The Journal of Biochemistry, Vol. 49, No. 6, 1961 Dedicated to K. Kodama on His 70th Birthday.

The Reciprocal Relationship between Melanization and Tyrosinase Activity in Melanosomes (Melanin Granules)

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(Received for publication, March 18, 1961)

In mammals, melanin pigment is synthesized in a speciffc cell, the melanocyte, by the action of a copper-containing oxidase, tyrosinase (*1*~*3*). This enzyme catalyzes the aerobic oxidation of tyrosine, the precursor of melanin, to a monomer, 5,6-dihydroxyindole; this monomer, in turn, is transformed into a large polymer which is probably attached through its quinone linkages to the amino or sulfhydryl groups of the protein matrix of the pigment granule. It has been shown that mammalian tyrosinase is attached to a specific cytoplasmic particle (*4*, *5*). The name "melanosome" has been proposed for these distinctive, enzymatically active particles which are the site of melanin formation and are found only within the cytoplasm of the melanocyte (*6*). Melanosomes have been shown by biochemical and electronmicroscopic studies to be different from mitochondia (*6*). Electronmicroscopy has revealed the morphology of melanosomes and melanin granules (*7*~*10*): Birbeck and Barnicot (*11*) and Seiji, Fitzpatrick and Birbeck (*6*) have reported that a series of steps can be seen in the formation of melanin granules within the melanocyte.

Recently we have become aware that there appears to be an inverse relationship between amount of tyrosinase activity and degree of melanization within melanosomes. The experiments described in this paper were performed in an effort to clarify this relationship. Melanosomes were obtained from two types of mammalian melanoma and from the retinal pigment-epithelium of the chick embryo. A decrease in reaction velocity was found to be associated with *in vitro* melanization of isolated melanosomes.

EXPERIMENTAL METHODS

Preparation of Large-Granule Suspension from Mouse Melanoma—B-16 and Harding-Passey mouse melanoma were serially transplanted in C-57-strain mice and Swiss-strain mice respectively. The entire, actively growing melanoma was excised when the diameter reached 1—1.5 cm. The tumor was promptly homogenized in 0.25 *M* sucrose sulotion at about 0°C. All subsequent processing took place at a temperature of about 3°C. The homogenate was centrifuged at 700 $\times g$ for 10 minutes. The resulting "low-speed" supernatant, when centrifuged at 11,000$\times g$ for 10 minutes, yielded a sediment which was resuspended in 0.25 *M* sucrose and recentrifuged at 15,000$\times g$ for 10 minutes. The sediment thus obtained was again suspended in 0.25 *M* sucrose to make the "large-granule suspension" used as starting material for density-gradient centrifugation in our experiments.

Preparation of Large-Granule Suspension from Retinal Pigment-Epithelium of Chick Embryos.—Retinal pigment epithelium, dissected from the eyes of Rhode-Island-Red chick embryo, was homogenized in 0.25 *M* sucrose solution. The homogenate was centrifuged at 300$\times$ *g* for 5 minutes and two more successive centrifugations were carried out at the same speed with the respective supernatants. The last supernatant was centrifuged again at 11,000$\times g$ for 10 minutes. The resulting sediment was then resuspended in 0.25 *M* sucrose solution and recentrifuged at 15,000$\times g$ for 10 minutes. The sediment thus obtained was suspended in 0.25 *M* sucrose to form the "large-granule suspension" used as the starting material for density-gradient centrifugation in our experiments.

Preparation of the Specific-Gravity-Gradient Tubes—Tubes of the Spinco swinging-bucket rotor (SW 39-L) were prepared by layering 0.5 ml. of eight different concentrations of sucrose solution in serial order, with

the most concentrated layer at the bottom of the tube (*12*). They were then allowed to stand overnight (about 18 hours) so that the gradient might become smooth. The sucrose concentrations used for separation of melanosomes (Fig. 1 A) were 2.6 *M*, 2.4 *M*, 2.2 *M*, 2.0 *M*, 1.8 *M*, 1.6 *M*, 1.55 *M* and 1.5 *M*. Immediately before centrifugation, 1 ml. of freshly prepared large-granule suspension from mouse melanoma or pigment-epithelium of the chick embryo was layered carefully over the top of each tube.

