PIGMENT FORMATION BY BACTERIUM SALMONICIDA

PHILIP J. GRIFFIN, S. F. SNIESZKO, AND S. B. FRIDDLE

Microbiological Laboratory, U. S. Fish and Wildlife Service, Kearneysville, West Virginia

Received for publication November 28, 1952

An extensive literature exists concerning the role of tyrosine and tyrosinase in the formation of melano-pigments. This interest reflects the widespread distribution of the enzyme and the conspicuousness of such pigments in man, animals, and plants. Several comprehensive reviews exist on these pigments and the various factors which influence their formation (Raper, 1928; Lerner and Fitzpatrick, 1950; Dawson and Tarpley, 1951). Much of the emphasis has been directed to the metabolism of tyrosine, an amino acid which plays an important role in melano-pigmentation. The formation of these darkly colored products from their precursors is a complex phenomenon, and detailed information concerning their identity and the sequence of chemical reactions which transform tyrosine to pigmented products is also incomplete.

Bacterium salmonicida, a pathogen of salmonoid fishes, produces a dark amber to brown-black pigment especially when grown on a tryptic digest of casein-yeast extract medium (Gutsell and Snieszko, 1949). Griffin (1952) reported that only a light brown color was produced in nutrient agar and that the addition of L-phenylalanine or L-tyrosine resulted in the formation of a deep amber pigment. The present report deals largely with the effect of compounds structurally related to tyrosine and phenylalanine on pigment formation. These studies suggest that *B. salmonicida* may be a convenient tool for investigating the nature and formation of pigments derived from phenylalanine and tyrosine.

METHODS

Culture and inoculum. Four strains of *B. salmonicida* were maintained at 6 C on a stock medium of the following percentage composition: trypticase 1.0, yeast extract (Difco) 0.5, NaCl 0.25, agar 1.5. All inoculations for the varied media employed in this study were made by utilizing the cells obtained from a second 24 hour subculture of the microorganism grown on the stock medium or basal medium (*vide infra*). The incubation temperature was 20 to 22 C.

Basal medium. The composition of the medium used for the investigations of substances structurally related to tyrosine or phenylalanine is presented in table 1. Slightly better growth occurred after the addition of 0.1 ml of a 10 per cent aqueous solution of autoclaved yeast extract to 10 ml of this medium. In experiments on compounds related to L-tyrosine or L-phenylalanine the basal medium as defined in table 1, with and without the yeast extract supplement, was used.

The effect of substituting compounds similar in structure to tyrosine or phenylalanine was noted by the presence or absence of a brown or amber-black pigment in the agar medium. The various additions were dissolved in distilled water, whenever possible the pH was adjusted from 6.5 to 6.8, and sterilization was accomplished by passage through Coors porcelain filters.

RESULTS

Effect of oxygen on pigment. Under aerobic conditions on stock medium slants, pigmentation usually appears after 28 to 30 hours' incubation at 22 C. At this time a very faint pinkish coloration can be detected in the medium at the extreme tip of the slant. A corresponding increase in the extent and intensity of coloration in the medium follows. Within 48 hours, the color changes from pink to red-amber and extends downward in the agar to approximately one-half of the slanted portion of the medium. At 72 hours, three-quarters of the tube contents is brown-amber in color, and at 96 hours or later substantially the color of the entire slant is deep coffee brown to black. Early in the pigmentation process, the colonies on the surface of the slant remain nearly colorless; after continued incubation the surface growth also becomes light brown.

Williamson (1928) reported that *B. salmonicida* does not produce pigment under anaerobic conditions. This was confirmed in our experiments in which oxygen was removed by using *Serratia marcescens* (Snieszko, 1930) or by utilizing a modification of the alkaline pyrogallol system of

Spray (1930). Growth under approximately 10 per cent CO_2 (candle-jar method) curtails pigment formation, and practically complete inhibition of pigmentation was obtained merely by tightening the tops of the screw-capped tubes in which the cultures were grown.

