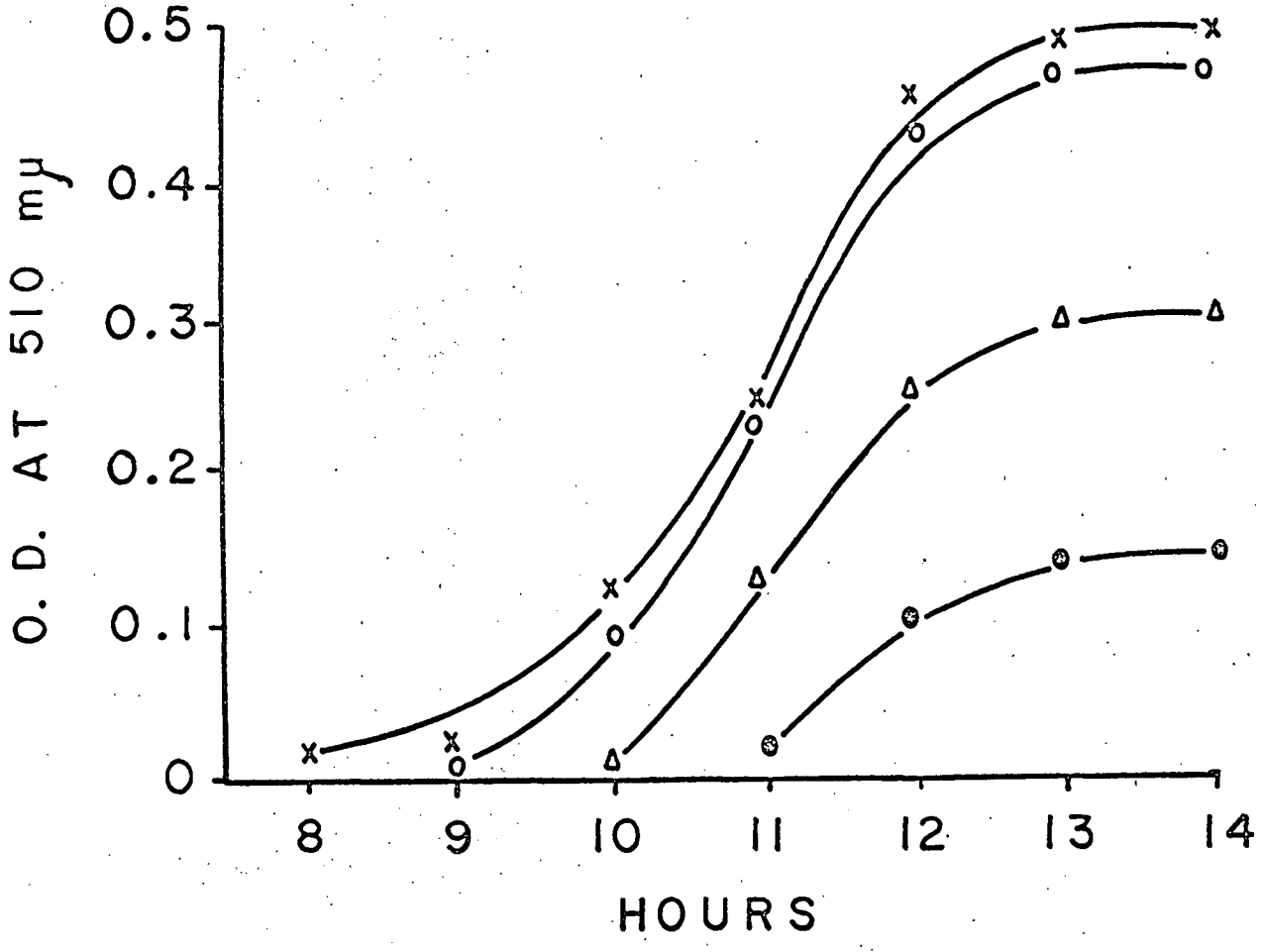

Figure 6. Effect of adding antrhanilic acid on the production of DHBG by strain B-1471. Anthranilic acid was added at zero time at levels of 10^{-4} M (O), 10^{-3} M (Δ), and 2 x 10^{-3} M (●). Identical curves were obtained when 10^{-4} M tryptophan was added to the anthranilic acid supplemented flasks. No aromatic addition was made to the control flask (X).

graphed on paper with three different solvent systems (29). A sample of the purified DHBG was hydrolysed in 6 N HCl for 16 hrs in a sealed, evacuated tube at 120 C. The hydrolysate was chromatographed in the above systems, and also was subjected to high voltage paper electrophoresis. Labelled DHB was not detected. The label was found only in the glycine moiety.

6. Variations among strains of B. subtilis

By use of chromatographic and spectrophotometric methods (see Materials and Methods), two other wild-type strains of B. subtilis were examined for compounds produced during iron-deficient growth. Strain WB-746 produced DHB and smaller quantities of catechol. Strain W-23 produced DHBG initially, but after about 10.5 hrs of growth, appeared unable to maintain the DHBG conjugation system and began producing DHB (Table V).

Strain W-23 exhibited the same responses to the concentration of iron, anthranilic acid, and aromatic amino acids in the medium as strain B-1471. Strain WB-746, however, exhibited significantly different responses. In this strain, DHB synthesis was independent of the level of added iron up to a concentration of 120 μg per litre. At 150 μg of added iron per litre, the quantity of DHB produced was almost as high as that obtained with the two other strains in the absence of added iron (Fig. 7). At higher levels of iron, DHB production by strain WB-746 was inhibited completely. Unlike strains B-1471 and W-23, production of DHB by strain WB-746 was not stimulated by low levels of tryptophan. In addition, the inhibition of production by high levels of tryptophan and by anthranilic acid was

Table IV. Distribution of glycine-1-^{14}C

Fraction	10^7 counts/min
Total culture	5.02
Cell supernatant fluid	2.03
Coproporphyrin III	0.590
Purified DHBG	0.600
Cells	2.96
Cold trichloroacetic acid-soluble	0.045
Alcohol-soluble	0.005
Ether-alcohol	0.000
Hot trichloroacetic acid-soluble	0.897
Residual	2.06

Table V. Characteristics of phenolic acid excretion by strains of *B. subtilis* in iron deficiency

Characteristics of phenolic acid excretion	Strain		
	B-1471	WB-746	W-23
Phenolic acid produced	DHBG	DHB	DHBG and DHB
R_F in solvent 1	0.83	0.88	0.83 0.88
R_F in solvent 2	0.47	0.69	0.47 0.69
Effect of aromatic supplementation on excretion:			
Control	100%	100%	100%
10^{-4} M tryptophan	124%	100%	120%
10^{-3} M tryptophan	75%	89%	75%
10^{-3} M tyrosine	48%	74%	50%
10^{-3} M phenylalanine	73%	90%	75%
10^{-3} M anthranilate	58%	84%	55%
2×10^{-3} M anthranilate	21%	78%	25%

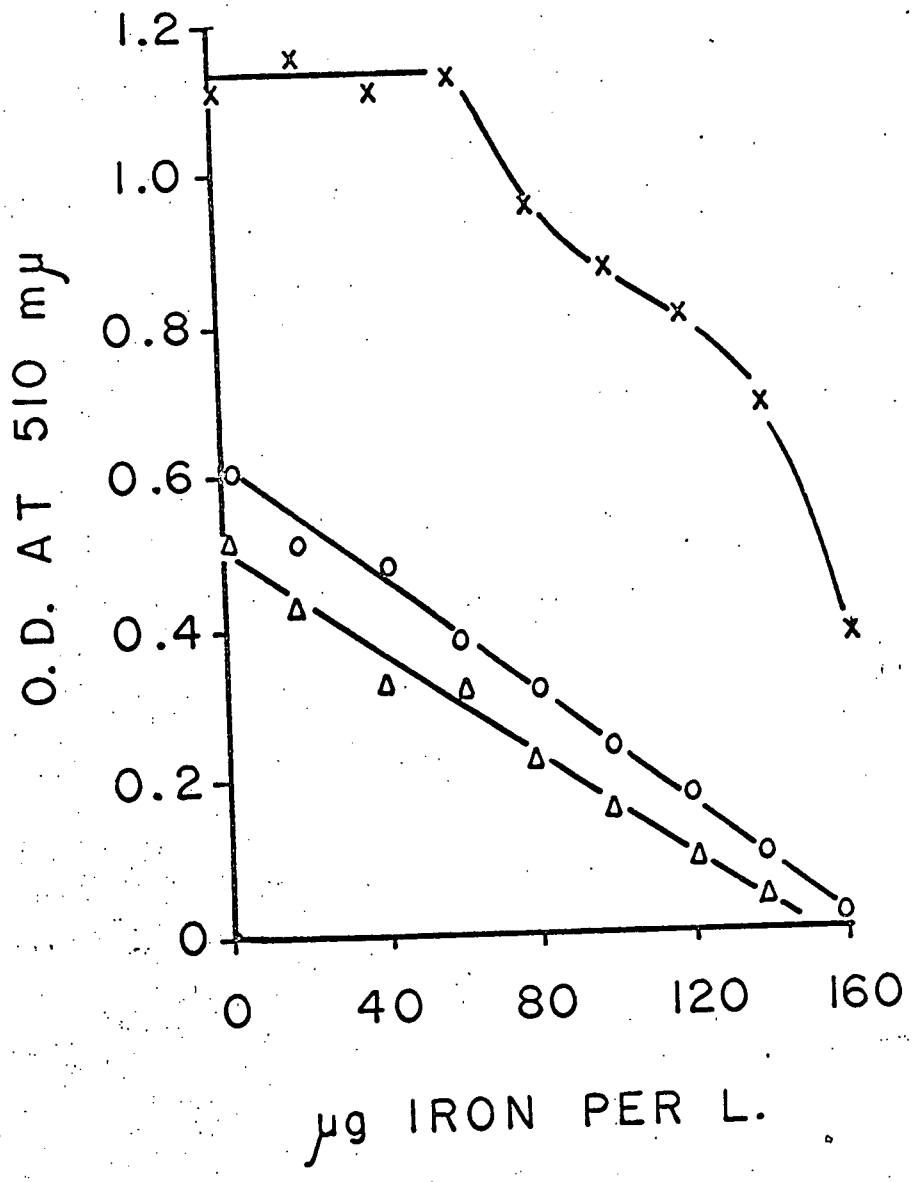

Figure 7. Effect of iron, added at zero time, on the production of DHB(G) by strains WB-746 (X), B-1471 (O), and W-23 (Δ) of Bacillus subtilis.

only about 50% of that observed in the two other strains (Table V).

7. Effect of histidine on phenolic acid excretion

When 20 μg histidine per ml was added at zero time to each of the wild-type strains, phenolic acid excretion was decreased about 25% in B-1471 and W-23, and about 50% in WB-746 (Table VI). Nester has demonstrated a regulative involvement of histidine in aromatic biosynthesis (43); in all cases, the effect of histidine supplementation was overcome by tyrosine supplementation at 20 μg per ml (43). Histidine inhibition of phenolic acid excretion was not relieved by tyrosine; instead, the inhibitory effect was additive (Table VI, Fig. 5). These results may reflect an additional unknown involvement of histidine in aromatic metabolism.

8. In vitro syntheses

(a) Formation of DHB

Cell-free extracts prepared from strains B-1471 or WB-746 converted chorismate to DHB when incubated under the conditions described by Young et al (67). (Table VII). WB-746 extracts were twice as active as B-1471 extracts. The addition of 10^{-3}M anthranilate completely inhibited the formation of DHB (Table VII).

(b) Formation of DHBG

Extracts of B-1471 formed DHBG from DHB or from chorismate in the presence of glycine when incubated under the conditions described by Brot et al (7). (Table VIII). There was no formation of DHBS when serine was substituted for glycine. The addition of Fe^{+3} had

Table VI. Effect of histidine on phenolic acid production in wild type strains

Strain	Supplement (20 µg/ml)	% Phenolic acids excreted
B-1471	-	100
B-1471	histidine	75
B-1471	histidine & tyrosine	55
W-23	-	100
W-23	histidine	70
W-23	histidine & tyrosine	50
WB-746	-	100
WB-746	histidine	50
WB-746	histidine & tyrosine	40

Table VII. In vitro synthesis of DHB

Extract Source	Reaction Mixture	DHB Formed (μmoles)
1. WB-746	Complete	0.32
2. "	- chorismate	0.02
3. "	Complete, at 0 C	0.03
4. "	Complete + 10^{-3} M anthranilate	0.01
5. "	Complete + 10^{-3} M of each of tryptophan, tyrosine and phenylalanine	0.13
6. B-1471	Complete	0.13

Synthesis of DHB from chorismate. The complete reaction mixture contained: crude extract (3.0 mg protein), 50 μmoles Tris-Cl buffer (pH 8.0), 1.0 μmole chorismate, 1.0 μmole NAD, and 5.0 μmoles $MgCl_2$ in a total volume of 1.0 ml.

Table VIII. In vitro synthesis of DHBG

Extract Source	Reaction Mixture	10^4 counts/min in DHBG
1. B-1471	Unsupplemented	0.08
2. "	+ chorismate	3.1
3. "	+ chorismate + Fe^{+3}	3.0
4. "	+ chorismate, at 0 C	0.10
5. "	+ DHB	8.2
6. WB-746	+ chorismate	0.05
7. "	+ DHB	0.04

Synthesis of DHBG. Each reaction mixture contained: crude extract (3.0 mg protein), 10 μmoles Tris-Cl buffer (pH 7.4), 0.1 μmoles ^{14}C-1-gly (0.2 μc), and 1.0 μmole ATP. Additions were made as indicated: 0.1 μmole DHB, 0.5 μmole chorismate, Fe^{+3} 0.05 μmole. The total volume of each reaction mixture was 1.0 ml. The incubation time was 1 hr at 37 C.

no effect on DHBG formation from chorismate. WB-746 extracts were incapable of DHBG formation (Table VIII).

9. Metabolism of DHB(G) by strain B-1471

When DHB was added to cultures of strain B-1471 at zero time, some of it disappeared from the medium. In the absence of added iron, this disappearance continued until the culture started to produce DHBG (Fig. 3). When iron, to a concentration of 1 mg per litre, was added with the DHB, a similar rate of disappearance was observed for 12 hrs (the OD at 510 mμ dropped from 0.55 to 0.35). No further change occurred during the next 8 hrs. If iron and DHB were added between 5 and 9 hrs there was no disappearance of DHB before 16 hrs (Fig. 4). The most marked disappearance of DHB(G) occurred in the early stationary phase. During this period, the rate of disappearance was proportional to the concentration of iron in the medium (Fig. 8).

Attempts were made to detect oxidation of DHB(G) in Warburg experiments. Washed cells were tested after growth with and without added iron and in the presence or absence of DHB(G). In no case was oxidation observed.

10. Metabolism of DHB(G) by strains W-23 and WB-746

Strain W-23 metabolized DHB(G) and the pattern of disappearance was similar to that seen in strain B-1471. Strain WB-746, however, did not metabolize DHB(G), not even when supplemented with 1 mg of iron per litre. Oxidation of DHB(G) was not observed with washed cells of either of these strains.

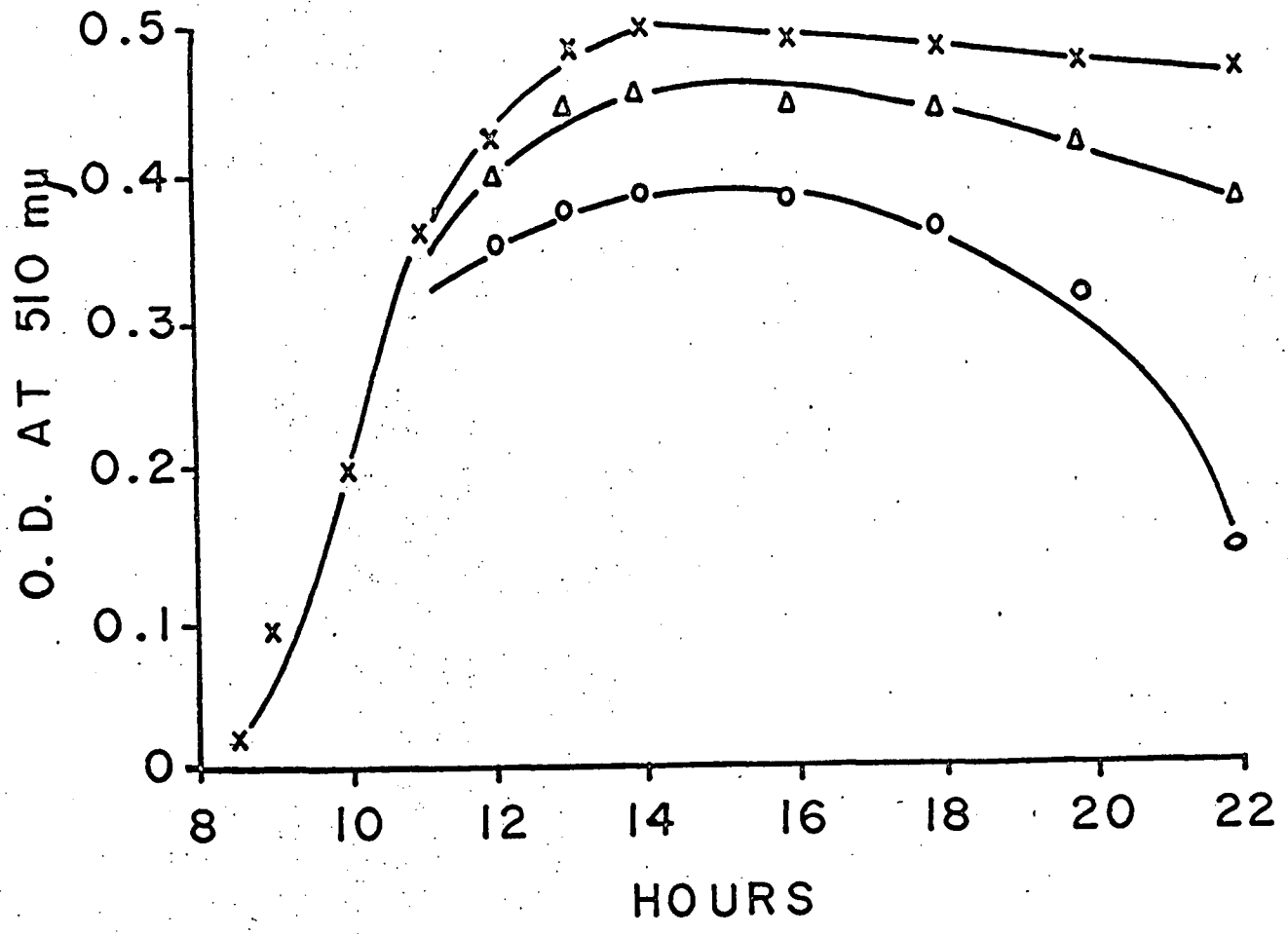

Figure 8. Metabolism of DHBG by strain B-1471 after addition of iron. An 80-μg amount of iron per litre (Δ) and 1 mg of iron per litre (O) were added to flasks at 10 hr. No iron was added to the control flask (X).

11. Properties of mutant strains

(a) 5-methyltryptophan resistant (MT^r) strains

Resistance to MT (37) in B. subtilis (45) causes derepression of the tryptophan biosynthetic enzymes (43). When MT^r mutants of B-1471 were grown in iron-deficient medium, all produced lower levels of phenolic acids than the corresponding parent strains (Fig. 9). MT^r mutants selected from anthranilate synthetase-less (ant^-) strains of B-1471 (Table II), produced the same level of phenolic acids as MT-sensitive ant^- strains (Fig. 9). Similar DHB(G) excretion patterns were observed in MT^r and ant^- mutants of WB-746 and W-23.

(b) Aromatic auxotrophs

Phenolic acid production by supplemented aromatic auxotrophs blocked at one or more steps after the synthesis of chorismate (Table II) was comparable to that of the corresponding parent strain. Multiple aromatic auxotrophs (Table II), however, produced no phenolic acids in iron-deficient medium supplemented with the required aromatic end-products. (20 μg per ml tryptophan, tyrosine and phenylalanine; 2 μg per ml p-aminobenzoic acid and p-hydroxybenzoic acid; and 20 μg per ml shikimic acid).