Isolation of the Melanosome Fraction after Centrifugation—At the end of centrifugation, the position of the strata in each tube was recorded (Fig. 1 B) and the fraction contained in the bottom of the tube (*i. e.*, between the bottom and a point 1 cm above the bottom) was isolated by means of a specially designed centrifuged-tube cutter (*13*). The fraction thus obtained constituted the "melanosome suspension."

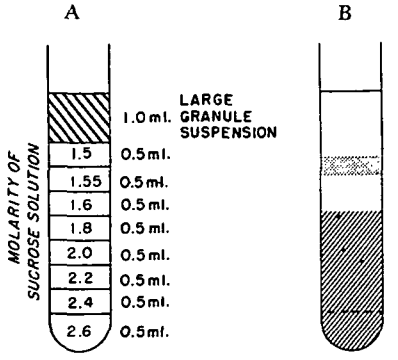


FIG. 1. A. Density-gradient tube before centrifugation, showing the layering of eight different concentrations of sucrose solution and the large-granule suspension (in 0.25 *M* sucrose). The gradient was prepared 18 hours in advance; the large-granule suspension was added immediately before centrifugation.

B. The two opaque regions appeared in the gradient tube after ultracentrifugation at 103,000 $\times g$ for 1 hour. The place where the tube was cut 1 cm. from the bottom is shown by a dotted line.

Determination of Tyrosinase Activity—Tyrosinase was determined manometrically by measurement of the oxygen consumption. L-Tyrosine, L-dopa, and a mixture of L-tyrosine and L-dopa in the 0.1 *M* phosphate buffer at pH 6.8 were used as substrates.

Protein-Nitrogen Determination—Protein was precipitated by adding 10% trichloroacetic acid to each sample. The precipitate was spun down, washed once with trichloroacetic acid and dissolved in *N* NaOH. The nitrogen-content of this alkaline solution was determined by the micro-Kjeldahl method.

Electronmicroscopy—For electronmicroscopy, suitable aliquots of each preparation were mixed at 0°C with 1% osmic acid tetraoxide buffered with acetate-veronal to pH 7.4, centrifuged at 11,000$\times g$ for 10 minutes, and kept at about 3°C for fixation. After fixation for 2 hours, the sediment was dehydrate in a graded series of ethyl alcohols and allowed to polymerize with Araldite resin. Sections cut with a modified Cambridge rocker-microtome were studied by means of a Siemens Electronmicroskop 1.

Estimation of the Optical Density of the Melanosome Suspension—The optical density of the melanosome suspension was measured by means of a Beckman spectrophotometer, Model DU, in the range between 400 mμ and 700 mμ.

RESULTS

Comparison of the Color Intensity of Melanosomes Isolated from Mouse Melanoma and from the Retinal Pigment-Epithelium of Chick Embryos—Fig. 2 shows that melanosome suspensions prepared from B-16 mouse melanoma are significantly darker than those prepared from Harding-Passey tumors. At 550 mμ, the optic-

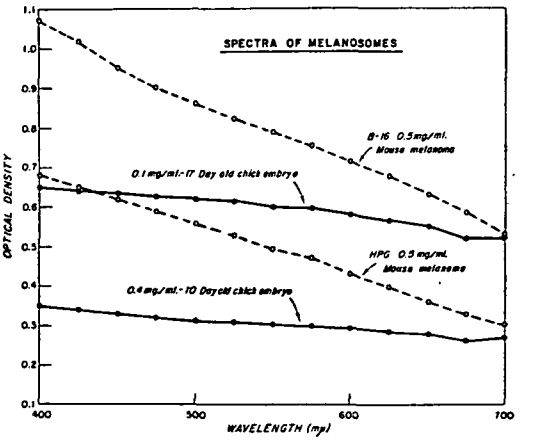


FIG. 2. Absorption spectra of melanosomes from mouse melanoma and retinal pigment-epithelium of Rhode-Island-Red chick embryos. The melanosomes from mouse melanoma and retinal-pigment-epithelium were isolated by the method described in the text, washed several times in distilled water, and resuspended in distilled water. The concentrations of these suspensions are expressed in milligrams of dry weight per milliliter. These readings were taken in a Beckman Model DU spectrophotometer.

al density of the melanosome suspension (0.1 mg. dry weight/ml.) isolated from retinal pigment-epithelium of the 17-day Rhode-Island-Red chick embryo is twice that of suspensions (0.4 mg. dry weight/ml.) isolated from the 10-day chick embryo.