Time and temperature. Excellent growth occurred in 18 hours on stock medium slants; however, the formation of a visible pigment in the medium usually required an additional 10 to 12 hour incubation period. This lag in the appearance of pigment may be analogous to the "induction period" described by other investigators for tyrosine-tyrosinase reactions in the presence of oxygen. Under the latter circumstances, there frequently is a lag period before the oxidation begins, as followed by O_2 uptake measurements. Experiments were initiated to determine whether the addition of 3,4 dihydroxyphenylalanine (dopa) or catechol accelerated pigment production by *B. salmonicida.* In these tests, 1.0, 0.1, 0.01, and 0.001 mg of dopa and catechol were added to tubes containing 10 ml of stock agar. At the end of 24 hours of incubation, growth was absent in those tubes containing 1.0 mg of catechol and growth was poor in the 1.0 mg dopa tubes. Growth occurred at all concentrations after prolonged incubation; however, the pigment was not formed more rapidly in any of the tubes containing dopa or catechol when compared with stock agar controls.

When 1 per cent "tween 40" was added to stock agar, pigmentation was delayed by as much as 4 to 6 days. A concentration of 0.1 per cent "tween 40" reduced the amount normally formed during the early stages of the pigment process, but lower levels were without effect. Lerner and Fitzpatrick (1950) previously showed that "tween 20" prolongs the induction period of the tyrosine-tyrosinase reaction.

Incubation at temperatures above 22 C reduced the intensity of pigmentation. At 32 C excellent growth occurred but a pigment did not appear. Pigment was not produced even after prolonged incubation when the microorganism was grown on stock agar slants at 22 C for 18 hours (approximately 10 to 12 hours before the usual pigmentation time) and then heated to 32 C in a water bath for 5 minutes and incubated at this elevated temperature. However, if the

TABLE 1

Basal medium for cultivation of Bacterium salmonicida

$MgSO_4$	200	mg	Adenine	10	mg
K_2HPO_4	25	mg	Guanine	10	mg
KH_2PO_4	25	mg	Uracil	10	mg
NaCl	5.0	g	DL-Alanine	500	mg
$CaCl_2$ (anhydrous)	50	mg	L-Arginine·HCl	500	mg
Salts solution*	1.0	ml	L-Asparagine	500	mg
Thiamin·HCl	0.1	mg	L-Glutamic acid·HCl	1.0	g
Nicotinic acid	1.0	mg	Glycine	500	mg
Nicotinamide	0.05	mg	DL-Histidine·HCl	200	mg
Calcium pantothenate	1.0	mg	L-Hydroxyproline	10	mg
Riboflavin	2.0	mg	DL-Leucine	500	mg
Pyridoxine·HCl	2.0	mg	DL-Lysine·HCl	60	mg
Pyridoxamine·2HCl	2.0	mg	DL-Methionine	40	mg
p-Aminobenzoic acid	1.0	μg	DL-Proline	100	mg
Biotin	2.0	μg	DL-Serine	100	mg
Choline·HCl	1.0	mg	DL-Threonine	50	mg
Inositol	1.0	mg	DL-Tryptophan	50	mg
Folic acid	2.0	mg	DL-Valine	50	mg
Putrescine	0.05	mg	DL-Isoleucine	30	mg
Vitamin B_{12}	15	μg	L-Cystine	3.0	mg
Noble agar (Difco)	15	g			

pH adjusted to 6.8; distilled water to 1,000 ml. Sterilized at 116 C for 10 min.

* Salts solution: $FeSO_4 \cdot 7H_2O$, 5 g; $ZnSO_4$, 0.1 g; $CoCl_2$, 0.1 g; $MnSO_4 \cdot H_2O$, 0.2 g; $CuSO_4$, 0.1 g; water to 250 ml.

cultures were placed at 32 C approximately 2 hours prior to the time at which pigmentation begins, a light brown coloration formed at the end of 96 hours. When the microorganism was incubated at 22 C until a very slight amount of pink coloration appeared at the tip of the slant and the cultures then heated and transferred to the higher temperature, pigmentation continued and at the end of 96 hours, over three-quarters of the slanted portion of the agar became dark brown. In general, after the initial formation of a pigment, the longer the incubation period at the lower temperature the more pronounced the pigmentation process was when the tubes were transferred to the higher temperature for further incubation. If the incubation was continued at 22 C until approximately the top 2.5 cm of the slant contained a red pigment, then pigment formation at 32 C was comparable to that which was produced at the lower temperature. The same process occurred at 37 C even though *B. salmonicida* did not grow at this temperature.