(c) HQ resistant mutants (HQ^r)

HQ is a powerful iron-binding compound (66). Growth of the three wild-type strains was inhibited by HQ at a concentration of 0.01 μg per ml. Spontaneous mutants of B-1471 were obtained, however, which were resistant to 10 μg HQ per ml (Table II). Five days were

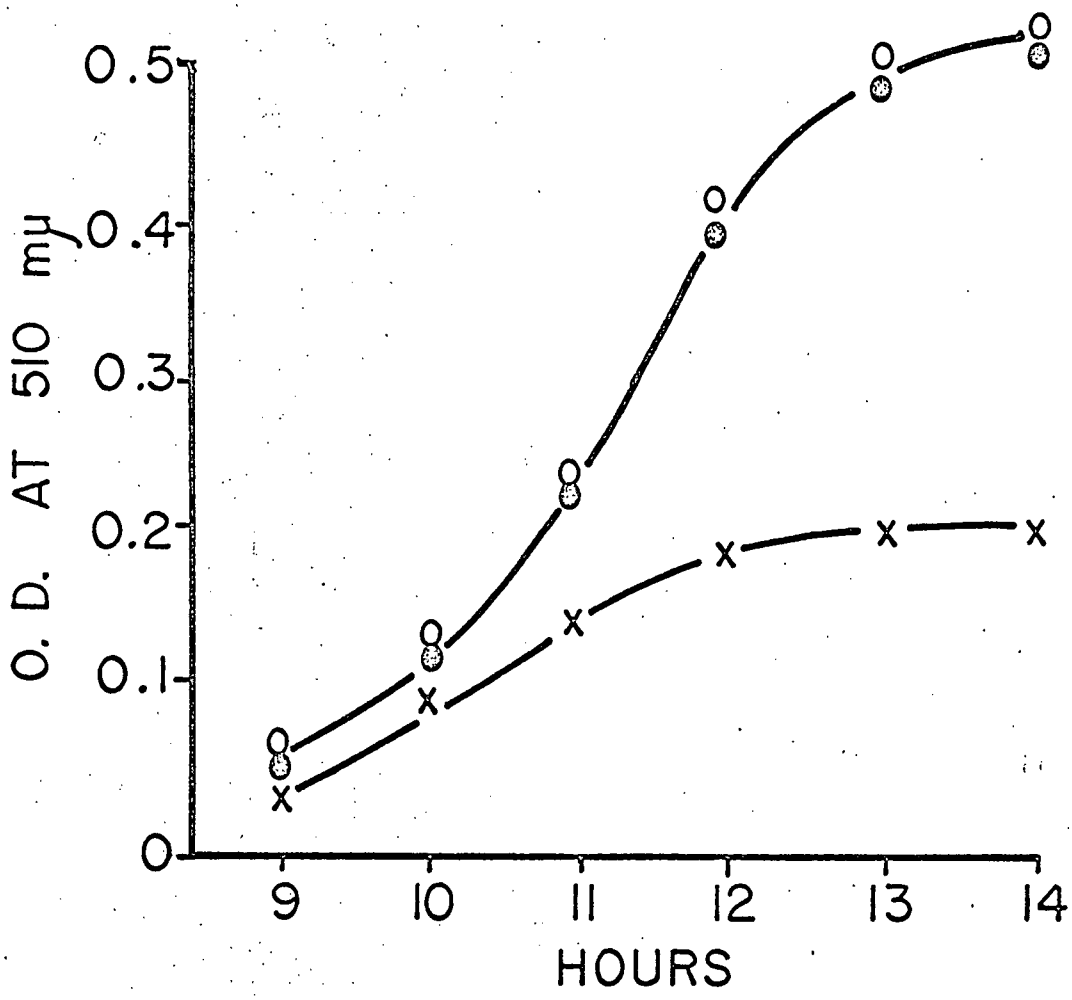

Figure 9. Production of DHBG by MT^r strains of B-1471. DHBG excretion was measured by: B-1471 (O); MT^r-1 and MT^r-2 (X); Trp-1 (●); and Trp-1-MT^r-1 (●).

required for colony formation under these conditions, although the growth rate of these mutants in the absence of HQ was the same as the parent strain. Mutants of W-23 or WB-746 resistant to 10 μg HQ per ml were not obtained, but mutants resistant to 0.1 μg HQ per ml were obtained (Table II). In all strains, one mutant colony was obtained for every 10^{-7} to 10^{-8} cells plated.

When HQ^r mutants of W-23 or WB-746 were grown in iron-deficient medium in the absence of HQ, normal levels of phenolic acids were produced. Under these conditions HQ^r mutants of B-1471 produced only very low levels of DHBG. Supplementation of media with 0.1 μg HQ/ml restored DHBG excretion in these mutants to the level of the parent strain.

Three series of spontaneous derivatives of B-1471 were selected which were resistant to: (a) albomycin (49), (b) actinomycin (49) and (c) catechol. These strains produced DHBG at the same level as the parent strain.

DISCUSSION

Under conditions of iron-deficiency, DHB(G) production by *B. subtilis* is associated with active growth of the organism. It is not a metabolic by-product accumulating during the stationary phase. The control of DHB(G) production by iron appears to involve the Fe^{3+}: $(DHB(G))_3$ complex, and a critical level of this effector complex is required to stop production. The complex may function directly, as a corepressor, a feedback inhibitor, or as both, or it may function indirectly, by facilitating the transport of iron into the cell.

The results of adding iron to iron-deficient cultures showed that after a certain time the cells are committed to the production of DHB(G), and the addition of iron does not reverse this commitment. These results also support the suggestion that the Fe^{3+}: $(DHB(G))_3$ complex is involved in the control of DHB(G) production. If iron is not added until the culture has started to produce DHB(G), production is stopped more effectively than by the addition of iron prior to the onset of production (Fig. 3). DHB and DHBG were equally effective as the Fe^{+3}:$(\text{phenolic acid})_3$ complex. *B. subtilis* utilized glycine as a nitrogen source, but was unable to cleave the glycine from DHBG for its nitrogen requirement. Therefore DHBG was not being converted to DHB under the conditions described.

From the levels of DHB(G) accumulated in the medium, it is obvious that an iron deficiency either (i) causes a severe distortion of aromatic biosynthesis in *B. subtilis,* or (ii) allows the cell

to utilize a metabolic pathway which is not functional in the presence of sufficient iron. The length of time required for the shutoff of DHB(G) production, in the presence of the Fe^{3+}: $(DHB(G))_3$ complex, suggests that repression is involved (see Fig. 3 and 4), thereby favouring the second alternative. This alternative agrees with the results obtained with E. coli, where the activities of extracts for the synthesis of DHB from chorismate were dependent on the level of iron in the growth medium (67),

The production of DHB(G) was influenced markedly by the exogenous levels of aromatic amino acids. These effects could be direct, the amino acids acting on the enzymes specific to DHB synthesis, or they could be indirect, decreasing the amount of chorismate available for DHB synthesis. A direct effect is unlikely because these amino acids do not inhibit DHB synthesis by extracts. (Table VII).

This suggests that the production of DHB in vitro is regulated by the mechanisms which form part of the general system of control of aromatic biosynthesis in B. subtilis (28, 44, 45). That portion of aromatic biosynthesis relevant to the present study is presented in Fig. 10. At low levels of exogenous tryptophan (Fig. 5) DHBG synthesis was initiated earlier and occurred to a higher extent than in the control flask. This phenomenon is expected, since low levels of tryptophan would inhibit anthranilate synthetase and prephenate (PPA) dehydratase (44). The increased levels of chorismate would then be available for DHB synthesis; and, if the enzyme(s) responsible for DHB synthesis (67) was derepressed, efficient utilization of chorismate might prevent PPA accumulation. High exogenous levels of tryptophan and the other aromatic amino

SCHEME OF AROMATIC BIOSYNTHESIS

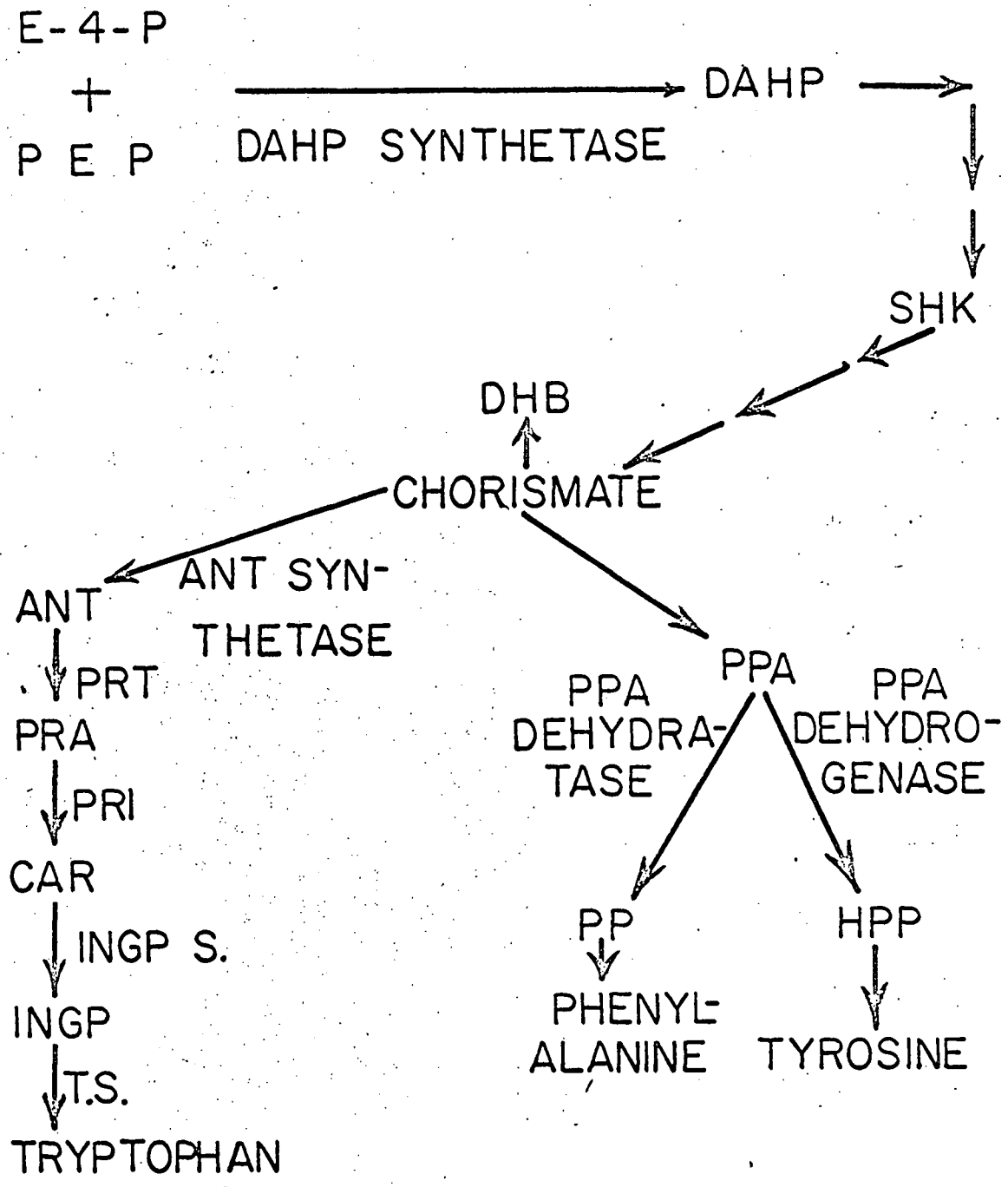

Figure 10. Abbreviations: E-4-P, erythrose-4-phosphate; PEP, phosphenol pyruvate; DAHP, d-arabinoheptulosonic acid-7-phosphate; SHK, shikimate; PPA, prephenate; PP, phenylpyruvate; HPP, 4-hydroxyphenylpyruvate; ANT, anthranilate; PRT, phosphoribosyl transferase; PRA, phosphoriboxylanthranilate; PRL, phosphoribosyl isomerase; CAR, 1-(o-carboxyphenylamino)-1-d-D-ribulose-5-phosphate; INGP S; indoleglycerol phosphate synthetase; T.S., tryptophan synthetase.

acids may have caused direct feedback inhibition and repression at the level of DAHP synthetase (28). The greater inhibition of DHBG accumulation by phenylalanine and tyrosine, when compared to tryptophan may reflect the higher sensitivity of DAHP synthetase to inhibition by PPA than by chorismate (28).

The inhibition of DHB(G) production by anthranilic acid was not caused by an indirect effect on tryptophan biosynthesis, since the inhibition was still observed in the presence of tryptophan. In addition, the inhibition of DHB(G) production was not caused by the conversion of anthranilate to tryptophan, because the inhibition has been observed in mutants blocked at any step between anthranilate and tryptophan. (Table II). Anthranilate, however, inhibited the synthesis of DHB from chorismate by extracts (Table VII).

Cell-free extracts of strains B-1471 and WB-746 converted chorismate to DHB. Extracts of B-1471 formed DHBG from chorismate or DHB in the presence of ATP. The addition of coenzyme A had no effect on DHBG formation. Extensive studies were not done on these in vitro syntheses because they were being conducted in other laboratories (6,7,21).

The formation of DHB but not DHBG, by strain WB-746 may result from the rigid control of glycine synthesis in this strain. The observation that WB-746 did not form appreciable amounts of coproporphyrin III under iron deficiency provides further evidence for this strict control. Strains B-1471 and W-23 did excrete coproporphyrin III under these conditions. It is also significant that strain WB-746 consistently produced greater quantities of DHB(G)

than strains B-1471 or W-23 (Fig. 8). Evidence is presented in Sections II and IV that glycine production may be related to an oxidatively functional tricarboxylic acid (TCA) cycle.

The disappearance of DHB(G) from cultures remains to be explained. An enzyme which decarboxylates DHB to catechol was purified from Aspergillus niger. Oxygen was not required for this reaction (61). A pseudomonad, capable of using DHB as the sole carbon source, cleaved the aromatic ring of DHB with the uptake of 1 mole of oxygen. Decarboxylation did not occur prior to oxidation (55). Although DHB(G) was metabolized by strains W-23 and B-1471, it was not possible to demonstrate either decarboxylation or oxidation of DHB by whole cells of these strains after growth under a variety of conditions. Cultures appear to be able to metabolize DHB(G) during the early log phase and the early stationary phase, but not during the late log phase, the time during which DHB(G) is produced in iron-deficient cultures (Fig. 3, 4 and 8).

DHB was present in a strain of A. aerogenes (blocked after anthranilate synthetase) after 11 hrs of growth in a quinic acid medium but not after 24 hrs. Catechol, on the other hand, was present after 24 hrs but not after 11 hrs (51). A series of B-1471 mutants (Table II), which were blocked at each of the steps of tryptophan biosynthesis, were examined for the compounds produced under iron-deficiency. Although each of these auxotrophs produced normal levels of DHBG and showed the usual responses to iron, none of them produced catechol during the 20-hr growth and incubation period.

The inability of MT^r strains to produce normal levels of phenolic

acids may reflect intracellular chorismate depletion by the derepressed tryptophan biosynthetic enzymes. In addition, excessive tryptophan production would tend to inhibit DAHP synthetase (28) further decreasing the chorismate available for DHB synthesis (67). This interpretation was supported by the observation (Fig. 9) that all ant$^-$ strains of B-1471 produced the same level of phenolic acids whether they were resistant to or sensitive to MT.

The excretion properties of HQ^r strains of B-1471 in the presence and absence of HQ indicated that these mutants may have a lower requirement for iron. This theory was supported by subsequent iron-uptake studies (Section IV).

Section II: - The production of coproporphyrin and its relationship to the production of phenolic acids.

Introduction

Strains B-1471 and W-23 produced DHBG and coproporphyrin during logarithmic growth (Table III, Fig. 1, p.43). Both products accumulated only in iron deficient medium. Since glycine is required for the synthesis of both compounds, strains auxotrophic for glycine were selected and were grown in iron deficient medium to determine alterations in their excretion capacities.

Since an iron requirement has been demonstrated in porphyrin biosynthesis (30,32), a strain blocked at the first step of porphyrin biosynthesis (δ-aminolevulinate synthetase) was selected. By growing this mutant in the presence and absence of δ-aminolevulinate (ALA), the effect of heme iron requirement on phenolic acid excretion was studied.

Strains lacking the ability to synthesize normal levels of phenolic acids were selected to further elucidate the relationship between phenolic acid and coprophorphyrin production.

1. Production of coproporphyrin and phenolic acids by wild-type strains

Strains B-1471 and W-23 showed similar responses to iron-deficiency, Phenolic acid production started when the cultures reached a density of about 7.0×10^8 viable cells per ml, and continued logarithmically, parallelling growth (Fig. 11 and 12). Coproporphyrin production started about 1 hr after the first appearance of phenolic acids and

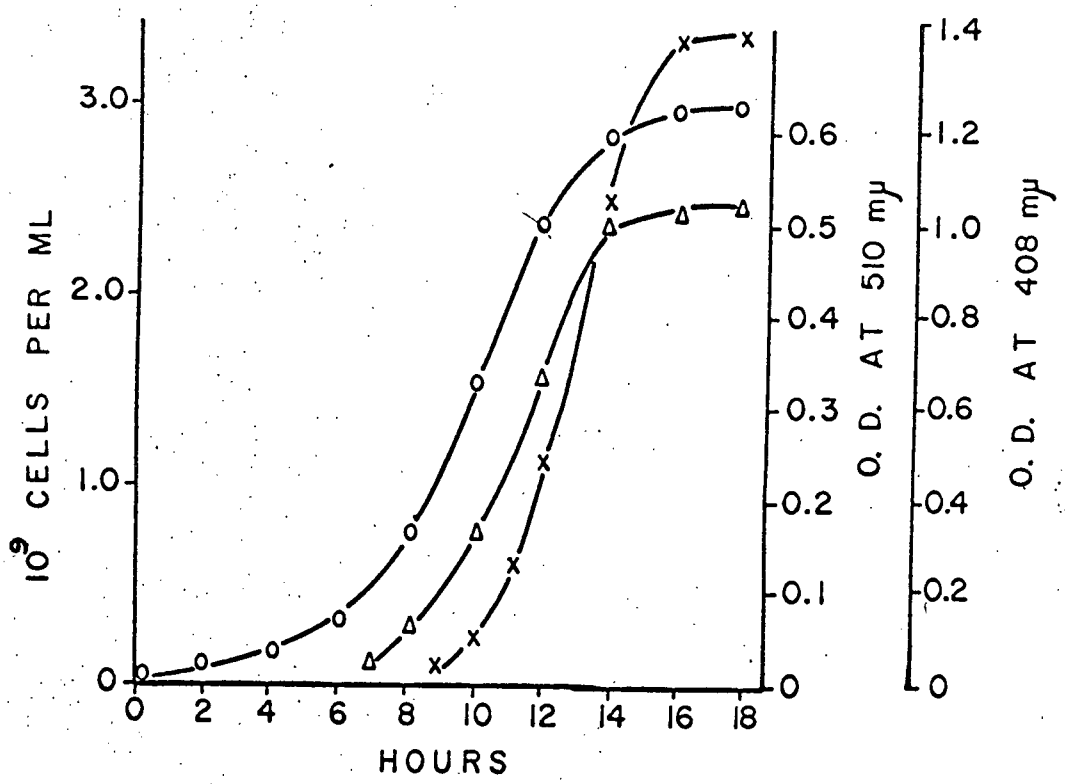

Figure 11. Growth and production of phenolic acids and Coproporphyrin by strain B-1471. Cell number (O); phenolic acids (Δ); and coproporphyrin (X).

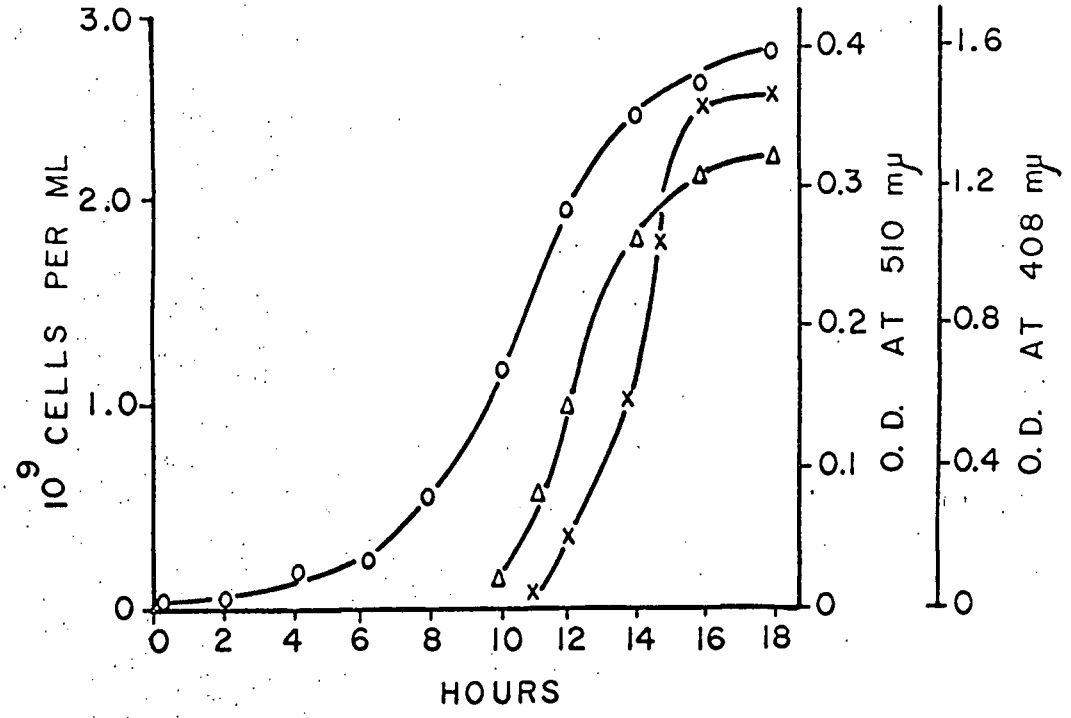

Figure 12. Growth and production of phenolic acids and coproporphyrin by strain W-23. Cell number (O); phenolic acids (▲); and coproporphyrin (X).