Comparison of the Tyrosinase Activity of Melanosomes Isolated from Mouse Melanoma and from the Retinal Pigment-Epithelium of Chick Embryos—Melanosomes isolated from Harding-Passey mouse melanoma contain three or four times more tyrosinase per milligram of protein-nitrogen than those from B-16 mouse melanoma (Table I). Comparison of melanosome suspensions obtained from 10-day and 17-day chick embryos (Table I) shows that the tyrosinase level of the suspension prepared from the pigment-epithelium of the 10-day chick embryo is twelve times higher than that of the suspension prepared from the corresponding tissue of the 17-day chick.

Some time ago, Miyamoto and Fitzpatrik (*14*) reported that the level of tyrosinase activity of pigment granules isolated from the retinal pigment-epithelium of Rhode-Island-Red chick embryos changes as embryonic development advances and that it reaches its highest level in the 10-day chick. The experimental data reported here confirm their observations.

TABLE I

Comparison of the Tyrosinase Activity

Experiment Number	Specific Activity μl. per hour per mg. protein-nitrogen	
	Harding-Passey	B-16
1	1114	210
2	1120	256
3	850	365
4		141
	Retinal Pigment-Epithelium	
	10-day chick embryo	17-day chick embryo
1	592	49.5

Comparison of the tyrosinase activity of melanosomes isolated from mouse melanoma and from retinal pigment-epithelium of chick embryos. Tyrosinase activity was estimated respirometrically by measuring the oxygen consumption, using a 10:1 mixture of L-tyrosine and L-dopa as substrate (1.77 μmoles) in the 0.1 *M* phosphate buffer (pH 6.8).

Effect of Incubation in L-dopa on the Tyrosinase Activity of Melanosomes—Keeping in mind the experimental results just described, the following *in vitro* experiment was designed to test the relationship between artificial melanization and changes of tyrosinase activity in melanosomes isolated from Harding-Passey mouse melanoma. These tumors are lighter in color than B-16 mouse melanoma and contain a high level of tyrosinase activity. Fig. 3 shows the experimental procedure.

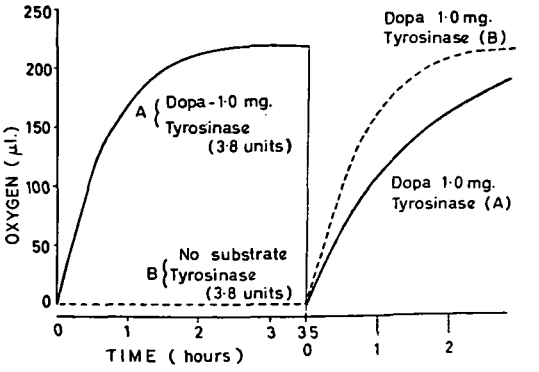


FIG. 3. Effect of dopa incubation on melanosomes isolated from Harding-Passey mouse melanoma. Melanosomes of experimental series *A* were incubated with L-dopa in Warburg apparatus at 38°C with 0.1 *M* phosphate buffer at pH 6.8; control group *B* was incubated without dopa under the same conditions. After the oxygen consumption of the experimental group reached its maximum, 1 mg. of L-dopa was added to the reaction vessels of both groups for measurement of tyrosinase activity.

Suspension A was incubated with 1 mg. of L-dopa. When the oxygen consumption had reached its maximum after 3.5 hours of incubation, the tyrosinase activity of the suspension was measured, using dopa as a substrate. Simultaneously with Suspension A, Suspension B was incubated under identical conditions, but without dopa, so that the tyrosinase activity of the two suspensions

TABLE II

Effect of Incubation in L-dopa on the Tyrosinase Activity of Melanosomes

Expt. No.	Dopa	Reaction Velocity of Original Melanosomes	Reaction Velocity of Original Melanosomes	Reaction Velocity of Incubated Melanosomes
	gm.	μl. per minute	μl. per minute	μl. per minute
1	0.5	3.8	3.1	2.7
2	0.5	3.4	3.3	3.1
3	0.5		2.8	2.5
4	1.0	4.3	4.1	3.1
5	1.0	4.6	3.8	2.8
6	1.0	3.8	3.4	2.1
7	1.0	3.8	3.1	2.8
8	1.0	3.4	3.3	2.3
9	1.5		3.1	1.1
9	1.2		3.1	1.6
9	0.9		3.1	1.9
9	0.6		3.1	2.4
9	0.3		3.1	2.8

The melanosomes isolated from Harding-Passey mouse melanoma were incubated with L-dopa. After the oxygen consumption reached a maximum, tyrosinase activity was measured by adding 1 mg. of L-dopa and was compared with the tyrosinase activity of untreated melanosomes; incubation was carried out under the same conditions, but without L-dopa.