Pigmentation progressed in essentially the same manner as described above at all temperatures when chloroform was added to the surface of those slants in which pigmentation had begun. Loopfuls of cells from these cultures were transferred to fresh broth, but growth did not occur. Cultures which had been grown at 22 C until a pigment appeared were heated in a water bath at 70 C for 15 minutes, quickly cooled to and incubated at temperatures of 22, 32, and 37 C. Within 12 hours a red-amber color developed which was more intense than that present in unheated controls maintained at 22 C. The initial pink or light red color also had changed to a purple-brown immediately after heating.

Lerner and Fitzpatrick (1950) indicated that the rate of enzymatic oxidation of tyrosine to melanin is more rapid above 30 C. In contrast, actively growing cells of *B. salmonicida* can initiate pigmentation at 22 C but not at 32 C. Recently Horowitz and Shen (1952) reported that tyrosinase production in *Neurospora* also is dependent on the temperature of cultivation and that at 35 C the cultures are practically devoid of enzymatic activity.

Cultures of *B. salmonicida* were incubated at 22 and at 32 C until a pigment formed in the upper half of the slant in those cultures held at 22 C. Some of the cultures grown at the higher temperature then were transferred to the lower temperature and incubated for a suitable period. A pigment eventually was formed in the lower half of both sets of cultures grown at 22 C but was absent in the upper half of those slants originally incubated at 32 C. Transfers from the higher to the lower temperature at various increased time intervals resulted in a corresponding decrease of pigment in the upper portions of the slanted surface. When maintained at 32 C until complete pigmentation occurred in the control tubes at the lower temperature, a pigment did not develop when the cultures were transferred from 32 to 22 C. One possible explanation of these observations is that the enzyme or enzyme system is formed at the higher temperature, but formation of the initial red pigment is either blocked or, alternatively, another path is in operation which leads to colorless products.

The increase in pigment at elevated temperatures is not a result of simple diffusion of the small amount of colored products originally formed at 22 C since the intensity and extent of pigment are so much greater at higher temperatures. This view accords with the fact that when cultures which contain the initial pigment are treated with formaldehyde, the pigmentation process stops and the original color present at the time of the addition of formaldehyde remains unaltered in intensity and extent at any temperature. One also could interpret the data to mean that the elevated temperatures accelerate an autocatalytic oxidation of precursor compounds previously formed only at the lower temperature. Once formed, no pigment forming enzyme need be present at this stage as the $CHCl_3$ suggests. Formaldehyde may inhibit this reaction by binding and preventing the autoxidation reaction. The formaldehyde treated cultures were found to be convenient controls and were used to compare the changes in pigmentation with the color of the originals.

Woiwod and Linggood (1949) reported the possible combination of tyrosine with formaldehyde as indicated from qualitative differences between chromatograms of acid hydrolyzate of highly purified diphtheria toxin and toxoid. Tyrosine was present in the chromatogram of toxin but not in toxoid. Fraenkel-Conrat *et al.* (1947) noted that the indole groups of gramicidin react rapidly with formaldehyde in alkaline solution, the formaldehyde bound being equivalent to the tryptophan content. Should the red product

initially formed by *B. salmonicida* prove to be an indole derivative (like dopachrome)[1] then a similar explanation might hold for the formaldehyde inhibition of pigment production by *B. salmonicida*.

Effect of pH. Although *B. salmonicida* produces pigment in stock agar at any pH allowing growth (approximately pH 5.5 to 8.5), pigment formed much sooner when the organism was grown in a medium adjusted initially at pH 6.5 to 6.8.

In broth cultures, a pigment develops at the surface only after approximately 10 to 12 days of incubation. An important factor contributing to the much slower pigment formation in broth as compared to agar is that in agar the pH rapidly changes within 24 hours from pH 6.7 to about 7.4, whereas in broth the pH change is from 6.7 to 6.3. At the end of 7 to 8 days of incubation the pH in broth usually rises to about 6.8, and at the time of pigmentation the reaction is alkaline.

In stock agar with 0.5 per cent maltose added, the onset of pigmentation is delayed by an additional 24 to 48 hours. This observation also points to the sensitivity to pH of the medium of pigment formation by *B. salmonicida*. At an initial pH of 7.0 with maltose added, the pH becomes acid. Without maltose, at 22 or 32 C, the pH quickly becomes alkaline, and at the time of incipient pigment production the first 2.5 cm of the slant containing the pigment is invariably pH 7.6 to 7.7. The alkalinity induced by growing cells of *B. salmonicida* appears to depend, at least in part, on NH_3 formation.