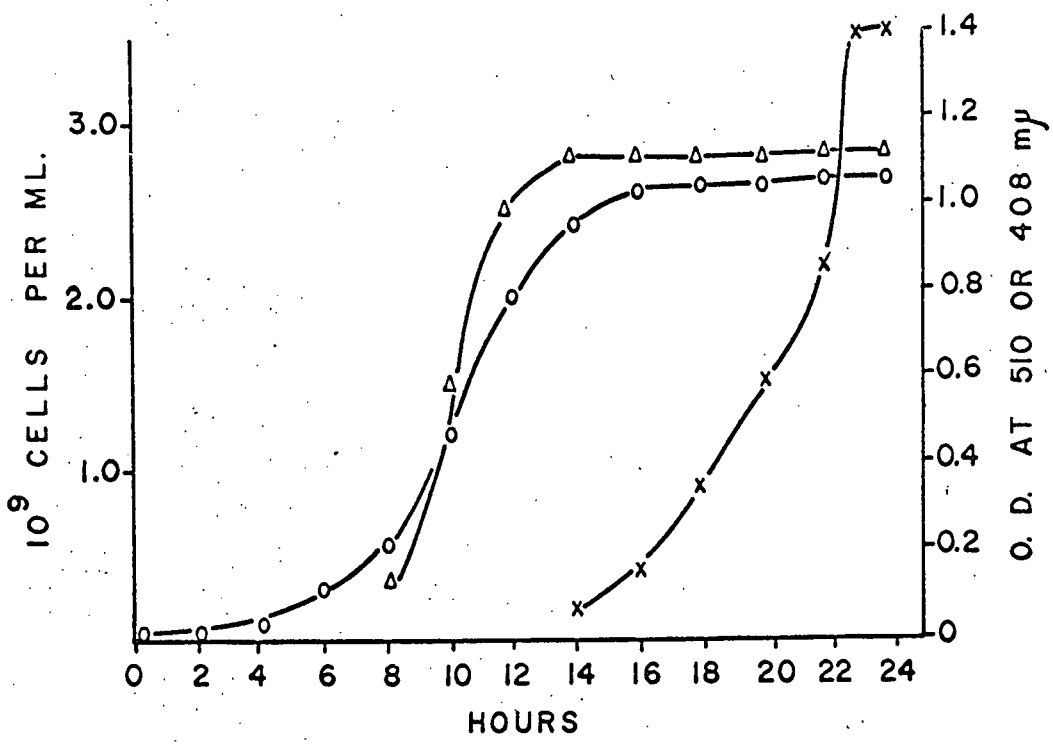

Figure 13. Growth and production of phenolic acids and coproporphyrin by strain WB-746. Cell number (X); phenolic acids (Δ); and coproporphyrin (X).

also continued logarithmically (Fig. 11 and 12). Phenolic acid production started earlier in strain WB-746, and continued at a faster rate than in the two other strains (Fig. 13). Conversely, coproporphyrin production started later, occurred at a slower rate, and did not parallel growth (Fig. 13).

2. Inhibition of coproporphyrin production by iron

The addition of various levels of iron at zero time to strain B-1471 showed that while 150 µg per litre completely inhibited phenolic acid production (Fig. 2), a level in excess of 200 µg per litre was required to inhibit coproporphyrin production. The rate of growth was not significantly altered by the addition of iron. As the level of added iron was increased above 200 µg per litre the time of appearance of coproporphyrin was delayed, and at the higher concentrations occurred in the stationary phase.

The addition of 1 mg iron per litre after 7 hrs incubation resulted in an 85% decrease in total coproporphyrin production by strain B-1471 (Fig. 14). If this amount of iron was added after 8 hrs incubation, production was decreased by 43%, and if added after 9 hrs, by 30% (Fig. 14). The addition of 3.5×10^{-4} M DHB or DHBG alone did not cause a significant decrease in coproporphyrin production. If 1 mg iron per litre was added with the phenolic acid, however, there was a rapid cessation of coproporphyrin production (Fig. 14).

3. Inhibition of coproporphyrin production by hemin

The addition of low levels of hemin to iron-deficient cultures

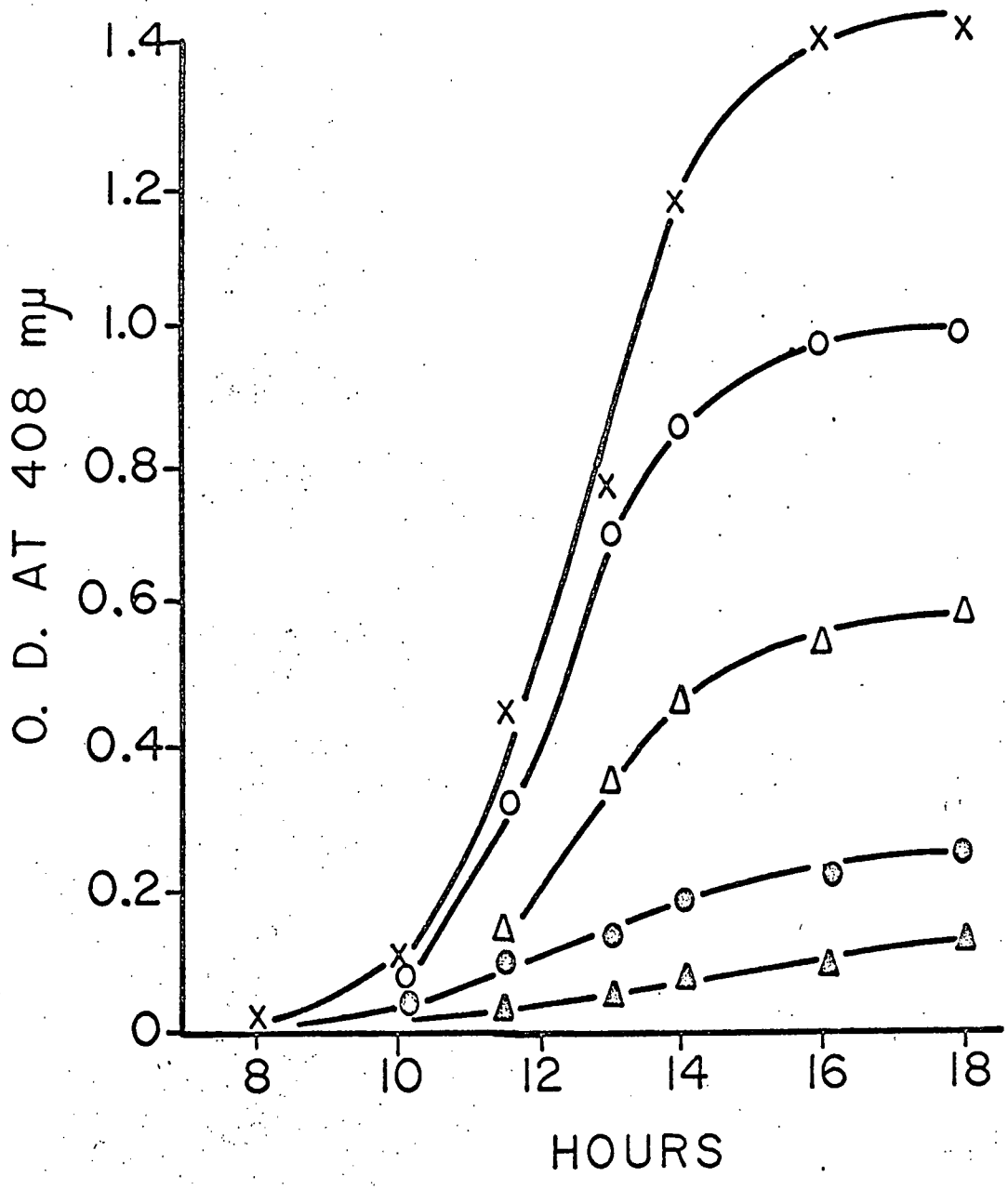

Figure 14. Effect on coproporphyrin production of adding iron and DHB(G). One mg iron per litre was added at 7 hr (●); 8 hr (△), or 9 hr (○). DHB(G) at 50 mg per litre and 1 mg iron per litre were added at 8 hr (▲). No additions were made to the control flask (X). The addition of 50 mg of DHB(G) alone had no effect (X).

of strain B-1471 at zero time resulted in later production of coproporphyrin and phenolic acid (Table IX). Under these conditions, coproporphyrin was eventually produced at the normal level but the level of phenolic acid accumulated was markedly reduced (Table IX). There was no inhibition of growth at 0.5 - 1.0 µg hemin per ml. Growth, however, was inhibited above 1.0 µg of hemin per ml.

4. Inhibition of coproporphyrin production by aromatic amino acids

Whereas accumulation of phenolic acids was inhibited by adding aromatic amino acids and anthranilic acid to the medium at zero time (Table V), coproporphyrin production was not influenced by supplementation of the medium with these acids at concentrations up to 2×10^{-3} M. Similarly, histidine supplementation decreased the excretion of phenolic acids (Table VI) but had no effect on coproporphyrin production.

5. Coproporphyrin and phenolic acid production by mutants of strain B-1471

(a) Glycine auxotrophs

Repeated attempts to isolate glycine auxotrophs were unsuccessful.

(b) Serine auxotrophs

Glycine is known to be synthesized via serine in E. coli (64). Therefore, attempts were made to isolate serine auxotrophs so that their capacity for coproporphyrin and phenolic acid production could be examined. Three absolute serine auxotrophs, ser-1, ser-2 and

Table IX. Inhibition of the production of phenolic acids and coproporphyrin by hemin in strain B-1471.

	O.D. 510			O.D. 408		
	14 hr	18 hr	24 hr	16 hr	20 hr	24 hr
1. Control	0.50	0.51	0.48	1.4	1.5	1.6
2. + 0.5 µg hemin per ml	0	0.3	0.35	0	0.9	1.5
3. + 1.0 µg hemin per ml	0	0	0.20	0	0.4	1.4
4. + 2.0 µg hemin per ml	growth inhibited					

Hemin was added to cultures at zero time. The growth rate was unaffected by hemin supplementation at 1.0 µg per ml.

ser-3, and one leaky serine auxotroph, ser-4, were isolated. Glycine would not replace serine as a growth requirement for any of the strains.

None of the strains produced phenolic acids, as determined by colorimetric assay, when grown under conditions of iron deficiency in minimal medium supplemented with 30 µg serine per ml. Coproporphyrin was produced by ser-1, ser-2 and ser-3 at about 10% and by ser-4 at about 15% of the level produced by the wild-type (Table X). When the culture supernatants from these experiments were concentrated and the phenolic acids extracted into ethyl acetate, chromatographic examination of the extracts revealed low levels of DHB but no DHBG (Table X).

These experiments were repeated using 50 - 100 µg glycine per ml in addition to the serine supplement. Under these conditions, the results were qualitatively and quantitatively similar to those observed with serine alone.

Spontaneous prototrophic revertants were selected from each of the serine auxotrophs. All revertants tested (2 for each auxotrophic strain) produced normal levels of coproporphyrin and phenolic acids under iron-deficiency. However, chromatographic examination of the culture supernatants showed that the revertants produced both DHB and DHBG (Table X).

(c) ALA auxotrophs

Only one ALA auxotroph, designated hem-1, was isolated in several attempts. In unsupplemented glucose medium, hem-1 had a doubling time of 190 min. When the medium was supplemented with

Table X. Production of coproporphyrin and phenolic acids by serine auxotrophs derived from strain B-1471.

Strain	Per Cent Production			
	Cop	Phenolic acids	DHB*	DHBG*
B-1471	100	100	0	100
Ser 1-3	10	5	100	0
Ser 4	15	5	100	0
Revertants of Ser 1-4	100	100	70-80	20-30

* Expressed as per cent of total phenolic acids produced

5.0 µg ALA per ml, the doubling time was 130 min. This strain would not grow in unsupplemented medium containing citrate as sole carbon source. When grown under conditions of iron-deficiency in the test (i.e. glucose) medium, hem-1 showed unusual behavior. In unsupplemented medium, neither phenolic acids nor coproporphyrin was produced. Supplementation of the medium with ALA led to the production of both coproporphyrin and phenolic acids. However, the responses to different levels of ALA showed that low levels led to the production of phenolic acids but not coproporphyrin, and that higher levels led to the production of coproporphyrin and a decreased quantity of phenolic acids (Fig. 15). Furthermore, when glycine or serine (50 - 100 µg per ml) was added to unsupplemented medium, phenolic acid excretion by hem-1 was restored to 10% of the level in the parent strain, while coproporphyrin accumulation remained unaffected. Chromatographic examination of culture supernatants showed that hem-1 produced only DHBG.

Protoporphyrin IX would not support the growth of hem-1 with citrate as sole carbon source, even in the presence of 1 µg of iron per litre. Similarly, supplementation of the glucose medium with 0.5 - 1.0 µg protoporphyrin IX per ml did not increase the growth rate, as seen with ALA, nor did it allow the production of phenolic acids and coproporphyrin during iron-deficient growth. It should be pointed out that the growth of strain B-1471 was inhibited by 0.5 µg per ml of protoporphyrin IX.

Ten spontaneous revertants of hem-1 were selected on citrate medium. All showed normal production of phenolic acids and coproporphyrin under iron-deficiency.

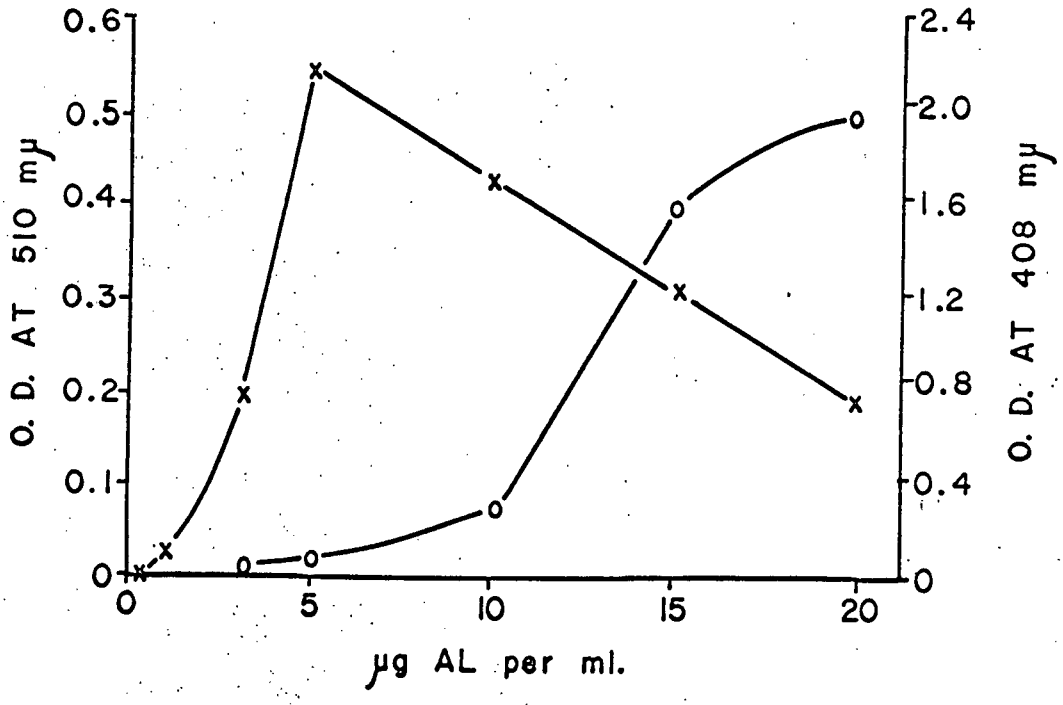

Figure 15. Effect of ALA supplementation on porphyrin and phenolic acid production by hem-1. The levels of coproporphyrin (O) and phenolic acids (X) were measured after 24 hrs growth.

(d) DHB auxotrophs

Only five strains, dhb-1, dhb-2, dhb-3, dhb-4 and dhb-5, which required DHB for growth were isolated in many attempts. When first isolated, supplementation of the minimal medium with 0.2 µg DHB per ml resulted in the appearance of normal colonies. However, all five strains rapidly lost their dependence on DHB for growth. The designations dhb-1, etc. were retained for these revertant strains because they produced low levels of phenolic acids. A "revertant" from each strain was screened for coproporphyrin and phenolic acid production under iron deficiency. In each case, the production of DHBG was considerably diminished compared to the production by the wild-type (Table XI). "Revertants" from four of the strains showed a decreased production of coproporphyrin (Table XI). A "revertant" from strain dhb-4, however, showed very interesting properties. Under conditions of iron-deficiency it produced a several-fold higher level of coproporphyrin than the wild-type (Fig. 16). Subsequently, it was found to produce coproporphyrin in the presence of iron, iron plus DHB(G), or ferrichrome (Table XII). The wild-type strain did not produce coproporphyrin under any of these conditions. Strain dhb-4 also had an extended lag period when inoculated into iron-deficient medium (Fig. 14). Supplementation of the medium with 2 mg iron per litre, 2 mg iron with 50 mg DHB(G) per litre, or 4 mg ferrichrome per litre did not shorten this lag. The lag was shortened by the addition of 0.5% yeast extract.

It should be pointed out that all the dhb-less strains grew more slowly than the wild-type strain in iron-deficient medium.

Table XI. Products excreted by "revertants" of DHB auxotrophs derived from strain B-1471.

Strain	Excretion level (per cent of parent strain)	
	DHBG	Coproporphyrin
Dhb-1	5	20
Dhb-2	20	8
Dhb-3	10	15
Dhb-4	5	300
Dhb-5	5	5

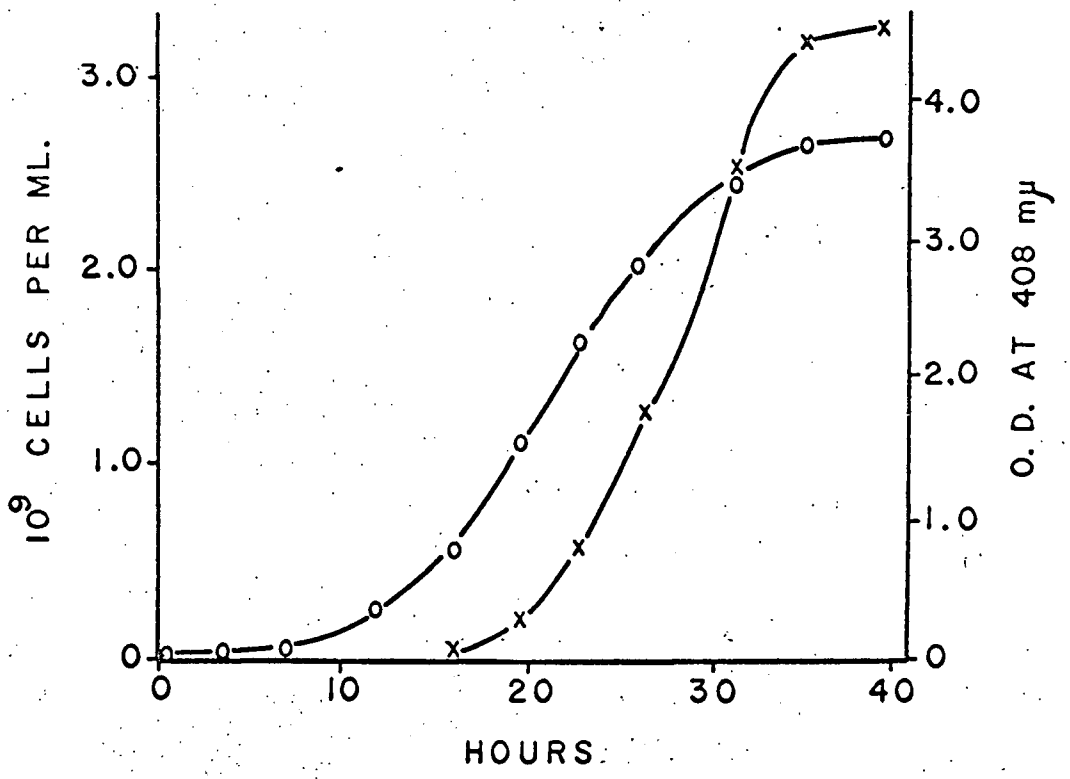

Figure 16. Growth and coproporphyrin production by dhb-4. Cell number (O); coproporphyrin (X). Note that the scale for O.D. at 408 mμ does not correspond to that given in Figure 11-13.

Table XII. Coproporphyrin production in Dhb-4.

	Per Cent
Control	100
+ 2 mg Fe^{3+} per litre	100
+ 50 mg per litre DHB(G) + 2 mg Fe^{3+} per litre	100
+ 4 mg per litre ferrichrome	100

Discussion

The results indicate that when cultures of B. subtilis strains B-1471 and W-23 became iron-deficient, the cells first started to produce phenolic acids. About an hour later the production of coproporphyrin was started. Phenolic acids have very strong affinities for ferric iron (26), and it has been suggested that they serve as iron solubilization factors (40). The production of DHB(G) may thus facilitate scavenging of the last traces of iron from the medium for the synthesis of heme and non-heme iron proteins. Once the available iron was taken up by the cell, hemin would not have been formed anymore, resulting in a loss of control over porphyrin biosynthesis, which was manifested by the production of high levels of coproporphyrin. In R. spheroides, hemin was shown to inhibit ALA synthetase (9). In addition, in the same organism (30, 32) and in T. vorax (30), iron appeared to be required for the conversion of coproporphyrinogen III to protoporphyrin IX (9). Loss of control over porphyrin synthesis in B. subtilis would be expected to lead to the accumulation of coproporphyrinogen III, which would oxidize spontaneously to coproporphyrin III, either before or after excretion. This interpretation is supported by the observation that phenolic acid production was initiated before coproporphyrin production, by the effect of hemin, and by the effect of iron at 150 µg per litre on phenolic acid and coproporphyrin production. It is supported also by the properties of hem-1; only phenolic acids were produced with low levels of added ALA, whereas both phenolic acids and coproporphyrin were produced with higher levels. Only the higher

levels of ALA would have allowed porphyrin synthesis in excess of iron.