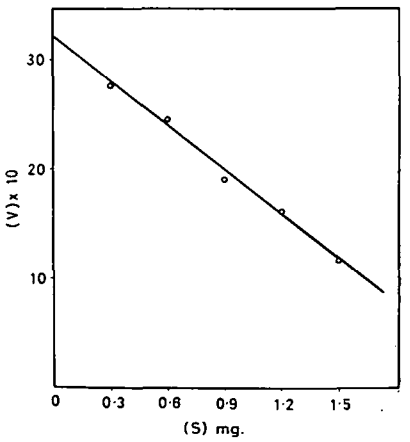
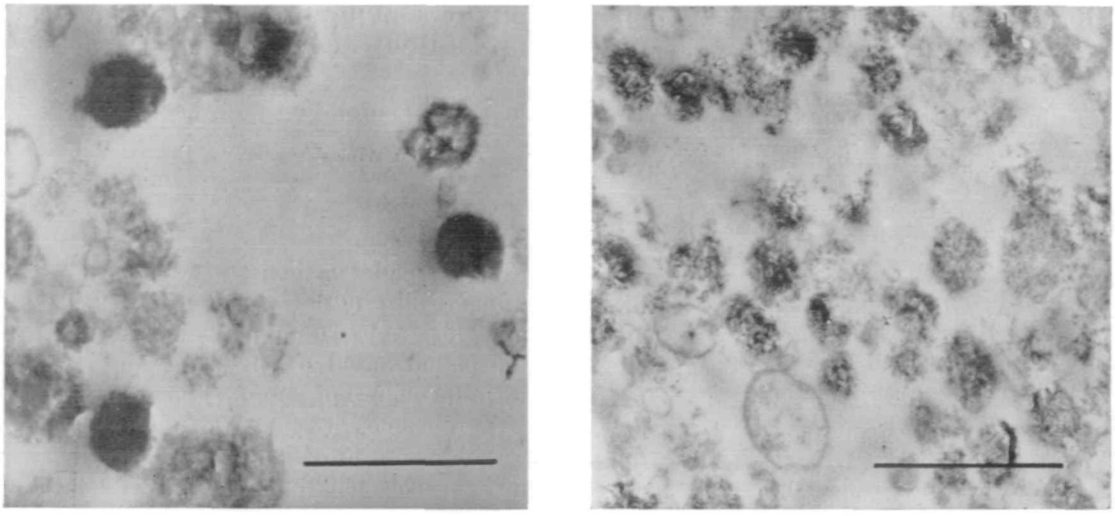


FIG 4. Relationship between dopa concentration (S) and reaction velocity (V) (rate of oxygen consumption, in microliters per minute) in melanosomes incubated in dopa.

could be compared. The color of the reaction mixture of A at the end of incubation was much darker than that of B. As can be seen in Table II, there is a significant decrease in the reaction velocity of tyrosinase in Suspension A after incubation. A linear relationship between the concentration of dopa in the incubation mixture and the reaction velocity of tyrosinase is shown in Fig. 4. (Experiment No. 9 in Table II).

Electronmicrographs of Melanosomes Which Have Been Melanized in Vitro—In order to demonstrate the morphological changes in melanosomes which follow incubation with dopa, electronmicrographs were taken after incubation of the dopa-treated and control large-granule suspension isolated from Harding-Passey melanoma (Figs. 5 A and B). Fig. 5 B is an electronmicrograph of the intensely electron-dense melanosomes which were never found in control suspensions (Fig. 5 A), but which appeared consistently in fractions which had been incubated with dopa. It is not possible to prepare good electronmicrographs, because of the effect of incubation with or without dopa on the fixation process, but those obtained were sufficiently clear to permit adequate identification of the elements present.



A B

FIG. 5. A. Electronmicrograph of large-granule suspension of Harding-Passey mouse melanoma after incubation without dopa (B in Fig. 3). Melanosomes and mitochondria can be identified. Magnification, ×32,000. The line in the lower part of each illustration denotes the length of 1 micron.