A micro-diffusion method was used to determine ammonia-N quantitatively. Several stock agar slants with and without 0.5 per cent maltose were inoculated and incubated in the manner described. At 8 hour intervals, one gram samples were cut away from the top portions of the agar slants, triturated, and diluted to 50 ml with distilled water. Two ml aliquots of the suspensions were transferred to the outer compartment of Conway units, and the procedure described by Fister (1950) then was followed. Toluene treated samples, prepared at the time of inoculation, served as the reagent blank and pH control. Readings were made in a Beckman Model B spectrophotometer at 625 mμ on samples contained in 10 mm matched cuvettes. Only data for one experiment with strain S-14 are presented in figure 1 as similar results were obtained with another strain and in repeat tests. Although at up to 40 hours' incubation there is some suppression of ammonia formation in maltose media, the amount of ammonia-N formed parallels that formed in the carbohydrate-free medium. This

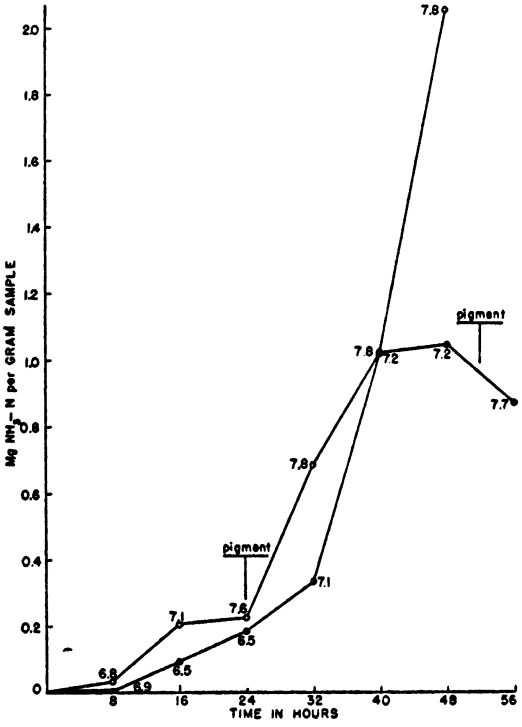

Figure 1. Relation of NH_3-N, pH, and time of pigment formation by *Bacterium salmonicida*, strain S-14. Open circles represent data obtained from media without maltose; filled circles, with maltose. Figures on the curves represent pH values of samples.

was unexpected on the basis of earlier observations from qualitative tests for NH_3 (Nessler's reagent). The lower pH values obtained in the presence of this carbohydrate may be explained, in part, by the formation of acids. It has been noted by Gale (1951) that in general, in the presence of fermentable carbohydrate, the liberation of ammonia usually is suppressed and the formation of deaminase is diminished markedly. In cultures of *B. salmonicida*, pigmentation correlated with pH. Other than the influence of NH_3 production on pH values, it appears likely that the pigmented products are not necessarily a

[1] Term suggested by Dr. A. B. Lerner. *Private communication.*

consequence of the deamination of amino acids such as tyrosine or phenylalanine since the intensified alkaline reaction also occurs in basal media devoid of either amino acid and subsequent sections of this paper present data indicating that the corresponding keto acids are not pigment formers.

Effect of compounds structurally related to tyrosine and phenylalanine. Since *B. salmonicida* acting on either L-phenylalanine or L-tyrosine gave rise to a blackened pigment, various substances which contained groups similar to those in tyrosine and phenylalanine were substituted in the basal medium to give a final concentration of at least 40 mg per cent.[2] Control tubes which contained similar concentrations of either L-phenylalanine or L-tyrosine were included as pigment controls. As noted earlier, either enantiomorph of phenylalanine is active in the pigment process; however, the natural isomer yields a darker pigment. L-Phenylalanine in peptide linkage with glycine (glycyl-L-phenylalanine) yielded a pigment which was even darker than that formed with L-phenylalanine itself. The color formed by substituting the analog β-2-thienylalanine was of particular interest. This compound alteration has the ring carbon atoms 2 and 3 of phenylalanine replaced by a sulfur atom. The color, a very bright salmon pink, formed throughout the medium and was reminiscent of the initial stages of the pigmentation process in stock agar in that the first visible color formed at the very tip of the slant is pink or salmon. Cyclohexyl-DL-alanine, phenylpyruvic acid, N-acetyl-L-phenylalanine, DL-β-phenyllactic acid, β-phenylserine and DL-mandelic acid were inactive. That the alanine side chain may be of prime importance in the pigmentation process is suggested by the absence of pigment "activity" by those compounds in which the amino group is missing or which contain a substituent for one of the hydrogen atoms of the α amino group or of the β carbon atom.