Phenolic acid production appeared also to be related to the demand for iron. In the absence of added ALA, hem-1 did not produce phenolic acids, suggesting that the iron available in the medium was sufficient to satisfy the non-heme iron requirements of the culture. In the presence of ALA, additional iron was required for heme synthesis, and phenolic acid production was initiated.

Compared with strains B-1471 and W-23, strain WB-746 showed an earlier onset of phenolic acid production, and a later onset of coproporphyrin production. It has been shown (Section IV) that the rate of uptake of iron by cells of strain WB-746 is much slower than the rates in strains B-1471 and W-23. This suggests that under the experimental conditions, iron was not made available to cells of strain WB-746 at a rate fast enough to satisfy non-heme iron requirements. In addition, the tricarboxylic acid (TCA) cycle in strain WB-746 appears to be oxidatively inoperative when high levels of glucose are present (Section III). This could lead to a reduced demand for heme iron, and a late initiation of porphyrin production.

The patterns of inhibition of coproporphyrin production by 1.0 mg iron per litre were in good agreement with those obtained for inhibition of phenolic acid production (Fig. 3). The results obtained by adding iron during growth showed that once a culture became iron-deficient, it was committed to the synthesis of some phenolic acid and coproporphyrin, even after the addition of iron.

The much more rapid inhibition obtained by the addition of iron and DHB(G) suggested that DHB(G) might help in carrying iron into the cell. Neilands (42) suggested some time ago that the addition of DHBG to the medium could make iron more available to cultures of B. subtilis. This has now been demonstrated (Section V).

The serine auxotrophs made no DHBG when grown in medium supplemented with serine, suggesting that the conversion of serine to glycine may have been the limiting step in the synthesis of DHBG from DHB (Table VIII). However, when these auxotrophs were grown in the presence of both serine and glycine, again no DHBG was formed. It has been shown that labelled glycine added to the medium was incorporated into the glycine moiety of DHBG (Table IV), implying that B. subtilis can take up glycine from the medium. The results obtained with the serine auxotrophs are difficult to interpret in light of this observation. However, it is possible that B. subtilis has a common active transport system for glycine and serine. If this system is inefficient, and transports serine in preference to glycine, the results could be explained by the cell being unable to transport glycine at a rate fast enough to support growth, and serine only at a rate fast enough to support growth but not DHBG production.

The fact that the inability to synthesize serine prevents the production of the normal level of phenolic acids and reduces the production of coproporphyrin suggests that there may be a linked system of control involving glycine-serine, phenolic acids and coproporphyrin. Since coproporphyrin is produced by the serine auxotrophs, although at low levels, it appears that glycine is used preferentially for porphyrin biosynthesis. The fact that supple-

mentation of the medium with glycine or serine led to the production of some phenolic acid by hem-1 is of significance in this regard.

The low level of coproporphyrin production by some strains defective in phenolic acid production again suggests a relationship between these compounds. The properties of strain dhb-4 cannot be explained at present, but they also support the existence of such a relationship. The fact that the extended lag period observed with this mutant in iron-deficient medium could not be reduced by supplementation of the medium with iron or iron-binding compounds suggested that solubilization of iron was not the factor limiting growth.

Genetic analyses were attempted, to further explain the relationship between the excretion capacities of mutant strains. DNA extracted from strains B-1471 and WB-746 was used to transform (35) the auxotrophs derived from B-1471, but none of the strains was competent, even after addition of the "competence inducing factor" isolated from B. subtilis 168 I (2).

Section III: - Inhibition of phenolic acid production in *B. subtilis*

Introduction

In bacteria, certain compounds inhibit growth by acting as false regulatory metabolites of biosynthetic pathways (37). Thus, in *E. coli* (37) and *B. subtilis* (46), 5-methyltryptophan has been shown to mimic tryptophan as a feedback inhibitor of anthranilate synthetase (37) and as a repressor of synthesis of the enzymes of the tryptophan pathway. Mutants resistant to this compound are either (i) insensitive to feedback inhibition or (ii) derepressed for the tryptophan biosynthetic enzymes (37, 46). Various compounds structurally related to DHB(G) were therefore tested for their capacity to inhibit phenolic acid production. Such inhibitors might be useful in the isolation of mutants defective in the control of phenolic acid synthesis.

DHB(G) production was related not only to the level of iron in the medium, but also to the level of iron required by the cell for heme biosynthesis. Because heme biosynthesis requires an operative TCA cycle, compounds known to inhibit the operation of this cycle in *B. subtilis* were tested for their effects on phenolic acid production.

1. Analogs of DHB

The compounds tested as analogs of DHB(G) varied widely in their effects on phenolic acid and coproporphyrin production. Table XIII lists the analogs in order of their effectiveness as inhibitors.

2. Compounds affecting the functioning of the TCA cycle

Some of the compounds tested are known to inhibit sporulation in

Table XIII. Effect of analogs of DHB on phenolic acid and coproporphyrin production.

Supplement (200 µg/ml)	Per cent accumulation					
	Strain B-1471		Strain W-23		Strain WB-746	
	DHBG	Cop*	DHB(G)	Cop	DHB	Cop
None	100	100	100	100	100	100
Benzoic acid	100	100	100	100	100	100
3-hydroxy-benzoate	50	100	50	100	55	100
2,3-dimethoxy-benzoate	65	100	50	100	75	100
2-fluoroben-zoate	65	100	40	100	65	100
3-fluoroben-zoate	0	40	0	80	10	100
Dipicolinic acid	0	55	0	45	20	40
3-hydroxyanth-ranilate	0	100	0	100	0	100

* Cop refers to coproporphyrin

Bacilli, possibly because they inhibit the functioning of the TCA cycle. Glutamic acid (62) and α-ketoglutaric acid (24) inhibited sporulation in B. subtilis by repressing the synthesis of aconitase. α-picolinic acid is an iron chelating agent which has been shown to inhibit sporulation in B. cereus (16) by preventing derepression of aconitase. m-Tyrosine inhibits sporulation in B. subtilis (4), but its mechanism of action is unknown. The compounds tested, and their effects on phenolic acid and coproporphyrin production are shown in Table XIV.

3. Effect of sideramines on phenolic acid and porphyrin production by strain B-1471.

Sideramines are ferric trihydroxyamates which can act as growth factors for certain bacteria (39), possibly by transporting iron into the cell (40). Micrococcus lysodeikticus will grow in minimal medium if a dihydroxyphenol, such as catechol (56) or protocatechuic acid (56), or a sideramine, such as ferrichrome (41), is present. Therefore, the effects of sideramines on phenolic acid and coproporphyrin production were examined in strain B-1471. There are two types of sideramine (49), and a representative of each group was chosen for study. When added at zero time, 0.4 μg ferrichrome per ml or 0.2 μg ferrioxamine per ml inhibited phenolic acid production completely, and delayed and reduced coproporphyrin production (Fig. 17, Table XV). The level of ferrichrome used was equivalent to the addition of 20 μg iron per litre. The addition of this much iron alone did not inhibit phenolic acid or coproporphyrin production by strain B-1471 (Fig. 2 and Section II). When the same levels of the

Table XIV. Effect of inhibitors acting at the level of the TCA cycle on phenolic acid and coproporphyrin production.

Inhibitors	Per cent accumulation					
	Strain B-1471		Strain W-23		Strain WB-746	
	DHBG	Cop	DHB(G)	Cop	DHB	Cop
None	100	100	100	100	100	100
0.05% α-ketoglutaric acid or glutamic acid	75	70	80	70	100	80
0.10% α-ketoglutaric acid or glutamic acid	55	45	60	50	100	50
α-picolinic acid	0	0	10	30	100	80
m - tyrosine	0	0	0	0	0	0
0 - tyrosine	60	0	20	80	60	85

All TCA cycle inhibitors were added at zero time. None of the supplements significantly affected growth except m - tyrosine, which retarded growth slightly in WB-746. Corrections were made for this growth impairment (see text).

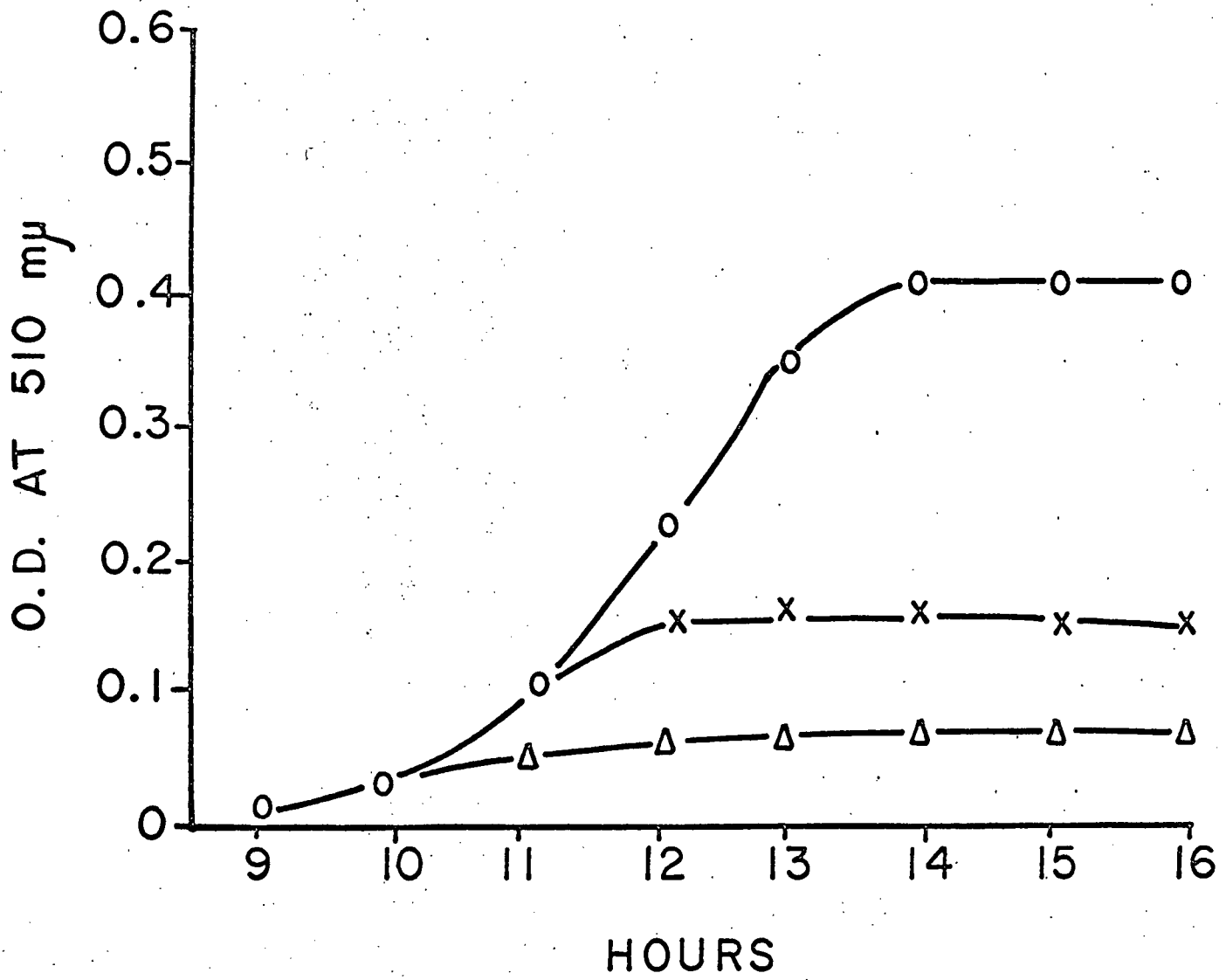

Figure 17. Effect of sideramine supplementation on phenolic acid excretion in strain B-1471. Ferrichrome (0.4 μg per ml) or ferrioxamine (0.2 μg per ml) was added at zero time (Δ) or after 8 hr growth (X). No additions were made to the control flask (0).

Table XV. Inhibition of the production of coproporphyrin by sideramines in strain B-1471

	O.D. at 408 mμ			
	12 hr	14 hr	18 hr	24 hr
1. Control	0.45	1.1	1.5	1.6
2. 0.4 μg ferrichrome per ml added at zero time	0	0	0.45	0.60
3. 0.4 μg ferrichrome per ml added at 8 hours	0	0	0.40	0.50
4. 0.2 μg ferrioxamine per ml added at zero time	0	0	0.65	0.90
5. 0.2 μg ferrioxamine per ml added at 8 hours	0	0	0.80	1.2

The growth rate was not affected by supplementation with ferrichrome or ferrioxamine.

sideramines were added to cultures after 8 hr growth under iron-deficient conditions, phenolic acid production was stopped rapidly (Fig. 17) and coproporphyrin production was delayed and reduced (Table XV).

Discussion

It appears that there are two classes of compounds affecting phenolic acid production in iron-deficient cells of B. subtilis. Compounds of Class 1 inhibit both coproporphyrin and phenolic acid production. Those of Class 11 inhibit only phenolic acid production.

The production of phenolic acids by B. subtilis is dependent upon the level of iron in the medium. It is dependent also on the iron requirements of the cell. If δ-aminolevulinate synthesis is blocked by mutation, phenolic acids are not produced under iron-deficiency, presumably because the non-heme iron requirements are met by the residual iron in the medium (Fig. 15). Therefore, it is not surprising that compounds affecting the functioning of the TCA cycle inhibit both coproporphyrin and phenolic acid production.

None of the compounds of Class 11 inhibited the growth of B. subtilis. This suggests that phenolic acids are not essential for growth under the experimental conditions used. Mutants isolated from B. subtilis strain B-1471 as DHB auxotrophs lost their dependence on DHB within one or two transfers. When examined, however, such "revertants" were found to produce very low levels of phenolic acid under iron-deficiency. Some of these strains also produced low levels of coproporphyrin, whereas one of them produced increased

levels (Table XI). Phenolic acids, therefore, are not essential for growth or coproporphyrin production, under the experimental conditions employed.

The most effective inhibitors in Class II are meta-substituted benzoic acids. Dipicolinic acid is, in effect, such a compound. It is interesting that anthranilate (Table V) and m-hydroxybenzoic acid inhibited phenolic acid production about 50 per cent, whereas 3-hydroxyanthranilic acid inhibited it 100 per cent (Table XIV). The effects of these inhibitors on DHB synthesis by cell-free extracts are being investigated.

The inhibitory effect of sideramines is probably due to their facilitating the uptake of iron by the cell, a conclusion (Section VI) which is supported by the failure of desferri-ferrichrome to produce the inhibition seen with ferrichrome. Since $Fe^{3+}:(DHB(G))_3$ is a more effective inhibitor of phenolic acid production than iron alone, it would seem that any compound able to form a ferric iron complex to which the cell is permeable will be able to inhibit phenolic acid production. This affords an experimental approach to the study of the specificity of the iron uptake mechanism(s) of B. subtilis (Section V).

Section IV: - Effects of aeration and glucose concentration on growth and phenolic acid excretion

Introduction

All three wild type strains which were studied extensively produced phenolic acids during logarithmic growth in iron-deficient medium containing high levels (1 per cent) of glucose (Table V), One of these strains (WB-746), however, excreted only DHB and no DHBG under these conditions (Table V). Unlike strains B-1471 and W-23, the accumulation of phenolic acid in WB-746 was not affected by inhibition of the TCA cycle (Table XIV). It was found subsequently that WB-746 excreted coproporphyrin at lower glucose levels, and that excretion was not initiated until the late stationary period (Fig. 13). These observations suggested that the TCA cycle in WB-746 was only partially functional under the conditions employed. Because high levels of glucose have been shown to repress the operation of this cycle in B. subtilis (11, 16, 17), phenolic acid and coproporphyrin production in WB-746 were studied in medium containing lower levels (0.3 per cent) of glucose. In addition, the effect of aeration on phenolic acid production was studied in the three strains.

1. Effect of aeration on phenolic acid production

When strains B-1471 and W-23 were grown in still cultures, containing 1 per cent glucose, the generation time was 190 min and the maximum cell density resulting over a 24 hr period was only 7.5×10^8 cells per ml (Table XVI). Under these conditions, no

Table XVI. Effect of aeration on growth and phenolic acid production in wild-type strains.

Strains	Aeration	Generation time (min)	Max. cell density (cells per ml)	O.D. at 510 mμ	O.D. at 408 mμ
B-1471	maximum	130	2.8×10^9	0.55	1.6
B-1471	still culture	190	7.5×10^8	0	0.2
W-23	maximum	130	2.8×10^9	0.45	1.6
W-23	still culture	190	8.0×10^8	0	0.3
WB-746	maximum	190	2.8×10^9	1.2	1.3
WB-746	still culture	190	2.7×10^9	1.1	0.05

Determinations were made over a 24 hr growth period, and the maximum values are reported.

DHB(G) and only 10 per cent of the normal level of coproporphyrin accumulated (Table XVI). Under aerobic conditions, the generation time of these strains was 130 min, the maximum cell density achieved was 2.8×10^9 per ml, and phenolic acids and coproporphyrin were produced (Table XVI). When WB-746 was grown in still culture, no coproporphyrin was produced; but the generation time, maximum cell density and production of DHB were identical to those obtained under aerobic growth conditions (Table XVI).

2. Effect of glucose concentration on phenolic acid excretion

When WB-746 was grown in medium containing 0.3 per cent glucose, a diauxic growth curve was observed (Fig. 17). The first 10 hr growth period was followed by a 5 hr plateau, during which there was no increase in cell number; this was followed by a second period of logarithmic growth. DHB production was initiated after 8 hr incubation, and continued logarithmically for 3 hr. The DHB concentration then decreased linearly during the plateau period. Coproporphyrin was produced only during the second logarithmic growth period (Fig. 18).

3. Oxidation studies

Because the growth medium contained 0.1% citrate and 0.3% acetate, the oxidation of these substrates by WB-746 was studied. Cells harvested and washed (see Materials and Methods) after 10 hr growth (Fig. 18) showed a lag of 1.5 hr before acetate and citrate were oxidized (Fig. 19). Even after this period, oxidation of these substrates proceeded very slowly. Extracts prepared from these

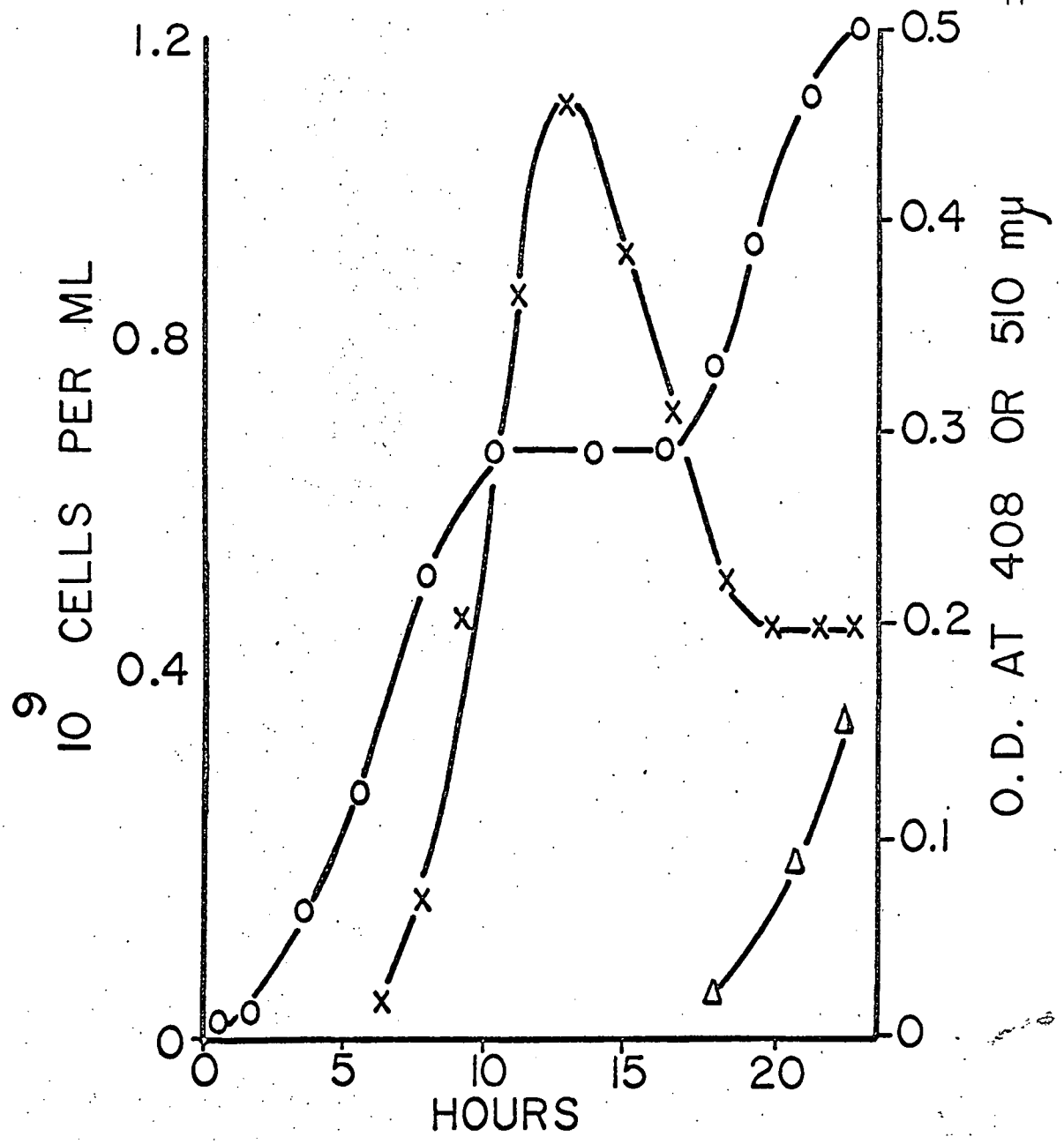

Figure 18. Growth of strain WB-746 in iron-deficient medium containing 0.3% glucose. Cell number (O), DHB (X) and coproporphyrin (Δ) were measured over a 24 hr incubation period.