B. Electronmicrograph of large-granule suspension of Harding-Passey mouse melanoma after incubation with dopa (A in Fig. 3). Intensely electron-dense melanosomes were never found in the control suspension, but appear consistently in fractions which have been incubated with dopa. Magnification, ×32,000.

DISCUSSION

The experimental results presented in Fig. 1 and Table I suggest that differences in the specific activity of tyrosinase may be related to degree of melanization of melanosomes. The decrease in reaction velocity following incubation with dopa of melanosomes isolated from Harding-Passey melanoma (Table II) appears to be related to the degree of *in vitro* melanization of melanosomes by incubation in dopa. In melanosomes incubated with dopa for three consecutive periods the tyrosinase activity is reduced 73 per cent below control levels, while in melanosomes incubated for the same period without dopa, the tyrosinase activity is reduced only 7 per cent below initial levels.

The question arises whether the decrease in reaction velocity might be explained by the type of reaction inactivation observed by Nelson *et al.* (*15*, *16*) in preparations of plant tyrosinase. These workers noted that orthodiphenolase or catecholase activity undergoes early and progressive inactivation as oxidation of the substrate proceeds. In preparations of mammalian tyrosinase, this effect has not been observed with the diphenolic substrate, dopa. The reaction inactivation of plant tyrosinase does not appear to be due to products known to be formed during the oxidation of catechol, but occurs at the time when catechol is oxidized.

The linear relationship between the concentration of dopa in the incubation mixture and the reaction velocity of tyrosinase clearly shows that the relationship between dopa concentration and tyrosinase activity is inverse. The same inverse relationship between enzyme activity and substrate concentration is found in melanosomes which have been incubated with tyrosine instead of dopa. Thus the reduction in the reaction velocity of tyrosinase (as shown in activity units of Hogeboom and Adams (*17*)) after incubation in tyrosine or dopa would appear

to result from a blocking of the active centers on the enzyme rather than from inactivation of the reaction. It is likely that the centers of tyrosinase activity in the melanosome are blocked by the quininoid intermediates (dopaquinone, indole-5,6-quinone) in a chemical process similar to tanning and that during melanization melanosomes are gradually converted from enzymically active particles (early stages) into masses of inert melano-protein (Fig. 6).

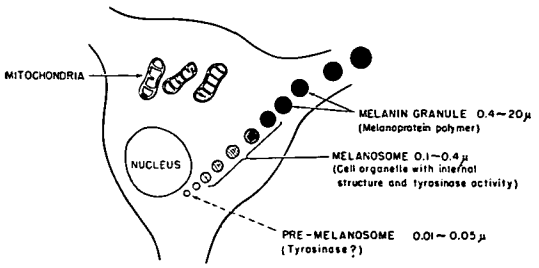


FIG. 6. Diagram showing development of melanin granules in the melanocyte. Melanin granules are distinctive cytoplasmic constituents of the melanin-forming cell and have their own characteristic enzyme (tyrosinase). The term "melanosome" is proposed for the distinctive, enzymatically active particle which is the site of melanin formation and is located within the cytoplasm of the melanocyte.

Electronmicroscopy has produced evidence to support this concept. Although the dense material seen in electronmicrographs may not be identical with melanin, it seems quite safe to consider that the changes seen after incubation with dopa may be due to *in vitro* melanization.

In order to separate the less melanosomes from more intensely melanized melanosomes, density-gradient tubes were prepared with sucrose solution (concentration 2.5 *M*—1.5 *M*) and the large-granule suspension isolated from Harding-Passey melanoma. The typical appearance of the tube after centrifugation in a horizontal rotor at 103,000×*g* for 1 hour is quite similar to that shown in Fig. 1 B: a narrow, brownish-gray, relatively tightly packed band is visible at the top of the gradient and a relatively clear zone lies between this layer and the brown or black suspension which fills almost the entire bottom half of the tube. The fractions containing melanosomes of low density had two or three times more activity than the fraction containing melanosomes of high density. Electronmicrographs of these fractions did not, however, show any significant differences in degree of melanization of melanosomes isolated from the upper fractions (low density) and the lower fractions (high density).

SUMMARY

1. Melanosomes, the distinctive, enzymically active particles which are the site of melanin formation, have been separated from B-16 and Harding-Passey mouse melanoma and from retinal pigment-epithelium of chick embryos by density-gradient centrifugation.