Curiously, when a pigment was formed in the basal medium which contained tyrosine or tyrosine ethyl ester, the intensity of the color was never as pronounced as that which formed in stock agar. Increasing the concentration of tyrosine ethyl ester from 10 to 75 mg per cent resulted in a corresponding increase in the depth of the color formed in the medium. Concentrations above 75 mg per cent were without apparent effect.

When glycyl-L-tyrosine was added to the basal medium, an intense brown-black color developed which resembled the pigment which was formed in stock agar. Additional tests, which included glycine alone and in combination with L-tyrosine, indicated that the addition of glycine did not increase the intensity of the pigment over that observed in the basal medium with the addition of only L-tyrosine. Paredrine, tyramine, N-formyl-L-tyrosine, N-acetyl-L-tyrosine, N-(*p*-hydroxyphenyl) glycine, *p*-hydroxyphenylacetic acid, *p*-hydroxyphenylpropionic acid, *p*-hydroxyphenylpyruvic acid, 3-amino-L-tyrosine, 3-nitro-L-tyrosine, 3-fluoro-L-tyrosine, diiodotyrosine, and 3,4-dichloro-β-phenyllactic acid were inactive as substituents for tyrosine. Again, compounds in which a hydrogen atom of the amino group was substituted were inactive in the pigmentation process; this suggests that the activity of the dipeptide may be a result of cleavage of the peptide bond. The results of these tests also indicate that removal of the carboxyl group (tyramine), replacement of this group with a methyl group (paredrine), or the presence of certain substituents in the 3 or 3,5 position in the ring results in "inactivity". Tryptophan, histidine, alanine, glycine, proline, hydroxyproline, and indole-3-acetic acid also were tested and proved inactive. A light brown color developed in the medium containing *p*-hydroxyphenylpyruvic acid after several days; however, uninoculated control tubes made alkaline with NaOH also developed essentially the same color. 3,4-Dihydroxyphenylalanine also was tested but its autoxidation led to equivocal results.

The literature indicates that the enzyme tyrosinase, obtained particularly from various plants and lower animals, does not show a high degree of substrate specificity. A number of monohydric and polyhydric phenols (e.g., phenol and catechol) are oxidized by such enzymes. The following substances, at levels of 10, 20, and 40 mg per cent, were tested for their "pigment activity" when incorporated in basal media upon which *B. salmonicida* was grown: phenol, *p*-cresol, *m*-cresol, *o*-cresol, and catechol. The higher concentrations retarded growth to some

[2] Cyclohexyl-DL-alanine was used at a concentration of only 20 mg per cent because of limited supply of substrate.

extent, and under no circumstance did the characteristic amber-black pigment form. Some darkening of the medium occurred in tubes containing catechol; however, the same condition developed in control tubes which had been adjusted to pH 7.6.

DISCUSSION

Tyrosinase is distributed widely in various animals and plants, including some microorganisms (Clark and Smith, 1939; Klykov, 1945). The conditions surrounding the formation of the darkly colored products noted in the present report are remarkably similar to those essential for pigment production in *Azotobacter chroococcum* as described by Ungerer (1934). The action on tyrosine by intact cells of *B. salmonicida* seems of particular interest in that the sequence of visible color change from pink or red through brown to amber-black parallels the color changes described for melanin formation in mammals.

Raper and Wormall (1923) and Raper and Speakman (1926), using tyrosinase preparations obtained from potato juice and mealworms, demonstrated that when tyrosine is oxidized by tyrosinase the first visible change is the formation of a red pigment (dopachrome). This compound undergoes a decarboxylative rearrangement, and apparently the reaction is base catalyzed (Mason and Wright, 1949).

The formation of pigment in *B. salmonicida* always is accompanied by a shift in the reaction of the medium to the alkaline side. Once a small amount of pigment is formed in the medium by *B. salmonicida* when grown at 22 C, pigment production continues at 32, 37, or even after heating to 70 C for 15 min. It appears that once a sufficient quantity of precursor compounds is formed at 22 C, the remaining steps of the pigmentation process are a consequence of autocatalytic oxidation reactions.