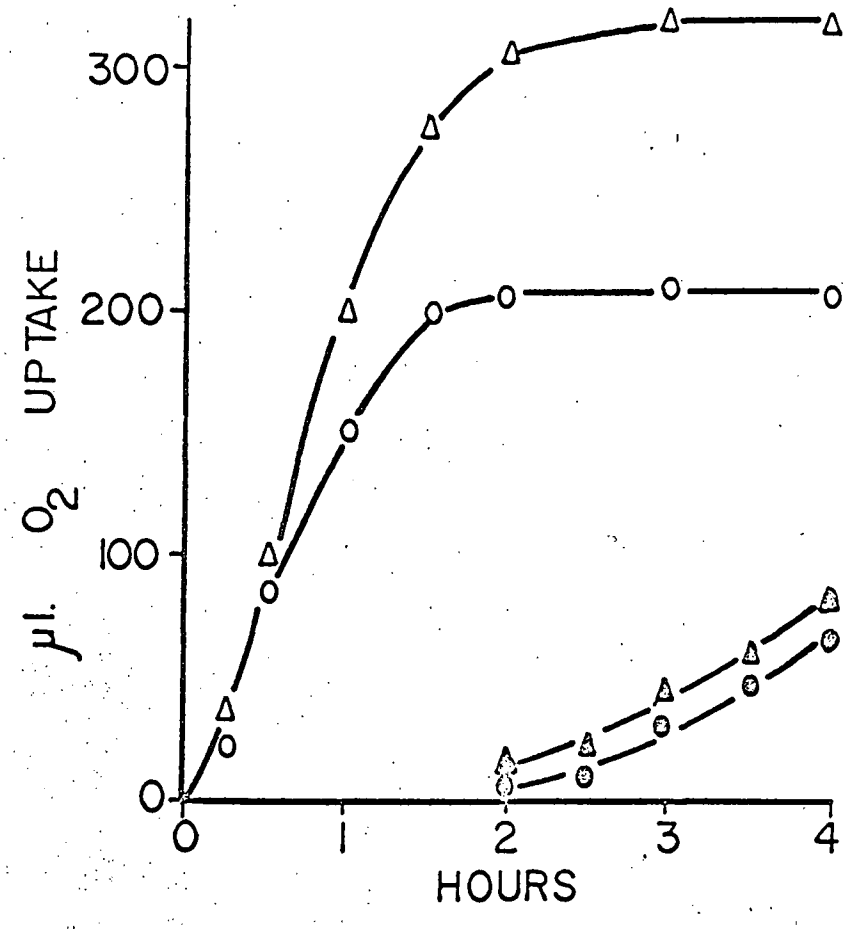

Figure 19. Oxidation capacities of strain WB-746 grown in iron-deficient medium containing 0.3% glucose. Cells harvested after 10 hr oxidized acetate (●) and citrate (▲) after a 90 min lag. Cells harvested after 17 hr growth oxidized acetate (○) and citrate without a lag (△).

10 hr cells were also unable to oxidize acetate or citrate. In contrast, cells harvested after 17 hr growth (Fig. 18) oxidized acetate and citrate without a lag. At no time during growth were cells able to oxidize DHB(G) in the presence or absence of 1 mg Fe^{+3} per litre. The extended plateau (Fig. 18) suggested that iron deficiency may have prevented the formation or functioning of TCA cycle enzymes. The addition of 1 mg iron per litre at 9 hr, however, did not reduce this plateau period.

Unlike strain WB-746, strains B-1471 and W-23 did not display diauxic growth when grown in medium containing 0.3 per cent glucose, and were able to oxidize acetate and citrate without a lag when harvested at any time after 7 hr incubation.

Discussion

The inability of strain WB-746 to produce coproporphyrin during logarithmic growth in iron-deficient medium (Fig. 13) appeared to be related to its inability to oxidize acetate and citrate in the presence of glucose. Prior to and during subsequent acetate and citrate oxidation, the level of DHB in the culture supernatant decreased; this decrease was independent of the level of iron in the medium. No oxidation of DHB was observed by cells harvested at any time during growth. It is possible that during the plateau preceding acetate and citrate oxidation (Fig. 17) DHB served, via the quinone form, as an electron transport by-pass, circumventing the normally operative cytochrome system.

Gray et al (23) have indicated that when an organism with a high aerobic and anaerobic rate of glycolysis was grown with adequate

glucose, enough ATP would become available from the Embden-Meyerhof pathway to minimize the role of the TCA cycle in energy production. These workers further suggested that the TCA cycle enzymes are induced or repressed in three groups, each under independent control: enzymes involved in the synthesis of (a) tricarboxylic acids, (b) 5-carbon dicarboxylic acids, and (c) 4-carbon dicarboxylic acids. In a medium containing adequate glucose, the synthetic portion (i.e. steps leading to the synthesis of α-ketoglutarate) of the cycle would predominate. This appears to be the case in WB-746. Glucose was much less inhibitory to the oxidative functioning of the TCA cycle in strains B-1471 and W-23.

The production of high levels of DHB but no DHBG in still cultures (Table XIV) again reflects the relationship of DHBG production to TCA cycle activity.

Section V: - Iron transport and phenolic acids

Introduction

Phenolic acids have very strong affinities for ferric iron (26), and it has been suggested that they may be involved in iron transport in B. subtilis (40). The production of phenolic acids was related not only to the level of iron in the medium (Fig. 2), but also to the iron requirements of the cell (see Section II). Therefore, an examination was made of the uptake of iron by B. subtilis, and the effects of phenolic acids on this process.

1. Preliminary experiments

Labelled iron was added to sterile medium and the mixture incubated as described in Materials and Methods. Samples were removed at intervals over a 50 min period and passed through 0.45 μ Millipore membranes. No radioactivity was retained on the membranes. Therefore, insoluble iron compounds, which might be retained on the membranes, were not being formed during the experiments described below. For uptake experiments, it was found that washing of cell samples with 10.0 ml medium removed no more iron from the membranes than washing with 2.0 ml. The smaller volume was used in all experiments.

2. Experiments with iron-deficient cultures. Iron transport as a function of culture age

The rate of iron uptake was proportional to cell concentration, i.e. culture age, up to a density of 2.0×10^8/ml. At higher

densities, the curve became sigmoid (Fig. 20). It should be emphasized that these experiments employed growing cells, and that the culture medium contained traces of iron. It was possible that the increased rate of transport observed above a cell density of 2.0×10^8/ml reflected the development of an increased capacity to take up iron in response to the approach to an iron-deficient state. All subsequent experiments were performed using a cell density of 5.5×10^8/ml. In the absence of added iron, cultures started to produce DHBG at a cell density of about 7.0×10^8/ml (see (Fig. 11).

3. Iron transport as a function of energy

The addition of iron led to an initial rapid binding of label to the cells (Fig. 21). This was followed by a slower, linear rate of uptake until about 30 min, after which time the rate decreased (Fig. 21). A mixture of sodium azide and iodoacetamide inhibited the slower phase of uptake, but was without effect on the initial rapid binding of iron (Fig. 21). The gradual decrease in bound iron in the presence of the inhibitors was the result of cell lysis.

4. The effect of temperature on iron transport

The binding of iron to the cells was temperature independent (Fig. 21). The slower rate of uptake was temperature dependent, with an optimum at 37 C (Fig. 22). Measurements at temperatures above 45 C were not made because of cell lysis.

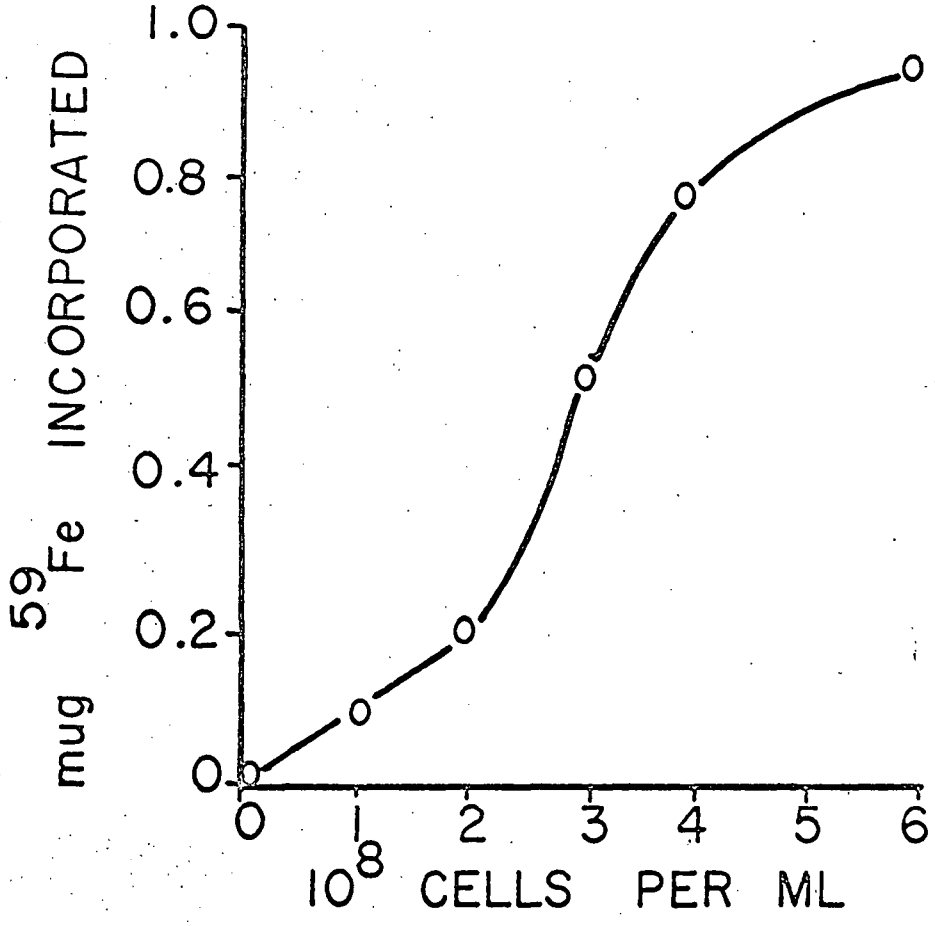

Figure 20. Iron uptake as a function of physiological age. Iron was added to culture samples after growth to the density indicated. Each point represents the rate of uptake measured over the interval 3-8 min after iron addition.

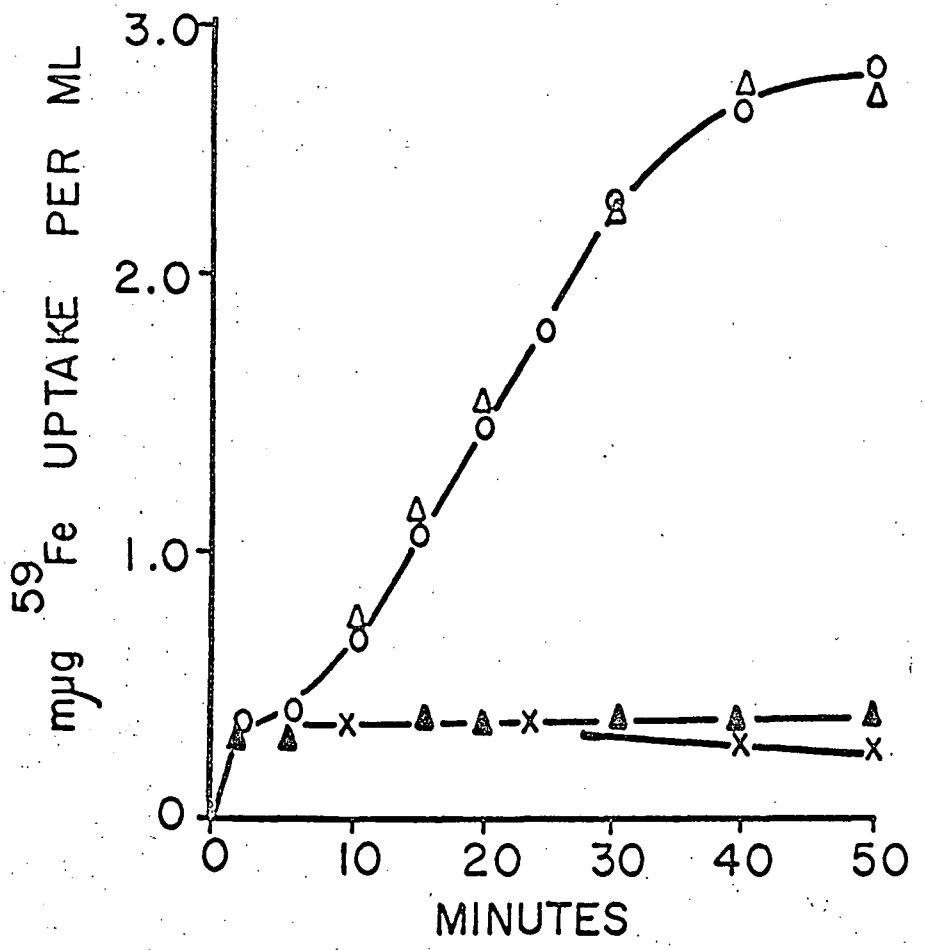

Figure 21. Iron uptake as a function of energy. The rate of iron uptake was measured at 37 C (O), o C (▲), at 37 C after pre-incubation for 30 min with 30 mM sodium azide and 1 mM iodoacetamide (X), and at 37 C after the addition of 0.1% citrate (Δ).

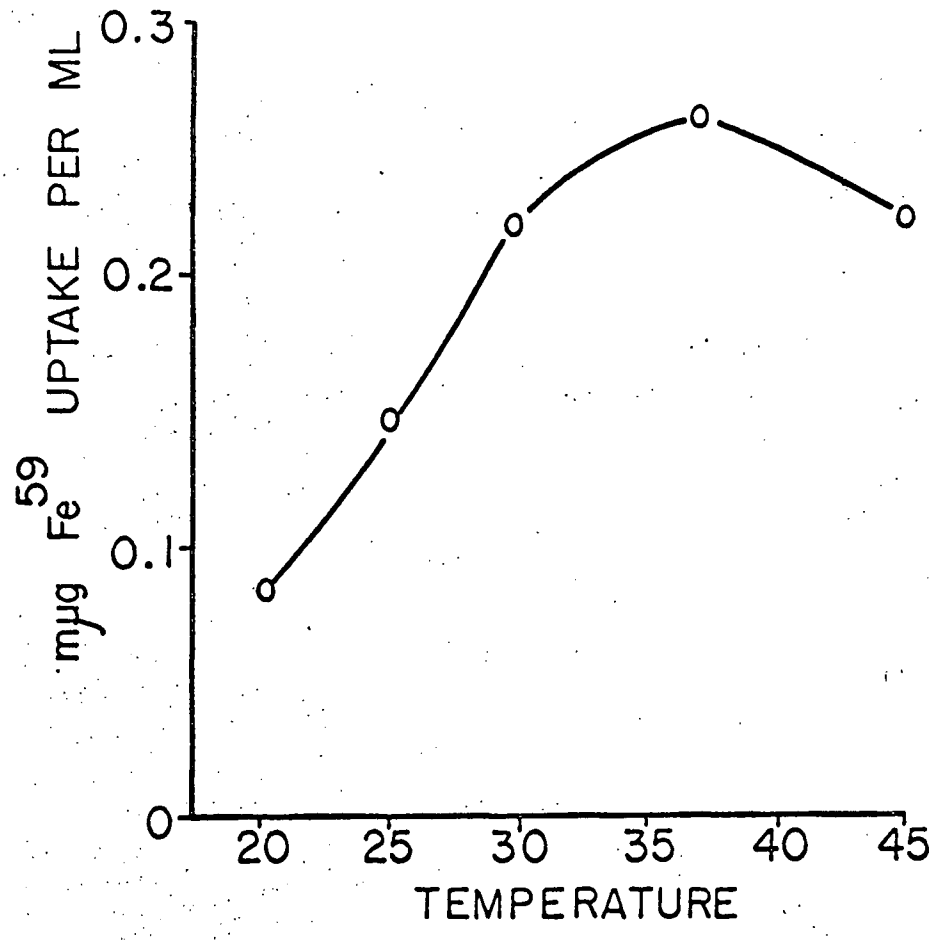

Figure 22. Effect of temperature on iron uptake. Culture samples were pre-incubated at the appropriate temperature for 10 min before the addition of iron.

5. Incorporation of iron into TCA-insoluble material

A significant fraction of the iron bound to the cells, and of that subsequently taken up, was insoluble in cold 5% TCA (Fig. 23). This was true also of the iron bound at 0 C.

6. Iron transport as a function of iron concentration

The rate of iron uptake at 37 C was proportional to the iron concentration, up to a level of 40 mμg/ml (Fig. 24). As the concentration was increased beyond 40 mμg/ml, the rate of uptake appeared to decrease (Fig. 24). Further examination of uptake at high iron concentrations showed that a period of very rapid uptake, lasting about 6-7 min, was followed by a period during which iron was lost very rapidly from the cells. This loss of iron was not observed at lower iron concentrations (i.e. at 5 mμg/ml), not even when cells were allowed to take up iron for 30 min, were filtered, washed with iron-free medium, and then incubated in iron-free medium for a further 30 min. The binding sites on the cells were saturated (i.e. ^{59}Fe uptake at 0 C) at an iron concentration of 200 mμg/ml (Fig. 25), while the maximum rate of uptake was obtained at 40 mμg iron/ml.

7. The effect of citrate on iron uptake

The medium used contained sodium citrate. It has been reported that citrate could replace the DHB requirement of a multiple aromatic auxotroph of Escherichia coli (67). When citrate was omitted from the medium, the rate of iron uptake by cells was increased about 4-fold, and the amount of iron taken up was increased about

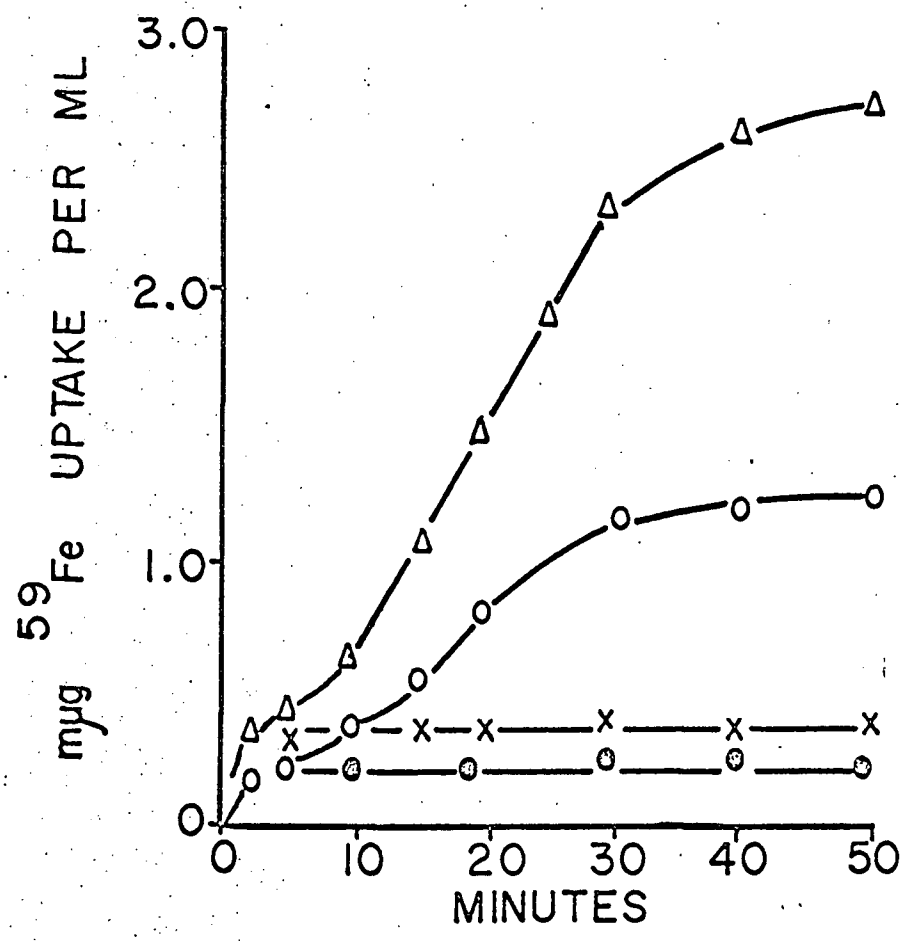

Figure 23. Incorporation of iron into TCA-insoluble material. Whole cells at 37 C (Δ), TCA-insoluble fraction at 37 C (O), whole cells at 0 C (X), and TCA-insoluble fraction at 0 C (●).