2. The color and tyrosinase activity per milligram of protein-nitrogen of melanosomes isolated from these tissues have been compared. Melanosomes isolated from B-16 mouse melanoma showed greater optical density and less tyrosinase activity than those of Harding-Passey melanoma. Melanosomes isolated from the retinal pigment-epithelium of the 10-day-old chick embryo showed less optical density and higher tyrosinase activity than those isolated from the retinal pigment-epithelium of the 17-day-old chick embryo.

3. Melanosomes isolated from Harding-Passey mouse melanoma were melanized *in vitro* by incubation with dopa. The reaction velocity of tyrosinase in melanosomes which had been thus melanized *in vitro* was significantly decreased. There was an inverse linear relationship between the concentration of dopa in the incubation mixture and the reaction velocity of tyrosinase.

4. Under the electronmicroscope, melanosomes which had been melanized *in vitro* had an intensely electron-dense appearance which suggested that their surface had been covered chemically and mechanically by dopa-melanin which had been produced during incubation.

5. Experimental results suggest that as successive layers of melano-protein accumulate

on the melanosome, the active centers of tyrosinase are blocked and that therefore there exists a reciprocal relationship between degree of melanization and level of measurable tyrosinase within the melanosome.

The authors wish to thank Dr. M. S. C. Birbeck of the Chester Beatty Research Institute of London, for taking the electronmicrographs and for his valuable suggestions. They also want to express their appreciation of the help given by Drs. H. Blaschko and R. Baker of Oxford University during the later phases of this investigation.

This investigation was supported in part by the Damon Runyon Cancer Fund and by Research Grant #8196-2 from the United States Public Health Service.

REFERENCES

(*1*) Lerner, A. B., and Fitzpatrick, T. B., *Physiol. Rev.*, **91**, 30 (1953)

(*2*) Mason, H. S., in "Pigment Cell Biology" edited by M. Gordon, Academic Press, Inc., New York, p. 563 (1959)

(*3*) Fitzpatrick, T. B., and Kukita, A., in "Pigment Cell Biology" edited by M. Gordon. Academic Press, Inc., New York, p. 489 (1959)

(*4*) Lerner, A. B., Fitzpatrick, T. B., Calkins, E., and Summerson, W. H., *J. Biol. Chem.*, **178**, 185 (1949)

(*5*) Herrman, H., and Boss, M. B., *J. Cell. & Comp. Physiol.*, **26**, 131 (1945)

(*6*) Seiji, M., and Fitzpatrick, T. B., *J. Invest. Dermat.*, in press.

(*7*) *a*) Wellings, S. R., and Siegel, B. V., *J. Natl. Cancer Institute*, **24**, 437 (1960)
b) Wellings, S. R., and Siegel, B. V., *J. Ultrastructure Research*, **3**, 147 (1959)

(*8*) Wissenfels, N., *Z. Zellforsch. u. mikroskop. Anat.*, **45**, 60 (1956)

(*9*) Charles, A., and Ingram, J. T., *J. Biophys. Biochem. Cytol.*, **6**, 41 (1959)

(*10*) Drochmans, P., *J. Biophys. Biochem. Cytol.*, **8**, 165 Vol. III (1960)

(*11*) Birbeck, M. S. C., and Barnicot, N. A., in "Pigment Cell Biology" edited by M. Gordon, Academic Press, Inc., New York, p. 549 (1959)

(*12*) Anderson, N. G., in "Physical Techniques in Biological Research" edited by Gerald Oster and Arthur W. Pollister, Academic Press, Inc., New York, Vol. **III** (1956)

(*13*) De Duve, C., Berthet, J., and Beaufay, H., *Prog. in Biophys. & Biophys. Chem.*, **9**, 325 (1959)

(*14*) Miyamoto, M., and Fitzpatrick, T. B., *Science*, **179**, 199 (1957)

(*15*) Nelson, J. M., and Dawson, C. R., in "Advances in Enzymology" Interscience Publishers, Inc., New York, Vol. **4**, p. 99 (1944)

(*16*) Ludwig, B. J., and Nelson, J. M., *J. Am. Chem. Soc.*, **61**, 2601 (1939)

(*17*) Hogeboom, G. H., and Adams, M. H., *J. Biol. Chem.*, **145**, 273 (1942)