A deamination of phenylalanine or tyrosine may not be requisite to the formation by *B. salmonicida* of pigmented products. Melano-pigments usually contain nitrogen, and the deamination of either or both of these amino acids obviously would lead to a nonmelanin product unless the product formed is like homogentisic acid. Additional suggestive evidence is that the corresponding keto acids are not active in pigmentation. N-substitution likewise prevented pigment formation, perhaps preventing oxidation to dopa analogues or ring closure.

The conversion of phenylalanine to tyrosine has been demonstrated repeatedly (Moss and Schoenheimer, 1940; Bernheim and Bernheim, 1944; Lien and Greenberg, 1952; Udenfriend and Cooper, 1952). The present data indicate that the addition of either L-tyrosine or L-phenylalanine to the basal medium results in pigment production; phenylalanine cultures show delayed pigmentation, and the color produced is not as intense as with tyrosine. Since either amino acid is active in pigmentation, it seems reasonable to suggest that phenylalanine is first converted to tyrosine, and delay in this conversion might account for the delayed pigmentation noted in phenylalanine cultures. But until such a conversion actually is demonstrated, the possibility of alternate pathways to pigmented end products must be considered: in *Escherichia coli* (Simmonds *et al.*, 1947; Davis, 1950) and in yeast (Gilvarg and Bloch, 1951) independent routes of phenylalanine and tyrosine synthesis occur and phenylalanine apparently is not converted to tyrosine.

The tyrosinases of plants and lower animals appear less specific towards substrate than is mammalian tyrosinase. The results with *B. salmonicida* were obtained with intact cells and may well have been influenced by permeability factors. However, the limited substrate specificity observed in these studies does parallel in many respects the data with mammalian enzyme systems (Lerner *et al.*, 1951).

The utilization of *B. salmonicida* offers promise for further studies of pigment formation since one of the chief difficulties hindering research in this field has been the absence of a convenient experimental object. Melano-pigments from animal sources are difficult to isolate and to purify because of limited solubility and close binding with tissue protein; *B. salmonicida*, grown on a synthetic medium, seems of value for such studies. *B. salmonicida* also may be a useful source of the enzymes involved in converting phenylalanine and tyrosine to pigmented end products.

ACKNOWLEDGMENTS

The authors wish to thank Dr. B. N. LaDu, Jr. and Dr. S. H. Hutner for suggestions concerning the manuscript, and Dr. A. B. Lerner and Dr. S. Udenfriend for their suggestions and

generosity in supplying some of the derivatives of amino acids used in the present investigation.

SUMMARY

Some factors determining the production of a melano-pigment by *Bacterium salmonicida* were investigated. Oxygen, hydrogen ion concentration, temperature, and amount of substrate were found to be important in pigment formation. In the absence of tyrosine and phenylalanine, pigmentation of the medium did not occur. The addition of either glycyl-L-phenylalanine, L-phenylalanine, or D-phenylalanine resulted in the production of a brown or amber-to-black pigment. A bright salmon pink color developed when β-2-thienylalanine was tested.

Compounds which contained an acetyl or chloracetyl substituent in place of a hydrogen atom of the α amino group, or a hydroxyl group in place of a hydrogen atom of the β carbon atom in the alanine side chain of phenylalanine were inactive. Saturation of the benzene ring also produced an inactive substance. Glycyl-L-tyrosine, L-tyrosine, and L-tyrosine ethyl ester were chromogenic. Substitution of the hydrogen atom of the α amino or the carboxyl group of the alanine side chain of tyrosine and substituents in the 3 position on the benzene ring yielded inactive compounds. *B. salmonicida* is proposed as a useful tool in studies of pigmented products from phenylalanine and tyrosine.

REFERENCES

BERNHEIM, M. L. C., AND BERNHEIM, F. 1944 The production of a hydroxyphenyl compound from L-phenylalanine incubated with liver slices. J. Biol. Chem., **152**, 481.

CLARK, F. E., AND SMITH, N. R. 1939. Cultural requirements for the production of black pigments by bacilli. J. Bact., **37**, 277–284.

DAVIS, B. D. 1950 Studies on nutritionally deficient bacterial mutants isolated by means of penicillin. Experientia, **6**, 41–50.