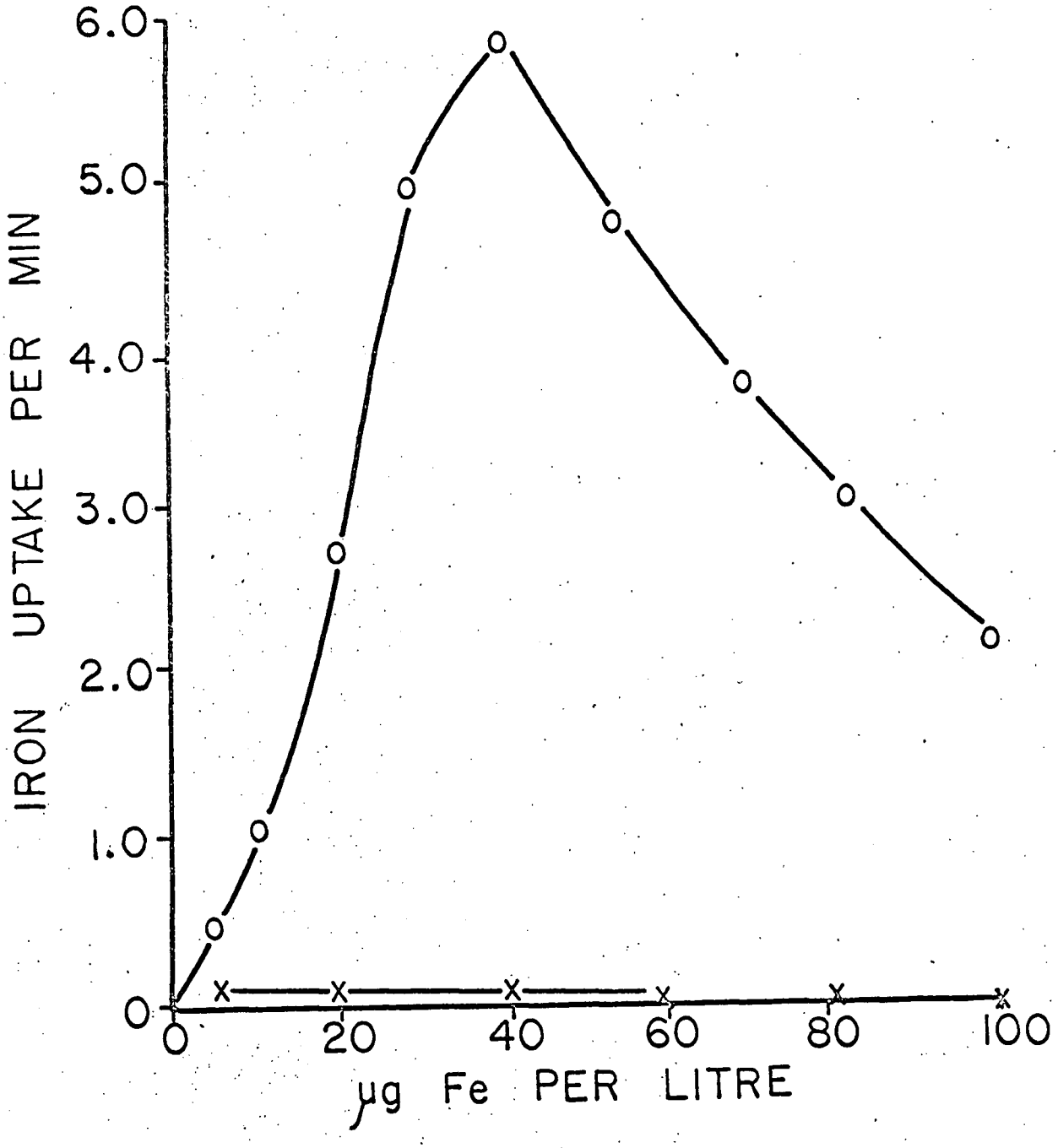

Figure 24. Rate of iron uptake as a function of iron concentration. Iron-deficient cells (O), iron-sufficient cells (X). Uptake rates were measured over the interval 2-6 min after iron addition.

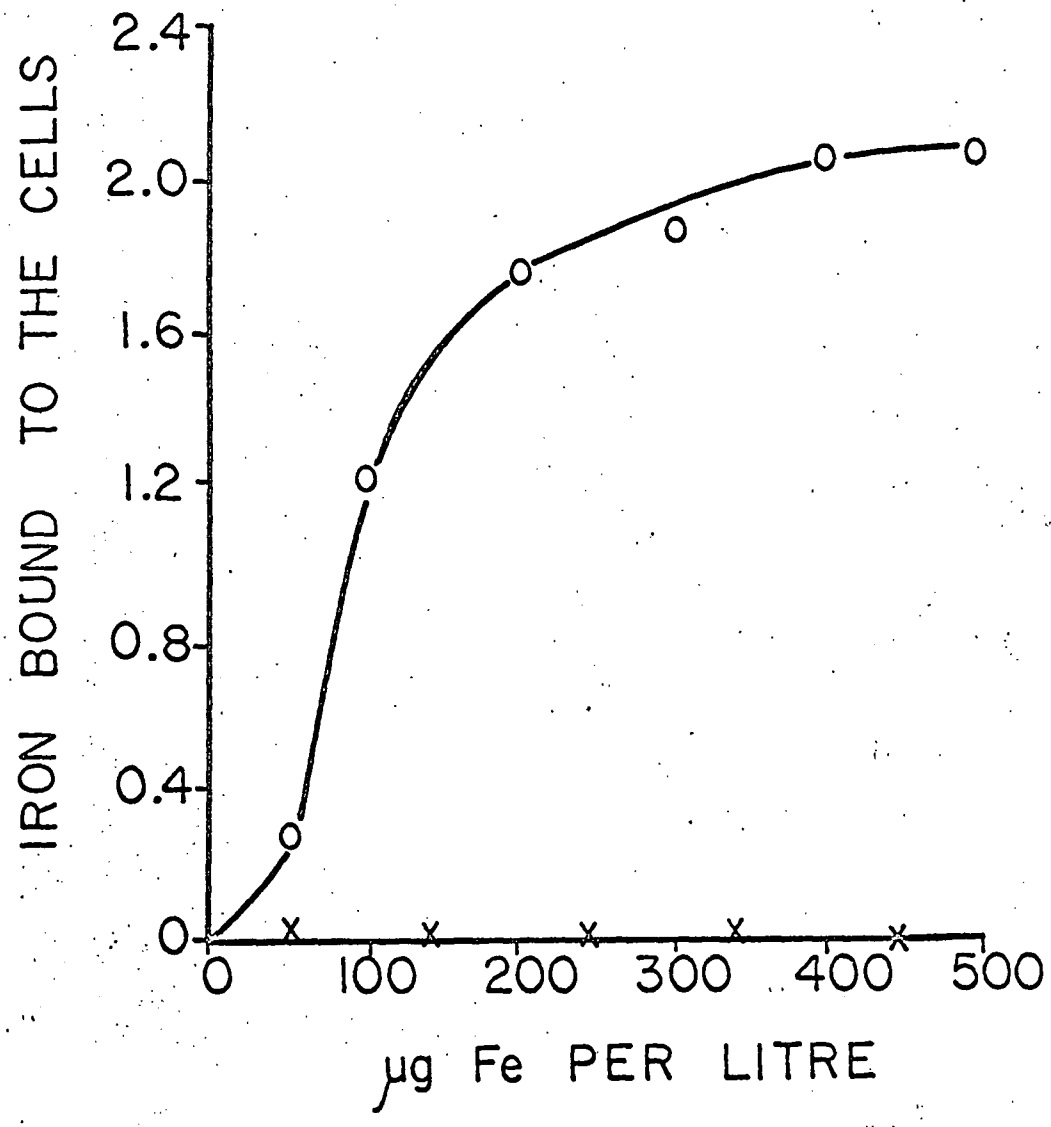

Figure 25. Binding of iron to cells at 0 C as a function of iron concentration. Iron deficient cells (O), iron sufficient cells (X). Uptake was measured 3 min after addition of iron.

40% (Fig. 26). There was also a 2-3 fold increase in the amount of iron bound by the cells at 0 C (Fig. 26). The addition of 5-300 μg DHB(G)/ml to cultures grown in the absence of citrate caused a 20% increase in the level of iron uptake (Fig. 26). The addition of 0.1% citrate at zero time or after 30 min incubation, however, did not affect iron uptake by cells growing in iron-deficient, citrate-containing medium (Fig. 21).

8. The effect of phenolic acids on iron uptake

The addition of 5 μg/ml of DHB or DHBG at the same time as 5 mμg of iron/ml resulted in a 4-fold increase in the rate of iron uptake (Fig. 27). Increased concentrations of phenolic acid up to 300 μg/ml did not give any further stimulation of the rate of uptake. It should be pointed out that the phenolic acids were mixed with the iron and left at room temperature to equilibriate for 15 min (26) prior to their addition to the cells. In the absence of a phenolic acid about 55% of the added iron was taken up by the cells. In the presence of a phenolic acid about 90% was taken up (Fig. 27). Phenolic acid in the range 5-300 μg/ml decreased the binding of iron at 0 C by about 50%. If less than 5 μg/ml phenolic acid was added, the rate of uptake was still elevated, but there was some decrease in the amount of iron taken up by the cells (Fig. 27). The addition of 5 μg/ml of phenolic acid with 0.5 mμg iron/ml did not affect the rate of uptake, but it did increase the amount of iron taken up from 30% to 70% (Fig. 28). The binding of iron at 0 C again was reduced in the presence of phenolic acid (Fig. 28).

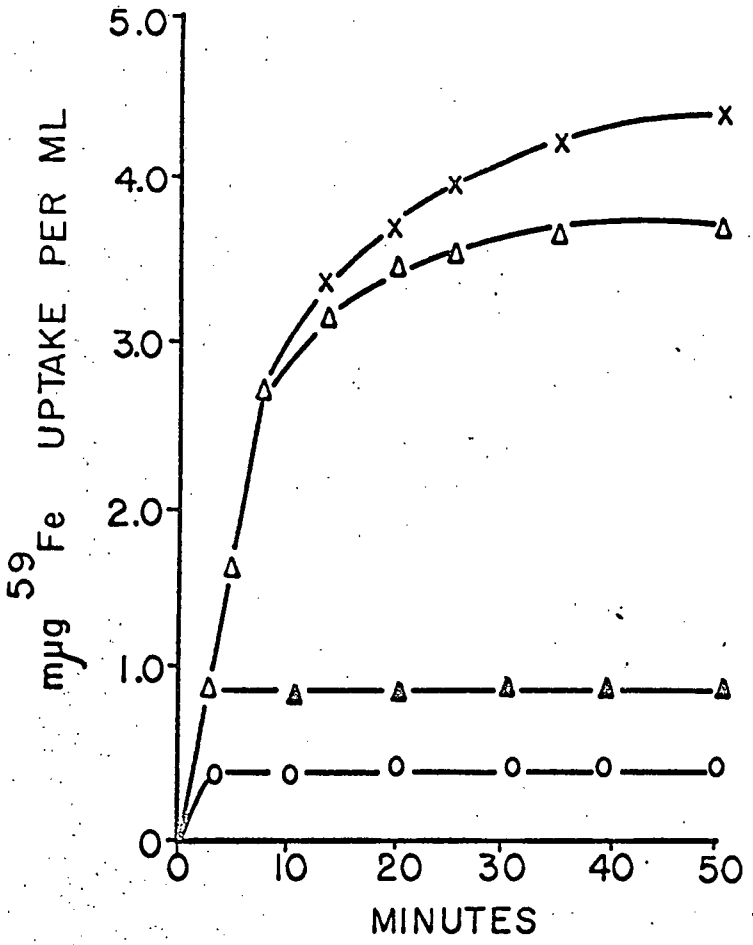

Figure 26. Effect of growth without citrate on iron uptake. Uptake was measured at 37 C with 5 μg DHB(G)/ml (X), and with no addition (Δ). Binding was at 0 C with 5 μg DHB(G)/ml (O), and with no addition (▲).

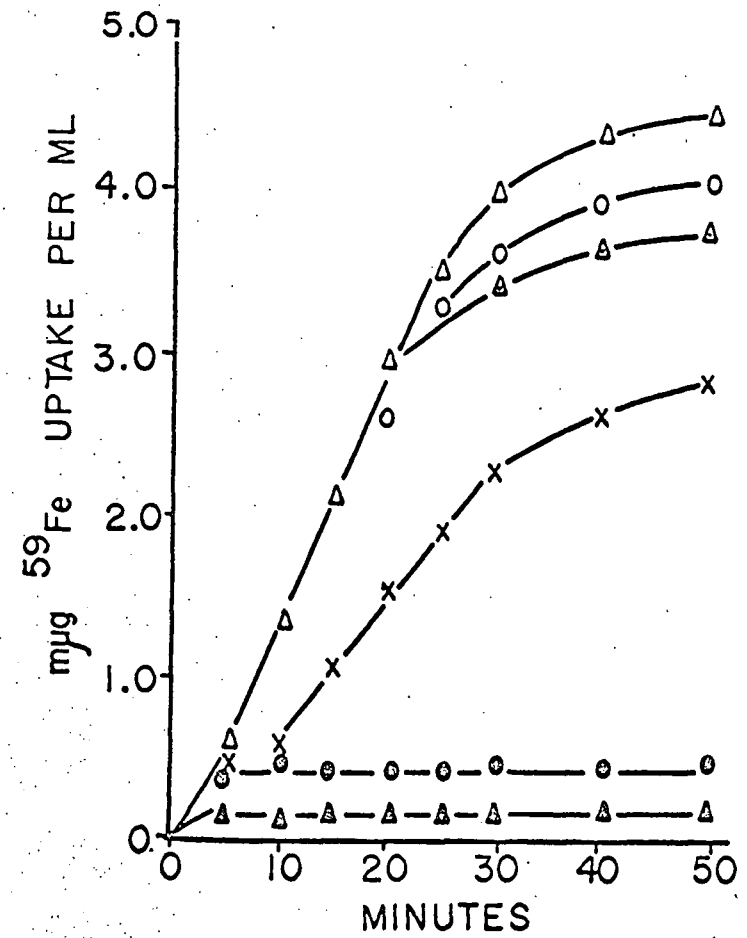

Figure 27. Effect of phenolic acids on iron uptake. Iron was added to all flasks at 5 mμg/ml. Uptake was measured at 37 C with 5-300 μg DHB(G)/ml (△), 1 μg DHB(G)/ml (O), 0.5 μg DHB(G)/ml (▲), and with no addition (X). Binding was measured at 0 C with 5-300 μg DHB(G)/ml (▲), and with no addition (●).

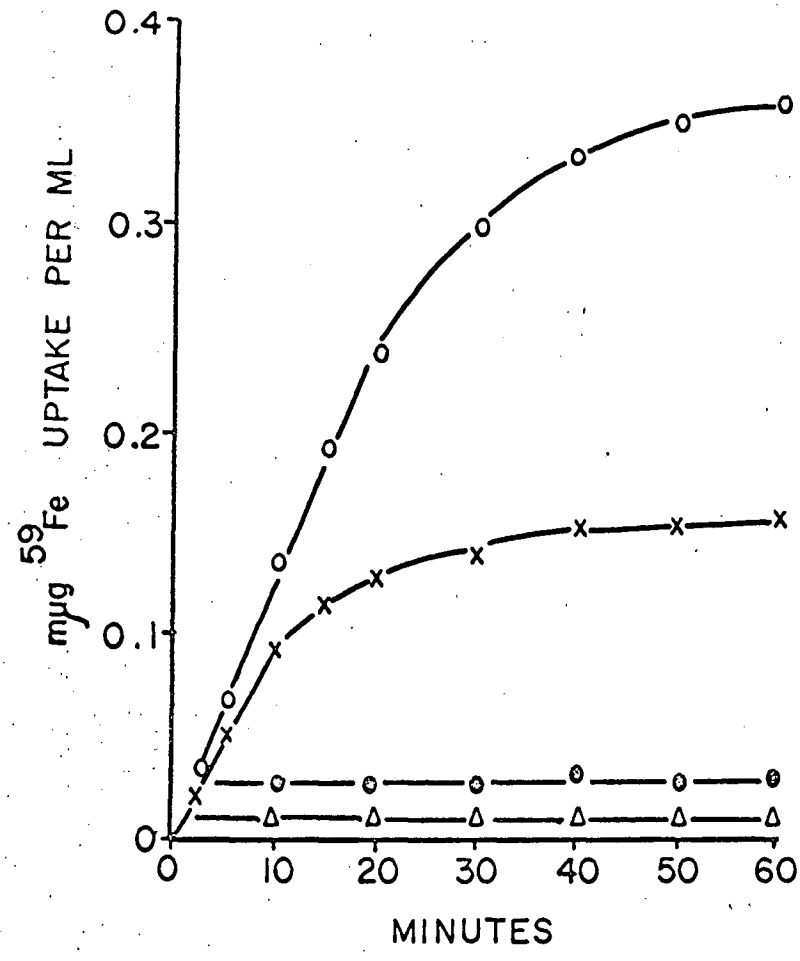

Figure 28. Effect of phenolic acids on uptake at lower levels of iron. Iron was added to all flasks at 0.5 mμg/ml. Uptake was measured at 37 C with 5 μg DHB(G)/ml (O), and with no addition (X). Binding was measured at 0 C with 5 g DHB(G)/ml (Δ), and with no addition (●).

9. Experiments with iron-sufficient cultures

Cells grown in iron-sufficient medium bound less than 5% of the level of iron bound by iron-deficient cells at 0 C (Fig. 25). In addition, the rate of uptake was greatly reduced as a consequence of growth in iron-sufficient medium (Fig. 24). The addition of phenolic acids to iron-sufficient cells did not affect either the binding or the rate of uptake of iron.

10. Experiments with mutant strains

After growth in iron-deficient medium, the rates of iron uptake in strains dhb-1, dhb-4 and dhb-5 were less than 20% of the rate observed in strain B-1471 (Fig. 29). The binding of iron at 0 C was reduced by 80% in these strains (Fig. 29). In the presence of 50 µg DHB(G)/ml, the rate of uptake was increased 10-fold in strain dhb-4, but the rates in strains dhb-1 and dhb-5 were unchanged (Fig. 29).

Discussion

The results indicate that B. subtilis possesses a system for the transport of iron, which has the properties of an active transport system (3), being temperature and energy-dependent. Growth under conditions of iron-deficiency results in a considerable increase in the capacity of the transport system. In fact, the iron-deficient cell develops this capacity to such an extent that eventually it can no longer retain the high levels of iron taken up when increased quantities are added to the medium. In addition to the transport system, cells of B. subtilis can bind iron at 0 C,

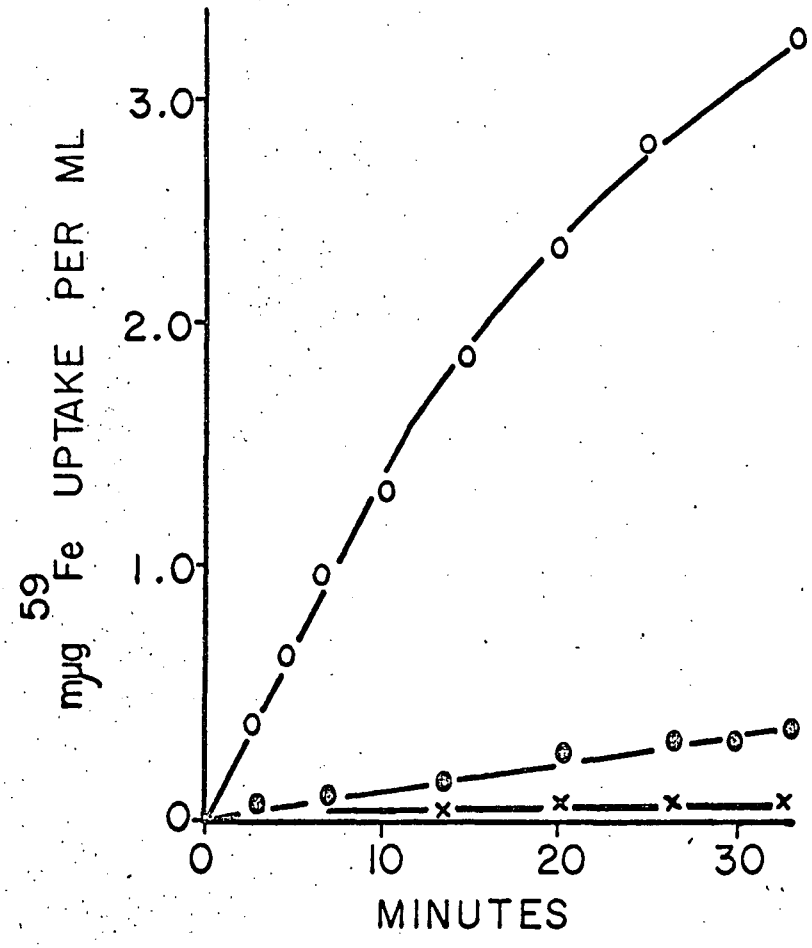

Figure 29. Iron uptake in mutant strains. Strain dhb-1, dhb-4, and dhb-5: without addition at 37 C (●), and at 0 C (X). Strains dhb-1 and dhb-5 with 50 μg DHB(G)/ml at 37 C (○). Strain dhb-4 with 50 μg DHB(G)/ml at 37 C (●).

and this binding also is increased considerably by growth under iron-deficiency.