DAWSON, C. R., AND TARPLEY, W. B. 1951 Copper oxidases. In *The enzymes*, edited by J. B. Sumner and K. Myrbäck. Vol. 2, Part 1, 454–498. Academic Press, N. Y.

FISTER, H. J. 1950 *Manual of standardized procedures for spectrophotometric chemistry*. Standard Scientific Supply Corporation, New York.

FRAENKEL-CONRAT, H., BRANDON, B. A., AND OLCOTT, H. S. 1947 The reaction of formaldehyde with proteins. IV. Participation of indole groups. Gramicidin. J. Biol. Chem., **168**, 99–118.

GALE, E. F. 1951 *The chemical activities of bacteria*, 3rd ed. Academic Press, Inc., New York.

GILVARG, C., AND BLOCH, K. 1951 Synthesis of phenylalanine and tyrosine in yeast. Federation Proc., **10**, 189–190.

GRIFFIN, P. J. 1952 Some factors influencing pigment production in *Bacterium salmonicida*. Bact. Proc., **1952**, 53.

GUTSELL, J. S., AND SNIESZKO, S. F. 1949. Dosage of sulfamerazine in the treatment of furunculosis in brook trout, *Salvelinus fontinalis*. Trans. Am. Fisheries Soc., 1946, **76**, 82–96.

HOROWITZ, N. H., AND SHEN, SAN-CHIUN 1952 *Neurospora* tyrosinase. J. Biol. Chem., **197**, 513–520.

KLYKOV, A. P. 1945 Tyrosinase enzyme in the causative agent of the black bacteriosis of cereals. Compt. rend. acad. sci. U.R.S.S., **47**, 597.

LERNER, A. B., AND FITZPATRICK, T. B. 1950 Biochemistry of melanin formation. Physiol. Revs., **30**, 91–126.

LERNER, A. B., FITZPATRICK, T. B., CALKINS, E., AND SUMMERSON, W. H. 1951 Mammalian tyrosinase. Action on substances structurally related to tyrosine. J. Biol. Chem., **191**, 799–806.

LIEN, O. G., JR., AND GREENBERG, D. M. 1952 Chromatographic studies on the interconversion of amino acids. J. Biol. Chem., **195**, 637–644.

MASON, H. S., AND WRIGHT, C. I. 1949 The chemistry of melanin. V. Oxidation of dihydroxyphenylalanine by tyrosinase. J. Biol. Chem., **180**, 235–247.

MOSS, A. R., AND SCHOENHEIMER, R. 1940 The conversion of phenylalanine to tyrosine in normal rats. J. Biol. Chem., **135**, 415–429.

RAPER, H. S. 1928 The aerobic oxidases. Physiol. Revs., **8**, 245–282.

RAPER, H. S., AND SPEAKMAN, H. B. 1926 The tyrosinase-tyrosine reaction. IV. Note on the identity of tyrosinase from different sources. Biochem. J., **20**, 69–72.

RAPER, H. S., AND WORMALL, A. 1923 The tyrosinase-tyrosine reaction. Biochem. J., **17**, 454–469.

SIMMONDS, S., TATUM, E. L., AND FRUTON, J. S. 1947 The utilization of phenylalanine and tyrosine derivatives by mutant strains of *Escherichia coli*. J. Biol. Chem., **169**, 91–101.

SNIESZKO, S. F. 1930 The growth of anaerobic bacteria in petri dish cultures. Zentr. Bakt. Parasitenk., II Abt., **82**, 110–111.

SPRAY, R. S. 1930 An improved anaerobic culture dish. J. Lab. Clin. Med., **16**, 203–206.

UDENFRIEND, S., AND COOPER, J. R. 1952 The enzymatic conversion of phenylalanine to tyrosine. J. Biol. Chem., **194**, 503–511.

UNGERER, E. 1934 Über die Entstehung des Pigmentes von *Azotobacter chroococcum*. Z. Pflanzenernähr. Düng. u. Bodenk., A., **36**, 287–290.

WILLIAMSON, I. J. F. 1928 Furunculosis of the *Salmonidae*. Fishery board for Scotland. Salmon fisheries, **5**, 1–17.

WOIWOD, A. J., AND LINGGOOD, F. V. 1949 Amino acid constitution of diphtheria toxin and toxoid. Nature, **163**, 218–219.