The addition of a phenolic acid to iron-deficient cells stimulated the rate and increased the level of iron transported. At lower iron concentrations, only the level of iron transported was altered by phenolic acid addition. Mutants unable to produce normal levels of phenolic acid showed a decreased rate of transport and a decreased binding capacity. This suggests that phenolic acids are involved directly in iron transport. At neutral pH, the 3:1 complex of DHBG:Fe^{3+} is favoured (Fig. 30) (40), so that the transport system would be able to recognize the complex in the presence of a great excess of free DHBG. The properties of the mutant strains suggest that phenolic acids may also be involved in the binding of iron to the cell.

The transport system may be inducible because the capacity for transport increases during iron-deficiency. If it is inducible, the question of the nature of the inducer becomes important. It is unlikely to be the $Fe^{3+}(DHB(G))_3$ complex because the increased capacity is the consequence of a lack of iron. An attractive alternative is that the system is repressible, with the corepressor being an iron complex, possibly $Fe^{3+}(DHB(G))_3$.

Citrate can bind iron, and could serve to make limiting iron more available to the cell, thereby postponing the onset of iron-deficiency. In the present case, the omission of citrate from the medium led to an increased capacity for iron transport.

Almost half of the iron taken up by the cells was insoluble in cold TCA. This was true also of the iron bound to the cells.

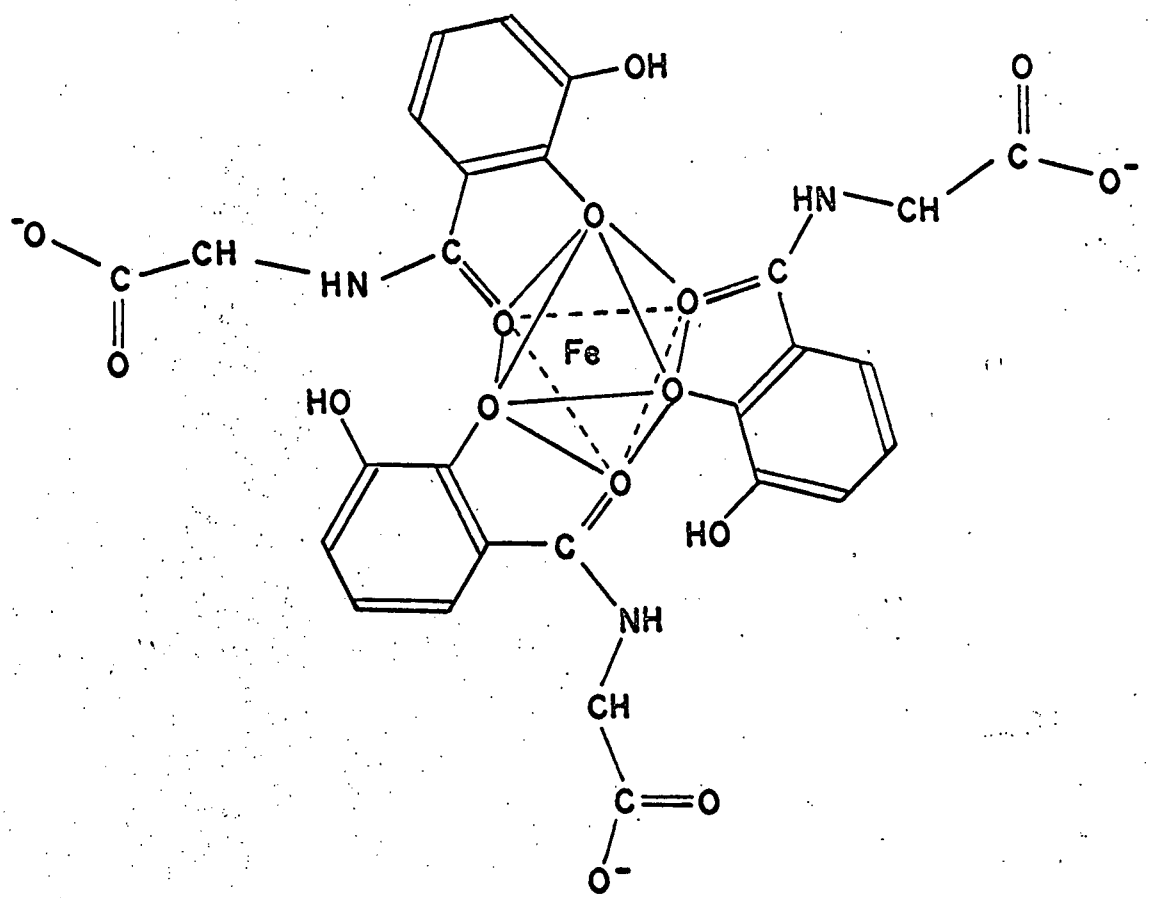

Figure 30. The Fe^{+3}:$(DHBG)_3$ complex

The nature of this "insoluble" iron is not clear at present, but it is being investigated. Attempts are being made to isolate mutants totally unable to synthesize phenolic acids so that their capacities for iron transport and binding may be studied.

Section VI: - Control of iron transport

Introduction

Phenolic acid production started earlier and continued at a faster rate in WB-746 than in B-1471 (Fig. 11, 13). Conversely, coproporphyrin production started later and occurred at a slower rate in WB-746 than in B-1471 (Fig. 11, 13). Oxidative functioning of the TCA cycle in WB-746 was inhibited by 0.3% glucose (Fig. 18), while that of B-1471 was not. Hem-1 had no oxidative TCA cycle in unsupplemented media, but the normal functioning of this cycle was restored following supplementation with ALA (Fig. 14, p.). These properties prompted an investigation of the relationship between iron transport capacity and heme-iron requirement.

1. Effect of heme-iron requirement on transport capacity

When uptake studies were conducted, the rate and extent of iron uptake in B-1471 greatly exceeded that in WB-746 or of hem-1 in unsupplemented medium (Fig. 31). When hem-1 was grown in medium supplemented with 5 μg ALA per ml, its iron uptake capacity approached that of the parent strain (Fig. 31).

2. Effect of ferrichrome

Addition of the phenolic acid Fe^{+3} complex inhibited phenolic acid (Fig. 4) and coproporphyrin production (Fig. 14) by strain B-1471. This was probably the result of phenolic acids increasing iron uptake (Fig. 27). The addition of low levels (0.4 μg per ml) of ferrichrome to iron-deficient cultures of B-1471 at zero time or after

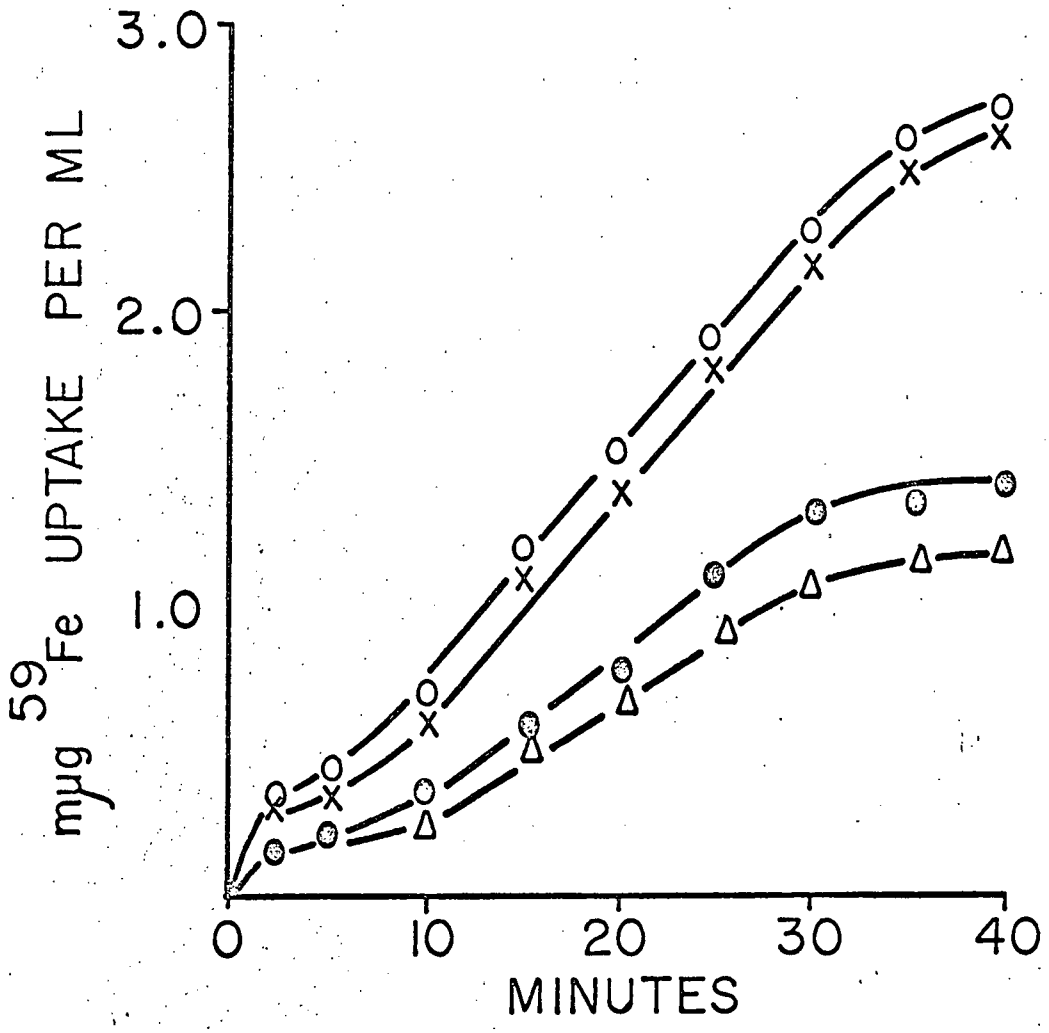

Figure 31. Strain differences in iron uptake capacities. Iron uptake was measured in strains B-1471 (O) and WB-746 (Δ). Hem-1 growing in the absence of ALA had a low iron uptake capacity (●). When hem-1 was grown in the presence of 5 μg ALA per ml, its uptake capacity (X) was comparable to that of the parent strain (O).

8 hr growth, inhibited DHBG (Fig. 17) and coproporphyrin production (Table XIV). The level of iron provided by this supplementation is about 20 μg per litre, a level which alone caused no inhibition of excretion (Fig. 2). Ferrichrome has been implicated as an iron solubilization factor for several microorganisms (39, 41). Ferrichrome and desferri-ferrichrome were tested therefore for their effects on iron transport. When cells were incubated with 5 mμg ^{59}Fe per ml, 55% of this iron was available to the cell over a 40 min incubation period (Fig. 32). When 0.4 μg ferrichrome per ml was added simultaneously with the radioactive iron, there was a 30% decrease in the amount of label incorporated into the cells. Preincubation of the ferrichrome with ^{59}Fe for one hr, which would allow about 22% of the ferrichrome-bound unlabelled iron to exchange with ^{59}Fe (34) resulted in only an 18% decrease in the amount of radioactive label incorporated compared to cells in the absence of ferrichrome (Fig. 32). The addition of desferri-ferrichrome simultaneously with ^{59}Fe, made 99% of the labelled iron available to the cells within 15 min incubation (Fig. 33).

3. HQ^r mutants

The properties of strains HQ^r-1-6 (Section I) suggested that these strains required less available iron than the parent strain. To check this theory, iron transport studies were conducted. When these strains were grown in the absence of HQ, the rate and extent of iron uptake were significantly increased (Fig. 34). Supplementation of media with 0.1 μg HQ per ml (which did not affect the growth rate) further increased the rate and extent of transport (Fig. 34).

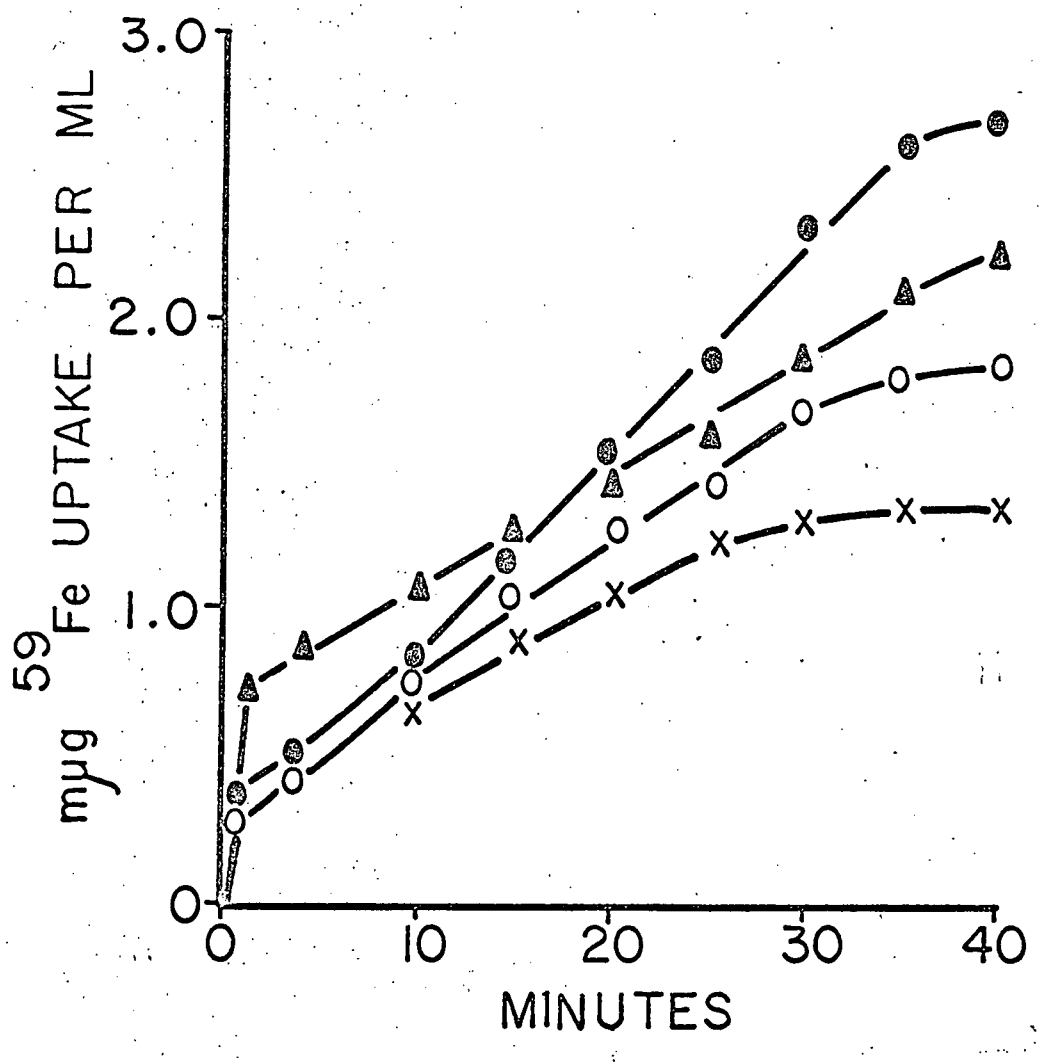

Figure 32. Effect of ferrichrome on iron transport. Ferrichrome (0.4 μg/ml) was pre-incubated for 1 hr with iron (▲), added simultaneously with iron (O), or ferrichrome (0.8 μg/ml) was added simultaneously with iron (X). No ferrichrome was added to the control flask (●).

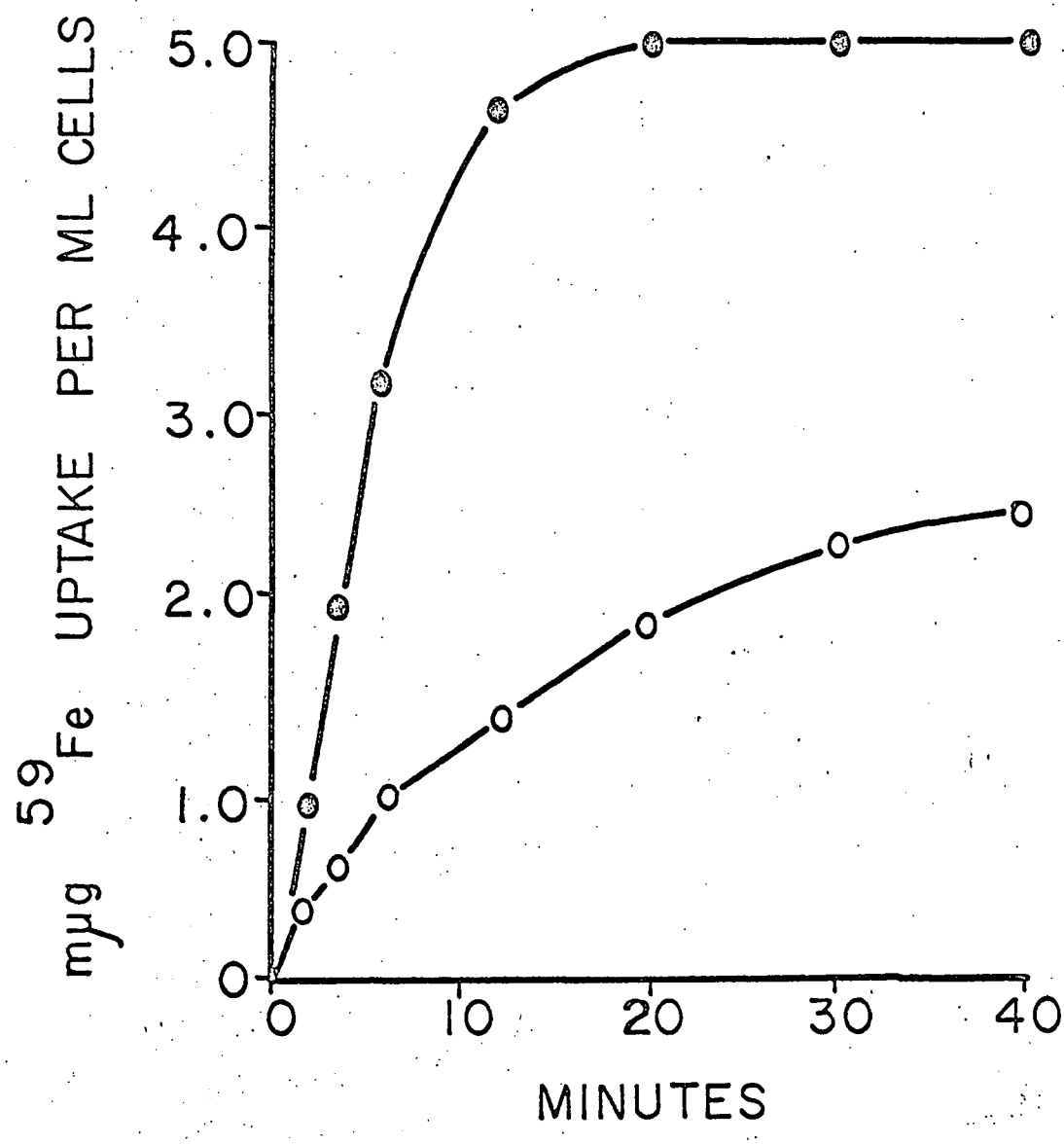

Figure 33. Effect of ferrichrome on iron uptake in strain B-1471. Cells were grown to a viable density of 5.5×10^8 per ml prior to study. ^{59}Fe (5 mμg per ml) and desferriferrichrome (0.4 μg per ml) were then added (at zero time (●). Ferrichrome was omitted from the control flask (○).

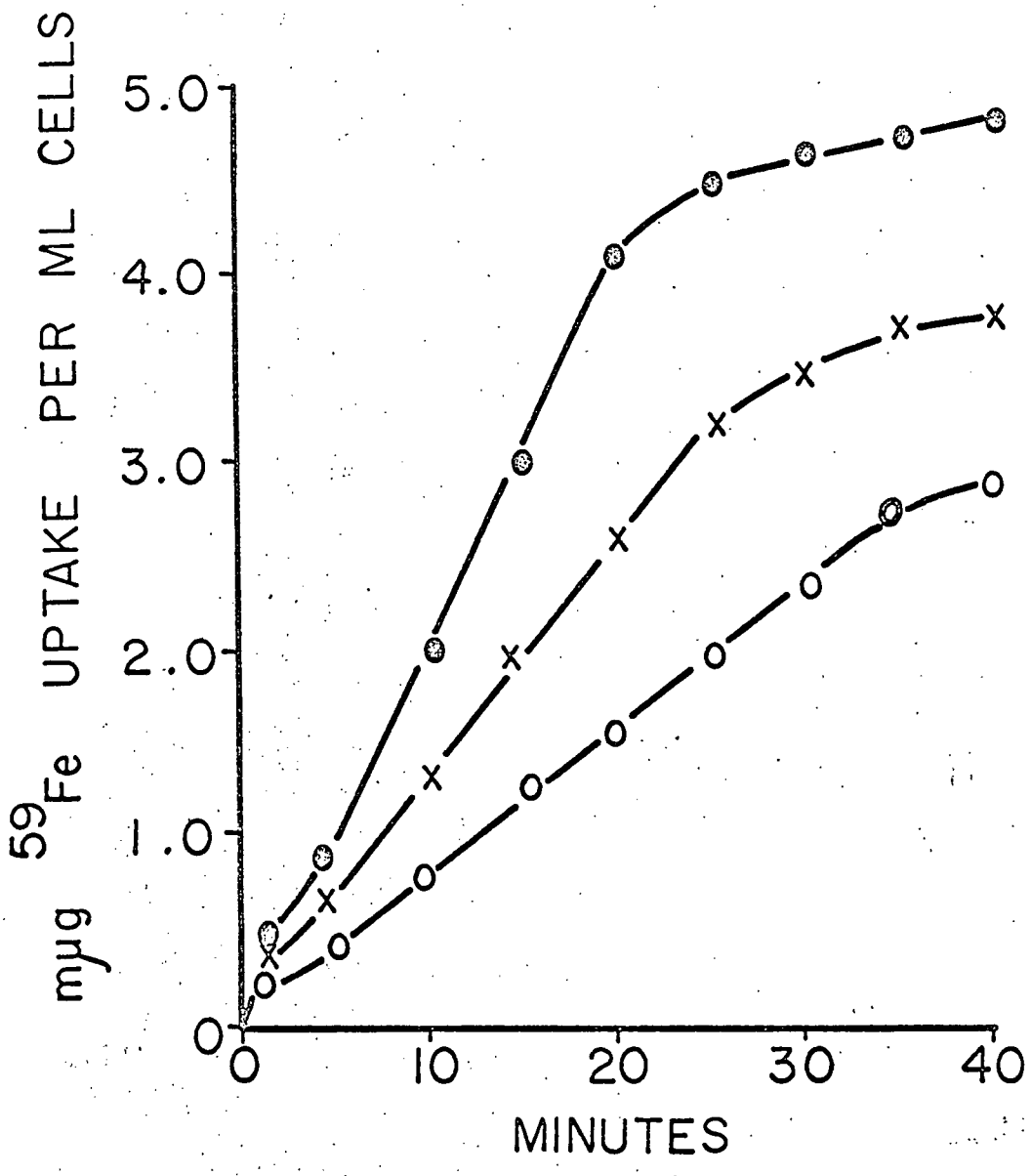

Figure 34. Iron uptake by strain HQ^r-1. Uptake of ^{59}Fe by strain B-1471 (O) was compared to uptake by strain HQ^r-1 grown in the absence (X) and presence (●) of 0.1 μg/ml HQ.

Discussion

A requirement for heme-iron appeared to cause an increase in the transport capacity of cells. The inhibitory effect of low levels of ferrichrome on DHBG and coproporphyrin excretion in B-1471 appeared to be related to its ability to drastically increase both the rate and extent of iron uptake. The observations that uptake of 5 mμg ^{59}Fe per ml was decreased by ferrichrome addition, and was increased by supplementation with desferri-ferrichrome may indicate that: (i) ferrichrome was competing at the active site for free iron transport, or (ii) upon entering the cell, ferrichrome caused inhibition of free iron uptake.

It has been suggested (41) that ferrichrome is active in the insertion of iron into porphyrin. Thus, ferrichrome may have provided iron directly for heme synthesis in B-1471, allowing normal iron sufficient porphyrin biosynthesis, thereby eliminating the requirement for DHBG synthesis. Results with B-1471 (Section II) indicated that if iron were available for heme synthesis, neither coproporphyrin nor DHBG would accumulate.

The extent of iron transport under the experimental conditions employed allows speculation concerning the level of extracellular iron remaining prior to the excretion of DHBG and coproporphyrin (Fig. 11) by this strain. Transport studies were conducted under conditions identical to those used for studying excretion products, except that 5 μg Fe^{+3} per litre were added to cells approximately one hour prior to the onset of phenolic acid excretion (Fig. 11). Of this level of added iron, 45% was unavailable to cells in the absence of phenolic acids (Fig. 26), so that the extracellular iron

concentration prior to DHBG excretion could be estimated to be 5 μg per litre. Excretion of DHBG by cells would then facilitate solubilization of about 90% of this iron (Fig. 26) preceding the onset of coproporphyrin production (Fig. 11). Only about 0.35 μg per litre of the remaining iron would subsequently be available to the cell (Fig. 27), which accounts for the excretion of very high levels of coproporphyrin (Fig. 11).

HQ^r mutants had an increased iron uptake capacity compared to the parent strain. When these mutants were grown in the presence of 0.1 μg HQ per ml, their iron transport ability was increased further. HQ^r-1-6 were unable to excrete normal levels of DHBG or coproporphyrin in the absence of HQ (Section I). In the presence of 0.1 μg HQ per ml, however, these products did accumulate in the medium (Section I). It remains to be determined if HQ forms a complex with iron which then (a) cannot be taken up by the cell, or (b) can be taken up by the cell but from which the iron cannot be released.

GENERAL DISCUSSION

Although the factor exerting the greatest effect on phenolic acid production by B. subtilis was the level of iron in the medium, production was influenced also by other aromatic compounds synthesized from chorismic acid and by the requirements of the cell for iron.

The effects of the aromatic amino acids in reducing phenolic acid production were to be expected because it is known that the aromatic biosynthetic pathway in B. subtilis is subjected to feedback inhibition by these amino acids (28, 33). Repression of the enzymes of the pathway has been demonstrated recently (43) and it is significant that it appears to be mediated most strongly by tyrosine, the amino acid exerting the greatest degree of inhibition of phenolic acid production. The aromatic amino acids would reduce the level of chorismic acid available for DHB synthesis. The marked inhibitory effect of anthranilic acid appeared to be directly on DHB synthesis from chorismic acid.

That the production of phenolic acids was related to the actual iron requirements of the cell was shown by the properties of strain B-1471 hem-1. This strain, blocked at the first step of porphyrin biosynthesis, did not produce phenolic acids under iron-deficiency. In addition, still cultures of strains B-1471 and W-23 did not produce phenolic acids under iron-deficiency. Such cultures would have had a low capacity for porphyrin biosynthesis. The production of DHB by strain WB-746 under these conditions may have reflected the decreased iron uptake capacity of this strain. It

was possible that, in spite of these observations, the relationship between phenolic acid production and a requirement for iron for hemin biosynthesis was more apparent than real, with phenolic acid and coproporphyrin production occurring independently in response to an iron deficiency. However, hemin inhibited phenolic acid production. It has been shown that hemin is probably unable to satisfy the non-heme iron requirements of certain bacteria (39), suggesting that it does not readily release its iron inside the cells. It was significant, also, that phenolic acid production started before coproporphyrin production. This aspect of the problem is to be extended by examining mutants blocked after ALA for their capacities for phenolic acid production. It is intended also, to use strain B-1471 hem-1 to determine the heme-iron, the non-heme iron and the total iron requirements of strain B-1471 under various conditions. The relative importance of iron for hemin biosynthesis can then be assessed and the relationship of phenolic acid production to porphyrin biosynthesis worked out in detail)

DHB and DHBG have strong affinities for ferric iron (26), so that they could chelate low levels of iron in the medium. The development of an increased capacity for iron uptake by cells grown in an iron-deficient medium, and the enhancement of this capacity by phenolic acids, supported the idea that the acids were serving to scavenge the last traces of iron from the medium. The production of phenolic acids could have been initiated when the level of iron inside the cell fell below a critical value. It remains to be determined what this value might be and whether it represents total,

free or bound iron.

Perhaps the most interesting aspect of this problem is the question of how the level of iron in the medium controls the production of phenolic acids. There are two major possibilities: the enzymes for DHB synthesis are either repressible or inducible. The nature of the corepressor or inducer is not clear at this point. It might be that iron itself is a corepressor, with a critical level being required to activate the repressor. The $Fe^{3+}:(DHB(G))_3$ complex might be the corepressor; in this case it would be necessary to postulate that sideramines inhibit phenolic acid production by carrying iron into the cell to allow formation of the $Fe^{3+}:(DHB(G))_3$ complex. Alternatively, the system might be induced by free DHB(G). In the presence of sufficient iron, all the DHB(G) could be in the $Fe^{3+}:(DHB(G))$ complex; as the level of iron falls below the critical level, free DHB(G) could appear to induce the system. It is unlikely that the m-substituted benzoic acids inhibited phenolic acid production by acting as corepressors since none of them could complex iron, and the medium was iron-deficient. They could, however, have competed with free DHB(G) to prevent induction.

The increased capacity for iron uptake seen in iron-deficient cells could have resulted from the appearance of a specific permease in response to the initiation of phenolic acid production. This point must be investigated further, and an attempt made to determine if such a permease is under the same system(s) of control as the enzymes involved in DHB synthesis. The system can be subjected to genetic analysis, using transformation and

or transduction. Attempts will be made to isolate mutants in which the production of phenolic acids is no longer controlled by iron.

Ferrichrome and ferrioxamine served as effective "inhibitors" of phenolic acid production, and ferrichrome served very effectively to carry iron into the cell. If a specific permease was involved in carrying ferric iron complexes into the cell, it might be characterized by being relatively non-specific with regard to the ligands binding the iron. The specificity of the iron uptake mechanism seen in iron-deficient cells could be examined using phenolic compounds closely and distantly related to DHB and DHBG.

LITERATURE CITED

1. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Conditions optimal for mutagenesis by N-methyl-N'-nitro'N-N-nitrosoguanidine in Escherichia coli K-12. Biochem. Biophys. Res. Commun. 18: 788-795.

2. Akrigg, A., S. R. Ayad, and G. R. Baker. 1967. The nature of a competence-inducing factor in Bacillus subtilis. Biochem. Biophys. Res. Commun. 28: 1062-1067.

3. Albers, R. W. 1967. Biochemical aspects of active transport. Ann. Rev. Biochem. 36: 727-756.

4. Aronson, J. N., and G. R. Wermus. 1965. Effects of m-tyrosine on growth and sporulation in Bacillus species. J. Bacteriol. 90: 38-46.

5. Baker, T. I., and I. P. Crawford. 1966. Anthranilate synthetase. Partial purification and some kinetic studies on this enzyme from Escherichia coli. J. Biol. Chem. 241: 5577-5584.

6. Brot, N., and J. Goodwin. 1968. Regulation of 2,3-dihydroxybenzoylserine synthetase by iron. J. Biol. Chem. 243: 510-513.

7. Brot, N., J. Goodwin, and H. Fales. 1966. In vivo and in vitro formation of 2,3-dihydroxybenzoylserine by Escherichia coli K-12. Biochem. Biophys. Res. Commun. 25: 454-461.

8. Burnham, B. F. 1961. Doctoral dissertation. University of California, Berkeley, Cal.

9. Burnham, B. F., and J. Lascelles. 1963. Control of porphyrin biosynthesis through a negative-feedback mechanism. Biochem. J. 87: 462-472.

10. Byers, B. R., M. V. Powell, and C. E. Lankford. 1967. Iron-chelating hydroxamic acid (schizokinen) active in initiation of cell division in Bacillus megaterium. J. Bacteriol. 93: 286-294.

11. Chasin, L. A., and J. Szulmajster. 1967. Biosynthesis of dipicolinic acid in Bacillus subtilis. Proc. N.A.S. 29: 648-654.

12. Cox, G. B., and F. Gibson. 1967. 2,3-dihydroxybenzoic acid, a new growth factor for multiple aromatic auxotrophs. J. Bacteriol. 93: 502-503.

13. Doy, C. H. 1966. Chemical synthesis of the tryptophan pathway intermediate 1-(O-carboxyphenylamino)-1-deoxy-D-ribulose-5-phosphate. Nature 211: 736-737.

14. Emery, T., and J. B. Neilands. 1960. Contribution to the structure of the ferrichrome compounds; Characterization of the acyl moieties of the hydroxamate functions. J. Am. Chem. Soc. 82: 3658-3662.

15. Falk, J. E. 1964. Porphyrins and metalloporphyrins. Elsevier Publishimg Company, New York.

16. Gallokata, K. G., and H. O. Halvorson. 1960. Biochemical changes occurring during sporulation by Bacillus cereus. Inhibitions of sporulation by α-picolinic acid. J. Bacteriol. 79: 1-8.

17. Garibaldi, J. A., and J. B. Neilands. 1955. Isolation and properties of ferrichrome A. J. Am. Chem. Soc. 77: 2429-2430.

18. Garibaldi, J. A., and J. B. Neilands, 1956. Formation of iron-binding compounds by microorganisms. Nature 177: 526-527.

19. Gibson, F. 1964. Chorismic acid: purification and some chemical and physical studies. Biochem. J. 90: 256-261.

20. Gibson, F. 1968. Chorismic acid. Biochem. Prepn. 12: 94-97.

21. Gibson, F. Personal communication.

22. Gorini, L., and R. Lord. 1956. Necessite des arthodiphenols pour la croissance de Coccus (Sarcina sp.). Biochim. Biophys. Acta. 19: 84-90.

23. Gray, C. H., and L. B. Holt. 1948. The isolation of coproporphyrin III from Corynebacterium diphtheriae culture filtrates. Biochem. J. 43: 191-193.

24. Hanson, R. S., and D. P. Cox. 1967. Effect of different nutritional conditions on the synthesis of tricarboxylic acid cycle enzymes. J. Bacteriol. 93: 1777-1787.

25. Hanson, R. S., J. Blicharska, M. Arnaud, and J. Szulmajster. 1964. Observations on the regulation of the synthesis of the tricarboxylic acid enzymes in Bacillus subtilis, Marburg: Biochem. Biophys. Res. Commun. 17: 690-695.

26. Ito, T. 1954. Doctoral dissertation. University of California, Berkeley, Cal.

27. Ito, T., and J. B. Neilands. 1958. Products of "low-iron fermentation" with Bacillus subtilis: isolation, characterization and synthesis of 2,3-dihydroxybenzoylglycine. J. Am. Chem. Soc. 80: 4645-4647.

28. Jensen, R. A., and E. W. Nester. 1965. The regulatory significance of intermediary metabolites: control of aromatic biosynthesis by feedback inhibition in Bacillus subtilis. J. Mol. Biol. 12: 468-481.

29. Lascelles, J. 1956. An assay of iron protoporphyrin based on the reduction of nitrate by a variant strain of Staphylococcus aureus: synthesis of iron protoporphyrin by suspensions of Rhodopseudomonas spheroides. J. Gen. Microbiol. 15: 404-416.

30. Lascelles, J. 1957. Synthesis of porphyrins by cell suspensions of Tetrahymena vorax: effect of members of the vitamin B group. Biochem. J. 66: 65-72.

31. Lascelles, J. 1961. Synthesis of tetrapyrroles by microorganisms. Physiol. Rev. 41: 417-441.

32. Lascelles, J. 1965. The synthesis of porphyrins and bacteriochlorophyll by cell suspensions of Rhodopseudomonas spheroides. Biochem. J. 62: 78-93.

33. Lorence, J. H., and E. W. Nester. 1967. Multiple molecular forms of chorismate mutase in Bacillus subtilis. Biochemistry 6: 1541-1552.

34. Lowenburg, W., B. B. Buchanan, and J. C. Rabinowitz. 1963. Studies on the chemical nature of Clostridial ferredoxin. J. Biol. Chem. 238: 3899-3913.

35. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the phenol reagent. J. Biol. Chem. 193: 265-275.

36. McCarthy, C., and E. W. Nester. 1967. Synthesis in newly transformed cells of Bacillus subtilis. J. Bacteriol. 94: 131-140.

37. Moyed, H. S. 1960. False feedback inhibition: inhibition of tryptophan biosynthesis by 5-methyltryptophan. J. Biol. Chem. 235: 1098-1102.

38. Neilands, J. B. 1952. A crystalline organo iron compound from the fungus Ustilago sphaerogena. J. Am. Chem. Soc. 74: 4846-4847.

39. Neilands, J. B. 1957. Some aspects of microbial iron metabolism. Bacteriol. Rev. 21: 101-111.

40. Neilands, J. B. 1961. Early stages in the metabolism of iron, p. 194-206. In J. E. Falk, R. Lemberg, and R. K. Morton (ed.)., Haematin Enzymes. Pergamon Press, Inc., New York, N.Y.

41. Neilands, J. B. 1967. Hydroxamic acids in nature. Science 156: 1443-1447.

42. Neilands, J. B., and J. A. Garibaldi. 1960. Coproporphyrin III tetramethyl ester. Biochem. Prepn. 7: 36-38.

43. Nester, E. W. Personal communication.

44. Nester, E. W., and R. A. Jensen. 1966. Control of aromatic biosynthesis in Bacillus subtilis: sequential feedback inhibition. J. Bacteriol. 91: 1594-1598.

45. Nester, E. W., J. H. Lorence, and D. S. Nasser. 1967. An enzyme aggregate involved in the biosynthesis of aromatic amino acids in Bacillus subtilis. Its possible function in feedback regulation. Biochemistry 6: 1553-1562.

46. Nester, E. W., M. Schafer, and J. Lederburg. 1963. Genetic linkage in DNA transfer: a cluster of genes concerned with aromatic biosynthesis in Bacillus subtilis. Genetics 48: 529-551.

47. Pittard, A. J., F. Gibson, and C. H. Doy. 1961. Phenolic compounds accumulated by washed cell suspensions of a tryptophan auxotroph of Aerobacter aerogenes. Biochim. Biophys. Acta 49: 485-494.

48. Pittard, A. J., F. Gibson, and C. H. Doy. 1962. A possible relationship between the formation of o-dihydric phenols and tryptophan biosynthesis by Aerobacter aerogenes. Biochim. Biophys. Acta 57: 290-298.

49. Prelog, V. 1963. Iron-containing antibiotics and microbial growth factors. Pure and Appl. Chem. 6: 327-338.

50. Ratledge, C. 1964. Relationship between the products of aromatic biosynthesis in Mycobacterium smegmatis and Aerobacter aerogenes. Nature 203: 428-429.

51. Ratledge, C. 1967. The production of N-acylanthranilic acid from shikimic acid and the effect of iron-deficiency on the biosynthesis of other aromatic compounds by Aerobacter aerogenes. Biochim. Biophys. Acta 141: 55-63.

52. Ratledge, C., and F. G. Winder. 1962. The accumulation of salicylic acid by Mycobacteria during growth in an iron-deficient medium. Biochem. J. 84: 501-506.

53. Ratledge, C., and F. G. Winder. 1966. Biosynthesis and utilization of aromatic compounds by Micobacterium smegmatis with particular reference to the origin of salicylic acid. Biochem. J. 101: 274-283.

54. Roberts, R. B., D. B. Cowie, P. H. Abelson, E. T. Bolton, and R. Y. Britten. 1957. Studies of biosynthesis in Escherichia coli. Carnegie Inst. Wash. Publ. 607: 13-14.

55. Ronald, W. P. Personal communication.

56. Salton, M. R. J. 1964. Requirement of dihydroxyphenols for the growth of Micrococcus lysodeikticus in synthetic medium. Biochim. Biophys. Acta 86: 421-422.

57. Schaeffer, P., J. Millet, and J. P. Aubert. 1965. Catabolite repression of bacterial sporulation. Proc. N.A.S. 53: 704-711.

58. Somerville, R. L., and R. Elford. 1967. Hydroxamate formation by anthranilate synthetase of Escherichia coli. Biochem. Biophys. Res. Commun. 28: 437-444.

59. Srinivasan, P. R., and A. Riviera Jr. 1963. The enzymatic synthesis of anthranilate from shikimate-5-phosphate and l-glutamine. Biochemistry 2: 1059-1062.

60. Srinivasan, P. R., and B. Weiss. 1961. The biosynthesis of p-aminobenzoic acid: studies on the origin of the amino group. Biochim. Biophys. Acta 51: 597-599.

61. Subba Rao, P. V., K. Moore, and G. H. N. Towers. 1967. O-pyrocatechuic acid carboxylase from Aspergillus niger. Arch. Biochem. Biophys. 122: 466-473.

62. Szulmajster, J., and R. S. Hanson. 1965. Physiological control of sporulation in Bacillus subtilis, p. 162-173. In W. W. Campbell and H. O. Halvorson (ed.)., Spores III. American Society for Microbiology.

63. Townsley, P. M., and J. B. Neilands. 1957. The iron and porphyrin metabolism of Micrococcus lysodeikticus. J. Biol. Chem. 224: 695-705.

64. Umbarger, H. E., M. A. Umbarger, and P. M. L. Siu. 1963. Biosynthesis of serine in Escherichia coli and Salmonella typhimurium. J. Bacteriol. 85: 1431-1439.

65. Vogel, H. J. 1956. A convenient growth medium for Neurospora (medium N). Microbiol. Gen. Bull. 13: 42-43.

66. Waring, W. S., and C. H. Werkman. 1942. Growth of bacteria in an iron-free medium. Arch. Biochem. 1: 303-310.
Also, Martell, A. E. 1964. Stability constants of metal ion complexes - Section II. Organic Ligands Chemical Society, London.

67. Young, I. G., G. B. Cox, and F. Gibson. 1967. 2,3-Dihydroxybenzoate as a bacterial growth factor and its route of biosynthesis. Biochim. Biophys. Acta 141: 319-331.

68. Young, I. G., L. M. Jackman, and F. Gibson. 1968. 2,3-Dihydro-2,3-dihydroxybenzoic acid as an intermediate in the biosynthesis of 2,3-dihydroxybenzoic acid. Biochim. Biophys. Acta 148: 313-315.